Lisa Solieri
Paolo Giudici *(Eds.)*

# Vinegars of the World

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Lisa Solieri • Paolo Giudici
Editors

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Preface by Wilhelm Holzapfel

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# Preface

Vinegars can be considered as acidic products of special importance for the enrichment of our diet, and resulting from the desired or controlled oxidation of ethanol containing (liquid) substrates. The traditional use and integration of vinegars in numerous cultures can be traced back to ancient times. In fact, the cultural heritage of virtually every civilization includes one or more vinegars made by the souring action (of micro-organisms) following alcoholic fermentation. It has been documented that the Egyptians, Sumerians and Babylonians had experience and technical knowledge in making vinegar from barley and any kind of fruit. Vinegar was very popular both in ancient Greece and Rome, where it was used in food preparations and as remedy against a great number of diseases. In Asia, the first records about vinegar date back to the Zhou Dynasty (1027-221 BC) and probably China's ancient rice wines may have originally been derived from fruit, for which (malted) rice was substituted later.

The historical and geographical success of vinegars is mainly due to the low technology required for their production, and to the fact that several kinds of raw materials rich in sugars may easily be processed to give vinegar. In addition, vinegars are well-known and accepted as safe and stable commodities that can be consumed as beverages, health drinks or added to food as preservatives or as flavouring agents. The majority of vinegars, especially from sugary and acidic fruits, are easy to make, and this explains the relatively slow development in their science and technology through time. However, there is an urgent necessity to increase scientific knowledge and improve the technology of vinegar manufacture, and thereby to ensure higher standards of quality and safety in an expanding and increasingly diverse world-wide market.

The quality of wine vinegar is determined by a network of factors, mainly the raw wine substrate, microorganisms involved, and the acetification process employed in its production. Attempts to characterise vinegars have been based on the control of these three features. When the final products are analysed, it is difficult to evaluate to what extent quality differences are due to the raw material or to differences in production methods and in the microbial starter employed. Thus, it is

necessary to determine the influence of each feature separately. The knowledge, prediction, and integrated control of any of these features constitute the challenge to obtain more efficient and predictable ethanol conversions, thus increasing stability, quality and processing efficiency.

It deserves special mentioning that this book also focuses on recent developments in the molecular characterisation of prokaryotes. The vital information is presented in a concise manner, and includes microbial genomics and multi-locus sequencing techniques. In addition, an unprecedented amount of molecular data is provided which, together with phenotypic information, forms the basis for a new roadmap of acetic acid bacteria systematics.

Recently, nomenclature and classification of acetic acid bacteria have been strongly revised, and new genera and species were recognised from different environment samples. This has culminated into an unexpectedly large group, now encompassing more than ten genera, including about fifty species. Yet, not all the species are involved in vinegar production, and those most frequently reported (still) belong to the genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter*.

Vinegar technologies could be grouped in three main domains: static, solid state and submerged "fermentation". The latter represents the most recent quick process, in which the typical parameters of oxidative conversion (temperature, oxygen, alcohol content, and acidity) are strictly controlled. This process requires a strong energy input, and is implemented for industrial vinegar production. By contrast, static and solid state fermentations are mainly applied to traditional vinegar production with a long fermentation phase, and entail low energy consumption.

Within the wide scope of this book, the editors succeeded in bringing together a group of internationally recognised experts as authors for the diverse areas and issues of importance to vinegars worldwide. About forthy scientists from ten countries have contributed to the compilation and preparation of the seventeen chapters, thereby giving a picture of the most representative vinegars in the world. The wide coverage includes both the history and global perspectives on vinegars, innovative and traditional technologies for manufacture, and the microbiology in the inclusive context of ecology and up-to-date taxonomy.

This book is unique in many respects. It can be considered as the most authoritative, documentation, both scientifically and practically, on vinegars hitherto. Its rational approach to vinegar production is based on recent scientific data on food science, technology, engineering and microbiology. The work is coherent, and all the chapters are well integrated and complementary in the context of its overall scope. General aspects regarding vinegar history, acetic acid bacteria, other micro-organisms, and technology were discussed separately in specific chapters. The main vinegars presently produced in the world are clearly exposed in synoptic tables giving evidence to raw materials, micro-organisms involved, and geographical distribution. Single chapters have been devoted to the most important vinegars, thereby providing a wealth of information on the wide variety of vinegars produced and consumed around the world.

It is my pleasure to congratulate the editors, Dr. Lisa Solieri and Prof. Paolo Giudici, with this outstanding achievement. I have no doubt that this book on vine-

gar will be an extremely valuable guide and standard work of reference to all scientists, technologists, engineers and scholars in this field. It will most definitely also provide exciting literature and pleasant reading experience to every consumer and particularly to the highly specialized connoisseurs and gourmets of our time.

November 2008

Wilhelm Holzapfel
Handong Global University

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# Chapter 1
# Vinegars of the World

**Lisa Solieri and Paolo Giudici**

## 1.1 General Overview

The history of vinegar production, which dates back to around 2000 BC, has taught us a great deal about microbial biotransformation. However, vinegar has been always considered a 'poor relation' among fermented food products: it is not considered to be a 'food', it does not have great nutritional value, and it is made by the transformation of richer and more nutritive fermented foods. Vinegar is used as a flavouring agent, as a preservative and, in some countries, also as a healthy drink. It can be made from almost any fermentable carbohydrate source by a two-step fermentation process involving yeasts as the first agent, followed by acetic acid bacteria (AAB): the most common raw materials are apples, pears, grapes, honey, syrups, cereals, hydrolysed starches, beer and wine.

Since vinegar is generally an inexpensive product, its production requires low-cost raw materials, such as substandard fruit, seasonal agricultural surpluses, by-products from food processing, and fruit waste. However, there are also some very expensive vinegars, produced from regional foods according to well-established methods, although these are the exception to the rule: examples include traditional balsamic vinegar from Modena in Italy, sherry vinegar from Spain, and oxos from Greece. There are also spirit vinegars obtained directly by acetic oxidation of ethanol derived from the distillation of fermented mashes or petrochemical ethanol. In addition, pyroligneous liquor (or 'wood vinegar'), collected during wood carbonization, is used as an agricultural feedstuff, an animal health product, an ingredient in cosmetics, and a traditional medicine in Japan and East Asia (Mu et al., 2003; 2006). In this book, these distilled solutions have not been considered as vinegars because no fermentative process occurs in their production. Another separate group consists of flavoured vinegars: herbal or fruit vinegars. Herbal vinegars are wine vinegars or white distilled vinegars flavoured with garlic, basil, tarragon, cinnamon, cloves, nutmeg or other herbs. Fruit-flavoured vinegars are wine and white vinegars sweetened with fruit or fruit juice to produce a characteristic sweet-sour taste. In these cases the name 'X vinegar' does not indicate the raw materials

L. Solieri, P. Giudici (eds), *Vinegars of the World*

**Figure 1.1** Fermentation of herbal flavoured vinegar in glass demijohns

used in vinegar fermentation but the ingredients added to obtain specific taste and flavour characteristics (Figure 1.1).

## 1.2 Vinegars: Raw Materials and Geographical Distribution

According to the international definition of vinegar, in this book we consider only vinegars derived from a two-stage fermentation process of agriculturally produced raw materials. A list of vinegars is presented in Table 1.1, but cannot be considered exhaustive, since many different varieties of vinegar are produced all over the world, and some of them are unknown outside their area of origin. Most vinegars have a plant origin, with two exceptions: those produced from whey or honey. Whey, which is the milk serum residual of the cheese-making process, is rich in lactose and/or its corresponding hydrolysed sugars, galactose and glucose, depending on the cheese-making technology. Furthermore, sour whey is heavily contaminated with lactic acid bacteria (LAB) and needs to be pasteurized before alcoholic and acetous fermentation. Honey is very rich in sugars (70-80% w/w), mostly sucrose, fructose and glucose, the proportions of which are influenced by the botanical origin of the nectar collected by the bees. Honey is always diluted before alcoholic fermentation occurs; honey wine contains up to 17% (v/v) ethanol (Steinkraus, 1996). This alcoholic beverage is well known around the world by different names, such as mead, ambrosia, metheglin, hydromel, aguamiel, medovukha and ogol, and is also used to produce vinegar.

**Table 1.1** Overview of vinegars from around the world: raw materials, intermediate product, vinegar name and geographical distribution

| Category | Raw material | Intermediate | Vinegar name | Geographical distribution |
|---|---|---|---|---|
| Vegetable[a] | Rice | Moromi | Komesu, kurosu (Japanese)<br>Heicu (Chinese) | East and Southeast Asia |
| | Bamboo sap | Fermented bamboo sap | Bamboo vinegar[b] | Japan, Korea |
| | Malt | Beer | Malt vinegar | Northern Europe, USA |
| | Palm sap | Palm wine (toddy, tari, tuack, tuba) | Palm vinegar, toddy vinegar | Southeast Asia, Africa |
| | Barley | Beer | Beer vinegar | Germany, Austria, Netherlands |
| | Millet | Koji | Black vinegar | China, East Asia |
| | Wheat | Koji | Black vinegar | China, East Asia |
| | Sorghum | Koji | Black vinegar | China, East Asia |
| | Tea and sugar | Kombucha | Kombucha vinegar | Russia, Asia (China, Japan, Indonesia) |
| | Onion | Onion alcohol | Onion vinegar | East and Southeast Asia |
| | Tomato | – | Tomato vinegar | Japan, East Asia |
| | Sugarcane | Fermented sugar cane juice | Cane vinegar | France, USA |
| | | Basi | Sukang iloko | Philippines |
| | | | Kibizu | Japan |
| Fruit | Apple | Cider | Cider vinegar | USA, Canada |
| | Grape | Raisin | Raisin (grape) vinegar | Turkey and Middle East |
| | | Red or white wine | Wine vinegar | Widespread |
| | | Sherry wine | Sherry (jerez) vinegar | Spain |
| | | Cooked must | Balsamic vinegar | Italy |
| | Coconut | Fermented coconut water | Coconut water vinegar | Philippines, Sri Lanka |
| | Date | Fermented date juice | Date vinegar | Middle East |
| | Mango | Fermented mango juice | Mango vinegar | East and Southeast Asia |
| | Red date | Fermented jujube juice | Jujube vinegar | China |
| | Raspberry | Fermented raspberry juice | Raspberry vinegar | East and Southeast Asia |
| | Blackcurrant | Fermented blackcurrant juice | Blackcurrant vinegar | East and Southeast Asia |
| | Blackberry | Fermented blackberry juice | Blackberry vinegar | East and Southeast Asia |
| | Mulberry | Fermented mulberry juice | Mulberry vinegar | East and Southeast Asia |
| | Plum | Umeboshi[c] fermented plum juice | Ume-su | Japan |
| | Cranberry | Fermented cranberry juice | Cranberry vinegar | East and Southeast Asia |
| | Kaki | Fermented persimmon juice | Persimmon vinegar | South Korea |
| | | | Kakisu | Japan |
| Animal | Whey | Fermented whey | Whey vinegar | Europe |
| | Honey | Diluted honey wine, tej | Honey vinegar | Europe, America, Africa |

[a] Vegetable is not a botanical term and is used to refer to an edible plant part; some botanical fruits, such as tomatoes, are also generally considered to be vegetables.
[b] Obtained by bamboo sap fermentation.
[c] Umeboshi are pickled *ume* fruits. *Ume* is a species of fruit-bearing tree of the genus *Prunus*, which is often called a plum but is actually more closely related to the apricot.

**Table 1.2** Botanical species and edible parts used in vinegar production

| Common name | Botanical name | Edible part | Main carbon sources[a] |
|---|---|---|---|
| Apple | *Malus domestica* | Fruits (pome) | Fructose, sucrose, glucose |
| Apricot | *Prunus armeniaca* | Fruits (drupe) | Sucrose, glucose, fructose |
| Bamboo | Species and genera of the family Poaceae, subfamily Bambusoideae | Bamboo sap | Sucrose |
| Banana | Species of the genus *Musa* | Fruits (false berry) | Sucrose, glucose, fructose |
| Barley | *Hordeum vulgare* | Seeds (caryopsis) | Starch |
| Carambola | *Averrhoa carambola* | Fruits | Fructose, glucose |
| Cashew | *Anacardium occidentale* | Fruits | Sucrose, inverted sugars |
| Cocoa | *Theobroma cacao* | Bean mucilage (sweatings) | Glucose |
| Coconut | *Cocos nucifera* and other species of the family Arecaceae | Coconut water (fibrous drupe) | Glucose, fructose |
| Date | *Phoenix dactylifera* | Fruits (drupe) | Sucrose |
| Fig | *Ficus carica* | False fruit (syconium) | Glucose, fructose |
| Grape | *Vitis vinifera* and other species of the genus | Fruits (berry) | Glucose, fructose |
| Oil palm tree | *Elaeis guineensis* | Sap (xylem fluid) | Sucrose |
| Onion | *Allium cepa* | Bulbs | Fructose, glucose, sucrose |
| Panicum | *Panicum miliaceum* and other species of the subfamily Panicoideae | Seeds | Starch |
| Pear | *Pyrus communis* and other species of the genus | Fruits (pome) | Fructose sucrose, glucose |
| Persimmon | *Diospyros kaki* and other species of the genus | Fruits | Fructose, glucose, sucrose |
| Pineapple | *Ananas comosus* | False fruit (syncarpel) | Sucrose, glucose, fructose |
| Plum | *Prunus domestica* | Fruits (drupe) | Sucrose, fructose, glucose |
| Potato | *Solanum tuberosum* | Tuber | Starch |
| Raphia palm | *Raphia hookeri* and *Raphia vinifera* | Sap (xylem fluid) | Sucrose |
| Ribes (Blackcurrant, Redcurrant, Gooseberry) | *Ribes* spp. | Fruits (berry) | Fructose, glucose |
| Rice | *Oryza sativa* and *Oryza glaberrima* | Seeds (caryopsis) | Starch |
| Sorghum | *Sorghum bicolor* and other species | Seeds (caryopsis) | Starch |
| Sugarbeet | *Beta vulgaris* | Roots | Sucrose |
| Sugarcane | Species of the genus *Saccharum* | Stalks | Sucrose |
| Wheat | *Triticum aestivum* and other species | Seeds (caryopsis) | Starch |

[a] Listed in order, from the largest to the smallest amount.

### 1.2.1 Botanical Species

Many botanical species can be used for vinegar production since they only need to have two main basic attributes; first to be safe for human and animal consumption, and second to be a direct or indirect source of fermentable sugars.

A non-exhaustive list of the main botanical species involved and their edible parts used is shown in Table 1.2. General classifications and groupings can be made on the basis of the chemical composition of the edible parts and their ease of fermentation:

- *Acid and easily fermentable*: pH <3.5, with glucose, fructose and sucrose as the main constituents, e.g. berries, grapes, apples, plums.
- *Moderate acid and easily fermentable*: pH 3.5-4.5, e.g. figs, dates.
- *Low acid and easily fermentable*: pH >4.5, e.g. palm sap.
- *Non-fermentable*: hydrolysis required before fermentation, e.g. seeds.

The chemical composition of the raw material exerts a strong selective pressure on microorganisms and determines the dominant species involved in acetification. Specific examples are given for the vinegars described in other chapters of this book.

The critical steps in vinegar production are the preparation and the fermentation of raw materials. Preparation of raw materials includes all the operations required to produce fermentable sugary and protein solutions, such as slicing and/or crushing to obtain fruit juice, enzymatic digestion of starch in cereals, as well as cooking and steaming in some cases. In general, fruits require less preparation than seeds. On the other hand, seeds are more easily stored and preserved, and consequently their use is independent of the harvest. Fruits are highly perishable, rich in water, and need to be processed very quickly; in some conditions, such as at high temperatures or in the case of damaged fruits, this will be immediately after harvest. These differences make seeds easier to transport and process in large factories, whereas fruits can be made into vinegar in small factories, with less technology, close to the production area.

### 1.2.2 Economic Importance

From an economic point of view, vinegar production is a small industry in the overall economy of industrialized countries (Adams, 1998). Global shares of the different kinds of vinegar in 2005 were balsamic vinegar (34%), red wine vinegar (17%), cider vinegar (7%), rice vinegar (4%), white vinegar (2%) and other vinegars (36%), as shown in Figure 1.2 (Vinegar Institute, 2006).

In the US market, white distilled vinegar has 68% of the unit share, cider vinegar accounts for 20%, and specialty vinegars account for 12%. In the specialty vinegar category, 39% comprises red wine vinegar, 30% balsamic, 13% all other wine, 12% rice vinegar, and 6% all other specialties (Vinegar Institute, 2006).

In Europe, the vinegar market was around $4.9\times10^8$ L in 2001 and $5\times10^8$ L in 2002, with business worth approximately € 268.6 million and € 234.3 million,

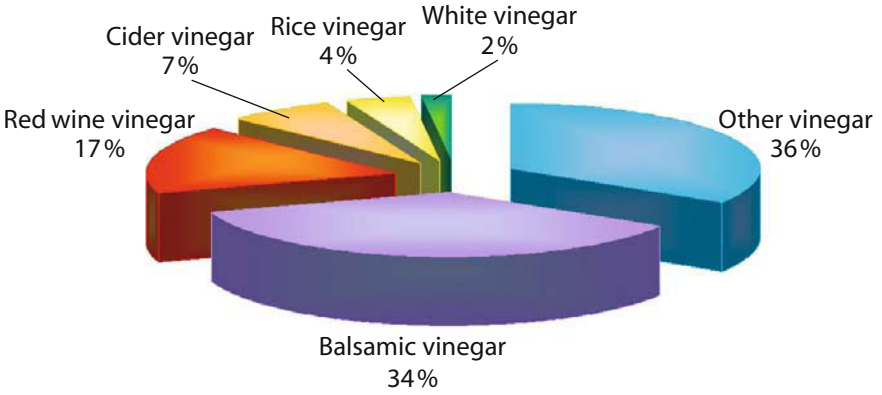


**Figure 1.2** Global shares of the different vinegar types in 2005 (from the Vinegar Institute, 2006, available at http://www.versatilevinegar.org/markettrends.html#2)

respectively. European vinegar shares are shown in Figure 1.3. The main vinegar-producing countries are France, Italy and Spain.

In China, white fruit and brewed vinegars are popular. Every year, $8.0\times10^9$ kg of distilled spirit vinegar and $2.0\times10^9$ kg of brewed vinegar are produced (Wei, 2001). There are at least 14 types of traditional brewed vinegars, among which five types are the most widespread: Zhenjiang aromatic vinegar, Sichuan bran vinegar, Shanghai rice vinegar, Jiangzhe rose vinegar and Fujian red rice vinegar (Liu et al., 2004).

In developing countries, where food preservation and technology options are limited, vinegar is an important agent for preserving fresh fruit and vegetables from rapid deterioration. Especially in the tropics, the environmental conditions accelerate food spoilage. Developing and improving small-scale vinegar production, and food fermentation technologies in general, is one of the goals of the FAO (Anonymous, 1995; FAO, 1998).

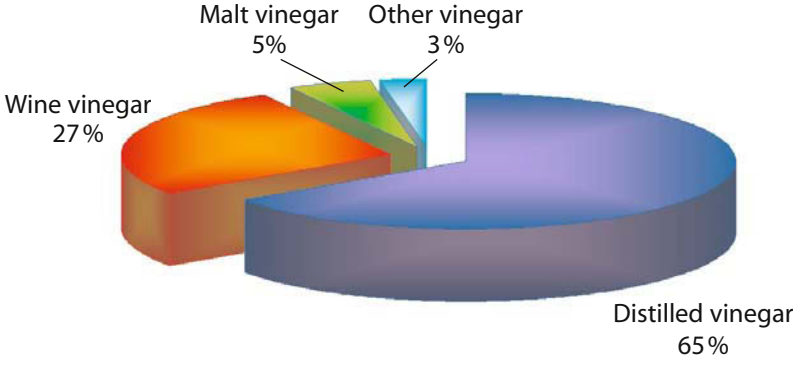


**Figure 1.3** European vinegar share in 2002 (from Comité Permanent International du Vinaigre, available at http://vinaigre.fr)

## 1.3 Vinegar Processing: the Role of Fermentation

As for any other food, the global view of vinegar processing from producer to market can be summarized as shown in Figure 1.4. In general, basic safe food operating principles, such as good agricultural practices (GAP), good manufacturing practices (GMP) and good hygiene practices (GHP), should be in place in all the steps, but in particular before starting fermentation, when environmental factors may permit the growth of dangerous microorganisms such as aflatoxin-producing moulds and harmful bacteria, especially since these steps are often carried out at room temperature. After acetification, there is no real danger of spoilage, since acetic acid has strong antibacterial activity at low pH. Vinegar also requires pack-

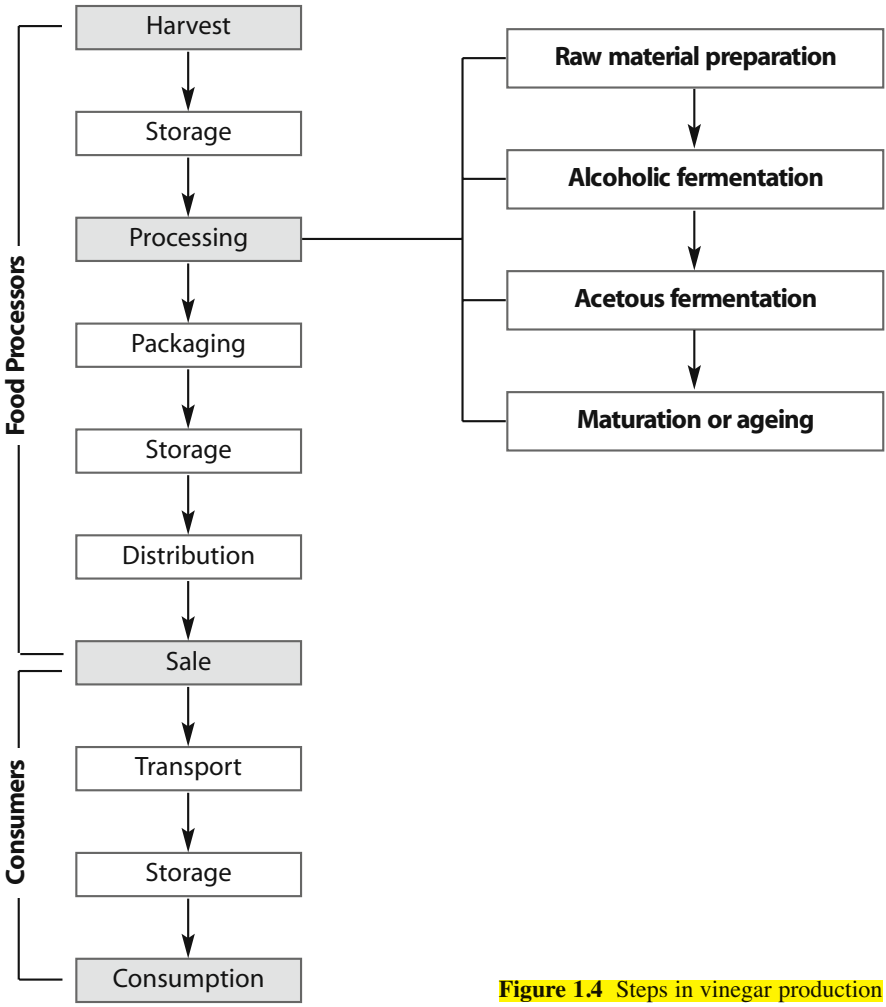


**Figure 1.4** Steps in vinegar production

**Table 1.3** The main microorganisms involved in vinegar production

| Vinegars | Moulds | Yeasts | LAB | AAB | References |
|---|---|---|---|---|---|
| Kombucha vinegar | – | *Z. kombuchaensis*, *Z. rouxii*, *Candida* spp., *Sc. pombe*, *S'codes ludwigii*, *P. membranaefaciens*, *B. bruxellensis* | – | *Ga. xylinum*, *Ga. intermedius*, *Ga. kombuchae* | Hesseltine, 1965; Liu et al.,1996; Boesch et al., 1998; Teoh et al., 2004; Dutta, Gachhui, 2007 |
| Beer/malt vinegar | – | *Saccharomyces sensu stricto* | *Lb. brevis*, *Lb. buchneri*, *P. damnosus* | *A. cerevisiae*, *Ga. sacchari* | White, 1970; Greenshields, 1975a,b; Fleet, 1998; Cleenwerck et al., 2002 |
| Coconut water vinegar | nd | *Saccharomyces* spp. | nd | *A. aceti* | Steinkraus, 1996 |
| Nata de coco | | *Saccharomyces* spp. | nd | *Ga. xylinus* | Steinkraus, 1996; Iguchi et al., 2004 |
| Fruit vinegars | – | *S. cerevisiae*, *Candida* spp. | nd | *A. aceti*, *A. pasteurianus* | Maldonado et al., 1975; Uchimura et al., 1991; Suenaga et al., 1993 |
| Honey vinegar | – | *S. cerevisiae*, *Zygosaccharomyces* spp., *Torulopsis* spp | *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Pediococcus* spp. | *Acetobacter* spp., *Gluconacetobacter* spp. | Adams, Nielsen, 1963; Snowdon, Cliver, 1996; Ilha et al., 2000; Bahiru et al., 2006 |
| Palm wine vinegar | – | *S. cerevisiae*, *S. uvarum*, *C. utilis*, *C. tropicalis*, *Sc. pombe*, *K. lactis* | *Lb. plantarum*, *Lc. mesenteroides* | *Acetobacter* spp., *Zymomonas mobilis* | Okafor, 1975; Uzochukwu et al., 1999; Ezeronye, Okerentugba, 2000; Amoa-Awua et al., 2007 |
| Rice vinegar | | | | | |
| Komesu | *Aspergillus oryzae*, *Aspergillus soyae*, *Rhizopus* spp. | *S. cerevisiae* | *Lb. casei* var. *rhamnosus* | *A. pasteurianus* | Hesseltine, 1983; Otsuka, 1990 |

(continued)

**Table 1.3** (continued)

| Vinegars | Moulds | Yeasts | LAB | AAB | References |
|---|---|---|---|---|---|
| Kurosu | *Aspergillus awamori*, *Aspergillus usami*, *Aspergillus oryzae* | *S. cerevisiae* | *Lb. fermentum*, *Lb. lactis*, *Lb. brevis*, *P. acidilactici*, *Lb. acetotolerans* | *A. pasteurianus* | Nanda et al., 2001; Haruta et. al., 2006 |
| Red rice vinegar | *Monascus purpureus* | *S. cerevisiae* | nd | *Acetobacter* spp. | Liu et al., 2004 |
| Sorghum vinegar | | | | | |
| African sorghum vinegar | nd | *S. cerevisiae* and other *Saccharomyces sensu stricto* | *Lc. meseteroides*, heterofermentative LAB | *Acetobacter* spp. | Steinkraus, 1996, Konlani et al., 1996; Naumova et al., 2003 |
| Chinese sorghum vinegar | *Mucor* spp., *Aspergillus orzyae*, *Monascus* spp. | *S. cerevisiae*, *Hansenula* spp | nd | *Acetobacter* spp. | Liu et al., 2004 |
| Traditional balsamic vinegar | – | *Z. bailii*, *S. cerevisiae*, *Z. pseudorouxii*, *C. stellata*, *Z. mellis*, *Z. bisporus*, *Z. rouxii*, *H. valbyensis*, *H. osmophila*, *C. lactis-condensi* | nd | *Ga. xylinus*, *A. pasteurianus*, *A. aceti*, *Ga. europaeus*, *Ga. hansenii*, *A. malorum* | De Vero et al., 2006; Gullo et al., 2006; Solieri et al., 2006; Solieri et al., 2007 |
| Whey vinegar | – | *K. marxianus* | nd | *Ga. liquefaciens*, *A. pasteurianus* | Parrondo et al., 2003 |
| Wine vinegar | – | *S. cerevisiae* | nd | *Ga. europaeus*, *Ga. oboediens*, *A. pomorum*, *Ga. intermedius*, *Ga. entanii* | Sievers et al., 1992; Sokollek et al., 1998; Boesch et al., 1998; Schüller et al., 2000 |

Abbreviations: nd, not determined; – not detected; A., *Acetobacter*; B., *Brettanomyces*; C., *Candida*; Ga., *Gluconacetobacter*; K., *Kluyveromyces*; Lb., *Lactobacillus*; Lc., *Leuconostoc*; P., *Pediococcus*; S., *Saccharomyces*; S'odes, *Saccharomycodes*; Sc., *Schizosacharomyces*; Z., *Zygosaccharomyces*.

aging; intermediate bulk containers and tanks should be manufactured of stainless steel, glass or plastic material resistant to corrosion. After raw material preparation, fermentation plays a key role in vinegar production. Different microbial species are involved at various stages of the fermentation process, such as LAB, yeasts, moulds and AAB, which often colonise vegetables, fruits and other raw materials used in vinegar production. From each microbial group, the main species associated with vinegars are listed in Table 1.3. The great microbial diversity reflects the variety of raw materials, sugar sources and processes, as well as the diversity of the physico-chemical characteristics (e.g. temperature, pH, water activity).

Two steps are common to all vinegars: alcoholic and acetic fermentation, due to yeasts and AAB, respectively, whilst other microorganisms, such as moulds and LAB, are involved only in specific vinegars. Among the yeasts, *Saccharomyces cerevisiae* is the most widespread species in fruit and vegetable vinegars; the lactose-fermenting yeast, *Kluyveromyces marxianus*, is the species responsible for whey fermentation; and a physical association of yeasts, LAB and AAB is involved in the fermentation of kombucha. Even though there are now ten generally recognized genera of AAB (Chapter 3), the majority of the species detected in vinegars belong to the genera *Acetobacter* and *Gluconacetobacter*. However, it is likely that several of the species and genera involved in vinegar production have not yet been described because of the difficulties in cultivating AAB. Furthermore, the taxonomy of the acetic acid bacteria is undergoing extensive revision at present, and many species and genera may soon be reclassified.

### 1.3.1 Spontaneous Fermentation

Fermentation can be induced either by spontaneous fermentation, by back-slopping, or by the addition of starter cultures. In spontaneous fermentation, the raw material is processed and the changed environmental conditions encourage the most appropriate indigenous microflora. The more stringent the growth conditions are, the greater becomes the selective pressure exerted on the indigenous microorganisms.

In a very acidic and sugary environment, such as some fruit juices, only yeast, LAB and AAB can grow. Spontaneous fermentation is suitable for small-scale production and only for very specific juices. However, the method is difficult to control and there is a great risk of spoilage occurring. In most spontaneous fermentations, a microbial succession takes place, and quite often LAB and yeasts dominate initially. These consume sugars and produce lactic acid and ethanol, respectively, which inhibit the growth of many bacteria species, determining prolongation of the shelf life of the goods. Moulds mainly grow aerobically and therefore their occurrence is limited to specific production steps or on crops before and after harvest. Moulds are a big safety concern, since some genera and species are aflatoxin producers. Therefore, the moulds used for starch hydrolysis of seeds should be GRAS (Generally Recognized As Safe). AAB are aerobic whole-cell biocatalysts involved in the conversion of ethanol to acetic acid. AAB are widespread on fruits and in many sugary and acid environments, and their growth is promoted by procedures

that increase the availability of oxygen after yeast fermentation. Examples are submerged culture and solid state fermentations (Chapters 9 and 15).

### 1.3.2 Back-Slopping Fermentation

Back-slopping uses part of a previously fermented batch to inoculate a new batch. This procedure increases the initial number of desirable microorganisms and

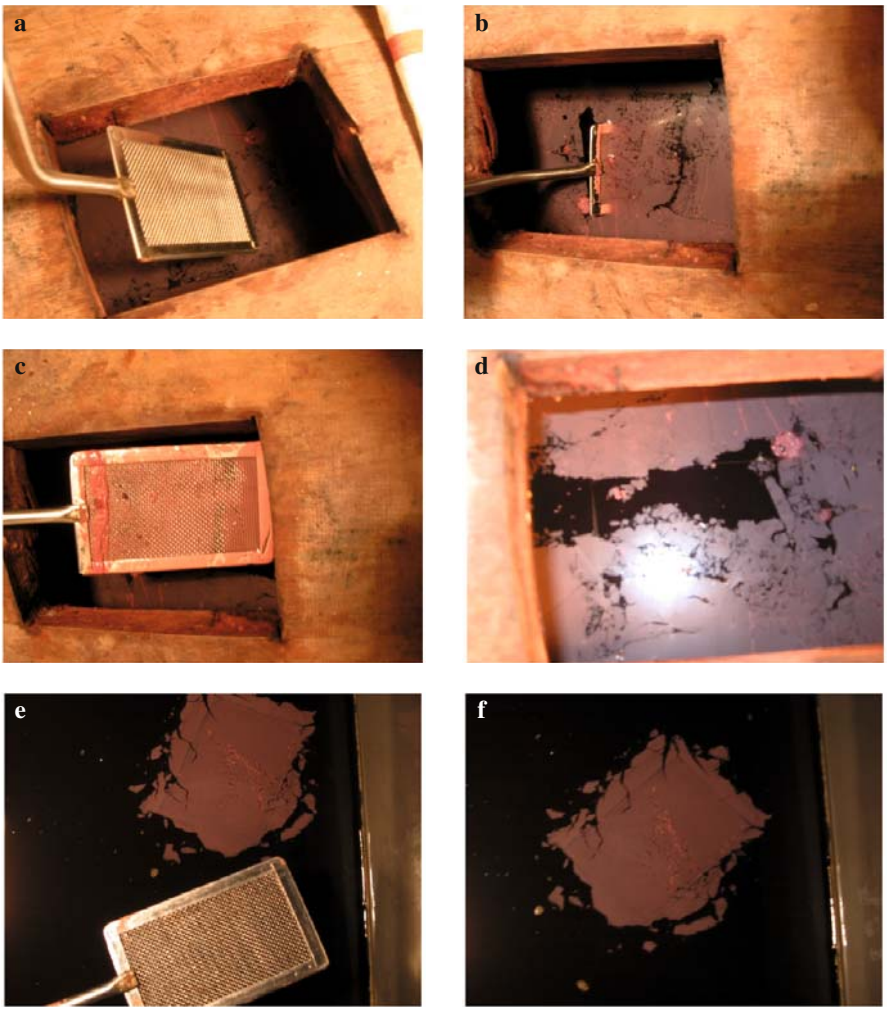


**Figure 1.5** Succession of steps in back-slopping to transfer the AAB film from a vinegar culture to a new wine barrel. **a** Tool to collect AAB film **b** Tool inside the vinegar barrel **c** Tool covered with the AAB film **d** Vinegar barrel after removal of an AAB film portion **e,f** Film transferred into wine barrel to start the acetification process (from Giudici et al., 2006)

ensures a more reliable and faster process than spontaneous fermentation. Back-slopping is a primitive precursor of the starter culture method, because the best-adapted species are seeded over the indigenous population (De Vuyst, 2000). Nevertheless, the manufactured goods are still exposed to the risk of fermentation failure, since mould growth or harmful bacteria spoilage can occur.

In general, back-slopping is considered a useful practice because it improves the growth of useful yeasts, while inhibiting the growth of pathogenic microorganisms and reducing spoilage, and in addition the laborious and time-consuming starter selection process is avoided. The back-slopping practice is particularly useful for inoculating AAB cultures, as they are very fastidious microorganisms that need special attention in order to produce true starter cultures. In the semi-continuous submerged acetification process, at least one-third of the vinegar is left in the fermenter to inoculate the new wine (Chapter 6), whereas in surface-layer fermentation a physical transplant of the AAB film can be easily done in order to preserve the integrity of the cell layer, as shown in Figure 1.5. This procedure assures a better implantation of inoculum on the indigenous microbial population in a new barrel.

### 1.3.3 Starter Culture Fermentation

A starter culture can be defined as a microbial preparation of a large number of cells of a microorganism (in some case more than one), which is added to the raw material to produce a fermented food by accelerating and steering its fermentation process (Leroy and De Vuyst, 2004). Starter culture development is strictly related to the 'pure culture' technique, which is a practice originally elaborated by Robert Koch for bacteria (Raineri et al., 2003). By using this approach each microbial colony is made up of cells that all originate from the same single cell. This ensures that the cultures are not a mixture of different unknown individuals and they can therefore be relied upon to produce the desired biochemical reactions.

The use of starter cultures in food production is a well-accepted practice, as it increases the safety, the stability and the efficiency of the process and reduces production losses caused by uncontrolled fermentation, eliminating undesired features. In some Asian vinegars, a mixed starter culture of undefined moulds and yeasts, called *koji*, is used to saccharify and ferment rice and cereals. However, *koji* cannot be considered to be a true starter, as its exact microbial composition is often unknown. In other cases, true starter cultures of oenological *S. cerevisiae* strains, selected for winemaking, are used for producing the alcoholic bases for vinegars, such as beer, wine and cider. *S. cerevisiae* var. *sake*, selected for sake production, is mainly used in rice vinegar fermentation.

Regarding acetous fermentation, the use of starter cultures is a long way from being applied on a large scale, for two main reasons: first, the AAB are nutritionally demanding microorganisms, which are difficult to cultivate and maintain in laboratory media, or to preserve as a dried starter; and, second, vinegar is generally an inexpensive commodity and therefore its manufacture does not warrant an expensive starter culture selection.

## 1.4 Vinegar Definitions and Legislation

Vinegar production is regulated through an extensive set of statutes, and the definition of vinegar itself differs from country to country. FAO/WHO defines vinegar as any liquid, fit for human consumption, produced exclusively from suitable products containing starch and/or sugars by the process of double fermentation, first alcoholic and then acetous. The residual ethanol content must be less than 0.5% in wine vinegar and less than 1% in other vinegars (Joint FAO/WHO Food Standards Programme, 1998).

In the USA, the Food and Drug Administration (FDA) requires that vinegar products must possess a minimum of 4% acidity. This qualification ensures the minimum strength of vinegars sold in the retail market. There are currently no standards of identity for vinegar; however, the FDA has established 'Compliance Policy Guides' that the Agency follows regarding the labelling of vinegars, such as cider, wine, malt, sugar, spirit and vinegar blends (Food and Drug Administration, 2007, FDA/ORA CPG 7109.22).

European countries have regional standards for the vinegar produced or sold in the area. Unlike the USA, the EU has established thresholds for both acidity and ethanol content. 'Vinegar of X' is a general definition used for products having a minimum of 5% (w/v) acidity and a maximum of 0.5% (v/v) ethanol. Wine vinegar is exclusively obtained by acetous fermentation of wine and must have a minimum of 6% acidity (w/v) and a maximum of 1.5% (v/v) of ethanol (Regulation (EC) No. 1493/1999).

In China, the term 'vinegar' is used to indicate both fermented and artificial vinegars, according to the Chinese National Standard definitions (CNS14834, N5239) (Chinese National Standard, 2005). In the previous National Industrial Standard for vinegar, vinegar was classified in three grades, depending on its concentration of acetic acid (3.5-4.5%, 4.5-6% and >6%). More recently a new National Standard Code for Condiments was issued by the Chinese State Administration Bureau for Quality and Technology, in which vinegar definitions are introduced and vinegars are classified as either brewed or artificial (acetic acid blended with other ingredients, such as flavourings). Moreover, each major vinegar type also has its own local quality criteria and grading system.

Considering the different laws on vinegar, it is clear that acidity and residual ethanol are the two main parameters used to establish an all-encompassing vinegar classification (Table 1.4). The acetic acid and ethanol contents change on the basis of raw materials used, the microorganisms involved in the fermentation process, the technology employed, but mainly on the basis of culture and 'vinegar lore'. Nowadays, it is a common practice in China to mix vinegar and wine to improve flavour and safety. In Europe, vinegar is considered as a flavouring or preservative and, with a few exceptions, it is generally sharp and sour. On the other hand, in Asia and Africa, vinegar is also a drink with a less sour taste. Several sweetened fruit vinegars characterized by low acidity and aromatic flavour are very popular in China and in East and Southeast Asia. In Africa, some fermented beverages can spontaneously acidify to produce alcoholic-acetous products, which are difficult to

**Table 1.4** Acidity and residual ethanol content in several vinegars

| Vinegar | Acidity (% w/v) | Ethanol (% v/v) |
|---|---|---|
| Malt vinegar | 4.3-5.9 | – |
| Cider vinegar | 3.9-9.0 | 0.03 |
| Wine vinegar | 4.4-7.4 | 0.05-0.3 |
| (semi-continuous process) | (8-14) | – |
| Rice vinegar | 4.2-4.5 | 0.68 |
| Chinese rice vinegar[a] | 6.8-10.9 | – |
| Cashew vinegar | 4.62 | 0.13 |
| Coconut water vinegar | 8.28 | 0.42 |
| Mango vinegar | 4.92 | 0.35 |
| Sherry vinegar | 7.0 | – |
| Pineapple vinegar | 5.34 | 0.67 |

[a] Chinese rice vinegar data were reported by Liu et al., 2004.
–, not reported.

Modified from Adams, 1998

classify either as alcoholic beverages or as vinegars. Similarly, in Japan, black rice vinegar is usually diluted with fruit juice and consumed daily as a healthy tonic beverage, with a share of 20% of the Japanese vinegar market, corresponding to 21.46 billion yen in the year 2004. In some western countries, mainly the USA and Canada, apple cider vinegar is a traditional folk remedy that is claimed to be beneficial in treating a long list of diseases; it is drunk mixed with fruit juice.

According to EU legislation and the FAO/WHO vinegar definitions, many fruit vinegars cannot be considered to be 'vinegar', since they have a low acetic acid content and, in addition, they may still contain ethanol. In the traditional wine-producing countries of Europe it is very easy to differentiate between wine and vinegar, since the names are well-established and have a precise meaning. Wine must have a minimal acetic acid content which is less than 1.2 $g \cdot L^{-1}$ and, for special wines, acetic acid must be less than 1% of the ethanol content. Vinegar must have more than 6% of titratable acidity and residual ethanol less than 1.5%. However, fermented alcoholic beverages and vinegars share many process steps and it is easy to envisage products that do not match the definition of either vinegar or wine. In our opinion, the grouping of vinegars first requires a complete picture of the kinds of vinegar available throughout the world, and then at least three parameters need to be established: the lowest threshold of acetic acid, the upper ethanol limit, and the lowest acceptable value of the acetic acid : ethanol ratio.

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# Chapter 2
# Vinegars Through the Ages

**Stefano Mazza and Yoshikatsu Murooka**

## 2.1 In the Beginning

The word *vinegar* has been in use in the English language since the 14th century, when it arrived in the British Isles from the French *vinaigre*, a word that simply means 'sour wine', and that came in turn from the Latin *vinum acre*, 'sour wine' or, more commonly, *vinum acetum*, 'wine vinegar'.

The word *acetum*, meaning 'vinegar' in the most proper sense, is derived from the verb *acere*, meaning 'to become pungent, go sour', and is similar to the ancient Greek ἀκμή [akmé], 'spike', while the Greek word for vinegar is ὄξος [óxos], sharing the same linguistic root and belonging to the semantic area of being sharp and pungent. Looking to another ancient culture, the Hebrew word for 'vinegar' in the Old Testament was *koe-metz* (also transliterated as *chomets*, *hometz*), meaning 'pungent' or 'fermented'.

These simple linguistic observations show us that since ancient times the words indicating vinegar are always associated with the idea of being pungent, strong-tasting and sharp or, more simply, the idea of being *acid*. Notwithstanding the long established knowledge of vinegar as an acid beverage obtained by fermentation, very little is known about its origin.

The birth of vinegar is lost in the dawn of human history, together with the beginning of agriculture and the discovery of alcoholic fermentation from fruits, cereals and vegetables, and it can hardly be distinguished from the origin of wine. Nowadays, we are accustomed to the fact that alcoholic and acetic fermentations are two distinct and separate processes, each leading to different products with particular and recognizable features. This discrimination, however, would not have been as evident to early men, simply because the two kinds of fermentation often follow on from each other when the process is not carefully controlled. It is obvious that what we nowadays call 'wine', 'beer', 'mead' and the like, cannot be compared to the analogous beverages obtained by our primeval ancestors, who became aware of the transformation of alcoholic drinks into vinegar only after a historical period of observation and improvement of their products.

L. Solieri, P. Giudici (eds), *Vinegars of the World*

It is a generally accepted idea that winemaking originated during the Neolithic period, approximately 8500-4000 BC, when humans underwent the transition from a hunter-gatherer society to breeding, farming and crafting stone tools, with Egypt and the Middle East being the major centres of this change. The Neolithic period is the first time in human prehistory when the necessary preconditions came together for the momentous innovation of viniculture, encompassing the knowledge of grape cultivation, winemaking and fermentation, along with methods for storage (Cavalieri et al., 2003).

Persia (now Iran), according to evidence from many sources, is the oldest winemaking country in the world. Large urns and vases have been excavated from the kitchen area of a mud-brick building in Hajji Firuz Tepe, a Neolithic village in Iran's northern Zagros Mountains. Shards from these jars dating back to 6000 BC were found to have a peculiar reddish-yellow coating. Using infrared spectrometry, liquid chromatography and a wet chemical test, a team from the University of Pennsylvania Museum found that the coating contained calcium salts from tartaric acid, which occurs naturally in large amounts only in grapes (McGovern et al., 1996). It is therefore likely that these vases were used to make or hold something similar to wine, although the early Persian version of wine may have been very different from our modern wines and vinegars.

What we can imagine is that the pressing methods used in prehistoric and ancient times were certainly crude, and the hot, dry climate of the desert encouraged a quick fermentation, rapidly turning grape juice into an indeterminate alcoholic-acidic beverage. It seems that acetic fermentation resulting in vinegar, although not entirely welcomed by the early farmers, was clearly very well known. For this reason, the first winemakers used to control fermentation by adding clay as a stopper, in order to prevent the wine – or other alcoholic beverage – from turning into vinegar. Further analyses of residuals left in those ancient jugs revealed the presence of resin from the terebinth tree (*Pistacia terebinthus*), a renowned natural preservative that would have helped to control the transformation of wine into vinegar (McGovern et al., 1996). It is, however, still uncertain whether terebinth resin was deliberately used for the control of fermentation or simply as an ingredient for adding flavour and taste to drinks.

## 2.2 Vinegar in Ancient Cultures

Of all the people of ancient times, the Egyptians were probably the first to discover and use true vinegar, because they had been brewing beer from barley, wheat or millet since the origin of their civilization. In 1988, the tomb of one of Egypt's first kings, who lived about 3150 BC and was probably named Scorpion I, was excavated at Abydos on the Middle Nile in Upper Egypt (McGovern, 1998). Hundreds of jars, which proved to have contained wine, were recovered. Several preserved grapes were found, and 47 of the jars contained grape pips, morphologically most similar to a domesticated subspecies, *Vitis vinifera vinifera* (Cavalieri et al., 2003). Therefore wine was known in Egypt before 3000 BC (Figure 2.1), and the Egyp-

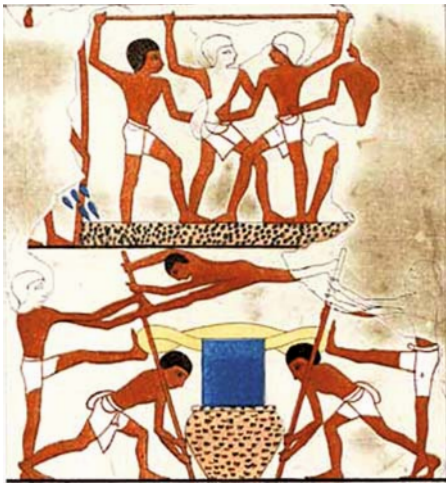

**Figure 2.1** Wine press in ancient Egyptian painting (from C.R. Lepsius, *Denkmäler aus Aegypten und Aethiopien*, 1897)

tians were aware of the fact that wine, once opened, undergoes sudden changes and becomes vinegar, which the Egyptians called *HmD* (usually pronounced 'hemedj'). Vinegar residues have actually been found in ancient Egyptian urns of 3000 BC and there is a line in the *Instruction of Ankhsheshonq*, dating to the Ptolemaic period (332-30 BC), that seems to recall the phenomenon of the acidification of wine when it is exposed to air and oxygen: 'Wine matures as long as one does not open it'.
Another important centre for the development of vinegar making was Mesopotamia, the food history of which is known from archaeological digs and written records on cuneiform tablets, including bilingual Sumerian-Akkadian word lists, where a sort of fermentation process is also described. It seems, for instance, that beer was a common drink for the Sumerians, whereas wine was better known in northern Mesopotamia and only in later times. Babylonian records of 5000 BC indicate that they were using the fruit of the date palm as the main raw material to obtain wine and vinegar, while figs and grapes were less common for this purpose. The Babylonians purposely used vinegar for pickling and preserving every sort of food (Bottero, 2004).

Moving northward to Anatolia, the legend tells that Helen of Troy used to bathe in vinegar or, more likely, in water mixed with vinegar in order to relax. We can't say much about the likelihood of this fact, but it tells us that vinegar was surely popular and widely used by the ancient Mediterranean cultures by the end of the 12th century BC, when the Trojan War presumably took place.

## 2.3 Vinegar in the Classical World

In ancient Greece, Hippocrates of Kos (460-377 BC), father of modern medicine, was the first to study the human digestive system and fix the principles of dietology, because he considered every food as a cause of illness or good health. Follow-

ing his theory, he prescribed vinegar as the main remedy against a great number of diseases, including the common cold and cough (Flandrin et al., 2000).

In the 3rd century BC, the Greek philosopher Theophrastus of Eressos (370-285 BC) described how vinegar acted on metals to produce pigments useful in art, including *white lead* (lead carbonate) and *verdigris*, a green mixture of copper salts including copper acetate.

From Xenophon's *Anabasis*, written in the 4th century BC, we learn that the Persians obtained wine and vinegar from palm trees:

> Πορευόμενοι δὲ ἀφίκοντο εἰς κώμας ὅθεν ἀπέδειξαν οἱ ἡγεμόνες λαμβάνειν τὰ ἐπιτήδεια. Ἐνῆν δὲ σῖτος πολὺς καὶ οἶνος φοινίκων καὶ ὄξος ἑψητὸν ἀπὸ τῶν αὐτῶν.
>
> They came to those villages where, following the indications of their guides, provisions should have been found. There was plenty of grain, wine of palm, and boiled vinegar from the same source. (Xenophon, *Anabasis*, II, 3:14)

One of the most famous vinegar-based recipes of the Greek world was surely μέλας ζωμός [mélas zomós], or Spartan broth, a rather unattractive kind of black soup made from vinegar, pork, salt and blood, which has become one of the symbols of the Spartan denial of luxury.

Another important and very popular Greek author, Plutarch, speaks to us about vinegar in his *Parallel Lives*. As an example, when relating the life of Marcus Cato, he writes that 'when suffering from parching thirst he would ask for some vinegar' (Plutarch, *The Parallel Lives*, Aristides and Cato the Elder).

Moving to Ancient Rome, we know that the Romans boiled soured wine in lead pots to produce an extremely sweet syrup called *sapa*. Even today, the Italian word *sapa* or *saba* indicates a similar product, the cooked grape must, obtained by heat concentration of grape juice in large vats. The Roman *sapa* was rich in lead acetate, also called 'sugar of lead' or 'sugar of Saturn', which was a sweet substance but contributed to lead poisoning among the Roman aristocracy. In fact, lead urns were also used by the Romans to sweeten wine and other beverages, because solubilized lead has a sweet taste. Unfortunately it is also very poisonous, as we now know.

At the beginning of the Second Punic War, in the year 219 BC, Hannibal achieved one of the greatest military feats ever attempted when he ventured through western Europe and crossed the Alps, invading Italy with his entire army (Figure 2.2). The exact route he took to cross such perilous mountains is not mentioned in historiographic reports, but there are many legends and stories telling how he did it. Even Titus Livius in his *Ab urbe condita libri* tells that when the Carthaginians found themselves between the narrow dales and rocky walls of the Alps, among the valleys of Monviso, Viù and Susa, they were not able to carry on with their journey. However, Hannibal was determined to march on and commanded his soldiers to collect a huge amount of timber around an enormous stone blocking the path and to set it on fire. As soon as the rock was heated by fire, Hannibal made his men drench it in vinegar: corroded and weakened by the acid, the stone could then be broken into small pieces and the Carthaginians had their way open:

**Figure 2.2** Roman marble bust of Hannibal found at Capua. It was apparently made in his honour during Hannibal's own lifetime (Naples, National Museum; courtesy of Soprintendenza Speciale per i Beni Acheologici di Napoli e Pompei)

> *Inde ad rupem muniendam per quam unam via esse poterat milites ducti, cum caedendum esset saxum, arboribus circa immanibus deiectis detruncatisque struem ingentem lignorum faciunt eamque, cum et vis venti apta faciendo igni coorta esset, succendunt ardentiaque saxa infuso aceto putrefaciunt*.
>
> The soldiers were then forced to find a passage through the cliff, which was the only traversable way, and because the rocks should be broken, they felled and cut many large trees growing around, erected a huge pile of timber and set fire to it with the favour of a strong wind that excited the flames and, pouring vinegar on the heated stones, they broke them into small crumbs. (Titus Livius, *Ab Urbe Condita Libri CXLII*, XXI, 37)

Dating from around AD 230, one the most exhaustive cookbooks of the Roman world was compiled: *De Re Coquinaria*. This book was ascribed to Marcus Gavius Apicius, a notorious Roman gourmet and lover of luxury who lived in the 1st century AD, even though he was not the real author. In *De Re Coquinaria*, vinegar appears as an ingredient in at least 150 recipes. This evidence shows us that vinegar was popular and common in Roman everyday life.

Vinegar was commonly used even during military campaigns and war time. From Caesar's *De Bello Gallico*, written between 58 and 50 BC, we learn that the Roman soldiers, and thus even the common citizens, used to drink vinegar mixed with water, obtaining a highly refreshing drink that was safer than water alone, because the vinegar acted as a disinfectant for the water taken from unknown sources in foreign lands.

A famous anecdote involving vinegar in the Roman world was recorded by Pliny the Elder (AD 23-79), who recounts that the Egyptian Queen Cleopatra held a luxurious banquet for the Roman triumvirate Mark Antony. To show her huge

**Figure 2.3** Cleopatra dissolves pearls in vinegar. Francesco Trevisani, *The Banquet of Mark Antony and Cleopatra*, 1702. Oil on canvas (Rome, Galleria Spada; courtesy of Soprintendenza Speciale per il Patrimonio Storico, Artistico ed Etnoantropologico e per il Polo Museale della città di Roma)

opulence, she won a wager that she could consume a fortune during a single meal by dissolving some pearls in vinegar, using its corrosive and dissolutive power (Figure 2.3).

> *Ex praecepto ministri unum tantum vas ante eam posuere aceti, cuius asperitas visque in tabem margaritas resolvit. Gerebat auribus cum maxime singulare illud et vere unicum naturae opus. Itaque expectante Antonio, quidnam esset actura, detractum alterum mersit ac liquefactum obsorbuit.*
>
> According to given orders, the servants set before her only a vase of vinegar, the sharpness and strength of which is able to dissolve pearls. She was wearing in her ears those choicest and most rare and unique productions of nature. Then, while Antony was waiting to see what she was going to do, she took one of them off, dipped it into vinegar and, once dissolved, she swallowed it. (C. Plinius Secundus, *Naturalis Historia*, IX, 58)

## 2.4 Vinegar in the Bible

An important ancient source, where vinegar is quite often mentioned, is the Bible, both in the Old and in the New Testament. During biblical times, vinegar became well known and was mainly used as a refreshing and energizing drink, often added to water, or as condiment for adding flavour to other foods, in the same manner as we use it today. In the Old Testament one may find vinegar cited in the Book of Proverbs:

> As vinegar to the teeth and smoke to the eyes, so is a sluggard to those who send him. (*Proverbs*, 10:6)

We also found a famous passage in the Book of Ruth, where Ruth is invited by Boaz to eat her bread dipped in vinegar:

> At mealtime Boaz said to her: 'Come over here. Have some bread and dip it in the wine vinegar.' (*Ruth*, 2:14)

From the Bible again we learn that Nazirites were not allowed to drink wine, wine vinegar, or malt vinegar:

> The Lord said to Moses: Speak to the Israelites and say to them: 'If a man or woman wants to make a special vow, a vow of separation to the Lord as a Nazirite, he must abstain from wine and other fermented drink and must not drink vinegar made from wine or from other fermented drink. He must not drink grape juice or eat grapes or raisins. As long as he is a Nazirite, he must not eat anything that comes from the grapevine, not even the seeds or skins.' (*Numbers*, 6:1-4)

Jesus, on the other hand, was offered vinegar to drink to quench his thirst while agonizing on the cross (Figure 2.4), in the passage reported in the Gospels, of which we report an example:

> Later, knowing that all was now completed, and so that the Scripture would be fulfilled, Jesus said, 'I am thirsty'. A jar of wine vinegar was there, so they soaked a sponge in it, put the sponge on a stalk of the hyssop plant, and lifted it to Jesus' lips. (*John*, 19:28-30)

It seems that what Jesus accepted to drink on the cross was actually a common drink for workers and soldiers named *posca*, which was a mixture of sour wine and water.


**Figure 2.4** Urs Graf, Christ on the cross given vinegar, 1520 (engraving)

Again in a religious context, St. Augustine of Hippo (AD 354-430) in one of his *Letters* writes the following statement:

> *Quia sicut acetum corrumpit vas, si diutius ibi fuerit, sic ira corrumpit cor, si in alium diem duraverit.*
>
> For as the vinegar corrodes its vase if kept for too long a time, so the wrath corrodes the heart if persists for a further day. (St. Augustine, *Letter 210*, around AD 423)

In this case the famous Christian philosopher refers to a chemical property of vinegar, of which people were already well aware at the dawn of medieval Europe.

## 2.5 Vinegar in the Middle Ages and Renaissance

The Middle Ages in Europe was a period of great circulation and distribution of culture, mainly in the form of books, parchments and other written and illustrated documents. In fact, many medieval authors tried to include in a single work all the learning and wisdom of their time, with the aim of being exhaustive on every possible topic: natural history, philosophy, mathematics, theology, etymology, architecture and every aspect of the available knowledge. One of the most complete and influential works in medieval Europe was surely the *Etymologiae*, the first known encyclopaedia of western civilization, compiled by the famous St. Isidore of Seville, who, in an astounding compilation of 20 volumes, epitomized all the erudition of his time. In his *Etymologiae*, Isidore did not mention vinegar, but some time later we can find a similar treatise in which vinegar appears as a well-known product: *De rerum naturis* by Rabanus Maurus Magnentius, a Carolingian erudite, Abbot of Fulda and Bishop of Mainz in Germany. He assembled *De Rerum Naturis* between AD 842 and 846.

> *Acetum uel quia acutum uel quia aquatum uinum, id est aquam mixtum cito in hunc saporem redigitur, unde et acidum quasi aquidum. Mystice autem acetum puritatem corruptam mentis significat, unde in Psalmo scriptum est: Et in siti mea potauerit me aceto.*
>
> Vinegar, whether from frank or watered wine, that is mixed with water, suddenly gets this taste for which it becomes sour, or else acidulous. In a mystical sense, vinegar itself represents the corrupted purity of mind, thus in the Psalm is said: 'and in my thirst they gave me vinegar to drink'. (Rabanus Maurus Magnentius, *De Rerum Naturis*, XXII)

It is interesting that Rabanus alludes to vinegar as a symbol of mental corruption; although this is justifiable according to the biblical interpretation of vinegar as a degradation and corruption of wine, thus becoming a metaphor for fraud and deceit, this seems to be in contradiction to its well-known culinary and medical properties.

During this historical period, the famous balsamic vinegar was invented in the Emilia region of Italy. It is difficult to know the precise location where it was originally produced: we do not know whether it was first made in Modena, Reggio Emilia, or in another Emilian town. In any case, the first mention of this precious,

home-made condiment is found in the poem *Acta Comitissae Mathildis*, written in the 12th century by the monk Donizo and later known as *Vita Mathildis*.

Going to the far northern latitudes of Europe, where the settlements of the Norsemen were located, we can find no written records in the Norse, or generally in the Old Germanic literature, about the usage or making of vinegar, even though this was a culture with a time-honoured tradition of alcoholic drinks such as mead, ale and even wine, so we would expect that the Nordic peoples must also have been aware of vinegar and acetic fermentation. The word for vinegar is of particular interest because it has several different variants in the Germanic languages: the Gothic *aket*/*akeit*, Old English *eced*, and Old Norwegian/Old Icelandic *edik*, to name just three. All of these variants are loan-words from the Latin *acetum* and spread northward via the Middle Low German *etik*. It is clear that the Gothic word came from Alemannic German in the 1st century, before viticulture spread to the Palatinate and the Middle Rhine in the 2nd century (Kortlandt, 2002). One of the earliest mentions of vinegar in the Germanic literature was in the Gothic Bible translated by Wulfila (ca. AD 310-388):

> *Jah suns þragida ains us im jah nam swamm fulljands* ***aketis****, jah lagjands ana raus draggkida ina.*
>
> Straightway one of them ran and took a sponge, filled it with vinegar, put it on a reed and gave him to drink. (Matthew 27:48 – from the *Wulfila Bible*, Codex Argenteus, 4th century)

Similarly, the first written attestation of the word *edik* in Old Icelandic is found in the translation of Matthew's gospel by Oddur Gottskálksson in 1538, published in Denmark in 1540. This was also the first printed book in the Icelandic language:

> *Gáfu þeir honum* ***edik*** *að drekka galli blandað.*
>
> They gave him to drink vinegar mingled with gall (Matthew 27:34 – from Oddur Gottskálksson's *Nýja testamentisþýðing*, 1538)

Vinegar itself was widely used by Scandinavians and other northern Europeans as a pickling agent to preserve meat and other food, with the main purpose of storing provisions in case of long journeys or famines. The main raw materials for the production of vinegar were the leftovers of mead and, less commonly, wine, that spontaneously turned sour. As proof of the common usage of vinegar, many recipes containing vinegar were known in the Norse culture and some of them have also been transcribed and preserved (Larsen, 1931; Bjornsson, 2002).

From the 14th century onwards, a series of medical and pharmacological handbooks in different Latin versions were circulated in northern Italy. These were based on an Arab treatise called *Taqwin al-Sihha*, literally 'Tables of health', transliterated in Latin as *Tacuinum Sanitatis*. These books described the various properties of foods and plants and offered suggestions about physical exercises, mental health, rest and general hints for a good health. The texts of the *Tacuina* were generally poorly detailed and very short, but they were profusely illustrated with brilliant miniatures that explained the various concepts.

**Figure 2.5** Vinegar drawing, from *Tacuinum Sanitatis Casanatense*

In the *Tacuinum Sanitatis Casanatense*, composed at the court of the Visconti family in Milan, Italy, and conserved at the Casanatense Library of Rome, we can see a picture of a lady climbing a ladder and filling a ewer from a cask of vinegar (Figure 2.5). An important detail is that the cask is kept above the roof of a small building, probably a stable or a shed. This picture illustrates the common habit of keeping the vinegar in high places, such as attics or other elevated parts of the house, and this custom remains even today.

Girolamo Cardano, also known by the Latinized name of Hieronymus Cardanus, was the author of one of the most interesting treatises on natural philosophy of the Italian Renaissance, *De Subtilitate Rerum*, published in Nuremberg by Johann Petreius in 1550. This book covers a wealth of natural phenomena, among which

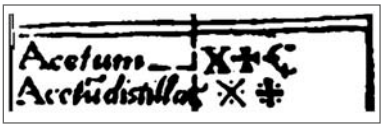

**Figure 2.6** Alchemical symbols for vinegar and distilled vinegar, from Basil Valentine, Last Will and Testament, London, 1671

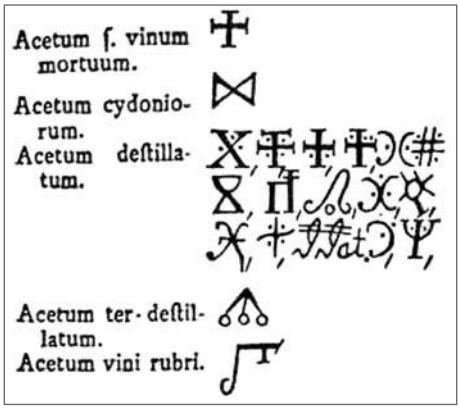


**Figure 2.7** Alchemical symbols for vinegar and various forms of vinegar as they appear in the lists from Medicinisch-, Chymisch- und Alchemistisches Oraculum, Ulm, 1755

are descriptions of how vinegar can be obtained in different conditions, and also analysing the possible causes of fermentation and identifying them in a sort of elemental theory, which was not so far from the various mystical and scientific research of the European alchemists of the Middle Ages and Renaissance. In fact, it seems that alchemists, in their pre-scientific investigations, worked extensively with vinegar, according to the various symbols under which vinegar and its modified or distilled derivatives appear in texts of the 17th and 18th centuries (Figures 2.6 and 2.7). They knew that vinegar and several distilled vinegar derivatives have the power to dissolve and solubilize metals. By pouring it over lead they also rediscovered the Roman 'sugar of lead', a solution of lead acetate which has already been mentioned in this chapter. The 'sugar of lead' was used to smooth other beverages when they were too harsh, especially cider. However, it was soon demonstrated that lead was lethal and this practice gradually disappeared.

### 2.5.1 Vinegar and Plague

The use of vinegar and the study of its medical properties remained very widespread during the Middle Ages, and it even gained in popularity as a remedy. For example, the famous Italian physician Tommaso Del Garbo, a professor of medicine at Bologna and Perugia, on witnessing an outbreak of plague in 1348, suggested in the first instance that everyone who was able should abandon the cities, where the disease had taken hold, and flee to places where the air was cleaner and healthier. But for those, like himself, who were obliged to stay in the cities, he suggested washing their hands, faces and mouth with vinegar. This suggestion was followed by many people during the bubonic plague, because it was a well-known practice all over Europe to pour vinegar on the body or on common tools as a protection against germs. Even during the Great Plague of 1665 in England, the money that

changed hands in the market-place at Derby was deposited in a great trough of vinegar placed on top of a stone, in order to disinfect the coins and prevent the spread of plague. The stone still stands at Friar Gate in Derby, known as the 'headless cross' or 'vinegar stone'.

In 1791, when the plague was spreading in France, a chronicle recounts that the homes of the disease victims were being raided by thieves. Nobody attempted to find them because they were expected to die soon, but as time went on the robberies did not stop and it was clear that the thieves were able to avoid catching the plague by using some kind of trick. The thieves were later identified: they were four boys and, when they were captured, the authorities offered to spare their lives if they revealed their secret weapon against the plague, so they had no choice. They revealed that their mother used to prepare a disinfectant brew made of vinegar, garlic, lavender, rosemary, mint and other herbs; after pouring it over their bodies or bathing in it, they were not infected by the killer disease. This concoction then became famous under the name of 'Four Thieves Vinegar' and is still manufactured today, mainly in France.

### 2.5.2 Early Industrial Experiences

From the beginning of the Middle Ages, as the use of vinegar increased because of its highly desirable properties, its availability on the markets started to decline. Until that time, it had always been a strictly home-made product obtainable only on a small scale, so it seemed necessary to expand its production to achieve a truly large-scale – or even *industrial* – production process. Many vintners thus began to produce vinegar along with wine, in order to meet this growing demand.

Probably the oldest corporation in the world specializing in vinegar making was established in Orléans, France, with an official statute recorded on 28 October 1394 under the name *Vinaigriers moutardiers sauciers distillateurs en eau-de-vie et esprit-de-vin buffetiers*. This corporation employed a number of alchemists and technicians who researched the various methods of obtaining vinegar, mustards and distillates, keeping them strictly secret. In France there is a common expression: '*c'est le secret du vinaigrier*' ['it's the secret of the vinegar maker'], used to indicate that something is mysterious or hard to understand, which surely comes from this historical period, when the mystery of vinegar making still seemed uncanny to the uninitiated (Bourgeois, 1999). The corporation of Vinaigriers soon had the monopoly for vinegar production and later developed the so-called 'Orléans process', or 'slow process', that is still in use today. The Orléans process is also called the 'continuous method', where the vinegar is obtained by starting from wine through a slow fermentation in wooden casks. To begin the fermentation in a new barrel, a small amount of the so-called 'mother of vinegar' is added to the wine mass. As the vinegar reaches the desired acidity and flavour, it is removed from the top, while new, fresh wine is periodically added; in this way the fermentation never stops and new vinegar is always available from the fermentation facility, even if this method requires several months to produce vinegar.

Another large-scale production facility was established in Southwark, London, in 1641, where the first 'vinegar yard' was situated, for the fermentation of ale, resulting in a product called *alegar* in Middle English, in analogy to 'vinegar', but nowadays more commonly known as *malt vinegar*. It is no surprise that, over a number of centuries, English vinegar was produced mainly from cereals, and associated with the brewing of beer (Conner and Allgeier, 1976).

Vinegar was not only used for culinary or medicinal purposes: Louis XIII of France (1601-1643) is reported to have paid 1.3 million francs for the vinegar used to cool the cannons of his army during just one of his many battles. When applied to the hot iron cannons, vinegar not only cooled them, but helped to clean the surface metal, while inhibiting rust formation.

Even in the New World, after the European discovery of America, the early colonists are said to have invented their own vinegar fermented from apple cider, by simply allowing the cider to ferment, first into alcohol, the so-called 'hard cider', and then into vinegar. Several varieties of wild crab apple are native to North America, but it was the western European settlers who introduced apple varieties that were more useful for producing cider.

In 1623, William Blackstone, an English clergyman, was credited with planting the first apple trees in Boston, and then in 1635 he planted an apple orchard in Rhode Island. The most common use of apple cider vinegar was for pickling vegetables, but it was also used as condiment and is still very popular in the USA. Similar to wine vinegars, apple cider vinegar was also used to fight diseases such as colds and arthritis.

## 2.6 Vinegar in the Islamic World

Another culture which accorded great importance to vinegar and its characteristics was the Muslim world. The Islamic alchemists of the 8th century actually seemed to have a deeper knowledge of the chemical properties of vinegar and were generally more skilled in science than their European counterparts. One of the most important men of science of the Middle Ages was the alchemist at the court of Caliph Harun Al-Rashid; this was Jabir Ibn Hayyan (ca. AD 721-815), more commonly known by the Latin name Geber, who has also been referred to as the 'Father of Chemistry' (Figure 2.8). Jabir made an extensive study of vinegar and was credited with the discovery of acetic acid, which he obtained from vinegar by distillation. Similarly, he extracted citric acid from lemons and other unripe fruits, and tartaric acid from wine-making residues. Jabir recorded his studies in several books that were soon translated into Latin and became standard reference works for European alchemists, for example the *Kitab al-Kimya* (entitled *Book of the Composition of Alchemy* in Europe).

Another important Islamic scientist, Ibn Sina (AD 980-1037), better known in the western world as Avicenna, in his famous medical treatise *Al-Qanoon fit Tibb* (*The Canon of Medicine*) also mentioned the properties of vinegar. According to Avicenna, vinegar can have several uses in medicine: it is a powerful clotting agent,


**Figure 2.8** European portrait of 'Geber', from *Codici Ashburnhamiani*, 1166

it heals burns and skin inflammations, and when applied to the head it relieves headaches caused by heat. He also considered vinegar a good digestive and suggested using it as an expectorant.

We know that vinegar has always been well regarded in the Muslim world and is also mentioned in Islamic sacred texts. According to the words of the Prophet Mohammed, vinegar is a good aliment: in the *Kitab Al-Ashriba* (*The Book of Drinks*), in chapter 28, eloquently titled 'The merit of vinegar as a condiment', we find the following episode:

> Jabir b. 'Abdullah reported: Allah's Messenger (may peace be upon him) took hold of my hand one day (and led me) to his residence. There was presented to him some pieces of bread, whereupon he said: Is there no condiment? They (the members of his household) said: No, except some vinegar. He (the Holy Prophet) said: Vinegar is a good condiment. Jabir said: I have always loved vinegar since I heard it from Allah's Apostle (may peace be upon him). Talha said: I have always loved vinegar since I heard about it from Jabir. (Sahih Muslim, book 23 *Kitab Al-Ashriba*, n. 5094. Translation by Abdul Hamid Siddiqui)

Vinegar has always been considered *halal* (‘allowed’) for Muslims even if produced from wine, which is strictly *haram* (‘forbidden’), when it is obtained by spontaneous and natural processes and not artificially manufactured.

## 2.7 Vinegar in the Age of Enlightenment

Le *secret du vinaigrier* – ‘the secret of the vinegar maker’ – claimed by the vintners of Orléans was not simply a commercial assertion, but an actual mystery whose complexity endured for millennia and which remained far from understood even in the 18th century. It was by then clear that vinegar had always been at man’s side since the early days of civilization, but nobody had yet found the reason why an alcoholic drink could turn into vinegar, acquiring its own distinctive taste and sharpness. Only with the development of modern experimental sciences did this issue start to become clearer to scientists.

In 1720 the illustrious Dutch humanist, botanist and physician, Hermann Boerhaave (1668-1738), proposed a quick method for vinegar fermentation: he observed that vinegar formation proceeded more quickly when a large surface of the starting liquid was exposed to the air. Boerhaave then suggested trickling wine through packed beech-wood chips in order to increase the available surface area exposed to the air and obtain a faster oxidation to vinegar. He also stated that a ‘flour’ or ‘vegetal substance’ was required in order for acetification to be successful, guessing that the so-called ‘mother of vinegar’ was of biological origin.

Probably building upon Boherhaave’s observations, the founder of modern chemistry, Antoine-Laurent de Lavoisier (1743-1794), went deeper into the study of vinegar making from a chemical point of view. He investigated the nature of air and the combination of various gases, rejecting the phlogiston theory and demonstrating that air was the principal cause of the phenomena of combustion and acidification. In 1778 he wrote *Considérations Générales sur la Nature des Acides*, in which he demonstrated that a component of the air was responsible for combustion and was also the source of acidity. He called this agent *oxygen*, whose etymology (from the Greek) means ‘acid-former’, also declaring that the sharp taste of acids is due to oxygen itself, while the other ‘air’ had no effect on matter; he called this component *azote*, meaning ‘lifeless’. On 22 June 1789, Lavoisier recorded an experiment on the alcoholic fermentation of a sugar solution in which he observed the presence of vinegar, together with alcohol, sugars, yeast, water and carbon dioxide. In his theory of acid formation, Lavoisier pointed out that oxygen was the agent of the transformation of wine into vinegar, because he demonstrated that vinegar was formed when alcoholic solutions were exposed to air.

Boerhaave’s original idea was later improved upon by the German scientists Karl Wilhelm Gottlob Kastner (1783-1857) and especially Johann Sebastian Schüzenbach (1793-1869), who applied it to the construction of a generator consisting of a wooden or metal-coated tank packed with wood shavings. The wine trickled from the top through the wood shavings, while a large volume of air was blown into the tank though perforations in the bottom. This generator became the

main basis for the modern industrialization of vinegar making, being known as the 'quick process', or also as the 'German process', and is widely used today.

## 2.8 Vinegar in Asia

Very little is known about the origins of vinegar in the history of Asia. The earliest records are found in China at the time of Zhou Dynasty (1027-221 BC), in texts such as *Tso Chuan* and *Mo Tzu*, which refer to a seasoning called *liu*, which has usually been interpreted as vinegar although its manufacture and ingredients remain unknown. It seems, however, that during the Zhou Dynasty a condiment obtained from plums and salt became very popular (Fong, 2000). In any case, it is beyond doubt that in China vinegar soon became a very important ingredient in the preparation of all sorts of dishes (Figure 2.9). Sour – along with sweet, salty, pungent and bitter – is one of the 'five flavours' of classical Chinese cooking (Simoons, 1991).

There is also a Chinese traditional painting called *The vinegar tasters* that depicts three men dipping their fingers into a vat of vinegar and tasting it (Figure 2.10). One of them, Confucius, reacts with a sour expression; the second, Buddha, reacts with a bitter expression; the third, Laozi, reacts with a happy expression. The

**Figure 2.9** Ancient bronze vessel used for fermenting Chinese rice vinegar during Zhou Dynasty (1100-221 BC)

**Figure 2.10** *The vinegar tasters*. Traditional Chinese painting

three men are also allegories for the 'three teachings' of China, and the vinegar they are sampling represents life.

Chinese rice wine (*sake, jiu*) was produced and fermented to rice vinegar (*cu*) in the Xia (ca. 2000-1500 BC) or Shang (Yin) period (ca. 1700-1027 BC) (Bo, 1988). In the book *Zhou Li*, which was published in the Western Han period (206 BC - AD 9), a public servant, Kei Nin, managed and recorded sake and vinegar fermentations during the Zhou imperial dynasty (1027-221 BC) (Ma, 2002). In this book, we also find the following description: 'When Song Jo's marquise died (ca. 700 BC), she was buried with vinegar in 100 ceramic pots as buried goods'.

Furthermore, China's ancient sake may have been derived first from fruits, with rice derivations later. Archaeological evidence indicates that the Southeast Asian people were the first to cultivate rice. Recently discovered Korean artefacts, with rice grain imprints, date back to 4000 BC.

In China's oldest agricultural treatise, *Qi Min Eyao Shu*, edited by Ka En-Kyou during the Northern Wei period (AD 386-533), Chinese characters for rice-*koji* (probably *Rhizopus* or *Aspergillus oryzae*) and acetic acid bacteria surface pellicles used in brewing sake and vinegar both appear (Bo, 1988). In this manual, the author noted that the surface pellicles, indicated as *shen gyi*, a Chinese compound word

**Table 2.1** Literature of ancient vinegar description in China

| Period | Christian year | Literature/ Book | Description/ Episode |
|---|---|---|---|
| **周** (Zhou) | 1027-221 BC | **周礼** (published in the Early Han dinasty, 206 BC - AD 9) | 醯人 (a public servant) managed and recorded sake and seven species of pickles and vinegar in the Zhou dinasty |
| **戦国時代** (Warring States period) | 475 BC or 403-221 BC | **韓非子** **四書五経** | 'Sake was acidified' 醯 means 酢 (vinegar) |
| 西漢 (Early Han) | 206 BC - AD9 | 法言 | 'The ceremony had been continued too long and then sake was acidified before drinking' |
| **後漢** (Late Han) | AD 25 - 220 | **釈名** | **苦酒** (bitter sake) means **酢** (vinegar) |
| **魏** (Wei) | AD 220 - 265 | **古今事物考** | 'Vinegar was produced from sweet sake' |
| **北魏** (Northern Wei) | AD 386 - 533 | **斉民要術** | **麦芽** (malt), **神麹** (god-koji, *Rhizopus*) and **女麹** (woman-koji, *Aspergillus oryzae*) were used to produce sake. Detailed protocol to brew sake and vinegar. |
| **唐** (Tang) | AD 618 - 907 | **四時纂要** | **米酢** (rice vinegar), **大麦酢** (barley vinegar) and **小麦酢** (wheat vinegar) production protocols |
| **宋** (Song) | AD 420 - 478 | **事林广記** | **麦黄醋法** (Wheat-koji vinegar brewing method)<br>**黄衣(黄麹)** (koji production from wheat)<br>**糟醋法** (sake-lees vinegar brewing method)<br>**梅醋法** (perm vinegar brewing method)<br>**麩醋法** (fusuma vinegar brewing method) |
| **元** (Yuan) | AD 1279 - 1368 | **民家必用事類全集** | **米醋** (rice vinegar)<br>**小麦醋** (wheat vinegar)<br>**大麦醋** (barley vinegar)<br>**梅醋** (perm vinegar)<br>**麩醋** (fusuma vinegar)<br>**糟醋** (sake-lees vinegar) |

From: Bo, 1988; Ma, 2002

Table 2.2 Literature of ancient vinegar description in Japan

| Period | Christian year | Literature/ Book | Description/ Episode |
|---|---|---|---|
| **奈良** (Nara) | AD 710 - 794 | **日本書紀**<br>**古事記**<br>**丹波国風土記**<br><br>**伊豆国正税帳**<br>**造物所作物帳** | **果実酒** (sake produced from fruits)<br>**米酒** (sake produced from rice)<br>'Excellent rice sake was brewed by a girl came down from heaven'<br>**酢分** (vinegar is a part of sake)<br>'11-**升** [about 20 litres] of vinegar was gifted' |
| **平安** (Heian) | AD 794 - 1192 | **和名抄** | **酢** (vinegar) is generally called **苦酒** (bitter sake) |
| **鎌倉** (Kamakura) | AD 1192 - 1333 | | **和泉酢** (Izumi vinegar) was produced in Osaka |
| **江戸** (Edo) | AD 1603 - 1868 | **本朝食鑑**<br>**宇津保物語** | **六月酢** (6-months vinegar)<br>'A millionaire in Kisyu (Wakayama) produced sake, vinegar, soy-sauce, pickles using ceramic pots in his house |

From: Otsuka, 1990

meaning a velvet coat of living organisms, should not be agitated during vinegar fermentation. Therefore, this Chinese scientist knew that living organisms were necessary for vinegar fermentation 1300 years before Pasteur's discovery of microbial fermentation in Europe. It is nowadays accepted that *shen gyi* is probably *Acetobacter xylinum* (Bo, 1988). The manual states that yellow-*koji* (*A. oryzae*) and god-*koji* (*Rhizopus*) were prepared from steamed rice and also from wheat in China (Table 2.1).

Rice vinegar is actually one of the most distinctive and typical Chinese condiments, which was certainly produced before the 1st century AD and was used for a meat or fish preparation very similar to *sushi* during the Song Dynasty (AD 960-1279). This later became better known as a Japanese speciality. Rice vinegar brewing techniques travelled from China to Japan and neighbouring countries around AD 300 (Otsuka, 1990).

Japan's rice vinegar, *komesu*, was originally produced in Izumi (Osaka), where brewing techniques were disseminated from China during the Oujin Dynasty (AD 270-310). In an old tax record from the Nara period (AD 710-794), we see that vinegar was taxed as a variety of sake in Japan (Table 2.2). Black rice vinegar was probably the first kind of vinegar brewed in China and Japan. Figure 2.11 shows the Chinese characters meaning vinegar used in both countries. Usually, rice was harvested in autumn, then steamed, kept in ceramic pots and finally mixed with water and koji rice (*A. oryzae*) to brew sake. This preparation was kept unconsumed until approximately the month of March.

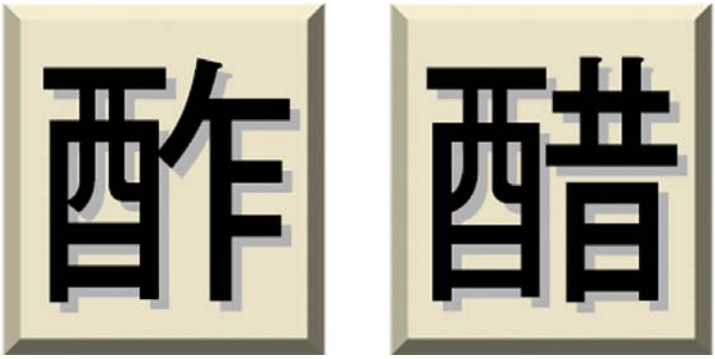

**Figure 2.11** Chinese characters, both meaning vinegar, used in Japan (left) and China (right). The left part of the letters, 酉, means sake; the letter originates from a brewing pot. The right parts of the letters, 乍 and 昔, mean production and old, respectively

Naturally occurring acetic acid bacteria fermented vinegar from sake. The resulting sour sake was decanted and the supernatant was transferred into dedicated pots that were kept underground for 4-6 months. This traditional brewing process for black vinegar is still used in Asian countries today. Figure 2.12 shows pictures of the traditional static vinegar fermentation method used in Fukui, Japan since 1710. In the Edo period (AD 1603-1868), commercially produced vinegar became quite popular in Japan. Since the earliest vinegars were brewed through the action of microorganisms occurring naturally in the environment, the species of koji moulds and acetic acid bacteria in China and Japan must have been different then (Otsuka, 1990).

Vinegar was believed to be a cleansing agent for the body, both inside and out. In 1368, Chia Ming's *Yin shih Hsu chih* (*Essential Knowledge of Eating and Drinking*) introduced the important concept of disease prevention and stated that vinegar can counteract the poisonous effects of fish, meat, fruit and vegetables. Chia Ming also stated that rice vinegar can damage the stomach, bones and flesh if used to

**a** **b** **c**

**Figure 2.12** Brewing of ceramic pot vinegar (tubonosu) at Tobaya-suten Co. Ltd., Fukui, Japan, using a method in use since 1710: **a** static fermentation using ceramic pots 2 days after inoculation of sweet sake with a crepe pellicle containing acetic acid bacteria; **b** 30 days fermentation after inoculation of the crepe pellicle; **c** ageing of tubonosu using ceramic pots covered with rice straw mats (pictures obtained from Tobaya-suten Co. Ltd.)

excess, although it is generally a very beneficial beverage. On the other hand, he believed that vinegar activated poisons and should never be taken in conjunction with medicines, particularly *fu ling* (pine root fungus), red ginseng, or whitlow grass (*Draba nemorosa*).

During the Tang Dynasty (AD 618-907), we know that vinegar was used as a preservative, in conjunction with leaves of the kumquat tree (*Fortunella japonica*), peach blossoms and sometimes brine, especially for conserving duck eggs.

In AD 1381, during the Ming Dynasty, the increasing demand for vinegar throughout the whole of China expanded the market, so that a dedicated bureau was established solely for the management of vinegar works. Later in the 17th century, it was believed that a Buddhist monk in Sichuan, who lived to be over 100 years old, reached this great age because of his daily intake of a special herb vinegar. This vinegar supposedly reduced his blood pressure, cured his colds, and prevented other epidemic diseases (Fong, 2000).

## 2.9 Vinegar after the Industrial Revolution

At the beginning of 19th century, European scientists carried out some key studies for the advancement of our knowledge of vinegar and, consequently, also for its production: Jean-Antoine Chaptal (1756-1832), French chemist and statesman, was the first to exhaustively describe the Orleans method, in 1807. The British chemist and physicist Humphrey Davy (1778-1829) demonstrated that acetification is due to the transformation of alcohol into acetic acid, also identifying its chemical formula.

In 1822 the Dutch scientist Christian Persoon identified in a microorganism the main agent of acetification, which he called *Mycoderma aceti*. Justus von Liebig (1803-1873) opposed this theory, maintaining that the cause of fermentation was strictly inorganic, but in 1862 Louis Pasteur (1822-1895) definitively confirmed the effectiveness of Persoon's studies. He demonstrated that the fermentation process is caused by the growth of microorganisms, and that the growth of microorganisms in nutrient broths is not due to spontaneous generation. In his work with yeast, Pasteur found that although air should be kept from fermenting wine, it was necessary for the production of vinegar. He also confirmed that *Mycoderma aceti*, now systematized as *Acetobacter aceti*, oxidized alcohol into acetic acid. Pasteur also suggested that vinegar production could be enhanced by adding a defined quantity of microorganisms to the fermenting mixture.

The vinegar industry of the 1800s and early 1900s took advantage of these scientific discoveries and many innovations were quickly applied to the productive processes and numerous patents were registered, although the manufacture of vinegar saw little improvement until the 20th century, when significant advances were observed in the industry as a result of new developments in equipment and pure culture techniques. In the meantime, vinegar was still used for many purposes; for example, World War I medics used vinegar to treat soldiers' wounds, because antibiotics were not yet available at the time and vinegar was still one of the most effective disinfectants.

A major improvement in the 'quick vinegar process' was achieved in 1929 with the introduction of the circulating generator. The production method is essentially similar to earlier methods, but in this case the materials are subject to a forced aeration which can afford a higher acetic acid concentration, optimizing space and production rate. The circulating generator is still used in many parts of the world and its design has been considerably improved since its invention (Conner and Allgeier, 1976).

Another great improvement in the acetification process was the submerged culture method, in which acetic bacteria are suspended in the fermenting medium and receive oxygen from an aerator placed at the bottom of the reservoir. This method allows a logarithmic growth rate for acetic bacteria, resulting in very high and rapid acetic acid yields. The submerged culture process for vinegar was first studied in 1923 but was applied only in the late 1940s, taking its inspiration from the methodology of used for the industrial production of penicillin. Further studies on submerged vinegar production were carried out to modify the process. By the end of the 1950s the air intake into the reactor was improved with the invention of the *cavitator*, although this was abandoned after a few years because it proved to have many technical problems. A very effective and highly automated fermenter was the Frings *acetator*, which facilitated the production of vinegar from any alcohol-containing raw material. It appears also that the Frings device eliminates any of the unexpected fluctuations in production rates and efficiencies which often occurred with older equipment.

In the early 1960s some processes for the production of concentrated vinegar were developed by Girdler Process Equipment Chemetron Corporation, a company in Louisville, Kentucky. This is essentially a freeze concentration method that has resulted in renewed interest in and new applications for vinegar, especially in the USA, because it allows transport costs to be reduced and is ideally suited to modern pickling methods.

## 2.10 Conclusions

It is worth noting that through the millennia, from the early days of agriculture until today, mankind has always used vinegar for the same purposes: as a condiment, as a pickling or preserving agent, as a disinfectant, as a cleansing agent and as a beverage, with virtually no exceptions for all the cultures in the world. We can then say that vinegar, in its various forms, is the most widespread and common product in the world, because it is available in every country in several varieties. It is also produced in a variety of forms, the extent and popularity of which mainly depend on the culture that invented them: flavoured, spiced, matured or aged, diluted or concentrated – it is always present in old-fashioned attics or in the cheapest supermarket and it serves the same purposes today as it did when mankind first discovered it.

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# Chapter 3
# Acetic Acid Bacteria Taxonomy from Early Descriptions to Molecular Techniques

**Maria Gullo and Paolo Giudici**

## 3.1 Introduction

The exploitation of acetic acid bacteria (AAB) has a long history in fermentation processes and now represents an emerging field in biotechnological applications, especially with regard to the biosynthesis of useful chemicals with a potentially high economic value and, in food science, through the standardization of microbiological processes for the manufacture of both vinegar and other fermented beverages.

Historically, AAB were recognized as 'vinegar bacteria' because the first studies were done on vinegar, and later on wine and beer spoilage. In fact, vinegar AAB are a subset of a larger AAB group, which includes bacteria that interact with flowers, fruits, the rhizosphere of plants, and even human beings (Table 3.1). It is generally recognized that AAB are fastidious microorganisms, which means that many of them are difficult to grow on laboratory media. Many efforts have been made to isolate and culture colonies of AAB. Several media have been suggested and tried, but none of them appears to satisfy the growth requirements of AAB. This has hindered the application of cultivation-based techniques to the study of AAB and, consequently, their taxonomic classification. However, the recent discovery of new culture-independent methods has opened up new horizons for the systematic study of AAB.

Current taxonomic studies are based on a polyphasic approach which, unlike traditional microbiological methods (morphological, physiological and biochemical), takes into account the information derived from metabolism, ecology, genome characterization and phylogeny.

These methods include 16S rRNA gene sequencing and its comparative analysis by phylogenetic trees, DNA-DNA hybridization studies with related organisms, analyses of molecular markers and signature pattern(s), biochemical assays and physiological and morphological tests. The new approach has resulted in several bacteria, which had previously been misclassified, being placed into new genera and species.

L. Solieri, P. Giudici (eds), *Vinegars of the World*

**Table 3.1** Currently recognized acetic acid bacteria (AAB) genera and species and their type strain isolation source

| Genus | Species | Source[a] | Reference |
|---|---|---|---|
| *ACETOBACTER* | | | Beijerinck, 1898[b] |
| *Acetobacter* | *aceti* | Vinegar | (Pasteur, 1864)<br>Beijerinck 1898 |
| *Acetobacter* | *cerevisiae* | Beer | Cleenwerck et al., 2002 |
| *Acetobacter* | *cibinongensis* | Fruit | Lisdiyanti et al., 2002 |
| *Acetobacter* | *estunensis* | Cider | (Carr, 1958)<br>Lisdiyanti et al., 2000 |
| *Acetobacter* | *ghanensis* | Cocoa bean | Cleenwerck et al., 2007 |
| *Acetobacter* | *indonesiensis* | Fruit and flower | Lisdiyanti et al., 2000 |
| *Acetobacter* | *lovaniensis* | Soil | (Frateur, 1950)<br>Lisdiyanti et al., 2000 |
| *Acetobacter* | *malorum* | Apple | Cleenwerck et al., 2002 |
| *Acetobacter* | *nitrogenifigens* | Kombucha tea | Dutta and Gachhui 2006 |
| *Acetobacter* | *oeni* | Wine | Silva et al., 2006 |
| *Acetobacter* | *orientalis* | Canna flower | Lisdiyanti et al., 2002 |
| *Acetobacter* | *orleanensis* | Beer | (Henneberg, 1906)<br>Lisdiyanti et al., 2000 |
| *Acetobacter* | *pasteurianus* | Beer | (Hansen, 1879)<br>Beijerinck and Folpmers 1916 |
| *Acetobacter* | *peroxydans* | Ditch water | Visser't Hooft, 1925 |
| *Acetobacter* | *pomorum* | Industrial vinegar fermentation | Sokollek et al., 1998 |
| *Acetobacter* | *senegalensis* | Mango fruit | Ndoye et al., 2007 |
| *Acetobacter* | *syzygii* | Organic apple juice | Lisdiyanti et al., 2002 |
| *Acetobacter* | *tropicalis* | Coconut | Lisdiyanti et al., 2000 |
| *ACIDOMONAS* | | | Urakami et al., 1989 emend.<br>Yamashita et al., 2004 |
| *Acidomonas* | *methanolica* | Yeast fermentation process | (Uhlig et al., 1986)<br>Urakami et al., 1989 emend.<br>Yamashita et al., 2004 |
| *ASAIA* | | | Yamada et al., 2000 |
| *Asaia* | *bogorensis* | Flower of orchid tree | Yamada et al., 2000 |
| *Asaia* | *krungthepensis* | Heliconia flower | Yukphan et al., 2004 |
| *Asaia* | *siamensis* | Flower of crown flower | Katsura et al., 2001 |
| *GLUCONACETOBACTER* | | | Yamada et al., 1997 |
| *Gluconacetobacter* | *azotocaptans* | Coffee plant | Fuentes-Ramírez et al., 2001 |
| *Gluconacetobacter* | *diazotrophicus* | Sugarcane | (Gillis et al., 1989)<br>Yamada et al., 1997 |
| *Gluconacetobacter* | *entanii* | High-acid industrial vinegar fermentation | Schüller et al., 2000 |
| *Gluconacetobacter* | *europaeus* | High acid vinegar fermentation | (Sievers et al., 1992)<br>Yamada et al., 1997 |
| *Gluconacetobacter* | *hansenii* | Vinegar | (Gosselé et al., 1983)<br>Yamada et al., 1997 emend.<br>Lisdiyanti et al., 2006 |

(continued)

**Table 3.1** (continued)

| Genus | Species | Source[a] | Reference |
|---|---|---|---|
| *Gluconacetobacter* | *intermedius* | 'Tea fungus' beverage (kombucha) | (Boesch et al., 1998) Yamada 2000 |
| *Gluconacetobacter* | *johannae* | Coffee plant | Fuentes-Ramírez et al., 2001 |
| *Gluconacetobacter* | *kombuchae* | Kombucha tea | Dutta and Gachhui, 2006 |
| *Gluconacetobacter* | *liquefaciens* | Dried fruit | (Asai, 1935) Yamada et al., 1997 |
| *Gluconacetobacter* | *nataicola* | Nata de coco | Lisdiyanti et al., 2006 |
| *Gluconacetobacter* | *oboediens* | Industrial red wine vinegar fermentation | (Sokollek et al., 1998) Yamada 2000 |
| *Gluconacetobacter* | *rhaeticus* | Organic apple juice | Dellaglio et al., 2005 |
| *Gluconacetobacter* | *sacchari* | Sugarcane | Franke et al., 1999 |
| *Gluconacetobacter* | *saccharivorans* | Beet juice | Lisdiyanti et al., 2006 |
| *Gluconacetobacter* | *swingsii* | Organic apple juice | Dellaglio et al., 2005 |
| *Gluconacetobacter* | *xylinus* | Mountain-ash berries | (Brown, 1886) Yamada et al., 1997 |
| *GLUCONOBACTER* | | | Asai, 1935 |
| *Gluconobacter* | *albidus* | Flower of dahlia | (ex Kondo and Ameyama, 1958) Yukphan et al., 2005 |
| *Gluconobacter* | *cerinus* | Cherry | (ex Asai, 1935) Yamada and Akita 1984 emend. Katsura et al., 2001 |
| *Gluconobacter* | *frateurii* | Strawberry | Mason and Claus, 1989 |
| *Gluconobacter* | *oxydans* | Beer | (Henneberg, 1897) De Ley 1961 emend. Gosselé et al., 1983 emend. Mason and Claus, 1989 |
| *Gluconobacter* | *thailandicus* | Flower | Tanasupawat et al., 2004 |
| *GRANULIBACTER* | | | Greenberg et al., 2006 |
| *Granulibacter* | *bethesdensis* | Lymph node culture from a patient with chronic granulomatous disease | Greenberg et al., 2006 |
| *KOZAKIA* | | | Lisdiyanti et al., 2002 |
| *Kozakia* | *baliensis* | Palm brown sugar | Lisdiyanti et al., 2002 |
| *FRATEURIA* | | | Swings et al., 1980 |
| *Frateuria* | *aurantia* | Flower and fruit | (ex Kondo and Ameyama, 1958) Swings et al., 1980 |
| *NEOSAIA* | | | Yukphan et al., 2005 |
| *Neosaia* | *chiangmaiensis* | Flower of red ginger | Yukphan et al., 2005 |
| *SACCHARIBACTER* | | | Jojima et al., 2004 |
| *Saccharibacter* | *floricola* | Flower | Jojima et al., 2004 |
| *SWAMINATHANIA* | | | Loganathan and Nair, 2004 |
| *Swaminathania* | *salitolerans* | Mangrove-associated wild rice | Loganathan and Nair, 2004 |

[a] Refers to type strain isolation source.

[b] Quoted in Buchanan et al. (1966).

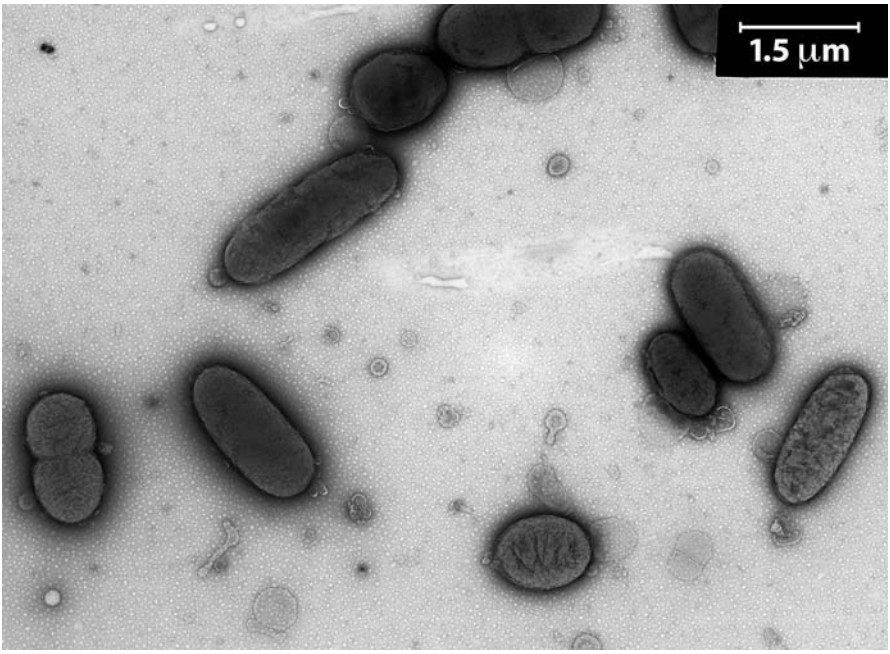


**Figure 3.2** SEM microscope image of acetic acid bacteria (courtesy of Giulio Petroni, Department of Biology, University of Pisa)

## 3.2 Acetic Acid Bacteria Taxonomy

Bacterial taxonomy is a scientifically dynamic area, whose main goal is to give a clear phylogenetic picture of microorganisms, and is an important tool for researchers, scientists and the biotechnological industries. Recent rapid developments in molecular biological techniques, the automation of DNA sequencing, advances in bioinformatic tools and access to sequence databases, has now made it possible for the first time to reveal the microbial 'identity' of microorganisms and has regenerated the study of taxonomy in terms of continuous reclassifications.

Since its discovery, the AAB group has been rearranged several times, with division, renaming, restoration and emendation of genera and species. The taxonomic grouping of AAB originated in 1837 when Kützing, who first observed the organisms in vinegar, described them as a kind of alga and named them *Ulvina aceti* (quoted in Asai, 1968).

Nowadays, ten genera of the *Acetobacteraceae* family are grouped under the collective name 'acetic acid bacteria' (class *α-Proteobacteria*): *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Kozakia*, *Neoasaia*, *Saccharibacter* and *Swaminathania*, and one genus, *Frateuria*, of the family *Xanthomonadaceae* (class *γ-Proteobacteria*). A phylogenetic tree showing relationships of type strains of the family *Acetobacteraceae* is shown in Figure 3.2.


**Figure 3.2**. A phylogenetic tree reflecting the relationships of the type strains of species belonging to the family *Acetobacteraceae* (courtesy of Wolfgang Ludwig; from Ludwig, 2007)

The current classification is the product of the long and continuous development of bacterial systematics. To obtain a chronological overview of this development, Stackebrandt (2006, 2007) proposed a division of the history of bacterial systematics into four chronological phases that summarize the progress of microbiology over the past 100 years. The first phase, 'early descriptions' (1872-1900), originated with the adoption of the Linnaean paradigm (for plants and animals) also for bacteria. According to this chronological division, the first description of AAB is dated 1868, when Louis Pasteur published the first systematic study, in which he described the 'mother of vinegar' as a mass of living microorganisms which caused acetic acid fermentation. The genus name '*Acetobacter*' appears before 1900, and is usually attributed to Beijerinck (see footnote to Table 3.2).

In the second phase (from 1900 to 1955), physiology began to have an impact on taxonomy, and bacteria were classified first according to morphology, and physiology was then used to discriminate between the most closely related organisms. At this time (1916), the AAB (as quoted in Asai, 1968) were classified by Janke on the basis of their ability to utilize inorganic ammonium salts and acetic acid as the nitrogen and carbon sources, respectively. Later, in 1935, Leifson observed the existence of peritrichous flagella in some *Acetobacter* species and proposed dividing it into *Acetobacter* (only peritrichous or non-flagellated strains with similar physiological characteristics) and *Acetomonas* gen. nov. (polarly flagellated or non-motile strains with similar physiological characteristics) (quoted by Asai, 1968).

In the same year, Asai proposed the division of AAB into two genera, *Acetobacter* and *Gluconobacter* gen. nov., on the basis of their capacity to oxidize ethanol and glucose. In the genus *Acetobacter* were included organisms that exhibited only peritrichous flagellation if motile, that oxidized ethanol strongly, oxidized glucose weakly or not at all, and oxidized acetate and lactate completely. The genus was divided into two subgenera: *Euacetobacter*, including strains able to oxidize ethanol and not glucose; and *Acetogluconobacter*, which included strains able to oxidize ethanol strongly and glucose weakly. The genus *Gluconobacter* included organisms that exhibited only polar flagellation if motile, oxidized glucose strongly, oxidized ethanol weakly or not at all, and did not oxidize acetate and lactate to carbonate. The new genus was also divided into two subgenera: *Gluconoacetobacter* (strains able to oxidize glucose strongly and ethanol weakly) and *Eugluconobacter* (strains able to oxidize glucose but not ethanol) (Asai, 1968). *Gluconobacter* was placed in the family *Pseudomonadaceae*. Since *Acetobacter* showed no close relationship with any existing family, it was placed in the family *Acetobacteraceae*, which had appeared previously in the fifth edition of *Bergey's Manual* (1939).

From 1953 to 1965 other proposals for the systematization of AAB were made by several authors, taking into account the increasing weight of physiology in strain discrimination (for a full report on grouping and proposals, see Asai, 1968). Rainbow and Mitson (1953) proposed the separation of AAB into two groups on the basis of nutritional requirements: (i) lactaphilic group (predominant lactate metabolism) and (ii) glycophilic group (predominant glucose metabolism). This systematization was similar to that proposed by Vaughn in which group I (capable

of oxidizing acetic acid) and group II (not capable of oxidizing acetic acid) corresponded to the lactaphilic and glycophilic groups, respectively.

The third phase of bacterial systematics (1955-1980) corresponds to the great revolution in taxonomy in consequence of the impact of the discovery of the DNA structure by Watson and Crick (1953), which made possible the introduction of appropriate techniques for its analysis and manipulation. Moreover, chemotaxonomy, in which the chemical structures of cell constituents are used to differentiate bacteria into relatedness groups, was integrated into species descriptions (Stackebrandt, 2006). In 1963, a new approach to measuring genetic relatedness among organisms was proposed by McCarthy and Bolton which suggested DNA-DNA hybridization as a powerful tool to distinguish between closely related species. During these years numerical phenotypic analysis and protein sequence analysis were applied to taxonomic research. With the improvement in molecular sequencing techniques, the idea of Zuckerkandl and Pauling (1965) to deduce the phylogenetic history of organisms by comparing the primary structures of macromolecules was able to be adopted.

At this point, it became clear that there was a need to integrate these independent approaches to support species descriptions in a more rational manner. The term 'polyphasic' in relation to bacterial taxonomy was coined by R.R. Colwell in 1968 at the International Conference on Culture Collections (Tokyo), when he documented the unreliability of a single feature as a sole diagnostic criterion for species definition. In 1970, two papers were published on the polyphasic approach to study *Vibrio*-related species (Citarella and Colwell, 1970; Colwell, 1970). From this date the polyphasic approach became more widespread among scientists as a basic and necessary tool to deal with bacterial systematics. With the polyphasic approach, both phenotypic and genetic characterization became the *core* of the 'comprehensive taxonomic' concept.

By the late 1970s, a new technique was available for scientists; this was based on small subunit (SSU) rRNA as an universal marker to measure phylogenetic relationships between microorganisms, as proposed by Carl Woese and co-workers (Woese and Fox, 1977). This revolutionary contribution suggested a natural relationship between microorganisms on which a new prokaryotic systematics could be based. Small subunit rRNA almost perfectly meets the basic requirements of a general phylogenetic marker: i.e. ubiquitous distribution, functional constancy, low frequency of lateral gene transfer and recombination, combined with the availability of a comprehensive database of primary structures. Genes encoding ribosomal RNA, comprising conserved and variable domains, were chosen for most phylogenetic studies and to act as the backbone of modern bacterial systematics (Ludwig, 2007). In the current edition of *Bergey's Manual of Systematic Bacteriology*, the taxonomic outline is derived from the phylogenetic information provided by comparative small unit rRNA analysis (Ludwig and Klenk, 2005).

In the years between 1955 and 1980, AAB taxonomy was subjected to considerable revision in the light of information provided by newly available systematic tools. In 1958, Kondo and Ameyama proposed a new system to classify the genus *Acetobacter* based on carbohydrate oxidation: Group 1 – does not oxidize gluconic

acid, sorbitol and mannitol; and Group 2 – oxidizes gluconic acid, sorbitol and mannitol, and does not oxidize acetic acid. To differentiate between them, several other physiological features were also considered as key elements. In 1960, Stouthamer confirmed the presence of the tricarboxylic acid cycle (TCA) in *Acetobacter* and its absence in *Gluconobacter*; in the same year, Shimwell and Carr proposed the inclusion of this characteristic in distinguishing AAB species.

Since then, a great contribution to AAB taxonomy has been made by the research of De Ley and co-workers. In 1961, De Ley published the first comprehensive study of carbohydrate metabolism, studying the oxidative behaviour of AAB on several substrates, and proposed a classification into three groups. De Ley and Schell (1963) then studied the base composition of DNA, suggesting a close relationship and a possible common phylogenetic origin for *Acetobacter* and *Gluconobacter*. One of the first attempts of AAB DNA hybridization was performed by De Ley and Friedman (1964), whose article started with the following assertion: 'An improved bacterial classification appears to be on the verge of emerging'. They stated that in spite of the great morphological, physiological and biochemical similarities among AAB strains, hybrids are only formed between strains of the same genus with DNA of almost the same base composition.

Starting from 1980, (fourth phase) DNA techniques were incorporated into species description. Consequently, the demarcation of bacterial species shifted away from arbitrary and artificial definitions to ecological and genetic entities sharing the same phylogenetic heritage (Stackebrandt, 2007).

This fourth phase of AAB taxonomy started with an extensive reinvestigation of the group, instigated by several Belgian scientists. An exploration of the internal taxonomic structure of *Acetobacter* and *Gluconobacter* genera and their relationship to each other and to other genera was provided by Gillis and De Ley (1980), who applied the DNA-rRNA hybridization method and reclassified a number of misnamed species in the *Acetobacter* and *Gluconobacter* genera. In the same year, Swings and co-workers re-examined the taxonomic position of strains previously identified as members of *Acetobacter aurantius* by Kondo and Ameyama (1958), and later defined as 'intermediate' strains by Asai because of the difficulty of assigning them to either *Acetobacter* or *Gluconobacter*. On the basis of DNA-rRNA hybridization, they removed the cluster formed by *Acetobacter aurantius* strains from *Acetobacter* and *Gluconobacter* and proposed the new genus, *Frateuria*, with *Frateuria aurantia* sp. nov. as the type strain.

In 1984, Yamada and Kondo divided the genus *Acetobacter* into two subgenera on the basis of differences in the ubiquinone system; the type subgenus *Acetobacter* was characterized by Q-9 quinone, and the type subgenus *Gluconacetobacter* was characterized by Q-10 quinone. In this latter subgenus two species were transferred: *Acetobacter* (*Gluconacetobacter*) *liquefaciens* and *Acetobacter* (*Gluconcetobacter*) *xylinus*. However, Swings (1992) criticized the establishment of the subgenus *Gluconacetobacter* because of the acetate-oxidizing AAB containing Q-10. The subgenus *Gluconacetobacter* was elevated to the generic level on the basis of partial 16S rRNA sequence analysis by Yamada and co-workers in 1997.

Urakami et al. (1989) proposed to transfer methylotrophic species of *Acetobacter* into a new genus, *Acidomonas*, incorporating *Acetobacter methanolicus* in *Acidomonas methanolica* comb. nov., on the basis of methylotrophic ability as a characteristic distinguishing it from other genera. In 1992, Bulygina et al. published a 5S rRNA-based taxonomic study of *Acidomonas*, *Acetobacter* and *Gluconobacter*, in which they supported the proposal of Urakami and co-workers, but criticized the use of chemotaxonomic features such as fatty acid and ubiquinone composition (which can be common to microorganisms belonging to closely related but different genera), affirming the limited value of these features for determining taxonomic rank. They suggested as the main distinguishing characteristic to justify the establishment of the genus *Acidomonas* the ability to utilize methanol, and used sequencing of 5S rRNA to provide evidence that methylotrophic strains of *Acetobacter* are correctly classified as *Acidomonas*. However, no DNA-rRNA data were taken into account when creating this new genus.

From the mid-1980s onwards, a great deal of applied research was done on AAB isolated from industrial vinegar, and two aspects of AAB were highlighted: (i) the need for a basic knowledge of vinegar AAB regarding isolation, cultivation of strains, and preservation of the phenotypic traits for which strains were selected; and (ii) the need for taxonomic clarification of vinegar-related species. In particular, Kittelmann et al. (1989) tested several media for cultivating AAB from industrial vinegar and developed a culture method based on the Wiame agar plate system. Meanwhile, Mariette et al. (1991) studied AAB from wine, spirit and cider acetators for industrial vinegar production, differentiating them by plasmid profile analysis performed on cells taken directly from acetators without intermediate cultivation. In 1992, Sievers and co-workers started to study the microflora of high-acid vinegar fermentation in Switzerland and Germany. The authors clarified the genomic relationship of strains by DNA-DNA hybridization using type strains of *Acetobacter* and *Gluconobacter*. They found very low (0-22%) DNA-DNA similarities with type strains and proposed *Acetobacter europaeus* as a new species. The main biochemical differentiation characteristics of this new species are its strong tolerance to acetic acid (4-8%) and the absolute requirement of acetic acid for growth.

Since 2000, many systematic studies have been done: Lisdiyanti et al. (2000) re-examined the taxonomic position of *Acetobacter* species, confirming the need for DNA-DNA relatedness data for the establishment of *Acetobacter* species. These authors identified taxonomic problems in the genus *Acetobacter* as being due several factors, including the broad range (9.7%) of G+C content of DNA in *Acetobacter pasteurianus*; no comprehensive data for DNA-DNA relatedness between strains identified as *Acetobacter aceti* and those identified as *Acetobacter pasteurianus*; and no consideration of the taxonomic significance of the ubiquinone system (Gosselé et al., 1983).

In the past 7 years, six new genera have been proposed: *Asaia* (Yamada et al., 2000), *Neoasaia* (Yukphan et al., 2005), *Saccharibacter* (Jojima et al., 2004), *Swaminathania* (Loganathan and Nair, 2004), *Kozakia* (Lisdiyanti et al., 2002) and *Granulibacter* (Greenberg et al., 2006). In the latest edition of *Bergey's Manual*, Sievers and Swings (2005) describe the following taxonomic allocation: AAB form

**Table 3.2** Main chronological phases of the study of AAB systematics

| Phase | Status | Reference |
|---|---|---|
| *1872-1900* | ***Early descriptions*** | |
| | First systematics study recognizing that 'mother of vinegar' was a mass of living microorganisms causing acetic acid fermentation | Louis Pasteur 1868 |
| | *Acetobacter* genus | Beijerinck 1898[a] |
| *1900-1955* | ***Bacterial physiology and ecology were first explored and described*** | |
| | Classification according to the capacity to utilize inorganic ammonium salts and acetic acid as nitrogen and carbon sources respectively | Janke 1916[b] |
| | Classification according to flagella and physiological traits | Leifson, 1935[b] |
| | Division into two genera *Acetobacter* and *Gluconobacter* gen. nov. | Asai, 1935 |
| *1955-1980* | ***Availability of new powerful techniques for bacterial description*** | |
| | Presence of tricarboxylic acid (TCA) cycle in *Acetobacter* and absence in *Gluconobacter* | Stouthamer, 1960[b] |
| | First comprehensive study on carbohydrate metabolism | De Ley, 1961 |
| | DNA-rRNA hybridization revealed several *Acetobacter* and *Gluconobacter* misnamed strains | Gillis and De Ley, 1980 |
| *1980 onwards* | ***Modern era*** | |
| | Description of a nitrogen-fixing acetic acid bacterium associated with sugarcane, namely *Acetobacter diazotrophicus* | Gillis et al., 1989 |
| | Nine new genera described: | |
| | *Frateuria* | Swings et al., 1980 |
| | *Acidomonas* | Urakami et al., 1989 |
| | *Gluconacetobacter* | Yamada et al., 1997 |
| | *Asaia* | Yamada et al., 2000 |
| | *Neoasaia* | Yukphan et al., 2005 |
| | *Saccharibacter* | Jojima et al., 2004 |
| | *Swaminathania* | Loganathan and Nair, 2004 |
| | *Kozakia* | Lisdiyanti et al., 2002 |
| | *Granulibacter bethesdensis* (first description of human pathogenic AAB) | Greenberg et al., 2006 |
| | Two full genome sequences available: | |
| | *Gluconobacter oxydans* (strain 621 H) | Prust et al., 2005 |
| | *Granulibacter bethesdensis* (strain CGDNIH) | Greenberg et al., 2006 |

[a] The *Index Bergeyana* (Buchanan et al., 1966) includes the following comment: 'Beijerinck used the vernacular name '*azijnbacterien*' for the acetic bacteria. Apparently at some time before 1900 the vernacular name was rendered into neo-Latin as *Acetobacter* and finally used in publication. There is no record of its formal proposal as a genus.' Kluyver (1940, p. 132), states: 'It is surprising that neither Beijerinck nor Hoyer proposed in their publications the creation of a new genus for the acetic bacteria ... There can be no doubt that, in any case morally, but probably also according to the code of Botanical Nomenclature, Beijerinck is to be considered as the author of the genus *Acetobacter* as it occurs today.' On 1 January 1980, the *Approved Lists of Bacterial Names* followed Kluyver's opinion and cited the genus name *Acetobacter* as *Acetobacter* Beijerinck 1898.

[b] Quoted in Asai (1968).

four major clusters, one containing the *Gluconacetobacter* species with a subcluster comprising *Gluconacetobacter europaeus*, *Gluconacetobacter xylinus*, *Gluconacetobacter intermedius*, *Gluconacetobacter oboediens*, *Gluconacetobacter entanii*, *Gluconacetobacter hansenii*, and one subcluster comprising *Gluconacetobacter sacchari*, *Gluconacetobacter liquefaciens*, *Gluconacetobacter diazotrophicus*, *Gluconacetobacter azotocaptans*, *Gluconacetobacter johannae*, and a second cluster containing *Acetobacter syzygii*, *Acetobacter lovaniensis*, *Acetobacter peroxydans*, *Acetobacter pomorum*, *Acetobacter pasteurianus*, *Acetobacter estunensis*, *Acetobacter aceti*, *Acetobacter indonesiensis*, *Acetobacter orleanensis*, *Acetobacter tropicalis*, *Acetobacter orientalis*, *Acetobacter cibinongensis*, and a third cluster comprising *Gluconobacter oxydans*, *Gluconobacter asaii*, *Gluconobacter cerinus* and *Gluconobacter frateurii*. *Kozakia baliensis* forms a sublineage separated from *Asaia bogorensis* and *Asaia siamensis*. *Acidomonas methanolica* represents a distinct phylogenetic line and the separate branching of this organism is supported by most of the treeing analyses.

For a chronological overview, some important milestones in AAB taxonomy are summarized in Table 3.2.

## 3.3 General Concepts and Applications of Taxonomic Techniques to Study AAB at Different Taxonomic Levels

The polyphasic approach to taxonomy is fed by phenotypic, genotypic and phylogenetic information, allowing a 'consensus' species description. However the combination of information from different techniques complicates the interpretation of results in comparison with a monophasic approach, where only one technique is used. Fortunately, scientists nowadays have the ability to standardize methods and apply them to specific cases of interest, allowing for an efficient clustering analysis.

Among the large spectrum of techniques available to polyphasic taxonomy (Table 3.3), some are essential, some are applicable but not necessary, some are interchangeable with each other, others are in some cases unsuitable. Techniques are useful if they are applied in a 'work strategy' that is appropriate for the individual case being studied and the taxonomic level being investigated. Below are described different categories of taxonomic techniques that can be used to study bacteria at different taxonomic levels.

### 3.3.1 Classical Phenotypic Analyses

Classical phenotypic analysis is the most common identification tool for bacteria and constitutes the basis for the formal description of taxa from species to family level. Classical phenotypic characteristics include cell morphology (shape, endospore, flagella, inclusion bodies, Gram staining reaction) and colony aspect (form, colour, size); physiology and biochemical features, comprising information

**Table 3.3** Techniques for studying AAB at different taxonomic levels

| Method | Notes |
|---|---|
| PHENOTYPIC | |
| *Morphological, physiological and biochemical features* | Basis for formal description of taxa from family to strain level<br>Provides descriptive information needed to recognize taxa<br>Highly standardized procedures are required to obtain reproducible results |
| Serotyping | Used for species and strain characterization<br>The need for living bacteria limits the power resolution |
| CHEMOTAXONOMIC | |
| *Cell wall composition, cellular fatty acids, isoprenoid quinines, whole-cell protein analysis* | Robust tools to describe phylogenetically closely related species as genera |
| GENOTYPIC | |
| *Genomic G+C content* | Considered part of the standard description of bacterial taxa |
| *DNA-DNA hybridization* | Acknowledged as the reference method for establishing relationships within and between species<br>Drawbacks:<br>(1) differences in genome size and DNA concentration influence results; (2) plasmid, chromosomal, housekeeping gene and DNA acquired by horizontal gene transfer are not discriminated; (3) results depend on the experimental parameters and are not cumulative; (4) different experiments cannot be compared directly; (5) the respective references (type strains) have to be included in each individual experiment |
| *RFLP* | The method is concerned with differentiation rather than identification of closely related strains |
| *RAPD* | Used for species- and strain-level identification<br>Strict standardization of experimental conditions is needed<br>Fragments are amplified with all ranges of efficiency, and the resulting patterns are very complex and difficult to interpret |
| *DGGE* | Short PCR products (<500 bp) are separated, which limits the phylogenetic information obtained by band sequencing.<br>When applied to complex bacterial communities does not provide reasonable estimates of diversity |
| *AFLP* | Irregular amplification of fragments can hamper the ability to reliably assign bands over multiple gels<br>Very laborious inspection of manual and computer-assisted band analyses |
| *ARDRA* | The main drawback is misinterpretation of pattern<br>Overcome by modern rapid sequencing rDNA |
| *rRNA comparative sequence analysis* | Gold standard for comprehensive phylogenetic analysis<br>Large availability of sequences in public databases |
| *rRNA FISH* | Effective identification of individual cells<br>Three-dimensional localization of cells inside samples |
| *DNA microarrays* | Allow profiling of differential gene expression<br>The main current limitation is the high cost |
| *MLST/MLSA* | Allow intraspecific-level identification<br>Database inconsistent with respect to other molecular markers |

on growth at different temperatures, pH values, salt concentrations or atmospheric conditions, growth in the presence of various substances, and data on the presence or activity of a variety of enzymes. Individually, many of these characteristics have been shown to be irrelevant as parameters for genetic relatedness, yet taken as a whole, they provide descriptive information enabling us to recognize taxa (Vandamme et al., 1996). From a technical point of view, highly standardized procedures are required in order to obtain reproducible results within and between laboratories. Nowadays, miniaturized and automated phenotypic fingerprinting systems have been introduced, which allow more reproducible results.

#### 3.3.1.1 Biotyping Analysis

Biotyping analysis is a combination of biochemical assays, phage typing, serotyping and growth in the presence of specific dyes used to characterize strains at an intraspecific level. Because of the need to use living bacteria, the power resolution is low and the results can be ambiguous, so these techniques are often replaced by DNA-based assays.

### 3.3.2 Chemotaxonomic Analysis

Chemotaxonomy includes the study of chemical constituents of cells (peptidoglycan structure, isoprenoid quinones, lipid and fatty acid composition of cells, polyamines, pigments and mycolic acids). The determination of chemical markers clarifies the cellular chemical composition and provides valuable properties that can be used to critically analyse the phylogenetic clustering of bacterial groups at genus level, thus strongly contributing to the application of a polyphasic approach. Of course, some methods are applicable to a great number of bacterial groups, while others are specific to particular taxa (e.g. peptidoglycan type is very informative, at both genus and species level, for Gram-positive bacteria, which have various types of this constituent; and less informative for Gram-negative bacteria, which have a simpler and more uniform peptidoglycan (Schleifer and Kandler, 1972).

### 3.3.3 Genotypic Methods

Genotypic methods include all the applications stemming from two important milestones in microbial systematics: the discovery of nucleic acids and the use of computers for handling biological data.

#### 3.3.3.1 Determination of the DNA Base Ratio (mol% G+C)

Determination of the DNA base composition (mol% guanosine plus cytosine) is one of the classical genotypic methods and is considered part of the standard description of bacterial taxa. Generally, the range observed is not more than 3%

within a well-defined species and not more than 10% within a well-defined genus. It varies between 24% and 76% in the bacterial world. The use of the G+C approach does not provide any phylogenetic information and does not allow an organism to be assigned to a particular taxon; instead it shows discriminating capacity. Different G+C contents indicate different organisms, whereas identical values per se do not necessarily characterize closely related taxa (Ludwig, 2007).

#### 3.3.3.2 DNA-DNA Hybridization

DNA hybridization is acknowledged as the reference method for establishing relationships within and between species (Harayama and Kasai, 2006). On the basis of DNA-DNA hybridization, Wayne et al. (1987) defined a species as an entity that includes strains sharing approximately 70% or higher DNA-DNA relatedness and with a difference of less than 5 °C in the DNA melting temperature between homologous and heterologous DNA hybrids. As cited by Ludwig (2007), a variety of alternative techniques and formats have been developed to measure the amount of heterologous hybrids (end-point measurement) and the kinetics of heterologous hybridization. Initially the technique was laborious and required large amounts of purified DNA, but it has now been miniaturized and microplate formats are now mainly used. Despite several drawbacks of the technique (see Table 3.3), DNA hybridization is the only generally applicable method for determining relationships at lower taxonomic levels where conserved phylogenetic markers fail to achieve resolution.

#### 3.3.3.3 DNA-based Differentiation: Fingerprinting Methods

These methods include indirect, rapid and simple techniques currently popular in many laboratories. RFLP (restriction fragment length polymorphisms), RAPD (random amplification of polymorphic DNA), AFLP (amplified fragment length polymorphism), ARDRA (amplified ribosomal DNA restriction analysis) and DGGE (denaturing gradient gel electrophoresis) are the most widespread techniques which allow the subdivision of species into a number of distinct types. All these techniques are based on the generation and visualization of target DNA fragments after amplification and, in some cases, digestion of amplicons with restriction enzymes. Several general and specific biases are associated with these approaches (see Table 3.3) and often they provide only differentiation information; for these reasons, in the modern taxonomy era, most of them have been replaced by rapid sequencing methods providing much more information for identification and phylogeny.

#### 3.3.3.4 rRNA Comparative Sequence Analysis

rRNA is currently the best target molecule for studying phylogenetic relationships and for creating practical identification systems because it is present in all bacteria, is functionally constant, is composed of highly conserved as well as more variable domains, is subject to a low frequency of lateral gene transfer, and because of the

availability of comprehensive databases containing more than 300,000 full and partial sequences (Ludwig et al., 2004).

When rRNA was first used in taxonomy, sequencing of the smaller subunit 5S molecule resulted in an accumulation of data for numerous bacteria, allowing the establishment of bacterial lineage. Later on, 16S and 23S rRNA gene sequence analysis by direct sequencing partial or whole subunits by using the PCR technique and selected primers, became a powerful tool for assessing the phylogenetic position of bacteria. In general, rRNA-based phylogenetic analysis includes the following steps:

1. DNA isolation from the target bacterium
2. amplification of a partial rRNA target gene sequence
3. alignment of the obtained rRNA sequence with other rRNA sequences available in databases
4. estimation of distances using evolution model
5. reconstruction of a phylogenetic tree from these distances
6. bootstrap analysis.

The large amount of data provided by rRNA comparative sequence analysis was the basis for the development of several probe-based techniques specific to different taxa-level studies. Among these are microarray technology, which shows great potential – offering the advantages of both sequencing and fingerprinting methodologies – and FISH (fluorescent in situ hybridization), which is now a widely applied technique in bacterial identification.

#### 3.3.3.5 Multilocus Sequence Typing/Analysis (MLST/MLSA)

Multilocus sequence typing (MLST) refers to a method for the genotypic characterization of prokaryotes at the intraspecific level, using the allelic mismatches of a small number (usually seven) of housekeeping genes; it is used for recognizing distinct strains within named species. In contrast, multilocus sequence analysis (MLSA) is described as a method for the genotypic characterization of a more diverse group of prokaryotes (including entire genera) using the sequences of single-copy and ubiquitous protein-coding genes, which evolve faster than rRNA (Gevers et al., 2005). At present, the major restriction using these approaches concerns database inconsistencies with respect to the taxonomic spectrum represented for other marker types (Ludwig, 2007). In the future, the availability of full genome sequences of bacteria will allow the application of MLST/MLSA to be considered for species description.

## 3.4 Final Remarks

Our knowledge of AAB began with vinegar as the main ecological niche, but in 2007, after about 200 years of studies, they were for the first time directly linked with human health with the description of the pathogenic species *Granulibacter*

*bethesdensis*. As shown in the taxonomic excursus, the amount of information at our disposal has increased with the development of new, robust and powerful tools of investigation, revealing a complex and fascinating scientific picture. To move forward in the field of AAB taxonomy, future research needs to address genomics, classification and characterization more fully. In this respect, the following two points need to be addressed in order to increase and regulate the stability of AAB taxonomy.

The availability of full genome sequences for all type strains would significantly advance the integration of genomic information into the understanding of microbial diversity and would enable researchers to map phenotypes to genomes. For the AAB, only two complete genome sequences are currently available, those for *Gluconobacter oxydans* 621 H (DSMZ 2343) (Prust et al., 2005) and the type strain *Granulibacter bethesdensis* CGDNIH1T (ATCC BAA-1260T=DSM 17861T) (Greenberg et al., 2006). Making available the entire genome sequences of type strains of the other species will be a vital step to a more complete understanding of the AAB.

The phenomenon of strain evolution affects bacterial species designation; although this drawback is currently known, no solution has been forthcoming to deal with microbial change during the time in laboratory conditions. This problem has crucial implications when clustering bacteria according to phenotypic traits. With respect to AAB, some authors have observed the loss of specific physiological activity as a consequence of spontaneous mutations. For instance, Kondo and Horinouchi, (1997) studied spontaneous high-frequency mutations, resulting from ISs family insertions, which were responsible for genetic instability, leading to deficiencies in various physiological properties of AAB, such as ethanol oxidation and cellulose production. This means that some phenotypic characters cannot be used for taxonomic purposes. Efforts are needed to evaluate microbial changes over time, as well as to guarantee appropriate tools for preserving ‘authentic’ strains.

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# Chapter 4
# Preservation of Vinegar Acetic Acid Bacteria

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## 4.1 Introduction

A starter culture is defined as a collection of microbial cells that are capable of initiating and completing a rapid fermentation process. The microorganisms used as starter cultures in industrial applications, such as lactic acid bacteria and yeasts, are usually conserved either in a frozen or a powdered form via the freeze-drying, spray-drying or fluidization processes (To and Etzel, 1997). With regard to acetic acid bacteria (AAB), three forms of starter culture are used in vinegar making:

- Liquid inoculum used in the culture surface method or Orleans method, submerged method, or immobilization method (Ohmori et al., 1982).
- Dried starter, as recently used by Sokollek et al. (1998) and Ndoye et al. (2007a) for submerged fermentation into Frings and Chansard acetators, respectively.
- Vinegar made from wine, using mixed strains from raw materials (Gullo et al., 2006).

In spite of attempts to standardize the process, a microbiologically defined AAB starter culture is not yet commercially available.

The lack of AAB starters causes several problems in vinegar production and, consequently, economic losses. For example, a reliable AAB starter could accelerate the start of oxidation, as well as decreasing phage problems (Sievers and Teuber, 1995; Sokollek and Hammes, 1997). Indeed, bacteriophages have been identified as the main cause of breakdowns in spirit vinegar production. Damage caused by phages or nematodes belonging to the species *Turbatrix aceti* in vinegar mashes leads to an interruption in production for at least a week, up to several weeks, depending on the type of vinegar making process involved (Sievers and Teuber, 1995).

Another important drawback of using the currently available AAB starters is related to the lack of dried cultures, which are superior to liquid preparations because they can more easily be manipulated to give a more rapid microbial activation. In addition, dried starters are easier to transport, as well as satisfying the stability criteria of long-term conservation (De Vuyst, 2000).

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The freeze-drying technique is a conservation process whereby a biological product is stabilized, generally by freezing, followed by sublimation of the water to form a solid shape. This technology has already been applied to many industrial microorganisms, such as lactic acid bacteria and yeasts (Sokollek and Hammes, 1997; Ziadi et al., 2005), whereas the conservation of AAB by freeze-drying is still a relatively recent development. *Acetobacter* strains were preserved by freeze-drying for the first time by Sokollek and Hammes (1997). Two novel strains were isolated by Sokollek and Hammes (1997) and their conservation was possible through a freeze-drying process for about 1 year without any loss of their original properties.

This chapter focuses on investigating the microbiological characteristics necessary for acetic acid bacteria to be conserved as a functional starter culture for vinegar production, especially in tropical and developing countries.

## 4.2 Starter Culture for Small-Scale Fermentation in Developing Countries

Small-scale food production in developing countries often requires traditional fermentation processes in order to improve food quality, safety and acceptability. These are often carried out by spontaneous fermentation, i.e. processes initiated without the use of a starter inoculum. The inoculation of the raw material with a small quantity of a previously performed successful fermentation, called back-slopping, optimizes spontaneous fermentation and represents a cheap and reliable preservation method in many developing countries. The use of spontaneous fermentation, as well as the back-slopping approach, is widespread in sub-Saharan Africa. Holzapfel (1989) gives, as one example of spontaneous fermentation, the baker's yeast commonly used in the fermentation of sorghum and other cereal beers in Africa.

However, the benefits of modern starter cultures as a means of improved hygiene, safety, and quality control are often not yet realized in small-scale fermentation. In developing countries under rural conditions, with limitations in infrastructure and low-technology production methods, there are problems associated with culture preservation, maintenance and distribution that would demand sophisticated logistics, and therefore economic considerations are one of the main obstacles to utilizing starter cultures (Holzapfel, 2002). Ndoye et al. (2006) report an example of vinegar obtained by dilution with imported acetic acid in Senegal (sub-Saharan Africa). WHO and FAO have recommended four research priorities for developing starter cultures:

1. assessment of the need for, and feasibility of, using starter cultures
2. establishment of an appropriate level of starter culture technology
3. development of appropriate starter culture delivery mechanisms
4. selection of microorganisms with desirable properties.

For a sustainable development of vinegar production in tropical areas, such as Asian and sub-Saharan Africa regions, conservation of the liquid inoculum does not fit in with the artisanal production of vinegar. For this purpose, solid acetic acid

bacteria starters must be used. Freeze-dried starter preparations may lead to optimal microbiological conditions, including a high level of biomass, a rapid revitalization for a new acetification process, and a long conservation period.

## 4.3 AAB as Functional Starter Cultures in Acidification Process

Nowadays, the use of a functional starter culture is one of the ways to improve food quality, safety, diversification and preservation. Leroy and De Vuyst (2004) have defined a functional starter culture as a starter that possesses at least one inherent functional property that offers one or more organoleptic, technological, nutritional or health advantages. A starter culture could be a microbial preparation consisting of a large number of cells of at least one microorganism to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process. Strains used as functional starter cultures must achieve a sufficiently high and active cell biomass for any preparation.

In the case of an acidification process involving the AAB, '-omics' techniques (genomics, proteomics, metabolomics, etc.) can promise the ability to select and improve bacteria with useful physiological and microbiological properties for vinegar production. Generally, the first selection of commercial starter cultures is based on a few basic features, such as rapid acidification, phage resistance, and adaptation to growth on a specific substrate or raw material (Sievers et al., 1992; Caplice and Fitzgerald, 1999; Ross et al., 2002; Giudici et al., 2005). For this reason, isolation of the AAB which naturally ferment the raw material is the first step to obtaining strains suitable for use as a starter. This approach has been applied to isolate thermotolerant AAB from mango fruit in Senegal for vinegar production in sub-Saharan Africa (Ndoye et al., 2007b), as well as to obtain osmotolerant strains for the production of extremely sugary traditional balsamic vinegar (Gullo et al., 2006).

Inoculations in vinegar manufacture can be done with either single-strain or mixed-strain cultures. Single-strain cultures offer advantages such as improved process control and predictability of metabolic activities within the culture. However, there may also be some disadvantages, such as an increased likelihood of spoilage through bacteriophage infection, spontaneous mutation, and loss of key physiological properties (Holzapfel, 2002). In contrast, mixed-strain cultures are less susceptible to deterioration and are therefore better suited to most small-scale fermentations. Nowadays, many modern vinegar industries use unknown mixed-strain cultures or 'seed vinegar' to start the fermentation process (Sokollek et al., 1998; De Ory et al., 2002), but no well-defined starter cultures of AAB are yet commercially available for the production of vinegar.

### 4.3.1 Cultivation Problem of AAB

The lack of defined AAB starters is, in the first instance, due to the problem of cultivation of these bacteria out of the acetators. Depending on the AAB strain and the

**Table 4.1** Some different media used to cultivate acetic acid bacteria

| Media | Viable counts (CFU · mL$^{-1}$) | Main components | References |
|---|---|---|---|
| YGM | $>1 \cdot 10^{11}$ | Yeast extract, glucose, mannitol | Ndoye et al., 2007a |
| RAE | $9.5 \cdot 10^{8}$ | Yeast extract, glucose, peptone, $Na_2HPO_4$ $2H_2O$, citric acid,·$H_2O$ | Sokollek and Hammes, 1997 |
| YEPG/Mg | $3 \cdot 10^{8}$ | Yeast extract, peptone, glucose, $Mg^{2+}$ | Blandino et al., 1996 |
| YEPE/Mg | $1 \cdot 10^{8}$ | Yeast extract, peptone, $Mg^{2+}$, ethanol | Blandino et al., 1996 |
| MAE | $>1 \cdot 10^{8}$ | Yeast extract, peptone, glucose, acetozym DS | Entani et al., 1985 |
| YGP | $>1 \cdot 10^{9}$ | Yeast extract, glucose, peptone | Ohmori et al., 1982 |

Abbreviations: YGM, yeast extract glucose medium; RAE, reinforced acetic acid ethanol medium; YEPG/Mg medium; YEPE/Mg medium; MAE, modified acetic acid ethanol medium; YGP medium.

design of the fermentation process and equipment, problems are encountered in strain isolation, culture maintenance, determination of the viable counts, strain preservation, and cultivation outside of the acetator; for instance, the transfer from liquid to solid and vice versa (Sievers et al., 1992).

To overcome these problems and develop a high cell biomass of AAB suitable for starter preparation, different media for cultivation have been designed, as summarized in Table 4.1. Sokollek et al. (1998) devised a reinforced acetic acid and ethanol medium (RAE) suitable for cultivating two *Acetobacter* strains (LTH 2455 and LTH 2459) and for investigating their growth parameters (length of lag phase and total counts) and metabolic characteristics, as well as for testing the efficiency of the methods for dried starter preparation. Some growth features of the two strains on RAE medium are shown in Figure 4.1. In particular, both *Acetobacter* strains grew without any appreciable lag phase up to total counts of $>10^9$ cells · mL$^{-1}$ and used glucose and ethanol as carbon sources. These results differed from those obtained for other vinegar-making strains, which showed a total count in fermenting vinegar of $2 \cdot 10^8$ cells · mL$^{-1}$ (Ebner, 1976; Muraoka et al., 1982).

Another two important AAB growth media, YEPD/$Mg^{2+}$ and YEPE/$Mg^{2+}$, differing in carbon source – glucose and ethanol, respectively – have been tested by Blandino et al. (1996), using an *Acetobacter aceti* strain (UCA1). As shown in Figure 4.2, YEPD/$Mg^{2+}$ medium allowed a final microbial cell mass greater than that on YEPE/$Mg^{2+}$ medium. From the point of view of biomass production, glucose was a more efficient carbon source than ethanol because growth on ethanol causes an expansion of the lag phase of 12 hours compared with the growth on glucose (Figure 4.2).

## 4.4 Freeze-Drying Process

In addition to the cultivation process, another important step in producing a dried AAB starter is to perform a freeze-drying protocol specifically to ensure the sur-

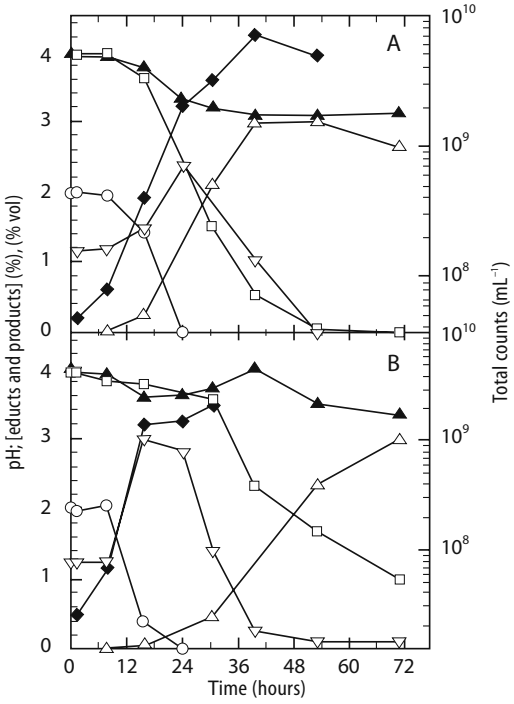


**Figure 4.1** Characteristics of growth and metabolism of isolates from: **A** fermenting wine (strain LTH 2455); **B** cider mashes (strain LTH 2459). Total counts ◆ ($mL^{-1}$); pH ▲; ethanol ▽ (%vol); glucose ○ (%); gluconic acid □ (%); acetic acid △ (%) (from Sokollek et al., 1998, with permission)

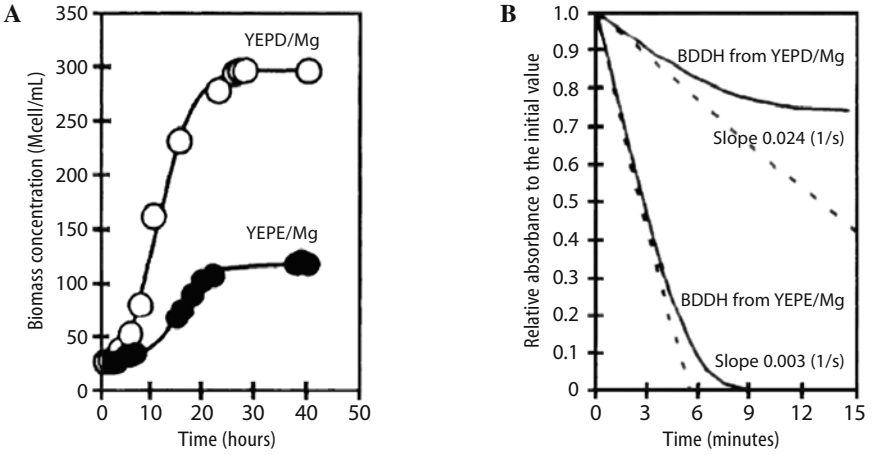


**Figure 4.2** Growth curves (A) and aldehyde dehydrogenase activity (B) of *Acetobacter aceti* strain UCA1, grown on different culture media (from Blandino et al., 1996, with permission)

vival and vitality of the AAB. Damage to biological systems resulting from freeze-drying can be attributed primarily to changes in the physical state of membrane lipids or to changes in the structure of sensitive proteins (Leslie et al., 1995).

Several physico-chemical factors affect the creation of freeze-dried bacteria, such as water activity ($a_w$), glass transition temperature ($T_g$), storage temperature ($T_s$), thermal inactivation mechanism, oxygen, moisture and light (To and Etzel, 1997; Sow et al., 2005). These factors may drastically influence the quality of the starters, especially in many developing countries where the industrial infrastructure for the safe storage of food, such as refrigeration, is not readily available.

Two particularly important parameters are strongly correlated both with the shelf-life and the microbial safety of food products obtained using these starters, i.e. glass transition temperature and water activity.

Glass transition temperature ($T_g$) is the temperature below which vitrification occurs.

Water activity ($a_w$) expresses the availability of water in the products and also its capacity to take part in reactions as a solvent or reagent. Therefore it is necessary to quantify $a_w$, as it greatly affects the rate at which the product will degrade (Schuck et al., 2004). Generally speaking, most food products are better preserved when $a_w$ has a value of ca. 0.2 (Le Meste et al., 2002).

Moreover, Sablani et al. (2007) have recently reported that, depending on the operating conditions, monitoring $a_w$ and $T_g$ could help to formulate, select and adapt the drying process. Roos (1993) suggested that certain physico-chemical and structural processes are better correlated with the glass transition temperature through plasticization by water or temperature. The overriding mechanism of deteriorative processes, including stickiness, crispness, collapse and amorphous-to-crystalline transformation, is the molecular mobility that relates directly to $T_g$ (Le Meste and Simatos, 1990; Sablani et al., 2007).

### 4.4.1 Use of Cryoprotectants

An important factor which improves AAB survival and vitality during congelation or desiccation is the addition of cryoprotectants. The cryoprotective capacity of a compound is dependent upon the number of lone-pair electrons that the compound contains, the spherical symmetry of the lone-pair electrons, and the solubility of the compound in water.

There are two classes of cryoprotectants:

- intracellular cryoprotectants (ICP) are substances which penetrate the cells
- extracellular cryoprotectants (ECP) are large molecules (typically sugars) that remain outside the cells (Turner et al., 2001).

The mechanisms of cryoprotectant action are not well understood, but the molecular bases of cryoprotectants, which avoid collapse, stickiness, crispness, etc., have recently been described by Sablani et al. (2007). In the case of a slow congelation, producing bigger crystals than high-speed methods, intracellular crys-

tal formation could be avoided by using cryoprotectants which increase the solute concentration (Carvalho et al., 2002). The ECPs with high molecular weight act as osmotic agents and facilitate dehydration, whereas those similar to glutamate or adonitol involve a strong retention of water, decreasing the amount of intracellular water available for ice formation.

The most well-investigated cryoprotectant is mannitol, which is a polyalcohol with four hydroxylic groups arranged along one side. This structure favours efficient interactions with the membrane bilayer. Therefore, mannitol helps to stabilize the cell membrane, particularly the phospholipid bilayer and membrane proteins, during cellular dehydration and freezing and, consequently, it improves the survival of the cells during conservation (Turner et al., 2001).

In addition to mannitol, the other most commonly used cryoprotectants are glycerol, malt extract, sucrose, maltose, $CaCO_3$ and sorbitol.

### 4.4.2 Basic Technology of the AAB Freeze-Drying Process

The influence of cryoprotectants upon AAB survival during freeze-drying and subsequent storage has to be evaluated when selecting a freeze-dried starter culture for vinegar making. However, not many studies have been carried out for this purpose. Recently, two thermoresistant AAB strains, belonging to *Acetobacter senegalensis* and *Acetobacter pasteurianus*, were isolated from over-producing crops of sub-Saharan Africa (Ndoye et al., 2006) and underwent conservation by the freeze-drying process (Ndoye et al., 2007a). Their survival and vitality were compared with those of a mesophilic reference strain, *Acetobacter cerevisiae*. Mannitol (20% w/w) was added as a cryoprotectant to the fresh cell pellets ($>1\times10^{10}$ CFU·g$^{-1}$ of dry cell weight) before freezing and freeze-drying, to evaluate its effect on the desiccation efficiency process and AAB viability. The yields of the freeze-drying process are given in Table 4.2.

Freeze-dying carried out using mannitol as a cryoprotectant did not affect the viability of the cells, whereas it increased the cell survival compared with the cells dried without a cryoprotectant (Figure 4.3). Without a cryoprotectant, the stability of the freeze-dried powder decreased rapidly, with a survival value of around 10% ($<1\times10^5$ powder CFU · g$^{-1}$ in the case of the control strain) during 6 months of conservation. The cryoprotectant maintains a residual viability of the cells of almost

**Table 4.2** Yield of freeze-drying process on cells

| Strains | Species | DCW (%) | | Survival % | |
|---|---|---|---|---|---|
| | | WC | C | WC | C |
| LMG 1625$^T$ | *Acetobacter cerevisiae* | 98±0.25 | 97±0.65 | 100±10 | 95±5 |
| CWBI-B418$^T$ | *Acetobacter senegalensis* | 98±0.75 | 97±0.85 | 100±15 | 97±10 |
| CWBI-B419$^T$ | *Acetobacter pastorianus* | 98±0.95 | 98±0.85 | 100±20 | 98±10 |

Abbreviations: DCW, dry cell weight; WC, with cryoprotectant; C, control

Data are presented as the average of two independent trials with SD.

From Ndoye et al., 2007a

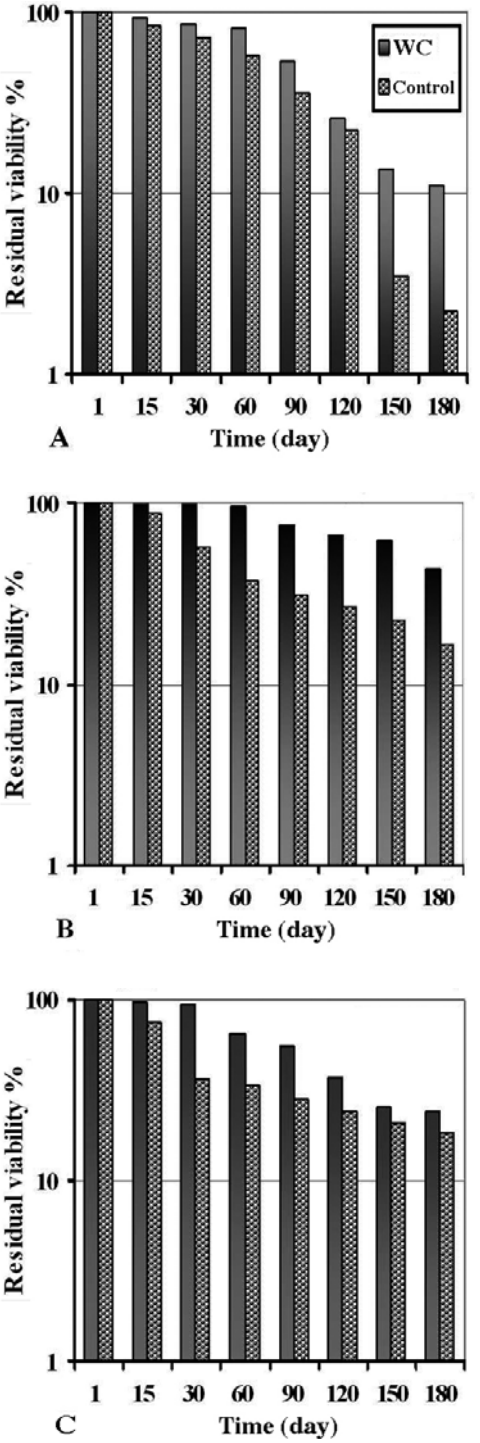


**Figure 4.3** Stability of freeze-dried powder, conserved at 4 °C in the absence of oxygen and moisture (WC, with cryoprotectant): **A** *Acetobacter cerevisiae*; **B** *A. senegalensis*; **C** *A. pasteurianus* (from Ndoye et al., 2007a)

**Table 4.3** Determination of the water activity $a_w$ and the glass transition temperature $T_g$ of powder after a freeze-drying process.

| Strains | Species | Water activity $a_w$ (0,001) | | $T_g$ (5 °C) | |
|---|---|---|---|---|---|
| | | WC | C | WC | C |
| LMG 1625$^T$ | *Acetobacter cerevisiae* | 0.17±0.0010 | 0.20±0.0011 | 87.68±2.71 | 85.69±2.32 |
| CWBI-B418$^T$ | *Acetobacter senegalensis* | 0.13±0.0017 | 0.18±0.0019 | 85.36±4.02 | 81.10±5.33 |
| CWBI-B419$^T$ | *Acetobacter pastorianus* | 0.17±0.0013 | 0.20±0.0009 | 86.06±3.11 | 85.18±2.71 |

WC, with cryoprotectant; C, control (without cryoprotectant).
The glass transition temperature was measured by DSC (differential scanning calorimetry).
Data are presented as the average of two independent trials with SD.
From Ndoye et al., 2007a

50 ± 1% after 6 months of conservation at +4 °C in the absence of oxygen and moisture by stabilizing the percentage dry cell weight at a value of ≥95%. In addition, the comparison between thermophilic and mesophilic strains showed that thermotolerant strains had a significantly higher viability. These results were confirmed by the intrinsic physico-chemical properties of the products after the freeze-drying process (Table 4.3). Water activity ($a_w$) and glass transition temperature ($T_g$) were stable even without the use of cryoprotectant. $T_g$ values were important, probably because of the accumulation of polysaccharides arising from sugars (mannitol or glucose) present in the cytoplasm.

## 4.5 Conclusions and Future Perspectives

The physico-chemical and microbiological characteristics of the freeze-dried starters described in this chapter may yield valuable information about the relationship between the vinegar environment and bacterial functionality, and contribute to optimal strain selection and process design. The selection and the construction of suitable strains with useful properties to be used as a new functional starter cultures may result in better process control, enhanced food safety and quality, and the reduction of economic losses. In the case of sub-Saharan Africa, the use of functional dried starters of acetic acid bacteria may contribute to the successful development of small- and medium-sized enterprises for industrial vinegar production.

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# Chapter 5
# Organisms Associated with Acetic Acid Bacteria in Vinegar Production

**Sandra Rainieri and Carlo Zambonelli**

## 5.1 Introduction

Vinegars are the product of scalar fermentations carried out by several groups of microorganisms acting at different moments in time. The initial phase is generally represented by an alcoholic fermentation commonly carried out by yeasts. Lactic acid bacteria (LAB) can also play a role in releasing ethanol and acetic acid from heterofermentative lactic acid fermentations. Depending on the nature of the substrate, the production of ethanol can be preceded by a transformation that induces the release of fermentable sugars from complex substrates. This is the case of rice vinegars, which require the action of some moulds of the genus *Aspergillus* to break the starch into fermentable sugars. The ethanol originating from the alcoholic fermentation is finally oxidized by acetic acid bacteria (AAB) and the alcoholic beverage is turned into vinegar (see Table 5.1). Even though acetic acid bacteria play the leading role in vinegar production, the metabolic activity of yeasts, moulds and lactic acid bacteria is also crucial for guaranteeing the manufacture of the product. These microorganisms, in fact, modify the fermentative substrates in order to allow the final stage of ethanol oxidation. This chapter provides an overview of their taxonomy, their nutritional requirements, their metabolic activity and their relevance in the vinegar manufacturing process (see Table 5.2). Brief descriptions of vinegar eels and *Drosophila* are also given to complete the variety of organisms that are involved in vinegar production.

**Table 5.1** Summary of the technological steps in vinegar production, the microorganisms involved, and their metabolic activity

| Step 1[a] | Step 2 | Step 3 |
|---|---|---|
| Saccharification | Ethanol production | Acetic oxidation |
| *Aspergillus* spp. | Yeasts (alcoholic fermentation) | Acetic acid bacteria (AAB) |
| | Lactic acid bacteria (LAB) (heterofermentative lactic fermentation) | |

[a] Necessary only for substrates containing complex sugars.

L. Solieri, P. Giudici (eds), *Vinegars of the World*

**Table 5.2** Microorganisms involved in the production of vinegars obtained from different substrates

| Substrate | Step 1 | Step 2 | Step 3 |
|---|---|---|---|
| Apple | NA | Yeast | AAB |
| Cassava | NA | Yeast/LAB | AAB |
| Grapes | NA | Yeast | AAB |
| Honey | NA | Yeast | AAB |
| Malted barley | NA | Yeast | AAB |
| Palm | NA | Yeast | AAB |
| Rice | *Aspergillus* spp. | Yeast | AAB |
| Tea | NA | Yeast | AAB |
| Whey | NA | Yeast/LAB | AAB |

NA, not applicable. AAB, acetic acid bacteria. LAB, lactic acid bacteria.

## 5.2 Yeasts

Yeasts are fungi with vegetative states that predominantly reproduce by budding or fission, which results in growth that comprises mainly single cells. The variety of yeasts is rather large: the current classification embraces over 700 species grouped in approximately 70 genera (Kurtzman and Fell, 1998).

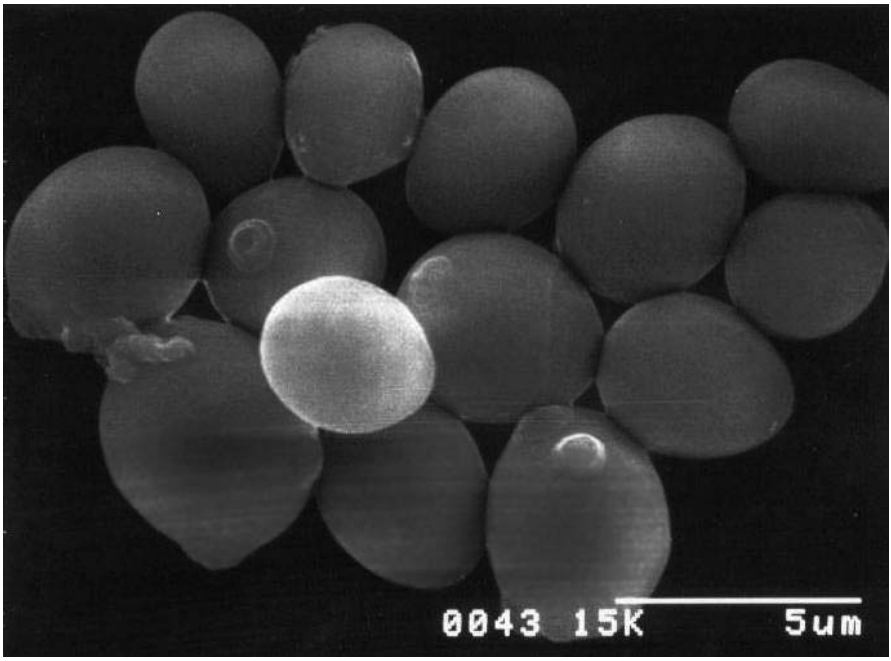


**Figure 5.1** *Saccharomyces cerevisiae* wine yeast cells photographed under a scanning electron microscope (SEM) at 6000× magnification

Most yeasts, as well as most fungi, can reproduce both sexually and asexually. Often only one method of reproduction is observable at a specific moment in time or under specific environmental conditions. A fungus that reproduces mainly sexually is referred to as teleomorphic; a fungus that reproduces solely asexually as anamorphic; and a fungal form combining both states is referred to as holomorphic. Figure 5.1 shows the cells of the typical yeast *Saccharomyces cerevisiae*.

### 5.2.1 Yeast Classification

Yeasts belong to the Kingdom of Fungi, or *Eumycotes*, which has traditionally been divided into the following five divisions, depending on the mode of sexual reproduction:

- *Ascomycota*
- *Basidiomycota*
- *Chytridiomycota*
- *Glomeromycota*
- *Zygomycota*

Recently some rearrangements of such a grouping has been proposed: the divisions *Ascomycota* and *Basidiomycota* have been grouped under a subkingdom named *Dikarya*, reflecting the putative synapomorphy of dikaryotic hyphae. Moreover, groups that had commonly been included in the phyla *Chytridiomycota* and *Zygomycota* have been shifted to new groups; in particular, taxa traditionally placed in *Zygomycota* are now distributed among *Glomeromycota* and in several subphyla *incertae sedis* (Hibbett et al., 2007).

The fungi that are of interest in vinegar production are placed within the phylum *Ascomycota*. In particular, yeasts are mostly part of the subphylum *Saccharomycotina*, which includes the class *Saccharomycetes*. The organization of this class is shown in Table 5.3.

**Table 5.3** Current classification of the class *Saccharomycetes*

| Subphylum | Order | Family | Genera |
|---|---|---|---|
| *Saccharomycotina* | *Saccharomycetales* | *Ascoidaceae* | 1 |
| | | *Cephaloascaceae* | 2 |
| | | *Dipodascaceae* | 4 |
| | | *Endomycetaceae* | 4 |
| | | *Eremotheciaceae* | 5 |
| | | *Lipomycetaceae* | 9 |
| | | *Metschnikowiaceae* | 6 |
| | | *Phaffomycetaceae* | 3 |
| | | *Saccharomycetaceae* | 53 |
| | | *Saccharomycodaceae* | 9 |
| | | *Saccharomycopsidaceae* | 7 |

From: Eriksson and Winka, 1997; Hibbett et al., 2007

#### 5.2.1.1 Genus

Yeast identification at the genus level is based on some simple determinations:

- mode of multiplication
- number of spores per ascus
- spore shape
- mode of sporulation (direct transformation of the cell into an ascus or by sexual conjugation followed by the conversion of the cells into asci)
- ability to ferment some sugars.

#### 5.2.1.2 Species

Until the 1980s, yeast species were defined exclusively on the basis of yeast phenotypic characteristics, the most important of which being the ability to ferment and assimilate a variety of carbon sources (e.g. glucose, galactose, maltose, sucrose, lactose, melibiose and raffinose). Yeast taxonomists have always been aware that the merely phenotypic characterization of cultures was not exhaustive for establishing the taxonomic status of yeasts; however, up to the classification of Kreger van Rij (1984), the determination of phenotypic characteristics was the only approach used in yeast taxonomic studies. Starting from the early 1990s novel approaches based on yeast genotypic characterization have been developed and successfully employed in yeast taxonomy. In particular, the development of molecular techniques, such as nucleotide sequencing and polymerase chain reaction (PCR), has allowed us to study the yeast genome in depth and to detect DNA regions with a large amount of interspecific polymorphism. The polymorphism of these regions, located in specific portions of the ribosomal DNA (rDNA), namely within the subunits 26S and 18S (the internal transcribed spacer – ITS; the non-transcribed spacer – NTS) and in a small portion of the subunit 26S (D1/D2 region), detected by sequencing or restriction fragment length polymorphism (RFLP) have recently been given a major taxonomic value. In addition, the determination of the chromosome banding pattern by contour-clamped homogeneous electric field (CHEF), the DNA composition (expressed as percentage of G+C), and the DNA reassociation value are considered useful tools in yeast taxonomy. The employment of a polyphasic approach in yeast taxonomy, which includes the determination of several genotypic characteristics, as well as a classical phenotypic characterization, is currently considered the most rational and accurate way to proceed in yeast species determination. For an overview of the techniques currently used for yeast species determination see Giudici and Pulvirenti (2002).

### 5.2.2 Growth and Nutritional Requirements

#### 5.2.2.1 Temperature

Yeasts are typical mesophilic organisms with an optimum temperature for growth ranging between 20 °C and 40 °C. Yeasts do not generally demonstrate optimal

temperatures for growth higher than 40 °C, and therefore cannot be considered as thermophilic microorganisms; or below 20 °C (with the only exception being a few strains isolated from Polar environments), and therefore cannot be considered as psychrophilic microorganisms.

However, some yeast species can still grow and be metabolically active at extreme temperatures. Some species of the genus *Kluyveromyces*, for example, maintain the ability to reproduce and to ferment far above 40 °C. Some species of the genus *Saccharomyces* have a very low minimum temperature for growth and can still ferment vigorously at approximately 0 °C (cold-fermenting yeasts). Some species of the genus *Zygosaccharomyces* are resistant to high temperatures and can survive at temperatures that are lethal for the cells and spores of all the other genera.

#### 5.2.2.2 pH

Yeasts prefer acidic substrates and grow well at pH values between 3 and 5. At neutral pH, they sometimes grow with difficulty.

#### 5.2.2.3 Growth Media Composition

The following compounds are essential for yeast growth; (i) carbon compounds; (ii) nitrogen compounds; (iii) growth factors (vitamins and minerals); (iv) minor elements.

***Carbon Compounds*** Like all fungi, yeasts have a respiratory metabolism and grow vigorously in the presence of oxygen, drawing energy from numerous carbon compounds: carbohydrates (monosaccharides, some disaccharides and trisaccharides); several organic acids (e.g. acetic acid, lactic acid and ketoglutaric acid); a number of alcohols (such as methanol and ethanol); dihydroxyacetone (DHA) and other substances. Some yeasts can also use carbohydrates with an anaerobic metabolism, thus carrying out alcoholic fermentation.

***Nitrogen Compounds*** Yeasts can use a great variety of nitrogen compounds, with the exception of elementary nitrogen and proteins. Ammonia can be metabolized by all yeasts, whereas only a few yeast species can metabolize nitrates. The ability of yeasts to grow in the presence of ammonia as the sole nitrogen source indicates that they are capable of synthesizing all the amino acids and DNA components. Generally, ammonia compounds are good sources of nitrogen, especially ammonium phosphate, which is perhaps the most important. This compound is frequently employed by fermentation industries to stimulate yeast growth. Ammonia represents the best source of nitrogen for yeasts; however, their growth is faster in media containing a variety of amino acids. Many yeasts can grow well even in media containing only one amino acid; particularly glutamic acid, aspartic acid or asparagine. Amino acid metabolism occurs by direct absorption or alternatively by a preceding transamination. Amino acids represent the starting point for the formation of a number of minor fermentation by-products.

***Growth Factors*** Yeasts have the ability to synthesize most vitamins except for biotin, pantothenic acid, thiamine, pyridoxine, nicotinic acid and inositol. Occasionally, some strains require parabenzoic acid, whereas folic acid and riboflavin are not generally required. Different yeast species have different nutritional requirements and in some cases such diversity can be detected even at strain level.

***Minor Elements*** Phosphorus, sulphur, potassium and magnesium are necessary for yeast growth under all conditions. In the absence of these elements, yeast growth is minimal and terminates when all the cell supplies have been exhausted.

#### 5.2.2.4 Oxygen

Yeasts are organisms with a respiratory metabolism that can grow in the presence of oxygen using a great variety of carbon compounds. Many types of yeasts in the absence of oxygen also show a fermentative metabolism especially when metabolizing monosaccharides with six atoms of carbon. This double activity was discovered by Pasteur, who established that, if provided with a sufficient amount of oxygen, yeast behaves like all other fungi growing actively; whereas, if deprived of air, yeasts grow very little but ferment instead. This means that in an abundance of oxygen (i.e. in forced aeration), from 4 g of sugar, 1 g of yeast cells (dry weight) are produced. In this case the sugar is metabolized following the classic respiration reaction:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2$$

totally exploiting the potential energy of 688 kcal · $mol^{-1}$.

In the absence of oxygen, yeasts grow very little, and 176 g of sugar are required to produce 1 g of yeast cells:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

Through fermentation, the potential energy of sugar is only partially exploited. In fact, the major product released, i.e. ethanol, is still rich in energy and develops 56 kcal · $mol^{-1}$.

A phenomenon known as the ‘Pasteur effect’ dictates that, with a sugar concentration above 5%, the fermentation activity of fermenting yeasts prevails over the respiratory activity. However, when the glucose concentration is higher, some *Saccharomyces* yeasts produce ethanol aerobically rather than producing biomass. This phenomenon is known as the ‘Crabtree effect’.

### 5.2.3 Alcoholic Fermentation

#### 5.2.3.1 Fermentation and Assimilation of Sugars

For almost a century, the ability to ferment sugars has been the major key to yeast species identification and differentiation. However, sugar metabolism has now lost its taxonomic value. Nevertheless, the knowledge of sugar fermentation metabolism is still valuable from a technological point of view; in fact, the sugars tested

for species identification are exactly those present in fermenting substrates used for the production of alcoholic beverages.

Yeasts ferment only carbohydrates with six atoms of carbon (hexoses). All fermenting yeasts can ferment glucose, fructose and mannose. In general, glucose and fructose are fermented with the same degree of vigour, whereas mannose is fermented more slowly. Galactose can be fermented by some yeasts. The speed and intensity of galactose fermentation depends on the species and on the strain. Disaccharides, particularly sucrose, maltose, lactose and melibiose, are fermented only by those yeasts possessing the specific hydrolytic enzyme. None of the fermenting yeasts possess the ability to ferment both maltose and lactose.

Raffinose is a trisaccharide composed of one molecule of fructose and one of melibiose; this sugar can be: (i) not fermented; (ii) completely fermented, by yeasts possessing both raffinase and melibiase; or (iii) partially fermented, by yeasts possessing raffinase but not melibiase. Starch is generally not fermented by yeasts, except for some species such as, for example, *Schwanniomyces occidentalis* (McCann and Barnett, 1986). Inulin (a polymer of fructose) can also be fermented by some yeast species, particularly those of the genus *Kluyveromyces* (Rouwenhorst et al., 1990).

Yeasts generally ferment sugars vigorously at concentrations up to 20%; at higher concentrations the fermentative metabolism slows down. If sugar concentration is above 50%, the osmotic pressure becomes excessively high and inhibits most yeasts. Only a few species, referred to as osmophilic, can grow in such conditions.

Several yeasts can assimilate certain sugars aerobically, but not anaerobically. This respiration-dependent sugar utilization is known as the 'Kluyver effect' and respiration-dependent species are referred to as Kluyver effect positive. A yeast can be Kluyver effect positive for some sugars and not for others. The cause of the Kluyver effect seems to be the low level of sugar transporters which, although it cannot sustain the high substrate flow required for fermentative growth, is sufficient to guarantee the energy-efficient respiratory growth that does not require a high rate of sugar uptake. *Saccharomyces cerevisiae*, which is predominantly fermentative, is Kluyver effect negative on most sugars (Fukuhara, 2003).

#### 5.2.3.2 Fermentation By-Products

The alcoholic fermentation produces two molecules of ethanol (PM $46 \times 2 = 92$) and two molecules of carbon dioxide (PM $44 \times 2 = 88$) starting from one molecule of glucose (PM 180). The ethanol yield is slightly above 50% (w/w) and above 65% (v/w). These yields are theoretical, as, in reality: (i) part of the glucose is used by the yeast to grow; (ii) in addition to ethanol and carbon dioxide, yeasts produces numerous minor compounds. Pasteur formulated the following balance for products formed by 100 g glucose:

- ethanol 48.4 g
- $CO_2$ 46.6 g
- glycerol 3.3 g
- succinic acid 0.5 g
- dried yeast 1.2 g

In current practice we assume that the ethanol yield is approximately 60% (60 mL per 100 g of fermented glucose). Ethanol that accumulates during the process of alcoholic fermentation exerts an inhibitory action on yeast growth and metabolic activity. Yeasts species show a different degree of ethanol tolerance; apiculate yeasts, such as those of the genus *Kloeckera*, cannot grow at an ethanol concentration higer than 4% (v/v), whereas more tolerant species, such as those of the genus *Saccharomyces*, can grow at up to 14% (v/v) ethanol.

During alcoholic fermentation, numerous other compounds are also released, generally at very low concentrations. These compounds can have three origins:

- they can originate from the alcoholic fermentation of sugars through anabolism: their formation occurs independently of the composition of the medium
- they can originate from the catabolism of some compound present in the medium
- they can originate via both anabolism and catabolism.

Table 5.4 shows the most important minor products of fermentation produced by *Saccharomyces cerevisiae*, the most common fermenting yeast.

*S. cerevisiae* is the yeast providing the purest alcoholic fermentation, achieving the maximum ethanol yield and the lowest concentration of minor fermentation compounds (Giudici et al., 1993; Antonelli et al., 1999; for a complete review, see Zambonelli, 2003).

Glycerol is quantitatively the third fermentation by-product. The amount produced is very variable among yeast strains, even within the same species. In alcoholic beverages, glycerol provides body and is generally regarded as a desirable compound. In addition to the compounds listed in Table 5.2, numerous other compounds are formed during alcoholic fermentation, such as lactic acid, fatty acids, acetic acid, succinic acid and esters. In many cases, nearly a hundred compounds can be found at the end of alcoholic fermentation. There is considerable variability between the different yeast species in the production of such minor fermentation compounds. For example, apiculate yeasts of the genus *Hanseniaspora* (*Kloeckera*) produce acetic acid at much higher concentrations than *S. cerevisiae* does. There is also a great difference between yeast species with regard to ethanol production.

**Table 5.4** Minor products of alcoholic fermentation produced by *S. cerevisiae*, with reference to 100 vol of ethanol produced

| Compound | Amount | Origin |
|---|---|---|
| Glycerol | 4-7 g | Sugar |
| Succinic acid | 0.30-0.6 g | Sugar |
| Acetic acid | 0.1-1 g | Sugar |
| Acetic aldehyde | 10-80 mg | Sugar |
| Propanol | 20-50 mg | Sugar and amino acids |
| Isobutanol | 30-90 mg | Sugar and amino acids |
| Amylic alcohol | 30-60 mg | Sugar and amino acids |
| Isoamylic alcohol | 100-300 mg | Sugar and amino acids |
| Phenylethyl alcohol | 10-100 mg | Sugar and amino acids |

Many species are inhibited when the ethanol concentration reaches levels that are far lower than those that can be tolerated by *S. cerevisiae*.

## 5.2.4 Important Genera and Species for Vinegar Production

Fermenting yeasts are of significant importance for vinegar production as they are responsible for the production of the alcoholic substrate from which vinegar is obtained. The most important groups of yeasts are the following:

- yeasts belonging to the genus *Saccharomyces*
- apiculate yeasts of the genera *Hanseniaspora* and *Kloeckera*
- lactose-fermenting yeasts of the genus *Kluyveromyces*
- osmophilic yeasts of the genus *Zygosaccharomyces*

### 5.2.4.1 The Genus *Saccharomyces*

The genus belongs to the family *Saccharomycetaceae* (see Table 5.1) and has the following general characteristics: multiplication by multilateral budding; elliptical or cylindrical cells that are generally diploid. Asci are formed without previous conjugation and contain from one to four elliptical or round smooth spores. Asci are persistent (do not release the spores into the medium). The genus is characterized by vigorous fermentative activity. Although the genus *Saccharomyces* is rather homogeneous; it has always been difficult to organize, especially at species level, and over the years it has undergone several changes. Since the 1970s, such species have been divided into three groups (Van der Walt, 1970):

- *Saccharomyces sensu stricto*, including *S. cerevisiae* and other species characterized by carrying out vigorous fermentations, showing good ethanol tolerance.
- *Saccharomyces sensu lato*, including less vigorous species with restricted habitat.
- The third group consist of just one species, *S. kluyveri*, which is phylogenetically distant from both *sensu stricto* and *sensu lato* groups.

***Saccharomyces sensu stricto*** Currently this group includes the following species: *S. cerevisiae*, *S. bayanus*, *S. pastorianus*, *S. paradoxus*, *S. cariocanus*, *S. mikatae* and *S. kudriavzevii* (Kurtzman, 2003).

*S. cerevisiae* is the most important yeast for the fermentation industry, being able to ferment efficiently almost all fermentative substrates, especially those originating from plants (fruit, vegetables, grains), but to a lesser extent also those of animal origin. *S. cerevisiae* is, in fact, the most vigorous and ethanol-tolerant yeast. Even though it is not naturally abundant in the wild, it is able to outcompete other microorganisms, including other yeasts that cannot tolerate high ethanol concentrations, to carry out the latest stages of alcoholic fermentation.

*S. bayanus* yeasts of interest for the fermentation industry are referred to as *S. uvarum*. These yeasts are frequently responsible for spontaneous fermentations that occur at low temperatures, as they can grow and ferment well even at 1-2 °C (Giudici et al., 1999). Generally they are isolated from the oenological environment

(Torriani et al., 1999; Naumov et al., 2002; Demuyter et al., 2004). They possess fermentation characteristics that differ from *S. cerevisiae* and generally produce a high concentration of glycerol, a low concentration of acetic acid and, interestingly, unlike *S. cerevisiae* strains they can synthesize malic acid, thus increasing the concentration of this compound in wine (Kishimoto et al., 1993; Castellari et al., 1994). They have been proposed as starter cultures for certain types of wine production (Giudici et al., 1995).

*S. pastorianus* is the typical yeast species used for lager brewing fermentations; it includes all lager brewing strains and is therefore important for the production of malt vinegar, which originates from the oxidation of fermented malt or beer. The species is thought to have originated through the natural hybridization between a *S. cerevisiae* and non-*S. cerevisiae* yeast similar to *S. bayanus* (see Kodama et al., 2006, and references therein). The genome of this species is indeed very complex and this reflects the metabolic properties of this organism. It grows and ferments well at low temperatures and produces a unique fermentation profile.

*S. paradoxus* is generally considered a yeast that is not of interest in the production of fermented foods; however, recent studies have highlighted its presence as the dominant agent of fermentation in Croatian wines, and it seems that one of the peculiarities of this species is its ability to metabolize malic acid efficiently, diminishing its concentration in the final product (Redzepovic et al., 2002, 2003).

***Saccharomyces sensu lato*** Currently this group includes the following species: *S. barnettii*, *S. castellii*, *S. dairenensis*, *S. exiguus*, *S. rosinii*, *S. servazzii*, *S. spencerorum*, *S. transvaalensis* and *S. unisporus*. More recently, three new species have been added: *S. naganishii*, *S. humaticus* and *S. yakushimaensis* (Mikata et al., 2001). Among this group only *S. unisporus* can be considered relevant for the production of fermented beverages. This yeast takes its name from the fact that it forms asci containing only one single spore. Yeasts belonging to this species do not ferment lactose; however, they ferment glucose and galactose, the latter more vigorously. Originally *S. unisporus* was named *S. mongolicus* and was detected in a number of foods of dairy origin such as whey, cheese, kefir and other fermented milks (Engel et al., 1986; Montanari et al., 1996). The reason for the presence of a yeast not fermenting lactose in substrates where the main source of carbon is actually lactose can be explained by the concomitant presence of some lactic acid bacteria (such as *Streptococcus thermophilus* and *Lactobacillus kefir*) that grow by metabolizing lactose, but using only the glucose fraction of the disaccharide, thus releasing the galactose fraction into the medium. This encourages the growth of the microorganisms that can ferment this monosaccharide more vigorously (Montanari et al., 1996). *S. unisporus* is important for the production of whey vinegars, obtained by the oxidation of fermented whey.

### 5.2.4.2 Apiculate Yeasts (Genera *Hanseniaspora* and *Kloeckera*)

Apiculate yeasts are named after the shape of their cells, which have extended extremities. This peculiar morphology is the consequence of the mode of multipli-

cation, which occurs by budding alternately at the two opposite extremes of the cell. They belong to the family *Saccharomycoidaceae* and have a corresponding anamorphic species in the family *Candidaceae*. The apiculate yeasts that more frequently occur in the production of alcoholic beverages are those of the genus *Hanseniaspora*, which corresponds with the asporigenous genus *Kloeckera* (*Candidaceae*). Currently the six species shown in Table 5.5 are recognized.

**Table 5.5** Species of *Hanseniaspora* and corresponding anamorphic species of *Kloeckera* currently recognized

| **Sporigenous apiculate species (*Hanseniaspora*)** | **Asporigenous anamorphic species (*Kloeckera*)** |
|---|---|
| *H. vinae* | *K. africana* |
| *H. uvarum* | *K. apiculata* |
| *H. guillermondii* | *K. apis* |
| *H. ospmophila* | *K. corticis* |
| *H. valbyensis* | *K. japonica* |
| *H. occidentalis* | *K. javanica* |

*Hanseniaspora* and *Kloeckera* yeasts are widespread in nature. The most frequent species, which are also the most active in natural alcoholic fermentations, are *H. uvarum* (*K. apiculata*) and *H. guillermondii* (*K. apis*). These yeasts have a vigorous fermentation activity limited to monosaccharides (maltose, sucrose and lactose are not fermented). They have a very low ethanol tolerance; the growth of *H. uvarum* stops when the ethanol concentration reaches 4% by volume. Alcoholic fermentations carried out by apiculate yeasts are rich in minor fermentation compounds, especially acetic acid, and for this reason they are generally considered negatively within the fermentation industries producing alcoholic beverages. In fact, they are responsible for one of the most frequent causes of wine spoilage due to their high level of volatile acidity they produce, often above the legal limits. The growth of apiculates, and of *H. uvarum* in particular, can be prevented by adding $SO_2$, to which these yeasts are very sensitive, or by using selected starter cultures of *S. cerevisiae*.

#### 5.2.4.3 Lactose-Fermenting Yeast (Genus *Kluyveromyces*)

The genus *Kluyveromyces* belongs to the family *Saccharomycetaceae* (see Table 5.3) and shows the following general characteristics: cells can be ovoid, ellipsoidal, cylindrical or elongated and they can produce a pseudomycelium. Reproduction occurs by multilateral budding. Conjugation may or may not precede ascus formation. One to four smooth, spherical, reniform, ellipsoidal spores are present in the ascus; one species is known to produce up to 100 spores per ascus. Glucose is fermented vigorously and nitrate is not assimilated. Currently this genus contains 15 species, some of which (*K. marxianus*, *K. lactis* and *K. thermotolerans*) show important technological characteristics such as the ability to ferment lactose, the ability to directly ferment inulin, and the ability to grow at high temperatures (over 40 °C).

*K. marxianus* is probably the most important species of the genus for the fermentation industry. This species comprises strains with different characteristics, and these are generally considered as varieties. The most important variety is *K. marxianus* var. *marxianus*, which includes strains that in the past were ascribed to two different species: *K. fragilis* and *K. marxianus*. Both these former species included strains growing well at high temperatures and possessing the ability to ferment inulin. However, only *K. fragilis* strains were able to ferment lactose. According to the currently accepted classification, both *K. fragilis* and *K. marxianus* are now grouped under the species *K. marxianus*. *Kluyveromyces marxianus* var. *lactis* can ferment lactose but not inulin.

All the strains of *K. marxianus* var. *marxianus* grow well at relatively high temperatures and can directly ferment inulin because they possess the specific enzyme that hydrolyses this polysaccharide (Rouwenhorst et al., 1990). Inulin is a polymer of fructose and has a wide distribution in nature; it accumulates in tubers, such as yam and cassava, as well as onions, which can therefore undergo fermentation. They can therefore intervene in the fermentation of such products, preparing the substrate for oxidation to yam, cassava and onion vinegars.

Some of the strains of the variety *marxianus* (those formerly referred to as *K. fragilis*) as well as all the strains belonging to the variety *lactis*, can ferment lactose. They are therefore able to grow in milk and to carry out an alcoholic fermentation from this substrate and are therefore of interest for whey vinegar production (Parrondo et al., 2003).

#### 5.2.4.4 Osmophilic Yeasts of the Genus *Zygosaccharomyces*

The genus *Zygosaccharomyces* belongs to the family *Saccharomycetaceae* and shows the following general characteristics: multiplication by multilateral budding; spheroidal, ellipsoidal or elongate cells that can form pseudohyphae. Cells are prevalently haploid, sporification is generally preceded by cell conjugation, more rarely between cells and buds. Asci are persistent and contain from one to four spherical or ellipsoidal spores. Glucose is fermented and nitrate is not assimilated. They provide a vigorous fermentation. Currently, nine species are acknowledged within this genus: *Z. bailii*, *Z. bisporus*, *Z. cidri*, *Z. fermentati*, *Z. florentinus*, *Z. mellis*, *Z. microellipsoideus*, *Z. mrakii* and *Z. rouxii*. More recently, based on the nucleotide sequence of the 18S subunit and of the ITS2 region of rDNA, a number of new *Zygosaccharomyces* type of yeasts have been proposed (Steel et al., 1999; Solieri et al., 2007), as well as a species of particular interest for vinegar production named *Z. kombuchaensis* (Kurtzman et al., 2001) which is typically isolated from 'tea fungus' – a complex of several yeast and *Acetobacter* strains that ferment sweetened tea, producing a vinegar beverage known as kombucha (Greenwalt et al., 2000).

*Zygosaccharomyces* yeasts are ubiquitous, always present in sugar-based substrates. They can predominate in some specific circumstances due to their distinctive characteristics. Their cells and spores are highly thermoresistant and some species of the genus can survive the thermal treatments to which some food products are subjected. *Zygosaccharomyces bailii*, in particular, is considered to be one

of the most frequent agents of spoilage of soft drinks, even after the products have been pasteurized (see Stratford, 2006). *Zygosaccharomyces* yeasts are osmophilic and can grow in media with a very high sugar concentration. *Zygosaccharomyces rouxii* and *Z. mellis* were originally isolated from fermented honeys with a sugar concentration close to 80%. Besides honey, they can grow in media such as syrups or concentrated grape must, which represent the fermentative basis for balsamic vinegar production (Solieri et al., 2006). Only a few other yeasts can grow in such environments, among which are yeasts of the genus *Hanseniaspora*.

## 5.3 Moulds

Mould is a generic name indicating a large group of multicellular organisms distributed in different divisions of the Kingdom of Fungi. Typically, they grow as filaments named hyphae of 5-10μm in diameter. Hyphae are surrounded by a wall and extend at their extremities, while drawing the protoplasm forwards as they grow. The hyphae branch repeatedly to form a mycelium and together they constitute the thallus or 'body' of the mould. Moulds are generally disseminated by spores that can be of many different varieties and produced by either an asexual or a sexual process. Moulds that are primarily of interest in vinegar production are mainly in the genus *Aspergillus*.

### 5.3.1 Classification

Moulds have traditionally been classified according to the morphology of the mycelium and of the spores. The moulds of industrial and biotechnological interest are basically grouped in the class *Ascomycetes* and are part of the subclass *Eurotiomycediade*, order *Eurotiales*, family *Trichocomaceae*, which includes the genera *Aspergillus* and *Penicillium*. Within the *Ascomycetes*, the genus *Aspergillus* is the most important for vinegar production. The classification of *Aspergillus* is primarily based on the morphology of some macroscopic (i.e. colony shape and colour) and microscopic features (i.e. shape and type of spores). The most relevant features of *Aspergillus* colonies are the following:

- colour of the vegetative mycelium and of the aerial portion
- pigmentation of the basal mycelium and of the substrate underneath
- texture of the basal mycelium
- development of concentric rings.

The numerous species that are part of this genus have been divided into subgroups or sections; the sections *Fumigati*, *Circumdati*, *Flavi* and *Nigri* contain the most important human pathogens, as well as the fungi of biotechnological interest. In particular the section *Flavi* includes both species used for rice vinegar production, namely *A. oryzae* and *A. sojae*, currently considered the domesticated forms of *A. flavus* and *A. parasiticus*, respectively.

The taxonomy of the genus *Aspergillus*, as well as most other fungi, has undergone several changes according to the taxonomic criteria adopted over time. As for most microorganism classification, the most recent trend is to apply a polyphasic approach that links the evaluation of the micro- and macromorphology of the fungus with an evaluation of its physiology and production of metabolites, as well as information on its genome (Samson et al., 2006).

### 5.3.2 Nutritional Requirements

All types of mould share a strictly aerobic metabolism and depend on pre-formed organic nutrients for energy as well as for the synthesis of cellular materials. They obtain these nutrients by absorbing simple, soluble nutrients (e.g. sugars, amino acids) through their walls, and by releasing extracellular enzymes to degrade polymers that they cannot absorb directly.

Moulds grow well in presence of a source of organic carbon for energy, a source of nitrogen for protein and vitamin synthesis, and several minerals; basically, they are ubiquitous.

### 5.3.3 Metabolic Activity

Moulds produce a wide range of secondary metabolites, including compounds with antibiotic properties, pigments, flavour and odour components. The release of a large variety of enzymes is a trait common to most moulds and this has been largely exploited in an industrial context. Moreover, the relatively high efficiency of growth of some moulds has made them the ideal organism to be used biotechnologically to extract a number of compounds such as organic acids.

Among the most important metabolites produced by moulds are potent toxins termed mycotoxins, which are highly toxic to some animals, causing liver and kidney damage and also showing carcinogenic potential (see Cary et al., 2000). The most notorious mycotoxins are aflatoxin and ochratoxin, which are primarily produced by *A. flavus* and *A. ochraceus*. However, with a different degree of toxicity, they can potentially be produced by other members of the *Aspergillus* genus as well. The topic is still the subject of numerous studies and some mycotoxins have not been identified yet; however, the spontaneous growth of moulds, especially on foodstuffs, is always considered undesirable.

### 5.3.4 Genera and Species Relevant to Vinegar Production

*Aspergillus oryzae* and *A. sojae* are the most common moulds associated with vinegar production. They constitute part of the so-called *koji*, a mouldy rice substance that has the function to break down the starch molecules of rice into simple fermentable sugars in the production of the alcoholic base for rice vinegars. *A. oryzae*

is considered the domesticated form of *A. flavus*; however, unlike *A. flavus*, it does not release mycotoxin and is therefore considered a safe organism to be used in foodstuff production (Matsushima et al., 2001). Due to its high biotechnological value, it is a very well-studied species used by the enzyme industry to produce numerous enzymes that are applied in a variety of fields. Its genome has been fully sequenced (Machida et al., 2005) and numerous cultures are being genetically manipulated in order to optimize the production of enzymes and other metabolites of industrial interest (e.g. Christensen, 1994).

## 5.4 Lactic Acid Bacteria

Lactic acid bacteria (LAB) represent a homogeneous group of microorganisms showing the following general characteristics: cells are regular, in the shape of cocci or rods, they are not mobile, Gram-positive and do not form spores. Figure 5.2 shows an example of lactic acid bacteria.

### 5.4.1 Classification

The earliest LAB classification was established by Orla-Jensen in 1919, and over the years it has undergone several modifications that, however, have maintained the

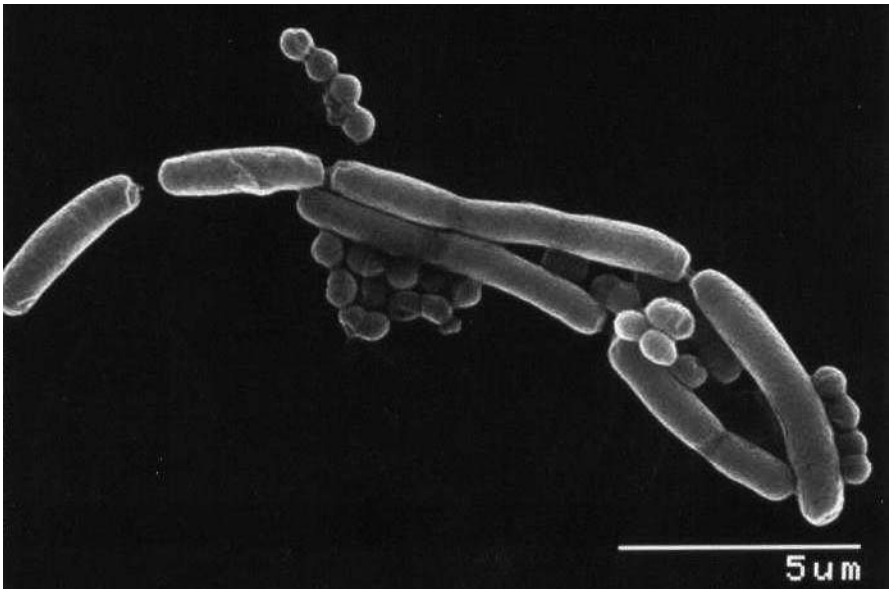


**Figure 5.2** Cells of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, typical LAB of fermented milk and whey, photographed under a scanning electron microscope (SEM) at 6000× magnification

**Table 5.6** Lactic acid bacteria classification according to Orla-Jensen (1919)

| Cell shape | Type of fermentation | Genus | Notes |
|---|---|---|---|
| Cocci | Homofermentative | *Streptococcus* | – |
| | Heterofermentative | *Betacoccus* | – |
| Rods | Homofermentative | *Thermobacterium* | Thermophilic |
| | | *Streptobacterium* | Mesophilic |
| | Heterofermentative | *Betabacterium* | – |

**Table 5.7A** Current status of cocci-shaped lactic acid bacteria classification

| Cell organization | Fermentation | Lactic acid | Genus of 1986[a] | Current genus |
|---|---|---|---|---|
| Chains | Homofermentative | L(+) lactic acid | *Streptococcus* | *Streptococcus*<br>*Lactococcus*<br>*Enterococcus* |
| Tetrads | Homofermentative | DL lactic acid | *Pediococcus* | *Pediococcus* |
| Chains | Heterofermentative | D(–) lactic acid | *Leuconostoc* | *Leuconostoc*<br>*Oenococcus*<br>*Weisella* |

[a] Sneath et al. (1986).

**Table 5.7B** Current status of rod-shaped lactic acid bacteria classification

| Genus | Group | Fermentation |
|---|---|---|
| *Lactobacillus* | Group I | Homofermentative |
| | Group II | Homofermentative, facultatively heterofermentative |
| | Group III | Heterofermentative |

original basic structure. From a physiological point of view, LAB are rather homogeneous and thus they have long been gathered into one single family. Since 1986 they have been grouped on the basis of their cell morphology (Sneath et al., 1986).

Tables 5.6 and 5.7 show the earliest and the currently acknowledged classifications (Stiles and Holzapfel, 1997), respectively.

## 5.4.2 Nutritional Requirements

Lactic acid bacteria are facultative anaerobes and have complex nutritional requirements. To grow they require a medium containing all the amino acids and many vitamins that they are not able to synthesize. They are catalase-negative, with the exception of a number of species of the genus *Pediococcus*.

Some LAB species are mesophilic and some are thermophilic. The latter have an optimal temperature of growth above 40 °C and are all homofermentative, with cells shaped as rods (genus *Lactobacillus*). Among the homofermentative cocci, the species *Streptococcus thermophilus*, in spite of having an optimal temperature of growth below 40 °C, can also grow at an extremely high temperature; it therefore grows well in the widest range of temperatures.

Lactic acid bacteria grow well at pH 7; however, they can also grow in habitats with very low pH values. Some species, either homo- or heterofermentative, can grow at pH values between 3 and 4. Moreover, LAB produce acids that lower the pH values of their growth substrate.

### 5.4.3 Metabolic Activity

Lactic acid bacteria can ferment sugars following two different metabolic pathways; (i) via the glycolytic pathway (Embden-Meyerhof-Parnas pathway) that, under standard conditions, causes the conversion of sugars almost exclusively to lactic acid; and (ii) via the 6-phosphogluconate/phosphoketolase pathway, through which sugars are converted into lactic acid, acetic acid, ethanol and $CO_2$. Lactic acid bacteria that metabolize sugars according to the first pathway are referred to as homofermentative; whereas lactic acid bacteria following the second pathway are defined as heterofermentative.

In homolactic fermentation, 1 mol of glucose yields 2 mol of lactic acid:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Lactic acid}}{2\,CH_3CHOHCOOH}$$

In heterolactic fermentation, 1 mol of glucose yields 1 mol each of lactic acid, ethanol and carbon dioxide:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Ethanol}}{CH_2H_5OH} + \underset{\text{Carbon dioxide}}{CO_2}$$

When glucose is used as carbon source, two fermentation patterns can be described for heterofermentative lactic acid bacteria (Nelson and Werkman, 1935):

The production of equimolar quantities of lactate, ethanol, and carbon dioxide, with occasional traces of acetate.

The formation of glycerol along with lactate, acetate and carbon dioxide.

Most LAB can ferment lactose and can therefore grow and reproduce in milk and dairy products. The physiology, metabolism and classification of LAB have recently been reviewed by Axelsson (2004).

### 5.4.4 Importance for Vinegar Production

Lactic acid bacteria are ubiquitous, widespread in soil and water, common contaminants of foodstuffs. Because of their characteristics they are widely employed in the fermentation and food industries. In particular, they play a major role in the production of several fermented foods, such as all cheeses, cured sausages (Italian salami), bakery products and preserved forages.

As a consequence of producing acid and lowering the pH of the environment, LAB can counteract putrid reactions; in fact, putrifying bacteria are very sensitive to low pH values and usually cannot live in the same habitat as LAB. Some LAB

inhabit the digestive tracts of mammals; 'probiotics' is a science based on the properties of this type of lactic acid bacteria. They are used industrially for the production of lactic acid obtained by fermentation.

The growth of some LAB is at times undesirable; this is the case, for example, for some *Enterococci* that, being thermotolerant, can survive after some fermented sausages are cooked and can be the cause of the souring of the final product.

#### 5.4.4.1 Lactic Acid Bacteria in Alcoholic Fermentations

Lactic acid bacteria can be of interest in the production of fermented beverages, as they can grow both before and after the fermentation process. On some occasions they are desired and are used as starter cultures; however, in some other cases their growth is considered detrimental.

In the case of beer, for example, specific LAB can be used to lower the pH of wort, guaranteeing the biological stability of the final product (Lowe and Arendt, 2004). In the case of wine, lactic acid bacteria can be helpful in reducing the total acidity by fermenting malic acid and encouraging the wine maturation process. Malolactic fermentation is generally determined by the heterofermentative *Oenococcus oeni*, often together with homo- or heterofermentative lactobacilli (Liu, 2002).

The growth of LAB in beer after wort fermentation is considered negatively, as it causes hazing of the product and increases the level of acidity excessively. In wines, their growth at the expense of residual sugars can cause spoilage, and the spoiled product can then only be employed in distillery.

For vinegar production, the growth of LAB in the above-mentioned alcoholic beverages is not necessary and does not affect the acetic acid bacteria (AAB) activity.

Milk and whey, a by-product of cheese making, are not ideal substrates for yeast growth, due to their high pH value and their sugar composition. Yeasts can grow in milk only if its pH is lowered to a value of 5, and this occurs as a consequence of a previous lactic acid fermentation carried out by LAB. Lactic acid bacteria therefore play a major role in the production of alcoholic beverages obtained from milk and whey. Lactic acid bacteria that use lactose as a sole carbon source are numerous and widespread in all genera and species. Some conditions, primarily temperature, can favour one or another species. Just after milking, at 37 °C, homofermentative bacteria with a high optimum or maximum growth temperature will grow: these belong to the species *Streptococcus thermophilus* and to some thermophilic *Lactobacillus* species, such as *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. lactis* and *Lb. helveticus*. At lower temperatures, up to 30 °C, *St. thermophilus*, a highly adaptable species, can still grow; however, mesophilic homo- and heterofermentative species generally prevail.

Several LAB, among which *St. thermophilus* and the heterofermentative *Lb. kefir*, are very frequent in fermented milks. They hydrolyse lactose but ferment only its glucose component and not galactose, which is therefore released into the medium and remains available as a nutrient together with some lactose. As a con-

sequence, both lactose-fermenting yeasts, such as those belonging to the genus *Kluyveromyces*, as well as galactose-fermenting yeasts such as *Saccharomyces* yeasts, can grow in such fermented milks (Giudici et al., 1996; Montanari et al., 1996).

## 5.5 Nematodes

The nematodes or roundworms are one of the most common phyla of animals, with over 20,000 different species described (including over 15,000 parasitic species). They are ubiquitous in freshwater, marine and terrestrial environments.

Nematodes are one of the simplest animal groups to have a complete digestive system, with a separate orifice for food intake and waste excretion; a pattern followed by all subsequent, more complex animals. Their nutrition generally depends upon the genus and species and includes bacteria, fungus, algae, protozoa, and animal and vegetal protoplasm. Reproduction is usually sexual; however, hermaphroditic and parthenogenetic species are also known.

Ecologically, they can be divided into free-living and parasitic forms. Free-living nematodes generally measure 1 mm in length, whereas parasitic species can be bigger (on average 8 mm of length). Taxonomically, nematodes can be divided into two classes (Klingler, 1986):

- Secernentea (or Phasmidiae), including most terrestrial free-living and parasitic species.
- Adenophorea (or Aphasmidia), generally represented by marine or free-living animals.

### 5.5.1 Nematodes in Vinegar Production

Nematodes are ubiquitous, inhabiting damaged fruit such as grapes and apples, and as a consequence they can often be seen swimming on the surface of vinegars. Not much is known about the role and the effect of vinegar eels on vinegar production and not many studies have been carried out to clarify this issue.

Shann (1987) reported for the first time the presence of a nematode (*Panagrellus zymosiphilus*) on damaged grapes of an Italian vineyard. He carried out a pioneer study in which demonstrated that such nematodes, besides carrying a yeast-like fungus (*Botryzyoma nematodophila*), also harbour a large amount of AAB on their surface. This saprophytic system seems to have an influence on the development of sour rot of grapes.

Recently, Buchholz et al. (2005) identified a large number of vinegar nematodes in traditional balsamic vinegar. The authors classified the nematode as *Turbatrix aceti*, formerly known as *Anguillula aceti* and colloquially referred to as the 'vinegar eel' or 'vinegar worm'. *Turbatrix aceti* is often found in vinegars obtained from apples or other fruits as well as in other fermented foods. Figure 5.3 shows a sample of *Turbatrix aceti* isolated from traditional balsamic vinegar.

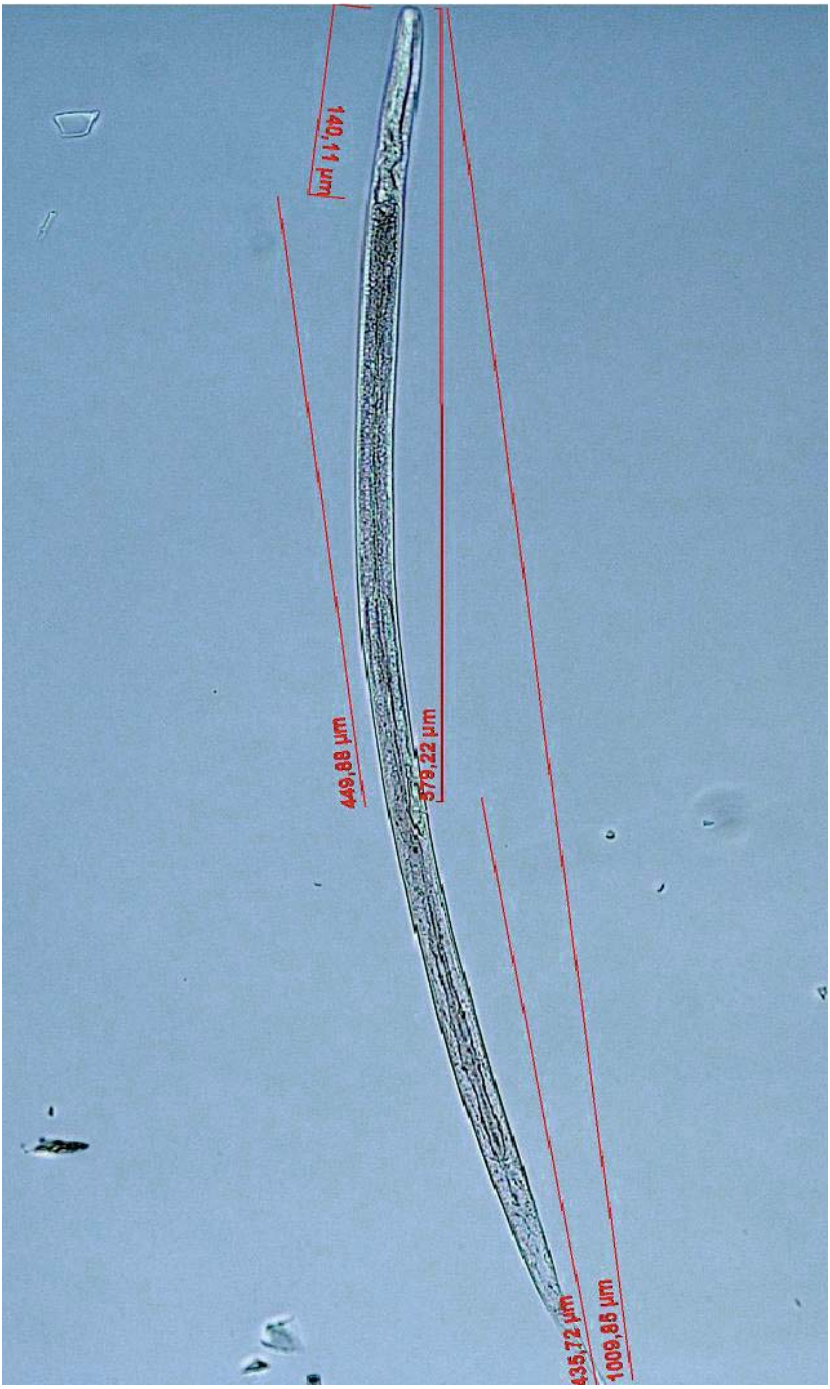


**Figure 5.3** The nematode *Turbatrix aceti*: body of the nematode with key measurements

## 5.6 Insects

Small flies belonging to the family Drosophilidae play a major role in vinegar production, especially in the case of wine and cider vinegars. The genus *Drosophila* hosts the most common flies that can be found in vinegar, and it also contains about 1500 species that are very diverse in appearance, behaviour and breeding habitat. One species of *Drosophila* in particular, *D. melanoganster*, has been extensively used as a model organism in genetic and developmental biology studies. *Drosophila* flies are commonly known as 'fruit flies' or 'vinegar flies'; in fact their existence is very often associated with the presence of such substrates.

### 5.6.1 The Role of Fruit Flies in Vinegar Production

Fruit flies feed on fresh as well as rotting and fermenting fruit and vegetables, and are therefore always found in such habitats. Generally they are considered as a negative, undesired element in food fermentations as well as in ethanol oxidation processes, as they compromise the sanitary quality of the final product. However, fruit flies play a relevant biological role both in fermentation and ethanol oxidation, as they are basically the vector to most microorganisms involved in such processes. Several studies have highlighted, for example, the finding that fermenting yeasts are often transported by insects such as fruit flies, which feed on them and carry yeast spores into their digestive tract (Mortimer and Polsinelli, 1999; Pulvirenti et al., 2002). This system allows the spreading of fermenting yeasts as well as other organisms. Shann (1987), in fact, reported that fruit flies were responsible not only for the transport of yeasts but also of nematodes that in turn were found to carry a large amount of AAB on their surface. Unfortunately, besides good fermenting yeasts and oxidising bacteria, other more undesirable microorganisms, responsible for fermentation defects or grape diseases and further oxidation processes, are also transported.

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# Chapter 6
# Vinegar Engineering

**Isidoro García-García, Inés María Santos-Dueñas, Carlos Jiménez-Ot, Jorge Eugenio Jiménez-Hornero and José Luis Bonilla-Venceslada**

## 6.1 Introduction

Decades of research, particularly as regards the study and development of submerged microbial cultures, have allowed the acetification process to be improved in such a way that it now provides vinegar of much higher quality and in substantially higher yields, all with a high reproducibility (Emde, 2006).

Accurate figures for vinegar production are difficult to obtain owing to the lack of official statistics in many countries and regions. In the European Union, production is estimated to amount to $5 \times 10^6$ hL of vinegar containing 10% acetic acid (Ebner et al., 1996a; Puyó, 2006).

Like any other modern industrial production process, acetification is necessarily a multidisciplinary process, requiring the integration of knowledge from various areas in order to solve, in an industrially feasible manner, the problems it poses.

Because the core process is a biochemical transformation effected by bacteria, optimizing the outcome requires the knowledge of a body of microbiological and biochemical concepts, in addition to the involvement of chemical, biochemical, civil, electronic, automation and control engineering methods. Also, because vinegar is primarily used as a food, its production must necessarily comply with legal and health regulations.

## 6.2 The Industrial Process of Vinegar Production

Although industrial acetification processes can vary slightly depending on the particular raw material and type of vinegar produced, all are essentially very similar. The process involves preparing the alcoholic substrate to be used as the culture medium, effecting its biological oxidation, and performing the separation and conditioning operations required to obtain the finished product. Figure 6.1 shows some selected operations that are required, depending on the particular type of vinegar to be obtained.

L. Solieri, P. Giudici (eds), *Vinegars of the World*

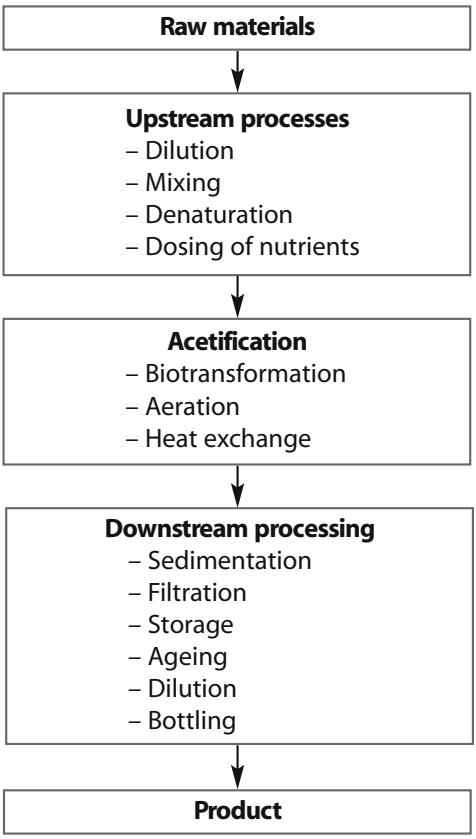


**Figure 6.1** General flow diagram for vinegar production showing the most usual unit operations

Because examining the whole body of operations performed at a vinegar production plant, or even thoroughly describing in full detail any individual operation, is beyond the scope of this chapter, the following sections focus on some of the more salient technological aspects of the process.

## 6.3 Biotransformation

For a number of reasons, the biotransformation of ethanol to acetic acid under the action of *Acetobacter* and *Gluconobacter* spp. (see Figure 6.2) is the most important step in the process.

$$C_2H_5OH + O_2 \rightarrow CH_3COOH + H_2O \quad (\Delta H = -493 \text{ kJ})$$

The bacteria convert the alcohol into the acid with a high efficiency (95-98% of the stoichiometric value, the remainder being lost through sweeping in the air

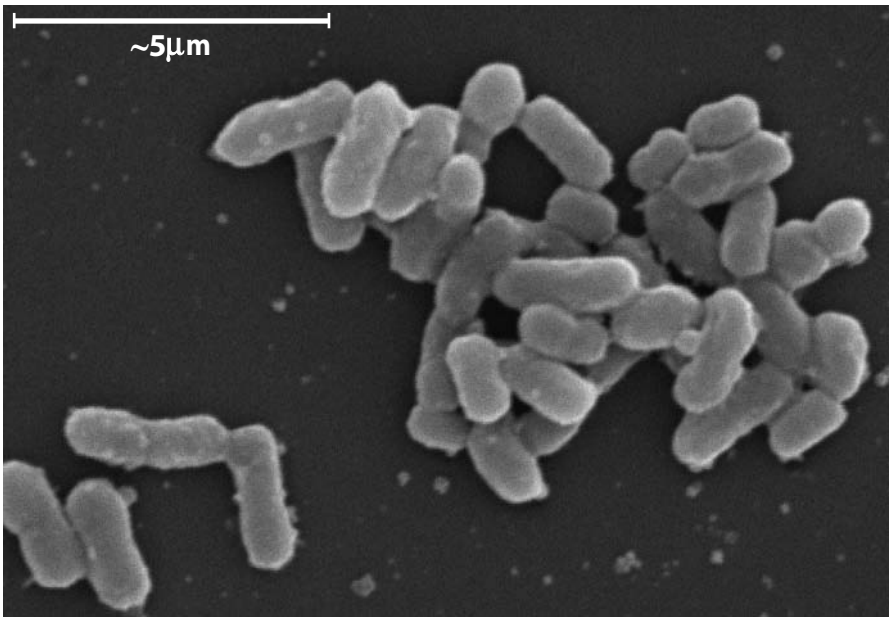


**Figure 6.2** Scanning electron microscopy (SEM) of acetic acid bacteria from a laboratory wine acetification bioprocess

stream used to oxygenate the medium). Based on the strictly aerobic nature of the bacteria and the amount of oxygen used in the reaction, the process exhibits a high oxygen demand. For example, an industrial tank holding 25,000 L of culture medium and operating at an acetification rate of 0.2% acetic acid · $h^{-1}$ will require about 20,000 L of oxygen (at 20 °C, 1 atm) per hour (i.e. 26.7 kg $O_2$ · $h^{-1}$). Because the oxygen is supplied from an air stream circulated through the culture medium, this would entail supplying about 95,000 L of air (at 20 °C, 1 atm) per hour; however, only 60-90% of the oxygen supply is used to oxidize the alcohol, so the volume of air at 20 °C and 1 atm required to meet the oxygen demand of the process is 100,000-150,000 L · $h^{-1}$. The risk of volatiles (particularly ethanol) being swept by such a massive air supply is therefore very high.

The sensitivity of acetic acid bacteria (AAB) to oxygen-deficient conditions (Nieto, 1991; Ebner et al., 1996b) can slow down the process or even render the bacteria unviable unless the medium is efficiently oxygenated on a continuous basis.

### 6.3.1 Oxygen Mass Transfer

Experience has shown that the overall acetification rate is controlled by mass transfer from the gas phase to the microorganisms. Based on the double film theory, oxygen must travel through various zones until it reaches the outer surface of the

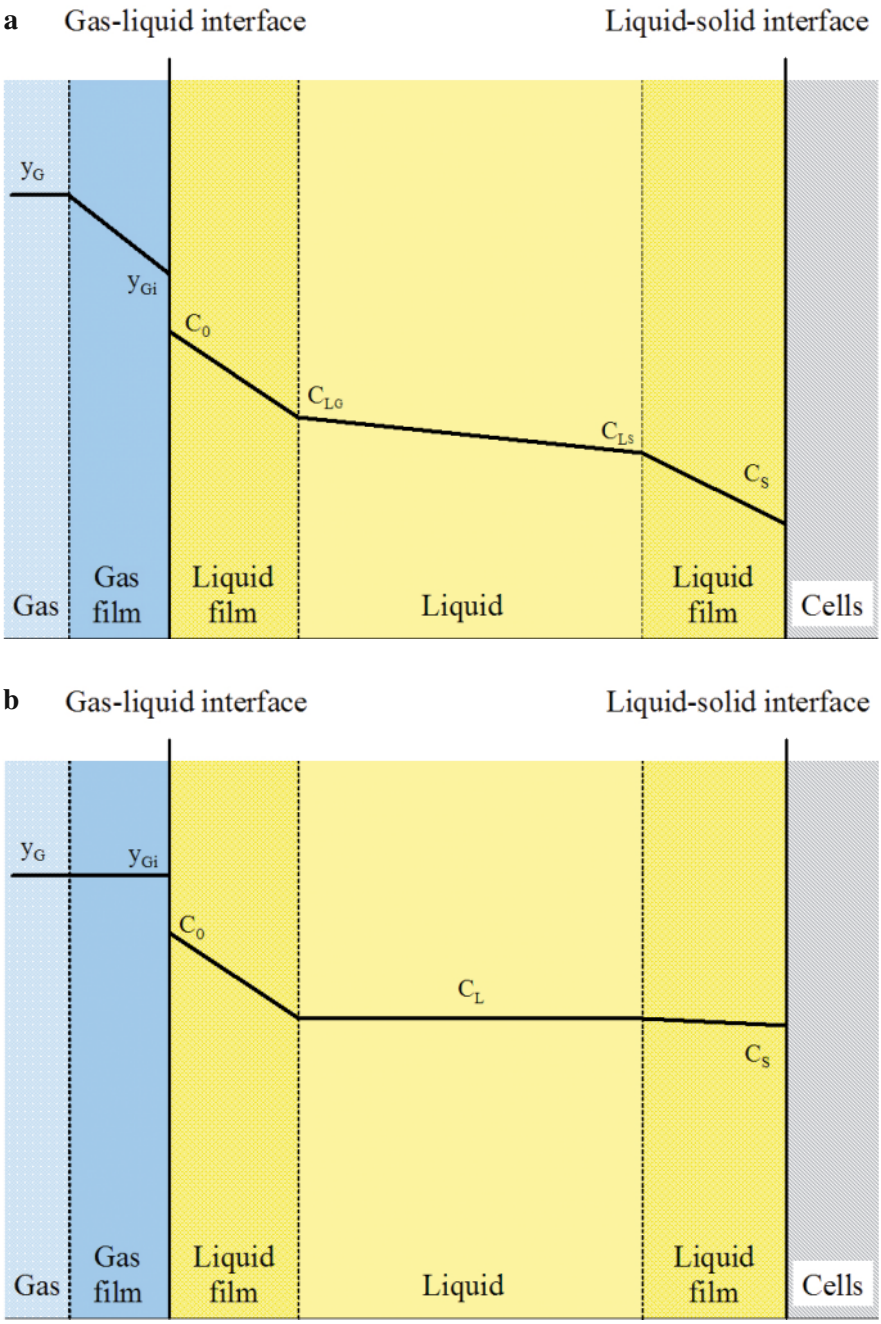


**Figure 6.3** Oxygen transfer from air to bacterial cells according to film theory: **a** concentration profile of oxygen taking into account all possible resistances; **b** concentration profile of oxygen when controlling the liquid film in contact with the gas-liquid interface

microbes (see Figure 6.3). In principle, oxygen transfer from the bulk gas phase to microbial surfaces can be hindered by (i) the gaseous film, (ii) the liquid film in contact with the gas-liquid interface, (iii) mass transfer in the bulk liquid phase and (iv) the liquid film in contact with the liquid-solid interface between the medium and the microorganisms (see Figure 6.3a).

Because the resistance to mass transfer within bubbles in this type of system is usually negligible (Blackebrough, 1967), the concentration at the interface is identical with that in the bulk gas phase (see Figure 6.3b). Also, because the concentrations on both sides of the interface are taken to be equilibrium concentrations, the interface is assumed to offer no resistance to mass transfer. On the other hand, the liquid film in contact with the gas-liquid interface does oppose mass transfer, to an extent dependent on a number of factors, and results in a concentration gradient from the interface to the film boundary. The reciprocal of the mass transfer coefficient for the film provides a measure of the film resistance. By virtue of the turbulence usually present in the medium, mass transfer from the outside of bubbles to the neighbourhood of the microorganisms is unconstrained. This ensures a largely uniform concentration of dissolved oxygen in the bulk liquid phase. Finally, the resistance of the liquid film in contact with the liquid-solid interface is negligible, as the interface surface area is typically several orders of magnitude greater than that of the gas-liquid interface. As a result, the volumetric mass transfer coefficient for the film in contact with the cells must also be much higher than that for the liquid film in contact with the gas phase.

#### 6.3.1.1 Liquid Film: Controlling Resistance

In summary, the only resistance to be considered in the overall process by which oxygen is transferred from the gas phase is that posed by the liquid film in contact with the gas-liquid interface, which is the sole region where a concentration gradient can exist (see Figure 6.3b). Therefore, the overall productivity will depend on the rate of mass transfer across such a film:

$$N_{O_2} = k_L a \, \Delta c \qquad \text{(Eqn 1)}$$

In those reactors where the gas phase undergoes no thorough mixing or where there are substantial differences in hydrostatic pressure between the bottom and top, $\Delta c$ is not constant throughout the reactor (Zlokarnik and Judat, 1988). In fact, under such conditions, different $y_G$ values can be found in the reactor from the bottom to the top; if, in addition, the gas pressure at the bottom and top of tank is different, then the equilibrium concentration, $c_O$, will also be different. Under these conditions, $\Delta c$ should be replaced with its logarithmic mean:

$$\overline{\Delta c} = \frac{\left(c_O^i - c_L\right) - \left(c_O^e - c_L\right)}{\ln\left(\dfrac{c_O^i - c_L}{c_O^e - c_L}\right)} \qquad \text{(Eqn 2)}$$

**Table 6.1** List of parameters and abbreviations

| | |
|---|---|
| $a$ | Surface area of the gas-liquid interface (Eqn 5) |
| $c_O^e$ | Oxygen saturation concentration in the culture medium under the conditions prevailing in the upper part of the reactor (Eqn 2) |
| $c_O^i$ | Oxygen saturation concentration in the culture medium under the conditions prevailing in the lower part of the reactor (Eqn 2) |
| $c_L$ | Concentration of dissolved oxygen in the culture medium (Eqn 2) (Fig. 6.3) |
| $c_{LG}$ | Concentration of dissolved oxygen in the culture medium close to the liquid film in contact with the gas-liquid interface (Fig. 6.3) |
| $c_{Ls}$ | Concentration of dissolved oxygen in the culture medium close to the liquid film in contact with the liquid-solid interface (Fig. 6.3) |
| $c_O$ | Oxygen saturation concentration in the liquid phase at the gas-liquid interface (Fig. 6.3) |
| $c_S$ | Concentration of dissolved oxygen in the liquid phase at the liquid-solid interface (Fig. 6.3) |
| $d$ | Particle diameter of the dispersed phase (Eqn 7) (Eqn 8) |
| $D_{32}$ | Sauter mean bubble diameter (cm) (Eqn 4) (Eqn 5) |
| $D_L$ | Diffusion coefficient for the continuous phase (Eqn 7) (Eqn 8) |
| DSHS | Dual-stage high-strength process |
| $E_{O_2}$ | Aeration efficency kg $O_2 \cdot kW^{-1} \cdot h^{-1}$ |
| $g$ | Acceleration due to gravity (Eqn 8) |
| $(hp \cdot V^{-1})$ | Agitator horsepower per vessel volume $(hp \cdot ft^{-3})$ (Eqn 4) |
| $k_L$ | Mass transfer coefficient for the liquid film in contact with the gas-liquid interface |
| $k_L a$ | Volumetric mass transfer coefficient across the liquid film in contact with the gas-liquid interface (Eqn 1) (Eqn 3) (Fig. 6.4) |
| $m$ | Multiplying factor (Eqn 3) (Fig. 6.4) |
| $N_{O_2}$ | Oxygen flow from the gas phase to the liquid phase (Eqn 1) |
| $N_{Ra}$ | Raleigh number (dimensionless) (Eqn 6) (Eqn 8) |
| $N_{Sh}$ | Sherwood number (dimensionless) (Eqn 6) (Eqn 7) |
| PLC | Programmable logic controller |
| $Q_{O_2}$ | Aeration efficency kg $O_2$ transferred $m^{-3} \cdot h^{-1}$ |
| $y_G$ | Oxygen concentration in the bulk gas phase (Fig. 6.3) |
| $y_{Gi}$ | Oxygen concentration in the gas phase at the interface (Fig. 6.3) |
| $\gamma$ | Specific gravity of the liquid (Eqn 4) |
| $\Delta c = c_O - c_L$ | Concentration difference or driving force between the interface and the bulk liquid phase (Eqn 1) |
| $\Delta \bar{c}$ | Logarithmic mean of the driving force between the upper and lower regions of the reactor (Eqn 2) |
| $\Delta H$ | Heat reaction, $kJ \cdot mol^{-1}$ |
| $\Delta\rho$ | Density difference between the dispersed and continuous phases (Eqn 8) |
| $\varepsilon$ | Fractional gas hold-up (Eqn 4) (Eqn 5) |
| $\mu_c$ | Viscosity of the continuous phase (Eqn 8) |
| $\mu_g$ | Viscosity of the gas (Eqn 4) |
| $\mu_l$ | Viscosity of the liquid (Eqn 4) |
| $\sigma$ | Surface tension $(dyne \cdot cm^{-1})$ (Eqn 4) |
| % acetic acid | g acetic acid $\cdot$ 100 $mL^{-1}$ of medium |
| % ethanol | ml ethanol $\cdot$ 100 $mL^{-1}$ of medium |

Therefore, the productivity of acetification essentially depends on the transfer coefficient and driving force. Experience has shown that the dissolved oxygen concentration is very low except during loading and unloading of the reactor. One should bear in mind that the air flow-rate should be low enough to avoid entrainment losses, but high enough to ensure efficient uptake. This entails ensuring a high oxygen transfer rate. Therefore, the factor driving force provides little room for accelerating the process; if the dissolved oxygen concentration is very low, however, the driving force at each point in the reactor will be very close to the maximum possible value.

Based on the foregoing, mass transfer in the reactor can only be optimized via the volumetric coefficient, which is in fact the product of two factors, namely the coefficient of mass transfer across the film, $k_L$, and the surface area of the gas-liquid interface ($a$). Usually, the two are determined jointly, as the volumetric coefficient of mass transfer, owing to the difficulty involved in their individual measurement. In principle, both should be as high as possible in order to ensure an increased flow of oxygen to be absorbed by the culture medium.

Mass transfer between phases may be strongly influenced by interfacial phenomena (Fair et al., 1973, 2001) of diverse origin, one of the most interesting of which is the increased interfacial area resulting from a low surface tension in the culture medium; in fact, the surface tension of the medium is typically lower than that of water (i.e. 50-60 vs 71 dyne · $cm^{-1}$ at 30 °C).

A low surface tension decreases coalescence of the bubbles formed in the device used to disrupt the incoming air stream. Any compounds in the culture medium with influence on the suppression of bubble coalescence will therefore prevent the interfacial area from reducing after the air stream leaves the disrupting device.

This phenomenon has been widely studied by adding various types of substances to pure solvents and examining their effect on volumetric coefficients of mass transfer via the ratio of the coefficient for the solution to that for the pure solvent (Zlokarnik, 1985):

$$m = \frac{(k_L a)_{\text{solution}}}{(k_L a)_{\text{pure water}}} \qquad \text{(Eqn 3)}$$

This parameter is known as the multiplying factor. As can be seen from Figure 6.4, which illustrates the influence of the ethanol concentration in water on it, raising such a concentration from ca. $2 \cdot 10^{-3}$ M to $5 \cdot 10^{-1}$ M increases the volumetric coefficient of mass transfer by a factor of 7.

Figure 6.5 illustrates the effect of the presence of ethanol in water on the number and size of bubbles formed in a Frings 8-L laboratory reactor. The reactor in Figure 6.5a contained pure water, whereas that in Figure 6.5b was supplied with a 1% ethanol concentration; with the aeration regime and all other conditions being identical in both cases.

These devices allow very small bubbles (0.1-1.0 mm) to be obtained. Bubble size depends on a number of factors, the most prominent of which include the surface tension, viscosity of the gas and fluid, agitator power input, and fraction of gas

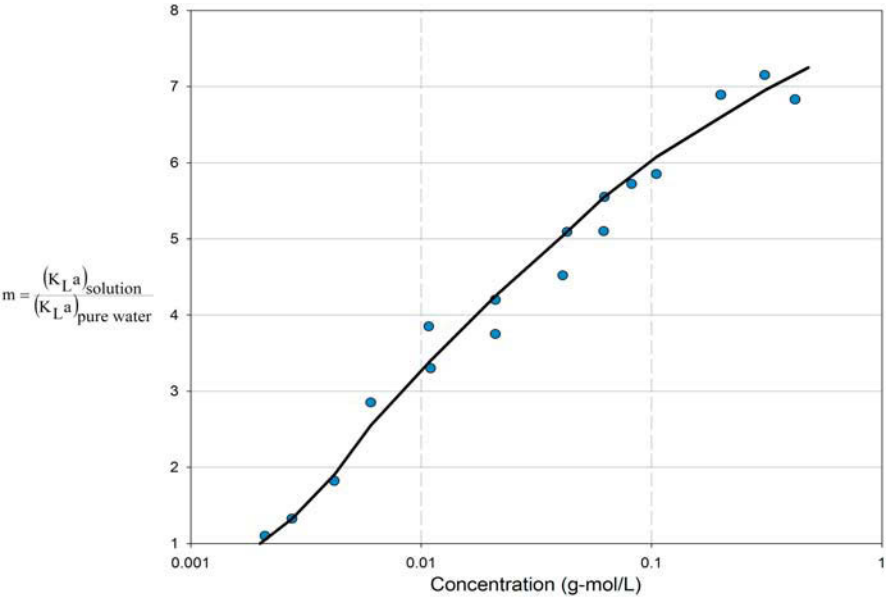


**Figure 6.4** Ratio between volumetric liquid film mass-transfer coefficients for ethanol solutions and pure water as a function of the ethanol concentration (Zlokarnik, 1985)

retained in the tank. An average value can be estimated by using the following equation (Fair et al., 1973):

$$D_{32} = 0.013 \frac{\sigma^{0.6}}{(hp/V)^{0.4}\gamma^{0.2}} \varepsilon^{0.65} \left(\frac{\mu_g}{\mu_l}\right)^{0.25} \qquad \text{(Eqn 4)}$$

This equation provides bubble diameters consistent with those experimentally determined for the culture media typically used to obtain vinegar.

For spherical bubbles (i.e. small bubbles), the interfacial area, $a$ ($cm^{-1}$), can be estimated from:

$$a = 6\frac{\varepsilon}{D_{32}} \qquad \text{(Eqn 5)}$$

Because the physical properties of the system provide virtually no room for alteration, the bubble diameter and interfacial area depend largely on the agitator power. Industrial agitators with a specific power input of 0.1-1 $kW \cdot m^{-3}$ usually result in interfacial areas of ca. 300-1000 $m^{-1}$; in any case, the area is strongly influenced by the specific type of equipment and the properties of the culture medium (Zehner and Kraume, 1992).

The problem of mass transfer in gas-liquid dispersions such as those formed in aerated mixed vessels was studied as early as 1961 (Calderbank and Moo-Young,


**Figure 6.5** Photographs that demonstrate the coalescence phenomena in the same bioreactor (Frings 8-L) under similar conditions except for the ethanol concentration: **a** using tap water; **b** using an ethanol (1%) solution in water

1961). For small bubbles (no larger than ca. 0.8 mm in diameter), $k_L$ can be related to the following parameters:

$$N_{Sh} = 2.0 + 0.31\, N_{Ra}^{1/3} \quad \text{(Eqn 6)}$$

$$N_{Sh} = \frac{k_L d}{D_L} \quad \text{(Eqn 7)}$$

$$N_{Ra} = \frac{d^3 \Delta \rho g}{\mu_c D_L} \quad \text{(Eqn 8)}$$

These equations provide a coefficient of mass transfer across the film, $k_L$, of ca. $1.5 \times 10^{-4}$ m · s$^{-1}$ – values typically range from $1 \times 10^{-4}$ to $2.5 \times 10^{-4}$ m · s$^{-1}$.

Based on the foregoing, the volumetric coefficient of mass transfer ($k_L a$), while dependent on a number of factors, typically ranges from 100 to 900 h$^{-1}$.

However, the choice of a specific bioreactor cannot rely solely on the volumetric coefficient of mass transfer; rather, the choice should consider the aeration efficiency, which is usually measured in terms of $Q_{O_2}$ (kg $O_2$ transferred · m$^{-3}$ · h$^{-1}$) or $E_{O_2}$ (kg $O_2$ · kW$^{-1}$ · h$^{-1}$). In practice, $Q_{O_2}$ typically ranges from 4 to 12, and $E_{O_2}$ from 0.9 to 4 (Voss, 1992).

In any case, one should bear in mind the need to efficiently use the oxygen fed to the system. This can be accomplished by using equipment with a high transfer coefficient, at the expense of an higher risk of increased volatile losses as a result of the high flow-rates needed. A compromise must therefore be made between the efficiency with which the amount of energy and oxygen used are employed.

### 6.3.2 Bioreactor

Ultimately, the best choice of bioreactor for the intended purpose is dictated by a number of factors. The primary goal is to achieve highly efficient oxygen transfer without significant yield losses through sweeping of volatiles.

Unsurprisingly, designing efficient aeration systems for acetators has raised an interesting scientific and technical challenge. Referring to the bioreactor in this context is essentially pointing to the aeration system. The firm Heinrich Frings has for decades led research in this field. More recently, several other firms including Cetocec Biotechnologie GmbH and Vogelbusch GmbH have joined the list of major acetator manufacturers.

The Frings bioreactors (Acetator) use a turbine consisting of a hollow body surrounded by a fixed piece called the stator. In spinning, the turbine generates a vacuum that aspirates air from the outside. This is thus a self-aspirating system and does not require the use of compressed air to oxygenate the medium. Aspirated air and the substrate are mixed between the stator and turbine prior to radial injection into the culture medium (see Figure 6.6); additionally, the aeration efficiency is close to the aforementioned maximum values for $Q_{O_2}$ and $E_{O_2}$. This ensures not only efficient oxygenation, but also efficient mixing of the culture.

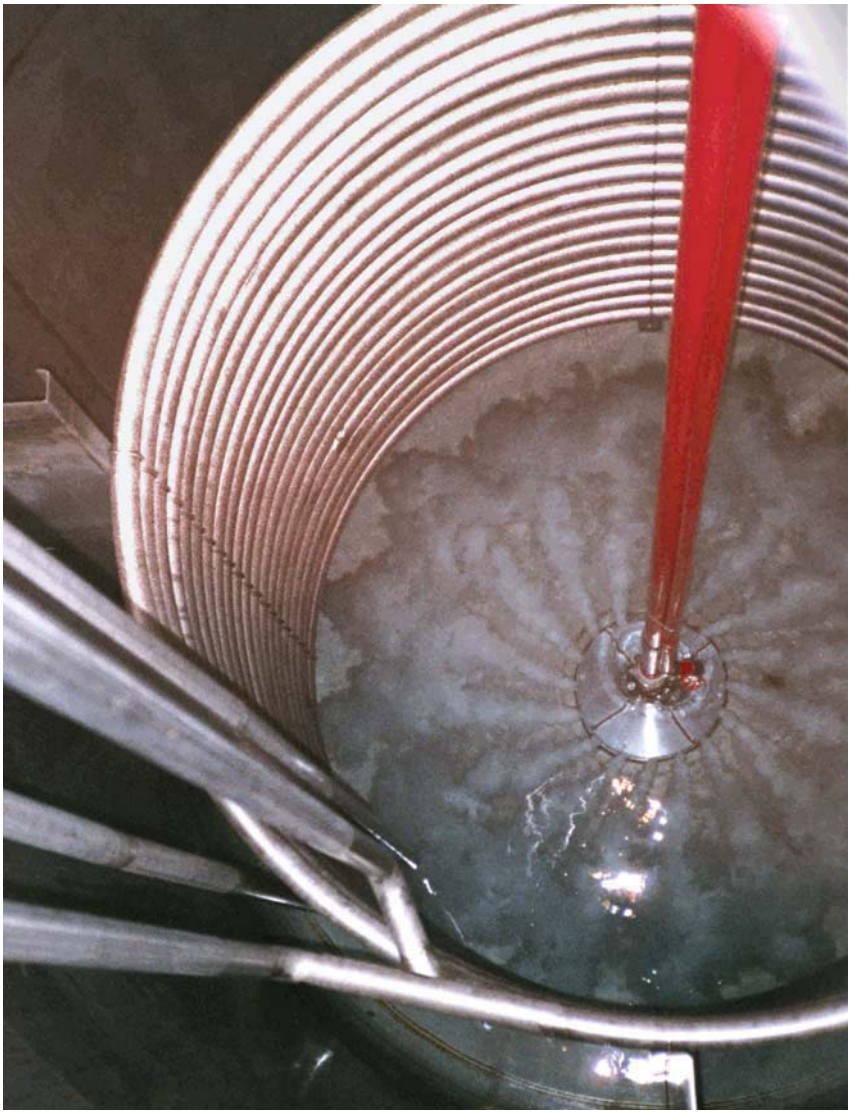

**Figure 6.6** Interior of an industrial Frings Acetator (courtesy of Heinrich Frings)

This turbine uses 60-90% of all the oxygen supplied. Despite the high air flow-rate used, ethanol conversion is in the region of 95-98% of the theoretical value. The system requires the use of a device to condense volatiles in exhaust gases (essentially ethanol and acetic acid swept by the gas stream leaving the tank).

Because the biotransformation is exothermal, the bioreactor also requires a heat exchanger in order to maintain the temperature within the range ensuring optimal operation. Figure 6.6 shows a coil used as heat exchanger.

### 6.3.3 Operational Modes

The way the bioreactor is operated is one other key to obtaining acceptable fermentation results. In principle, the bioreactor can be operated in a batch, semi-continuous or continuous way. The choice depends on many factors. Thus, depending on the specific purpose, the bioreactor should be operated under the best possible environmental conditions for acetic acid bacteria to develop. Economy and end-product quality are obviously two major considerations here.

One can hardly choose an appropriate operational mode without an accurate knowledge of the way in which the main variables of the process influence the activity of acetic bacteria. This topic has been widely documented in ample compilation, review and modelling work (Nieto, 1991; Ebner et al., 1996b; Tesfaye et al., 2002; Garrido-Vidal et al., 2003; González-Sáiz et al., 2003; Emde, 2006; Sellmer, 2006).

The list of specific factors influencing the development of acetic bacteria includes, but is not limited to, temperature, ethanol concentration, acetic acid concentration, the combination of the previous two (total concentration), and dissolved oxygen availability.

Studies in this context have clearly revealed that the previous variables are strongly interdependent. In fact, one cannot examine the influence of any individual variable in isolation. Thus, the sensitivity to oxygen deficiency depends on the total concentration delivered by the culture medium, the specific time in the bacterial cycle or stage, and the acidity present.

Furthermore, ethanol can have adverse effects. Thus, a high concentration of the alcohol results in strong bacterial inhibition. On an industrial level, ethanol concentrations above 50 $g \cdot L^{-1}$ are known to adversely affect the acetification rate. On the other hand, too low an ethanol concentration can have a more or less marked effect on cell viability depending on the acidity level of the medium. This is important with a view to using the bacteria as a starter in subsequent cycles. As a rule, the ethanol concentration in the medium should never fall below 0.2% by volume. Too little ethanol in the medium can additionally result in over-oxidation of acetic acid to carbon dioxide and water, the likelihood of which increases with decreasing total concentration of the medium.

The sensitivity of acetic bacteria to acetic acid hinders the production of high-strength vinegar. In fact, it precludes continuous operation when producing vinegar containing more than 8-9% acetic acid.

The previous factors are all temperature-dependent. Thus, the acidity resistance decreases with increasing temperature.

In any case, the operating conditions should provide a medium that is as non-aggressive to the bacteria as possible. Such conditions, and the operational mode of choice, vary depending on the particular type of product to be obtained. Thus, producing vinegar containing up to 15% acetic acid entails operating in a semi-continuous mode. Specifically, the fermenter should be partly unloaded at the end of each cycle and the remainder of its contents used as a starter in the next cycle, the unloaded volume being replenished with fresh medium.

Typically, 40-50% of the reactor volume is unloaded when the ethanol concentration in the medium falls to 0.2-0.3%. The tank should be unloaded as rapidly as possible in order to avoid depletion of the medium, and then slowly loaded to avoid abrupt changes and excessively high local concentrations of ethanol. Depending on the loading rate used, the final load concentration can be in the range 5-6% ethanol and 7-10% acidity (as acetic acid).

Alternatively, the tank can be loaded by monitoring ethanol in order to avoid introducing an excessive concentration in the medium.

As a rule, the semi-continuous operation mode is the most widely used for several reasons, namely:

(i) It allows part of the biomass produced in a cycle to be used in the next.

(ii) Cell changes caused by variations in the composition of the medium during the loading and unloading operations can be controlled via the operating conditions with a view to maintaining the ethanol and acetic concentrations within acceptable limits, which facilitates self-selection of the most suitable bacteria for the specific medium used.

(iii) It is compatible with combined concentrations of ethanol and acetic acid spanning a wide range, which facilitates adjustment to the needs of a variety of product compositions.

The demand for higher-strength vinegars, using ethanol as raw material (particularly those for use as preservatives by the food industry), has promoted the development of operational modes yielding products with acetic acid concentrations above 20%.

Depending on the final acidity to be obtained, it may be necessary to use two fermentation tanks in synchronous operation rather than a single one. Thus, if the maximum acidity to be reached is 18-19% as acetic acid, one can use a single tank and gradually raise the total strength (total concentration) by adding ethanol in amounts not exceeding a given level. Once the total strength required to obtain the maximum acidity level tolerated by the bacteria is reached, ethanol addition is stopped and the process continued until the ethanol concentration falls to about 0.5% by volume. The deleterious effect of the high acidity prevalent near the end of the cycle can be lessened by lowering the temperature.

After an appropriate fraction of the culture medium has been unloaded, the tank is reloaded with a solution reducing the total concentration to levels below those reached at the final stages of the previous cycle. In this way, the bacterial population is periodically subjected to stress periods which it would be unable to withstand on a continuous basis. Figure 6.7 (Sellmer, 2006) illustrates this operational mode.

Obtaining higher-strength vinegar entails using two fermenters (dual-stage high-strength process: DSHS) (Ebner et al., 1996b; Emde, 2006). One is used to gradually raise the total concentration while the biotransformation reaction takes place; once the acidity reaches a predetermined value (e.g. 15%), a portion of its contents is unloaded into the second fermenter and depleted. The total concentration available in the medium results in such a high acidity at the end of the cycle that it facilitates the slow depletion of all ethanol unloaded but renders the bacteria useless for

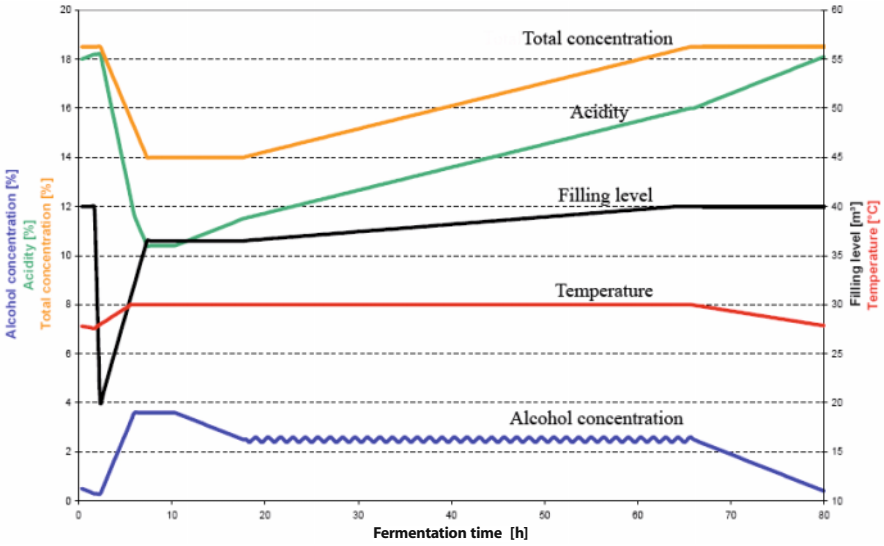


**Figure 6.7** Ethanol, acidity, volume, temperature and total concentration profiles through an industrial fed-batch high-strength alcoholic vinegar process (courtesy of Cetotec Biotechnologie GmbH)

cost-effective recycling. These procedures require the use of highly resistant biomass and effective control procedures.

Obviously, producing high-strength vinegars has the advantages of decreased storage and transportation costs – although this comes at the expense of decreased productivity relative to lighter vinegars. In fact, the enormous stress to which bacterial cells are subjected detracts from the overall acetification rate.

Finally, if the raw material to be acetified contains little ethanol (8-10% by volume) and the aim is to maximize productivity, one can use a continuous operational mode. The need for no reactor loading-unloading cycles and the stability of the acetification environment result in a high throughput (typically above 50 L ethanol $\cdot$ $m^{-3}$ $\cdot$ 24 $h^{-1}$) (Emde, 2006).

### 6.3.4 Modelling

The foregoing, based on experimental studies aimed at optimizing the operating conditions for specific applications, confirms the complex interdependence of the variables influencing the development and activity of acetic bacteria. Such complexity has fostered studies with the aim of modelling, in a quantitative manner, the relationships between the major variables of the process. An accurate model can be a highly useful tool with a view to predicting the results one might obtain by using alternative conditions, and hence to optimizing and controlling the production process.

Acetic fermentation is one salient example of a bioprocess challenging our scientific modelling abilities. Some mathematical models for the acetification process

rely on a structured approach (González-Sáiz et al., 2003; Jiménez-Hornero and García-García, 2006) that uses equations of balance relating the equilibrium concentrations needed and the kinetic laws providing an algebraic formulation of the influence of the state variables of the system concerned. Often, such models consist of complex systems of differential equations relating the state variables and the parameters needed in order to consider the influence of the experimentally observed variables.

The parameters in question must be estimated from experimental data in order to fit the model to the physical facts to be reproduced. Because fitting usually involves using mathematical algorithms, the model should not only reproduce as accurately as possible the influence of the variables considered, but should also exhibit various mathematical properties such as structural and practical identifiability (Jiménez-Hornero and García-García, 2006). Examining such properties allows one to confirm whether the modelled parameters can be estimated in an unequivocal manner from available experimental data, with due provision for their amount and quality, and also for the intrinsic structure of the model.

A model not meeting the previous requirements cannot fit the experimental facts with a single set of parameter values, so it can never provide the true values sought. Therefore, it can afford no reliable conclusion as regards the body of parameters obtained with an estimating algorithm and the parameter values in question, as it will be impossible to confirm whether the values were real. Also, strictly speaking in mathematical terms, the absence of identifiability makes it enormously difficult for estimation methods to find the optimum parameter values (i.e. those allowing the model to closely fit the experimental data).

Once the previous parameters have been estimated, the model can be used to identify the specific operation conditions maximizing or minimizing a given target function or, for example, for process control purposes.

The difficulty of the analysis and the inability to consider every potential source of influence has led to the use of so-called black-box models, which, rather than explaining as far as possible the influence of diverse variables on the basis of physico-chemical principles, seek to find as simple as possible a relation between the operational variables and a given target function used as goal. In this context, it is customary to use polynomial equations of varying complexity depending on the number of variables considered and polynomial order used, but still algebraic in nature (Garrido-Vidal et al., 2003). The use of second-order polynomials, which provide quadratic surfaces, has proved effective for solving many optimization problems.

This type of model is much easier to fit and is rarely subjected to the typical problems of phenomenological models. Also, it allows one to correlate such variables as the loading rate or fraction of medium to be unloaded, which are directly related to specific operational modes. Also, the conclusions derived from an optimized target function can be readily applied to routine work with fermentation tanks.

Despite the advances in modelling, the extreme complexity of relationships involving the response of living organisms and the ability of acetic bacteria to

adjust to changing – occasionally extreme – conditions hinders the practical application of existing models. Some authors (Arnold et al., 2002) have chosen to use an alternative approach involving the transfer to expert systems such as those based on fuzzy logic of the experience and knowledge of experienced workers at vinegar production plants capable of ensuring the reliable yield of vinegar with a high productivity. This allows a 'virtual vinegar brewer' to be developed in order not only to adjust the microbes to productivity-boosting conditions, but also to facilitate automatic control of the process.

### 6.3.5 Automation

Any operational mode, but particularly the semi-continuous one, needs to be monitored and controlled. For this to be possible, automation is absolutely necessary.

Fermentation cycles are known to vary in duration depending on the particular conditions; also, cycles differ in duration even under identical conditions. This entails obtaining on-line information in order to accurately identify the points in time where a given action (e.g. unloading of the tank) should be performed.

Automating an acetification system therefore requires using equipment capable of obtaining such information on-line (sensors) in addition to actuators, controllers and appropriate interfaces, and also a monitoring system which usually consists of a computer running Supervisory Control and Data Acquisition (SCADA) software. Automation technology has by now developed to such an extent that describing the wide range of choices currently available is clearly beyond the scope of this chapter. Some comments on a few selected elements are warranted here, however.

In most cases, the variables to be unavoidably measured and controlled include the volume of medium, concentration of ethanol in it, and temperature.

It is essential to use some device in order to control the operating volume. In fact, a semi-continuous acetification cycle requires accurately measuring the volume of medium present in the reactor at different points in time in order to determine when loading and unloading are to be stopped. Differential pressure sensors are specially precise and robust for measuring volumes of medium (Yokogawa Iberia S.A.). Figure 6.8 shows a typical example.

One other essential element is a device for measuring the ethanol concentration. Ideally, it should provide a continuous measurement of such a concentration in order not only to facilitate monitoring of nutrient uptake, but also to determine the exact time the reactor should be unloaded or supplied with further ethanol during a cycle.

Although a variety of methods for determining ethanol exist, the most suitable for on-line measurement of its concentration are based on the response of specific sensors to an air stream used to collect ethanol diffusing from the culture medium across a semi-permeable membrane. The alcohol can be determined by using a semi-conductor responding to its concentration or by analysing its near infrared (NIR) absorption spectrum. In any case, the analytical equipment used for this

**Figure 6.8** Differential pressure transmitter (courtesy of Yokogawa Iberia SA)

purpose should be calibrated by using a reference method (e.g. gas chromatography). The previous sensors can be supplemented with others affording measurement of specific variables with a view to characterizing a given process, especially for research purposes.

In any case, the information acquired by the sensors should be delivered to the controlling element (the core of the automation process), which is responsible for triggering the actions required as a function of the state of the process. The controlling element can take various forms, the most usual of which is a programmable logic controller (PLC). In fact, PLCs have been specially designed to operate in industrial environments (and are thus highly robust), possess increased I/O (input/output) capabilities to interact with processes, and allow the whole system to be controlled in an unattended manner. Also, they allow recording of the variation of any target parameters.

One alternative to PLCs is provided by PCs in the loop. These are industrial computers running dedicated controlling software. In addition to the usual functions of a PLC, these systems can perform a host of others, thanks to the flexibility of computers. For this reason, they tend to be more frequently used by research laboratories.

Obviously, any automated system must include some element facilitating monitoring of the target process. As noted earlier, PCs running dedicated SCADA software are the norm here, as they allow operators in a control room to follow the course of the process or even interact with it, as well as recording useful information and storing it in a database. The computers are connected to the controlling elements via a network (e.g. industrial Ethernet, Profibus), the particular choice being dictated by various factors, including the presence or absence of electromagnetic noise and the distance between elements. Also, large production plants often use

tactile panels on-site to display information about specific zones and facilitate intervention by operators; the panels are also connected to the controlling elements and higher-level SCADA PCs. In this way, a hierarchical surveillance and control structure can be constructed if needed.

## 6.4 Post-Treatments

As noted earlier, dealing with every single major aspect of vinegar production engineering is beyond the scope of this chapter. However, filtration is worth a mention here as it constitutes the most important post-fermentation treatment with a view to ensuring stability and safety in the finished product. In fact, meeting the quality requirements for vinegar for human consumption entails removing any suspended solids in addition to colloids, which might otherwise detract from its stability. This is usually accomplished by prior sedimentation, clearing – which is only necessary with some types of vinegar – and subsequent filtration.

### 6.4.1 Filtration

Upon fermentation, the culture medium is a complex suspension essentially containing microbes and their cell debris in its solid fraction. Before filtering, the suspension should be decanted in order to reduce the solid concentration as far as possible. Also, clearing with clay (e.g. bentonite) often helps remove colloids – which, as stated above, can render the finished product unstable by causing precipitation of solids during storage and after bottling. Obviously, these operations facilitate subsequent filtering of the product.

#### 6.4.1.1 Normal Filtration

The traditional filtering method, which has been increasingly replaced by tangential filtration, involves using filters to pass the feed flow in the same direction as the filtrate (see Figure 6.9a); this requires using a filter aid such as diatomaceous earth in order to prevent the filtering bed from collapsing. With this type of filter, the filtration operation is typically performed in two steps. The first is conducted with candle filters of a fairly large pore size (ca. 50 μm) and the second, which can have sterilizing effects, with cartridges of pore size smaller than 1 μm.

This filtering approach requires the formation of a pre-layer in order to facilitate filtering across the candles and prevent clogging of their pores by small solid residues in the substrate (see Figure 6.9a). This increases the useful life of the candles, expedites the obtainment of a clear liquid, and facilitates periodic cleaning. Filtration proper starts after the pre-layer has formed. During the process, a porous bed consisting of solids in the medium and the filter aid – which has been added previously or supplied in a continuous manner to the suspension before it reaches the filter – gradually builds up. By virtue of its structural strength, the filter aid

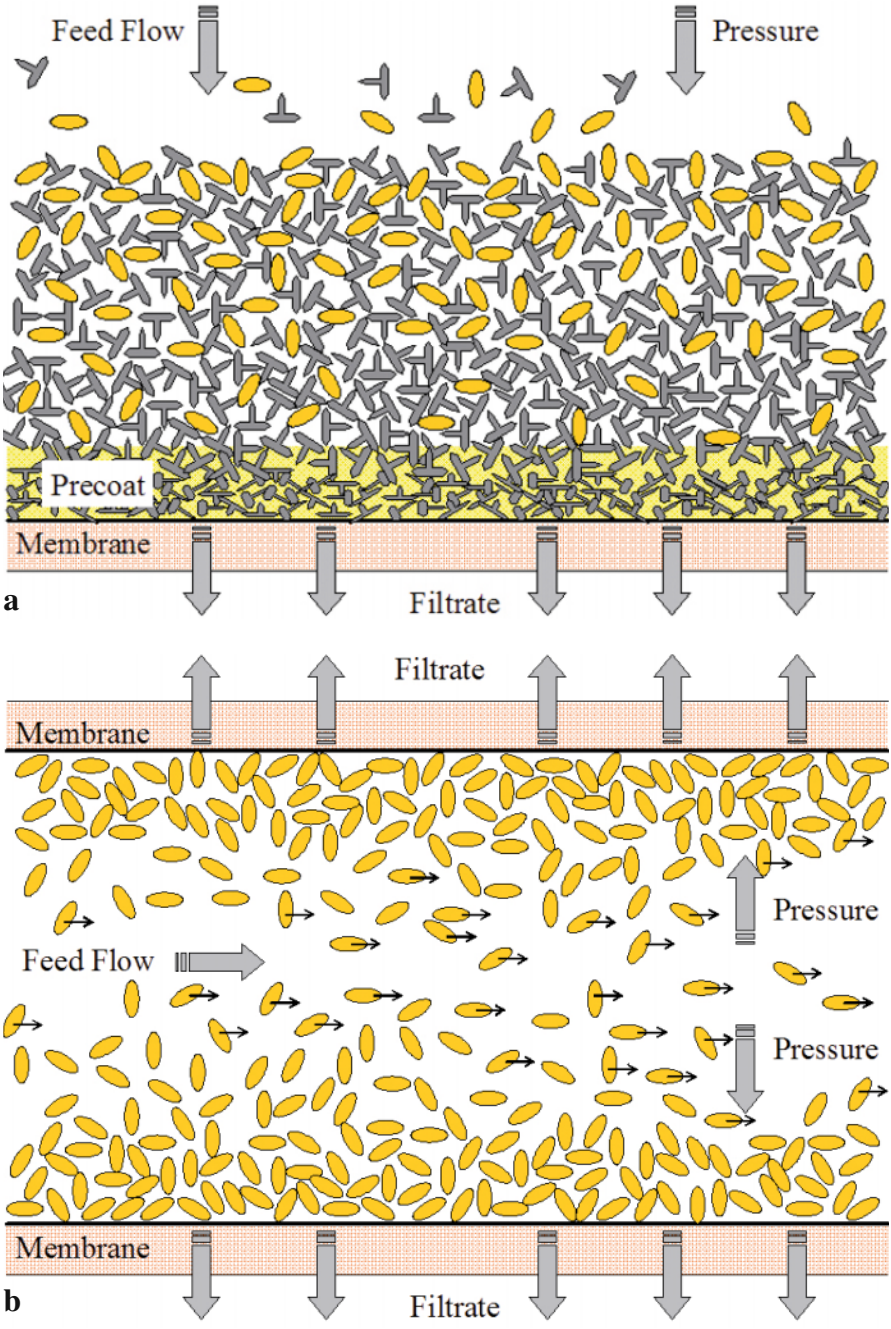


**Figure 6.9** Illustration of filtration procedures for biological suspensions: **a** normal flow filtration; **b** tangential flow filtration (courtesy of Millipore Corporation)

Key: , cell; , diatomaceous earth

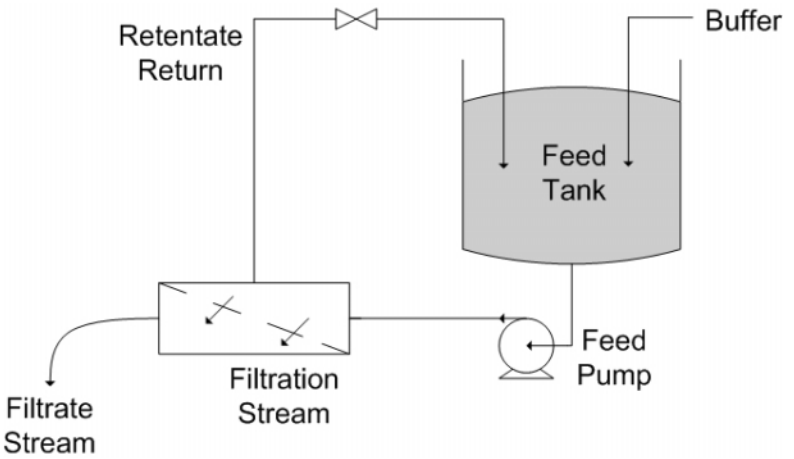


**Figure 6.10** Diagrammatic representation of a tangential flow filtration process

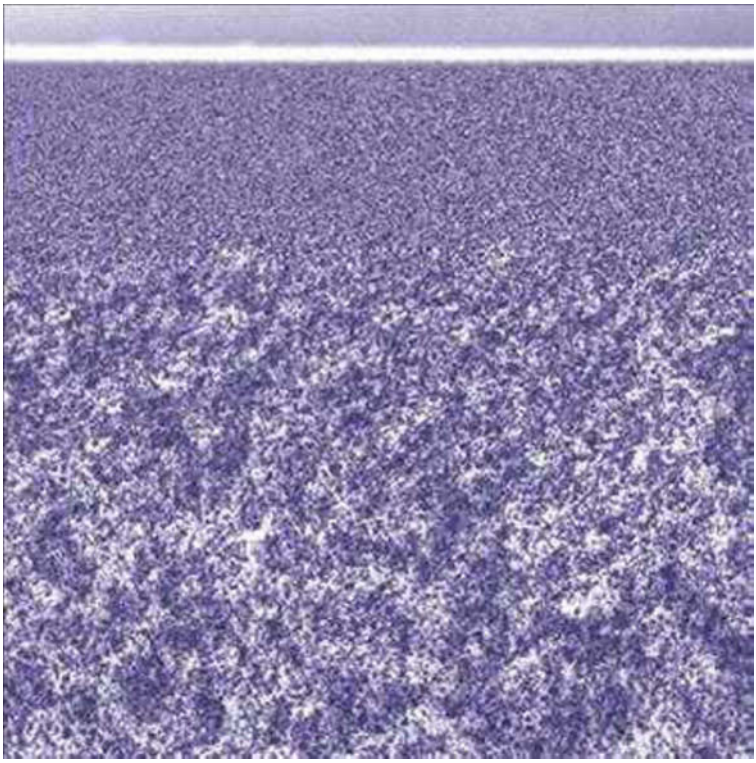

**Figure 6.11** Scanning electron microscopy (SEM) of a cross-section of a membrane based on polyethersulphone (courtesy of Millipore Corporation)

helps preserve the porosity of the bed. If the bed consisted solely of cells and cell debris in the suspension, it would rapidly collapse by effect of their malleability and the pressure exerted to facilitate filtering. The ultimate purpose of the filter aid (diatomaceous earth) is to act as a backbone capable of withstanding pressure on the filtering bed and extend the life of the filter as a result.

As noted earlier, the second step is better performed with cartridge filters, which require no additive such as a filtering aid. The low concentration of solids in the medium following pre-filtering allows them to be efficiently retained on the surface of the cartridges. Also, cleaning – or even sterilization – requirements are easier to meet with cartridges by virtue of their high flexibility as regards material and pore size; in fact, cartridges can be made in easily sterilized, physically strong and chemically inert forms. Interested readers can find detailed technical information about filtration equipment elsewhere.

#### 6.4.1.2 Tangential Filtration

As stated above, tangential filtering has now superseded the previous traditional filtration scheme. The procedure involves circulating the suspension to be filtered through a flooded chamber in tangential contact with the surface of the filtering membrane. Overpressure in the chamber with respect to the other side of the membrane causes part of the liquid to pass through the membrane. Figure 6.9a,b shows the differences between tangential and traditional filtering.

The type of flow used prevents solids from accumulating on the membrane; this avoids the need for a filtering aid, since solids are continuously swept by the outgoing stream. Depending on its pore size, the membrane can retain solids of variable size, from microbial cells to dissolved compounds (e.g. buffering salts).

Figure 6.10 depicts a straightforward tangential filtration system. The first choices to be made in constructing this sort of system include the membrane material and overall design. The ability of some materials to adsorb specific substances such as proteins can dictate their choice. Regenerated cellulose and polyethersulphone are among the most widely used materials for filtering membranes (e.g. those from Millipore Corporation). Figure 6.11 is a scanning electron micrograph (SEM) of a polyethersulphone membrane from Millipore; this membrane can withstand extreme pH conditions.

Using an appropriate filtering design is crucial with a view to ensuring proper, economical functioning. One of the most effective designs in this respect is that based on cartridges packed with many hollow fibre membranes (e.g. those from Koch Membrane Systems). The suspension to be filtered is fed through the centre of the hollow fibre and permeate passes through the fibre wall to the outside of the membrane, which facilitates control of the flow and reduces membrane clogging. Figure 6.12 shows a photograph of a membrane fibre, an operational scheme, and cartridges containing many packed fibres. Figure 6.13 shows a tangential filter from Heinrich Frings specifically designed for filtering vinegar.

Tangential filtering has superseded traditional filtering for a number of reasons (see, e.g., Koch Membrane Systems), namely:

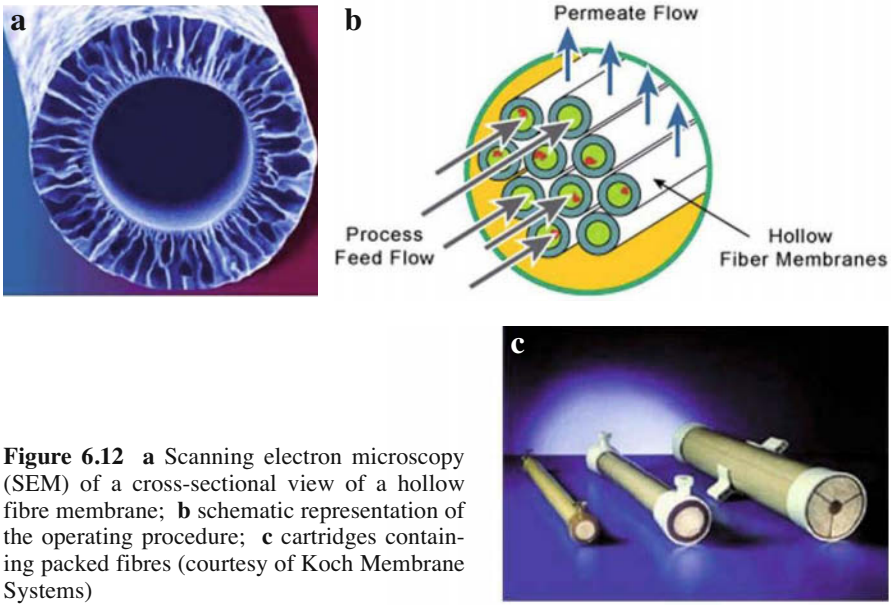


**Figure 6.12** **a** Scanning electron microscopy (SEM) of a cross-sectional view of a hollow fibre membrane; **b** schematic representation of the operating procedure; **c** cartridges containing packed fibres (courtesy of Koch Membrane Systems)


**Figure 6.13** Fully automatic tangential flow filtration plant (courtesy of Heinrich Frings)

- It provides a product of increased quality, as none of the typical colour or aroma impurities of vinegar are retained.
- It involves fewer steps and hence less extensive manipulation of the product.
- It reduces product losses by about 70% with respect to conventional filtration and recovers more than 99% of the filtered liquid.
- It is easily automated, which reduces labour costs.
- Membranes have a long useful lifetime (up to several years if used properly).
- It requires no filtering aids (i.e. diatomaceous earth), so it produces no additional waste and avoids the health risks resulting from operators breathing hazardous dust.
- It is highly flexible in terms of configuration and capabilities.
- Because it saves time, labour, material, energy and maintenance work, it is much more economical than traditional filtering.

## 6.5 Concluding Remarks

Vinegar engineering now has reached a highly developed state. Bioreaction stage and various steps in the process can now easily be performed in an automated manner. This provides great advantages in terms of throughput and product quality and safety. However, further efforts are still required with a view to improving the existing technology and adapting it for compliance with increasingly strict legal and health regulations and requirements.

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# Chapter 7
# Rice Vinegars

**Yoshikatsu Murooka, Kumiko Nanda and Mitsuo Yamashita**

## 7.1 Introduction

Rice vinegar is a traditional seasoning condiment used in China, Japan and Korea. Rice vinegar is made from fermented rice wine (*sake* in Japanese). Fermentation methods for rice vinegar include a traditional static method and recently developed continuous-culture and batch-culture methods. Chinese and Japanese rice vinegars are slightly milder and sweeter than Western vinegars.

There are three types of Chinese/Japanese rice vinegars: amber (clear/pale amber), used mainly in sweet-and-sour dishes; red, a popular accompaniment to boiled and steamed crab in China; and black, used mainly as a table condiment and health drink (Table 7.1). *Komesu*, an almost colourless Japanese rice vinegar, is used in a variety of Japanese dishes, such as *sushi* rice and a popular seaweed

**Table 7.1** Variety of rice vinegars

| **Type of vinegar** | **Colour** | **Ingredient** | **Source of alcohol** (spp. of *koji* mould) | **Acidity**[a] (%) | **Country** |
|---|---|---|---|---|---|
| Amber | Pale amber | Polished rice | Fermented (*A. oryzae*, *Rhizopus*) | 4.2-4.5 | Japan, Korea, China |
| | | | Sake lees | 4.2-4.5 | Japan, China |
| | | | Synthetic ethanol | 4.2-4.5 | China, Japan |
| Red | Pale red | Glutinous rice | Fermented (*Monascus purpureus*) | 7.0-8.0 | China |
| Black | Dark black | Unpolished rice | Fermented (*A. oryzae*, *A. awamori*, *Rhizopus*) | 4.2-4.5 | Japan, Korea |
| | | Glutinous rice | Fermented (*A. oryzae*, *Rhizopus*) | 4.2-4.5 | China |

[a] Percentages of acidity shown are Japanese rice vinegar except red vinegar. Generally, acidities of Chinese and Korean vinegars are stronger than Japanese vinegars but no data are available.

L. Solieri, P. Giudici (eds), *Vinegars of the World*

salad, *sunomono*. Chinese black vinegar may be thought of as a substitute for balsamic vinegar. However, their dark colour and ageing process are the only similarities between these two vinegars. Korean vinegars have a stronger flavour than Japanese rice vinegars. Rice vinegars are sold in Asian markets and some US and European markets.

## 7.2 Variety of Vinegars in Asia

Rice vinegars are popular in China and some neighbouring countries sharing a similar culture, including Japan and Korea. Rice vinegars are fermented from rice wine (sake) to acetic acid. Vinegar production records have been found in Chinese literature dating back to the Zhou period (1027-221 BC) (see Chapter 2). China's ancient sake may have been derived first from fruits, with rice derivation later.

Rice vinegars are less popular in Southeast Asian countries, although rice vodka is produced in Vietnam. Coconut vinegar, made from the sap or 'toddy' of the coconut palm, is produced and used extensively in Southeast Asia, particularly in the Philippines and India. One month is required to produce coconut vinegar by static fermentation. A cloudy white liquid, coconut vinegar has a particularly sharp, acidic taste (4% acetic acid) with a slightly yeasty flavour. In the Philippines, cane vinegar and nipa-palm vinegar are popular (Kozaki and Sanchez, 1974). Nipa-palm vinegar is mainly produced by a traditional method using ceramic pots like those used in Chinese black vinegar production. Cane vinegar (*sukang iloko* in Tagalog), made from sugarcane juice, is most popular in the Ilcos region of the northern Philippines. It ranges in colour from dark yellow to golden brown and has a mellow flavour – a somewhat fresher taste than rice vinegar. Contrary to expectation, it is no sweeter than other vinegars and contains no residual sugar. Distilled alcohol is used to convert the acetic acid after fermentation. Cane vinegar produced by the traditional static fermentation method requires a month, with a shorter period being needed for the modern method using an acetator (Kozaki and Sanchez, 1974).

## 7.3 Defining Rice Vinegars

In Japan, vinegars are classified as fermented and synthetic vinegars. Fermented vinegars are further classified into polished rice vinegar (*komesu*), unpolished rice vinegar (*kurosu*), sake-lees vinegar (*kasuzu*) and other grain vinegars. These grain vinegars must contain 40 $g \cdot L^{-1}$ of rice grains among their ingredients. Komesu and kurosu must contain more than 40 $g \cdot L^{-1}$ and 180 $g \cdot L^{-1}$ of rice grains, respectively. These fermented vinegars are generally produced by static fermentation requiring more than one month. Fast-produced, acetator-fermented vinegars (from alcohol), known as synthetic vinegars, are prohibited from being labelled as fermented or brewed vinegar, according to JAS (Japanese Agricultural Standard) rules.

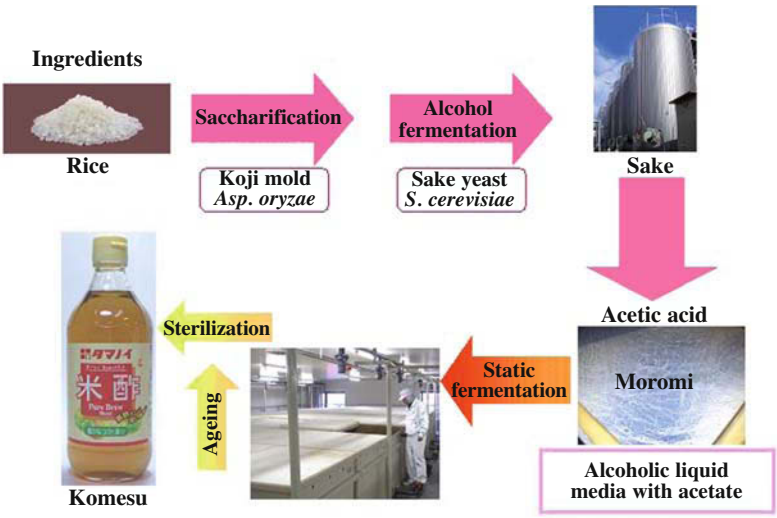


**Figure 7.1** A flow sheet for Japanese rice vinegar fermentation. Komesu and kurosu are produced from polished and unpolished rice, respectively, by the same process; saccharification of rice starch by koji mould and alcohol fermentation by sake yeast. An alcoholic liquid broth with vinegar wash and acetic acid bacteria called moromi is used to further process rice vinegar by the static surface fermentation method (pictures provided by Tamanoi Vinegar Co. Ltd.)

## 7.4 Amber Rice Vinegar: Komesu

Amber rice vinegar is pale amber, less acidic, and milder in flavour than distilled vinegar. China's amber rice vinegars have a hint of sweetness due to the Chinese glutinous rice content. The higher acetic acid content in China's amber rice vinegars make them ideal for sweet-and-sour dishes and for pickling vegetables. They generally work well in stir-fries too. The Pearl River Bridge brand is quite popular. Japan's amber rice vinegar, komesu, has a plain taste (4.2-4.5% acidity) and is thus ideal for preparing sushi and the vinegary seaweed salad, sunomono. Komesu is produced from polished non-glutinous rice, '*Japonica*', and imported '*Indica*' in Japan. Komesu was originally produced in Izumi (Osaka), then Owari (Aichi), and eventually spread to other areas throughout Japan (Otsuka, 1990).

### 7.4.1 Alcohol Fermentation

In China and Japan, alcohol fermentation follows the same process as that for rice wine, or sake, production. In ancient times, Chinese sake was made from malt and a koji mould variety. Recent sake production uses *koji* moulds, usually *Aspergillus oryzae* or *Rhizopus*, together with a rice variety. In 1982, a German scientist, Dr. Calmette, isolated several *Rhizopus* species from Chinese sake broths. One of these

species had a strong saccharification capability. Thus, instead of malt, this strain was incorporated in Europe's alcohol production (Bo, 1988a). In addition to *Aspergillus* and *Rhizopus*, *Monascus* and *Mucor* are also known as koji-forming moulds. In Japan, *A. oryzae* has been widely used in the saccharification of polished rice, and sake yeast (*Saccharomyces cerevisiae*) for alcohol fermentation (Figure 7.1). A tremendous number of reports on sake production have been published in Japan.

### 7.4.2 Acetic Acid Fermentation

Vinegar is industrially produced by two main methods: a slow process involving static surface acetic acid fermentation, and a fast-producing, submerged fermentation process. Generally, the static fermentation method is used in traditional vinegar production. This technique is not costly in terms of factory investment, and product quality is good, although a relatively long period is required to complete fermentation (Nanda et al., 2001). A liquid containing alcohol and vinegar wash (*moromi*) is fermented with an acetic acid bacteria layer in tightly covered containers, in order to prevent bacterial contamination during fermentation (Figure 7.1). After a few days, crepe pellicles of acetic acid bacteria cover the moromi surface. Fermentation proceeds, with completion in about one month. No strict sterilization procedures are used. More recently in Japan, however, large-scale rice vinegar production is being managed by computer-controlled acetators (Yanagida, 1990).

### 7.4.3 Acetic Acid Bacteria

*Acetobacter* strains are usually involved in vinegar production. However, in China, naturally occurring acetic acid bacteria without purification are used. The main species of acetic acid bacteria used in vinegar production are most usually *Acetobacter aceti*, *Acetobacter pasteurianus* and/or *Gluconacetobacter xylinus*; however, no detailed taxonomic reports have been found for the bacteria used in the production of Chinese vinegars. Identification of the species and characterization of the dominant strains in static acetic acid fermentation are desirable for the stabilization of fermentation procedures and strain improvement. Until recently, strain differentiation for similar species involved in traditional static fermentation had not been possible. No method was available. However, in recent years, the enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) method (Nuswantara et al., 1997), random amplified polymorphic DNA (RAPD) fingerprinting analysis (Trãek et al., 1997), and repetitive extragenic palindromic (REP) element-PCR methods (Nuswantara et al., 1997) are being used for the taxonomic grouping of bacteria, including acetic acid bacteria.

Nanda et al. (2001) investigated acetic acid bacterial strains isolated from samples obtained during commercial komesu production in Japan. They used the ERIC-PCR and RAPD methods. Bacterial strains were isolated from komesu samples fermented by the traditional static method. These fermentations had not been

**Table 7.2** Flora changes in polished rice vinegar (*komesu*) and unpolished rice vinegar (*kurosu*) fermentation

| Source | Fermentation period (days) | Number of isolates (appearance rate %) Group A | Group B |
|---|---|---|---|
| Polished rice vinegar (*komesu*) | Early (1-10) | 49 (100) | nd |
| | Middle (11-20) | 28 (100) | nd |
| | Late (21-32) | 26 (100) | nd |
| | Post-fermentation (>32) | 23 (98) | 2 (8.0) |
| Unpolished rice vinegar (*kurosu*) | Early (1-10) | 16 (100) | nd |
| | Middle (11-20) | 22 (100) | nd |
| | Late (21-32) | 12 (100) | nd |

Bacterial strains were isolated from samples of komesu and kurosu fermented by the traditional static method and analysed by the ERIC-PCR and RAPD methods (Nanda et al., 2001).
nd, not detected.

inoculated with a pure culture, since the vinegar production began in 1907. A total of 126 isolates were divided into groups A and B on the basis of DNA fingerprinting analyses. The 16S ribosomal DNA sequences of strains belonging to each group showed similarities of more than 99% with *A. pasteurianus*. Group A strains overwhelmingly dominated all stages of fermentation (Table 7.2). Nanda et al. (2001) concluded that the appropriate strains of acetic acid bacteria had spontaneously established nearly pure cultures after a century of komesu fermentation.

## 7.5 Black Rice Vinegar: Kurosu

### 7.5.1 Varieties of Black Rice Vinegar

In China and Japan, black rice vinegars (*heicu* in Chinese and *kurosu* in Japanese) are produced from unpolished rice. These vinegars are known for their health benefits, such as an aid for hypertension and cancer prevention.

Black rice vinegar, in particular Chinkiang vinegar, is very popular in southern China. Black rice vinegar is made from glutinous rice (also called sweet rice or sticky rice) and *uruchi* rice in China and Japan, respectively, although in China, millet and sorghum are used too. Black rice vinegar is dark in colour, with a deep, smoky flavour. Resembling China's black vinegar, Zhenjian vinegar is produced in Hong Kong. For approximately 200 years, black rice vinegar in Japan has been produced in Fukuyama-Cho, in northeast Kagoshima City. Similar types have been produced by a modernized version of the static fermentation method in Izumi/Nara, Handa, and other areas of Japan. Since kurosu is produced from unpolished rice, it contains more amino acids, vitamins and soluble metal ions (Mori, 2000) than komesu, and thus is considered a health drink in Japan. Acidity is 4.2-4.5%.

### 7.5.2 Scientific Evidence for Kurosu as a Health Drink

One kurosu extract was recently shown to suppress lipid peroxidation (Ohigashi, 2000). This extract expressed stronger antioxidative activity in a radical scavenging system than other vinegar extracts (Nishikawa et al., 2001). DPPH radical-scavenging compounds, such as dihydroferulic acid, dihydrosinapic acid, ferulic acid and sinapic acid, were isolated from kurosu (Shimoji et al., 2002). Kurosu extract was also shown to prevent hypertension in rats, and actually lowered blood pressure in rats (Nishidai et al., 2001) and in humans (Sugiyama et al., 2008). Kurosu also promoted anti-tumour activity in mouse skin carcinogenesis and anti-tumour activity in a variety of cultured tumour cells (Nanda et al., 2004). Generally acetic acid reduces cholesterol (Fushimi et al., 2006) and hyperglycemia (Sakakibara et al., 2006). With increasing public interest in health, effective health-related elements of traditional vinegars are being carefully researched.

### 7.5.3 Technology

Black rice vinegar, like amber rice vinegar, is produced by rice saccharification, alcohol fermentation, and the oxidation of ethanol to acetic acid (Figure 7.1). Although some vinegar companies produce unpolished rice vinegars using a modern submerged fermentation process, most kurosu is produced by the traditional

**Figure 7.2** Traditional kurosu brewing in Fukuyama-Cho, Japan: ageing of kurosu using ceramic pot (picture provided by Sakamoto Brewing Co. Ltd.)

**Figure 7.2** Traditional kurosu brewing in Fukuyama-Cho, Japan: monitoring the kurosu fermentation process in a ceramic pot (picture provided by Sakamoto Brewing Co. Ltd.)

static fermentation method. Traditionally, ceramic pots have been used for the sake-to-vinegar production process in China and Japan. Ceramic pots are covered with ceramic lids in Japan or with a tightly-woven rice straw mat in China. For example, Chinkiang vinegar is produced from glutinous rice with rice grain shells (*momigara* in Japanese) by a combinational parallel fermentation process (Bo, 1988b). Rice grain shells are occasionally added during fermentation, and the moromi pellicles are agitated for aeration before being transferred from one ceramic pot to the next sequentially. In this fermentation process, pots are covered with vinyl sheets to exclude the air. Fermentation requires about 6 months. However, many modified methods are being used in China's factories today (Bo, 1988b).

Fukuyama kurosu is produced from non-glutinous rice with yellow or *kome koji*, *Aspergillus oryzae*. Inoculations of acetic acid bacteria are not necessary, since these bacteria reside in the ceramic fermentation pots. In one ceramic pot (43 cm diameter × 62 cm high, 54 L capacity), steamed rice, *kome koji* and water are mixed and fermented for several months using the combinational parallel fermentation process (Figure 7.2).

For the Tamanoi Vinegar Company's vinegar produced in Nara, Japan, fibreglass reinforced plastic (FRP) containers are used (see Figure 7.3). The alcohol liquid medium, sake, is produced from unpolished rice and *kome koji*, *A. oryzae*. This sake, together with vinegar wash and acetic acid bacteria, create the *moromi* which is used to process the kurosu vinegar by static surface fermentation. In static fermentation, no purified strain is inoculated once the vinegar fermentation process begins. The acetic acid bacteria layer covering the moromi surface is scooped up with a mesh bowl and then gently introduced into a new batch of moromi (Murooka et al., 2005).

### 7.5.4 Microbiology

The first detailed analysis of microorganisms during kurosu fermentation was reported by Nanda et al. (2001). A total of 40 strains isolated from the early, mid-

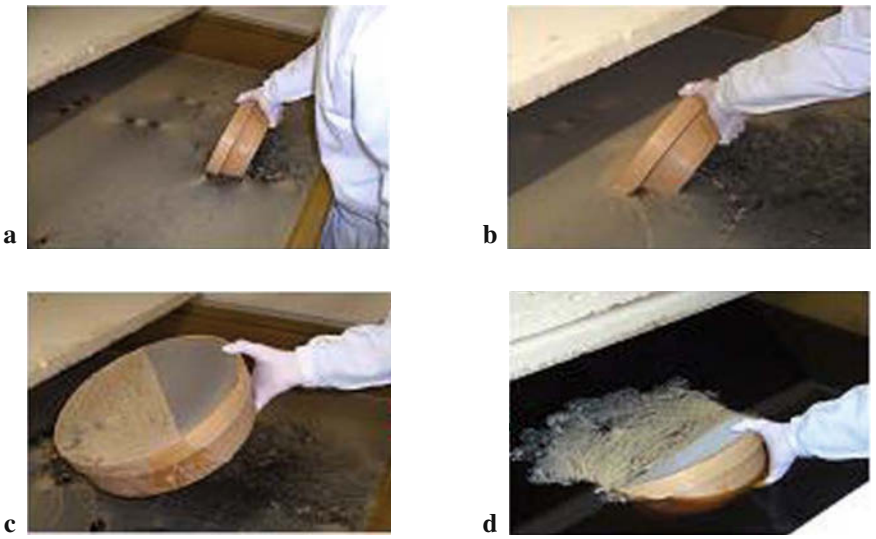


**Figure 7.3** Transferring inoculation of a crepe pellicle of acetic acid bacteria. In static fermentation, no purified stain is inoculated once vinegar fermentation begins. The crepe pellicles of acetic acid bacteria that cover the moromi surface are scooped up with a mesh bowl and then gently floated into the pellicle layer of a new moromi (pictures provided by Tamanoi Vinegar Co. Ltd.)

dle and late phases of static surface fermentation (Figure 7.3) were identified to be one group by the ERIC-PCR method and RAPD DNA fingerprinting analyses (Table 7.2). The 16S rDNA strain sequences belonging to the group showed similarities of more than 99% with *A. pasteurianus*. No purified strain was inoculated once vinegar fermentation began, which in some cases had been continued without the inoculation of a pure culture for more than 100 years.

In ceramic pot kurosu fermentation at Fukuyama-Cho, lactic acid bacteria such as *Lactobacillus fermentum* and *Pediococcus acidilactici* were observed during the first days of fermentation, and *A. pasteurianus* and *Lactobacillus acetotolerans* were detected after 15 days. Analysis of the eukaryotic 26S rDNA detected *Aspergillus* and *S. cerevisiae* on the first 15 day of fermentation (Haruta et al., 2006).

## 7.6 Sake-Lees Vinegar: Kasuzu

In China and Japan, some vinegars are produced from sake lees by traditional static fermentation. The sake-lees vinegar production was mentioned in the Chinese literature during the Song Dynasty (AD 420-478) (Ma, 2002). A world-renowned vinegar, Zhen Jian Xiancu, is produced from yellow-sake lees (huang jiu zao in Chinese). In Japan, this kind of vinegar is known as kasuzu and has been widely produced since the Edo period, i.e. for about 300 years. Kasuzu acidity is 4.2-4.5%.

### 7.6.1 Technology

Sake-lees vinegar (kasuzu) is fermented from extracts of sake lees and has a strong yet tasty flavour, and is thus generally used at home and in food factories. Since sake lees are the waste material resulting from Japanese sake production, kasuzu has been economical and valuable as a commercial product since the middle of the Edo period (ca. AD 1700) in Japan.

No alcohol fermentation process is necessary for kasuzu production. Sake lees are ripened for 2 years and then dissolved in water. Extracts of sake lees are mixed with alcohol (final alcohol content is about 5%) and acidified with vinegar to make moromi. Then crepe pellicles of acetic acid bacteria are added to the moromi and fermented by the traditional static method to form acetic acid (Murooka et al., 2005). Traditionally, wooden barrels were used, but more recently plastic tubs (30-50 kL) have been used.Kurokoji Moromisu, produced in Okinawa, uses black koji (*A. awamori*) to produce the traditional *shochu*, *awamori*, and lees from the distilled awamori. In the case of Kurokoji Moromisu, the main component of the acidic material is citric acid, which produced by *A. awamori*, and thus this product is not defined as vinegar, although it is marketed as a health drink in Japan.

### 7.6.2 Microbiology

Acetic acid bacteria were isolated from samples of Japanese sake-lees vinegar (kasuzu) produced from an extract of sake-lees and ripened for 2 years. A total of 210 isolates were analysed by the ERIC-PCR method. These isolates were classified into four groups: A, B, C and D (Figure 7.4). These groups were further confirmed by the REP-PCR method.

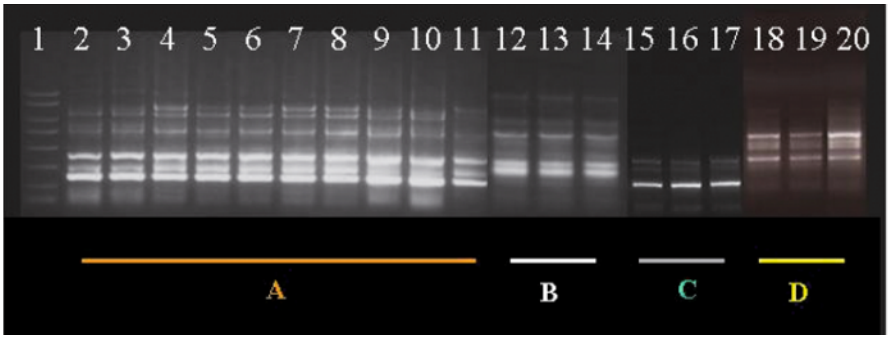


**Figure 7.4** ERIC profiles of selected acetic acid bacteria strains from sake-lees vinegar (*kasuzu*). Lanes 1, 11 and 23: PCR markers (Novagen); Lanes 2-10: TN-542, TN-543, TN-644, TN-646, TN-648, TN-655, TN-657, TN-574, TN-575; Lanes 12-20: TN-308, TN-309, TN-592, TN-687, TN-688, TN-613, TN-614, TN-617. These data were obtained by M. Taniguchi, K. Nanda, S. Ujike, Y. Simoji, N. Ishihara, K. Uenakai from Tamano Vinegar Co. Ltd. and H. Ono and Y. Murooka from Osaka University

**Table 7.3** Characteristics of differentiating the type strains and isolated strains from rice vinegar fermentations

| Characteristics | *A. pasteurianus* ATCC33445 | Group A TN-27 | Group B TN-1 | Group C TN592 | Group D TN613 | *Ga. xylinus* IFO15237 | *Ga. intermedius* DSM11804 |
|---|---|---|---|---|---|---|---|
| Gram stain | Negative | Negative | Negative | Negative | Negative | Negative | Negative |
| Shape | Rod | Rod | Rod | Rod | Rod | Rod | Rod |
| Catalase | + | + | + | + | + | + | + |
| Oxidase | – | – | – | – | – | – | – |
| O-F test | Oxidation | Oxidation | Oxidation | Oxidation | Oxidation | Oxidation | Oxidation |
| G+C (mol%) | 53.4 | 54.1 | 54.3 | 62.0 | 63.5 | 61.7[a] | 61.6[c] |
| Oxidation: | | | | | | | |
| Lactate | + | + | + | + | + | –[b] | –[c] |
| Acetate | + | + | + | – | – | –[b] | –[c] |
| Growth on: | | | | | | | |
| GY medium | nd | nd | nd | + | – | – | + |
| MRS medium | nd | nd | nd | + | + | + | + |
| Isolation on agar medium | nd | nd | nd | + | – | – | + |
| Formation from glucose of: | | | | | | | |
| 2-ketogluconic acid | – | – | – | + | + | – | –[c] |
| 5-ketogluconic acid | – | – | – | – | + | – | –[c] |
| Quinone type | Q-9 | Q-9 | Q-9 | Q-10 | Q-10 | Q-10 | nd |
| Growth on $(NH_4)2SO_4$ | – | – | – | + | + | +[b] | nd |

[a] Toyosaki et al. (1995). [b] Tanaka et al. (1998, 2000). [c] Boesch et al. (1998). nd, not determined.

Group A to D strains were isolated from sake-lees (*kasuzu*) vinegar fermentation. Group A and B strains were also found in polished (*komesu*) and unpolished rice (*kurosu*) vinegar fermentations. These data other than a, b and c were analysed by M. Taniguchi, K. Nanda, S. Ujike, Y. Simoji, N. Ishihara and K. Uenakai from Tamano Vinegar Co. Ltd., and H. Ono and Y. Murooka from Osaka University. *Gluconacetobacter xylinus* and *Gluconacetobacter intermedius* have been transferred from genus *Acetobacter* (Yamada, 2000).

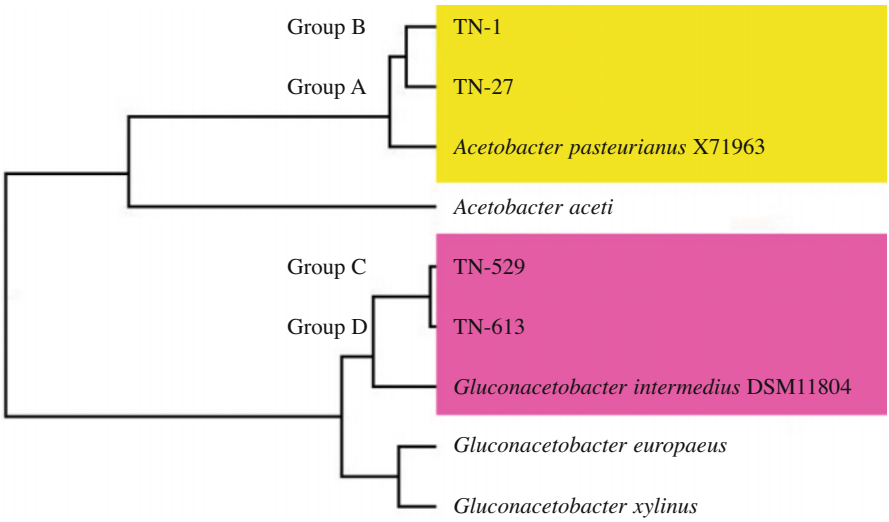


**Figure 7.5** Phylogenetic tree of type strains of acetic acid bacteria and isolated strains from rice vinegar fermentations. Groups A and B, isolated from komesu, kurosu and kasuzu fermentation processes, belong to *Acetobacter pasteurianus*; groups C and D, isolated from the kasuzu fermentation process, belong to *Gluconacetobacter intermedius*

Groups A and B of acetic acid bacteria from kasuzu showed the same PCR band patterns as those isolated from polished rice vinegar (komesu) and unpolished rice vinegar (kurosu) fermentations. They were identified as *A. pasteurianus*. Group C and D strains were found only in samples from the late phase of kasuzu fermentation. The 16S rDNA sequences of representative strains from each group showed similarities of more than 99% with *Gluconacetobacter intermedius*. However, the physiological characteristics for Groups C and D strains were different from the type culture strain, DSM 11804$^{T}$, of *Ga. intermedius* (Table 7.3). ERIC-PCR patterns for Groups C and D were also different from that of the *Ga. intermedius* strain, and therefore Murooka et al. (2005) classified them into a new subspecies, *Gluconacetobacter intermedius* ssp. nov. *tamanoi*. Phylogenetic diagrams for these acetic acid bacteria are shown in Figure 7.5.

## 7.7 Red Rice Vinegar

Red rice vinegar is darker than amber rice vinegar, and paler than black rice vinegar. Its distinctive red colour comes from the red koji, which is cultivated with the mould *Monascus purpureus*. This vinegar's distinctive flavour is due to the red mould. The famous red rice vinegar, Yongchun Laocu, has been produced in China for more than 200 years. Steamed rice is saccharified and made into sake with red koji. This moromi is transferred to a one-year fermented vinegar batch and pre-

served for about 3 years. The acid level will reach 8.0% by static surface fermentation (Bo, 1988b). No analysis of the acetic acid bacteria involved in red rice vinegar fermentation has been reported.

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# Chapter 8
# Malt and Distilled Malt Vinegar

**Bill Grierson**

## 8.1 History

The origins of malt vinegar in Britain go back several hundred years. As Britain is a beer-drinking nation, the early vinegars were simply beer that had turned sour; it was left and encouraged to acetify completely, and then processed, packed and sold in the area around the local beer brewery. The area of sales covered only a few miles radius from the brewery, as this was as far as a horse could travel and return in a day.

Malt vinegar was in those days frequently referred to as 'alegar' – a combination of the words 'ale' (beer) and 'vinegar'. It was some time later that the vinegar brewers of the day had to learn how to brew their own 'barley wine', as the beer brewers had discovered the reasons for their beer going sour and had taken steps to correct the process.

## 8.2 Definition

There are several varieties of malt vinegar available, which include distilled, light and dark malt vinegar.

Malt vinegar is a vinegar produced, without intermediate distillation, by the process of double fermentation – alcoholic and acetous – from malted barley, with or without the addition of cereal grain, the starch of which has been converted to sugars by the natural enzymes of malted barley. It is well known that the popular vinegar of a region follows the popular beverage of that region. Thus it is the popular choice of Great Britain, the eastern USA and some areas of Europe. Malt vinegar is a pale straw-coloured liquid, with a strong acetous flavour, whereas dark malt vinegar has a dark brown colour. The dark colour is derived from the addition of barley extract or caramel to the malt vinegar.

Distilled malt vinegar is obtained by the distillation of malt vinegar (as defined above) under reduced pressure. It contains only the volatile constituents of the malt

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vinegar from which it is derived. Distilled malt vinegar is colourless and is sharper in flavour and mildly aromatic.

## 8.3 Raw Materials

The essential raw materials for malt vinegar production are malted barley, water and the microorganisms responsible for fermentative and oxidative transformations; yeasts and acetic acid bacteria, respectively.

## 8.4 Production

Barley provides sugars (maltose and glucose) for alcoholic fermentation by yeasts and the nitrogen source (amino acids) necessary to support yeast and acetic acid bacterial growth. In raw barley, substances are present as starch and proteins, both not directly metabolized by yeasts. Some initial processing steps are necessary to permit the subsequent fermentation and acetification.

### 8.4.1 Brewing

#### 8.4.1.1 Milling

The first stage of the brewing process is to prepare the malted barley by milling. That means breaking open the corn in a precisely controlled fashion. This task is undertaken using a series of rollers in a malt mill (Figure 8.1). These rollers are set

**Figure 8.1** Malt mill

very precisely to within one-thousandth of an inch. A double roller mill is common, but more than two sets of rollers may be present. The first set of rollers reduces the size of the grain by splitting it, preferably longitudinally, with the second set reducing the size further. A typical specification for the grain exiting the mill would be: husk 28-30%, coarse grits 60-70%, fine grits 2-4%, and flour 1-2%. This material is now known as 'grist'.

This milling can be carried out using any one of several types of mill. Double roller mills are most commonly used in the UK, but ball mills, pin disc mills or hammer mills may also be used.

#### 8.4.1.2 Mashing

The second stage in the brewing process is to convert the grain starch to sugars in order to permit the alcoholic fermentation by yeasts. This is the function of the mashing process. The mashing vessel should be preheated by covering the perforated bottom plates with hot water and heating the remainder of the vessel by steam. The hot water is run off immediately prior to starting the mashing process. The milled grain is then initially mixed with hot water at a precisely controlled temperature by means of a Steel's masher; this consists of a set of revolving spikes that produces a thorough admixture of the grist and the hot water to a fairly thick consistency (0.019-0.023 hL of liquor per kilogram of grist) which is then allowed to flow directly into a mash tun (Figure 8.2). The temperature of the water (called the striking heat) should be approx 70 °C; this should give an initial temperature in the mash tun of around 63-66 °C. Temperature and time profiles of mashing are important parameters in the brewing process as they affect the enzyme activity and therefore the proportions of the various sugars and amino acids in the wort.

At this stage the grain/water mixture, called 'goods', is allowed to stand for at least 1 hour. During this time the grain 'floats' above the false bottom of the tun and the enzymes – alpha and beta amylase, protease and beta glucanases – convert the starch to fermentable sugars. When at least an hour from the end of mashing has elapsed, we obtain a sugar solution, called 'sweet wort'. This sweet wort is run off from the bottom of the mash tun and cooled. It is not boiled, as in beer brewing. When a controlled volume has been run off, more liquor is sparged onto the grain

**Figure 8.2** Mash tun

**Figure 8.3** Fermenting vessels

bed at an approximate temperature of 77 °C. The amount of liquor needed can be calculated depending on the quantity of grain used per brew. It is normally split into two additions. The run-off is then continued until the requisite volume has been collected at the required gravity. This sweet wort is cooled to 25 °C as it is run off from the tun and is then transferred directly to the fermentation vessel (Figure 8.3). The spent grain is then removed from the tun and normally sold for animal feed as 'wet grains'.

### 8.4.1.3 Alcoholic Fermentation

When a small volume of sweet wort has been collected, brewing yeasts belonging to the species *Saccharomyces cerevisiae* are added at a pitching rate of approx 0.03% by weight to obtain a suitable starting population. The fermenting wort is maintained at a controlled temperature of 20-30 °C (Figure 8.4) by means of a circulation system, which will introduce air to the fermenter. During this fermentation process, the growth of the yeast population by about five- to six-fold occurs. Wort sugars, glucose, fructose, maltose and maltotriose are fermented by the Embden-Meyerhof-Parnas pathway to ethanol, carbon dioxide and some traces of higher alcohols, esters and aldehydes. After 2-4 days the fermentation has been completed and the circulation is closed down. Finally the fermented wort is transferred to storage via the separators.

**Figure 8.4** Temperature control panel

### 8.4.2 Separation

At this stage in the process, the yeasts are inactive and begin to autolyse, promoting strongly reducing conditions which will hinder the acetification process. It is therefore imperative to remove the yeast cells by high-speed centrifugal separation (the normal filters used for live yeast are useless in this situation) (Figure 8.5). The 'bright wort' is stored to await acetification, and the yeast is discharged to waste. This waste yeast can be added to the wet grains from the mash tun as it goes for animal feed.

During the storage of this fermented wort the maturing process starts to take place due to the esterification of the alcohols and the acetic acid which begins to form in the stored wort.

**Figure 8.5** Centrifugal separator

### 8.4.3 Acetification

The conversion of the alcoholic liquid produced by the above processes into 'vinegar' can be accomplished by two types of process; either by acetifiers, which give an average yield of 90-94%, or by acetators, with a yield of 93-97%.

Acetifiers are vessels designed with an open trellis-work platform constructed approximately two-thirds of the way up the vessel, on to which is packed woodwool. This material provides a suitable habitat for the *Acetobacter* species. Air enters the system below the platform and, together with the alcoholic liquid, facilitates the growth of the Acetobacter species, which convert the alcohol to acetic acid via two successive catalytic reactions of a membrane-bound alcohol dehydrogenase (ADH) and a membrane-bound aldehyde dehydrogenase (ALDH). The space below the platform houses the fermenting material, with this being continuously circulated, by way of a cooling system, into a spray mechanism above the woodwool packing, and then being allowed to trickle back to the bulk. The air is introduced into the system immediately below the platform and is drawn through the woodwool by the increased temperature of fermentation; it is allowed to exit

through the top of the vessel. This is a fairly slow process and has largely been superseded by the acetator.

### 8.4.4 Storage

The 'rough stock vinegar', as the liquid is called after acetification, is pumped to large storage vessels and allowed to stand for at least 3 months (Figure 8.6). At this phase we have a high level of acetic acid and a low level of alcohols, which produce esters. The concentrations and relative amounts of these compounds have a significant impact on the unique flavour and bouquet of malt vinegar. The pH of the vinegar has by this time dropped to below 3 and various polyphenols etc. will slowly drop out of solution, giving the final vinegar a much more stable character.

**Figure 8.6** Storage tanks of malt vinegar after acetification

### 8.4.5 Clarification

The vinegar at this stage needs to be filtered bright; this process may be carried out in different ways. Ultra-high-speed centrifuges are available with which to remove any particulate matter and produce a bright, straw-coloured liquid, which is fairly stable.

Plate and frame filtration is an alternative method of clarifying the rough vinegar. This process consists of mixing filter powder, usually diatomaceous earth, with the vinegar, which is then pumped through the filter, with the powder building up a fine filter bed on the plates and with the frames filling with powder, giving a depth of filter bed to enhance the clarity of the vinegar. Membrane filters may also be employed to produce the required clarity.

### 8.4.6 Making up

This is the stage where the vinegar is 'made up' to whatever strength and colour is required for sale to customers (Figure 8.7). For bottled vinegar the normal strength

**Figure 8.7** Making-up vessels

is 5% of total acidity and the colour is amber; water is therefore added to adjust the strength to this level, and colour is added to give the required degree of colour. The colour may be caramel or barley extract. This is also the stage when flavours may be added to produce special vinegars. The flavours are normally essential oils of the herb or fruit desired. It is also possible to utilize synthetic flavours at this stage.

### 8.4.7 Final Filtration and Packaging

The finished vinegar is normally filtered using sheet filters packed with clarifying grades of sheet (Figure 8.8) and passed directly to a heat exchanger for hot filling, or to a pasteurizer for bulk supplies. Bottled vinegar is hot-filled at 60 °C directly into the bottle. This ensures a sterile product, as all the machine components are sterilized by the hot vinegar on its way into the bottle.

The completed bottles are stacked on pallets and allowed to cool naturally; they are then forwarded to storage areas to await delivery to the customer. Vinegars for the catering trade are also packed at this stage and would consist of 5-, 10- or 25-litre packs.

Vinegars for the bulk trade are taken direct from the make-up vessels through clarifying sheet filters (or cartridge filters) to a pasteurizer, and they are then filled

**Figure 8.8** Sheet filters

into the final containers for delivery to manufacturing customers. These bulk containers can be barrels, intermediate bulk containers (1000 L), or road tankers.

### 8.4.8 Distribution

Vinegar distribution is carried out by the normal schedules of road and rail transport to its required destinations – bottled vinegar on pallets and bulk vinegars either on pallets or palletized IBC's. Road tankers take higher-strength vinegars direct to other manufacturers for the production of pickles, sauces and similar products.

## 8.5 Distilled Malt Vinegar

After acetification and before the maturation storage, vinegar is passed direct to the distillation plant (Figure 8.9) for the production of distilled malt vinegar. The distillation plant consists of a steam-heated pan in which the vinegar is vaporized and then passed through a water-cooled condenser, all under high vacuum. The final product is a completely clear water-like liquid, containing only the volatile constituents of the original malt vinegar.

The distilled malt vinegar is sometimes referred to as 'white' vinegar and is approximately 0.2% weaker than the starting vinegar, as the fixed acid derived from the malt is non-volatile and remains behind in the still pan. This thick brown, residual, acidic liquid is on occasion sold for the production of flavouring for snack foods such as potato crisps.

**Figure 8.9** Vinegar distillation plant

## 8.6 Vinegar Powder

A version of 'dried vinegar' can also be made. This powder is produced by initially mixing malt vinegar of at least 10% acidity with malto-dextrin powder, using a high sheer mixer. Malto-dextrin makes it easier to powderize the liquid. The resultant fairly viscous liquid is then fed into a conical spray-drying unit using an inlet

temperature of 190 °C and an outlet temperature of 98 °C. The air supply is at ambient temperature and the atomizer speed is approx 11,000 rpm using a curved vein atomizer.

The resultant product is a pale-coloured powder used in many 'dry mixes', such as sweet-and-sour recipes, soups and many other ready-meals.

Testing of the final powders consists of acidity levels and a measure of occupational density. This is a measure of the weight of powder per volume and is usually expressed in grams per litre.

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# Chapter 9
# Wine and Grape Vinegars

**Sylvia Sellmer-Wilsberg**

## 9.1 Wine and Grape Vinegars

### 9.1.1 Definition and Description

According to the legal definition of the European Union, vinegar is the product obtained from alcoholic and subsequent acetous fermentation of liquids or other substances of agricultural origin (ethanol, wine, cider, perry, or liquors obtained from cereals, including malted barley). In the USA, this definition is extended to include synthetically produced alcohol used for acetous fermentation.

Some kinds of vinegar are: spirit vinegar, wine vinegar, fruit vinegar, cider vinegar, grain vinegar and malt vinegar. Mashes obtained by the alcoholic fermentation of natural sugar containing liquids also serve as raw material. The vinegar is designated according to the particular raw material used. For instance, wine vinegar is produced by acetic acid fermentation of grape wine, cider vinegar is produced from fermented apple juice, etc.

Regional speciality vinegars such as traditional balsamic vinegar from Modena (Italy) and sherry vinegar from Jerez (Spain) are described in the following chapters.

Wine vinegar is made from red or white wine, and is the most commonly used vinegar in the households of the Mediterranean countries and Central Europe. As with wine, there is a considerable range in quality. Better-quality wine vinegars are matured in wood for up to 2 years and exhibit a complex flavour. The most expensive wine vinegars are made from individual varieties of wine, such as Champagne, Sherry or Pinot Grigio.

### 9.1.2 Composition

As well as acetic acid and ethanol, vinegar contains secondary constituents which play an important role with regard to its smell, taste and preservative qualities.

L. Solieri, P. Giudici (eds), *Vinegars of the World*

These constituents have their origin in the raw material, in the added nutrients, and in the water used for dilution. They are also formed by acetic acid bacteria, or they are a product of the interaction of the different components.

Wine vinegars contain the same spectrum of amino acids as spirit vinegar, but in larger amounts. Galoppini and Rotini (1956) and Rotini and Galoppini (1957) found that during acetous fermentation, acetylmethylcarbinol develops in varying quantities. In an ether-pentane extract of wine vinegar, Kahn et al. (1972) identified 42 compounds. Besides the substances which had been found in spirit vinegar, compounds derived from higher alcohols, such as isopentyl acetate, isovalerylaldehyde, or β-phenethyl acetate are of particular interest. Garcia et al. (1973) analysed 20 types of wine vinegar. Most of them contained acetoin and butylene glycol, but only seven contained diacetyl.

Polyphenolic compounds have been shown to be of great interest with regard to the stability of wine vinegars. Galvez et al. (1994) identified polyphenolic compounds by using HPLC separation. They stated that, as a rule, vinegars obtained from wines exhibit a greater number and content of polyphenolic compounds than vinegars obtained from apples or honey. Those originating from Rioja wines showed the maximum number, followed by those derived from sherries. In wine vinegars the following substances were identified: gallic acid, *p*-OH-benzaldehyde, caffeic acid, vanillic acid, syringic acid, *p*-coumaric acid, anisaldehyde, epicatechin, sinapic acid and salicylaldehyde. The phenolic compounds are generally contributed by the solid parts of the grapes. Therefore, in the case of wines kept in longer contact with the grapes (as in the case with Rioja wines), a larger amount of polyphenols will be extracted.

## 9.2 Technology

### 9.2.1 Fermentation

Nowadays, wine vinegars are mostly produced in the same way as spirit (alcohol) vinegar using the semi-continuous submerged process. Stainless steel fermenters (Figure 9.1) of a size between 1000 and 110,000 L working volume equipped with a self-aspirating aeration system (Figure 9.2), ensuring a short and highly efficient mixing of alcohol, water and nutrients, are used most frequently. Usually wine vinegar is produced in smaller fermenters (20-40 $m^3$) than those used for the production of alcohol vinegar.

In the semi-continuous process, wine vinegar with an acetic acid concentration of 8-14% is produced (see Figure 9.3). Each fermentation cycle takes about the same time as the preceding and following cycles. The starting concentration of each cycle is 7-10% acetic acid and about 5% ethanol. When an alcohol concentration between 0.05% and 0.3% has been reached in the fermentation liquid, a quantity of vinegar is discharged from the fermenter. Refilling with new mash of 0-2% acetic acid and 12-15% alcohol leads to the starting concentrations for the


**Figure 9.1** Example of a submerged vinegar fermenter with an annual production capacity of 18 million L of vinegar containing 10% acetic acid

**Figure 9.2** Highly efficient aeration system in a fermenter for wine vinegar production

new cycle mentioned earlier. Discharging must be carried out quickly to avoid complete alcohol depletion. Charging must be done slowly under constant fermentation temperature and rapid mixing. The duration of a cycle is between 18 and 30 hours, depending on the total concentration and the efficiency of the aeration system.

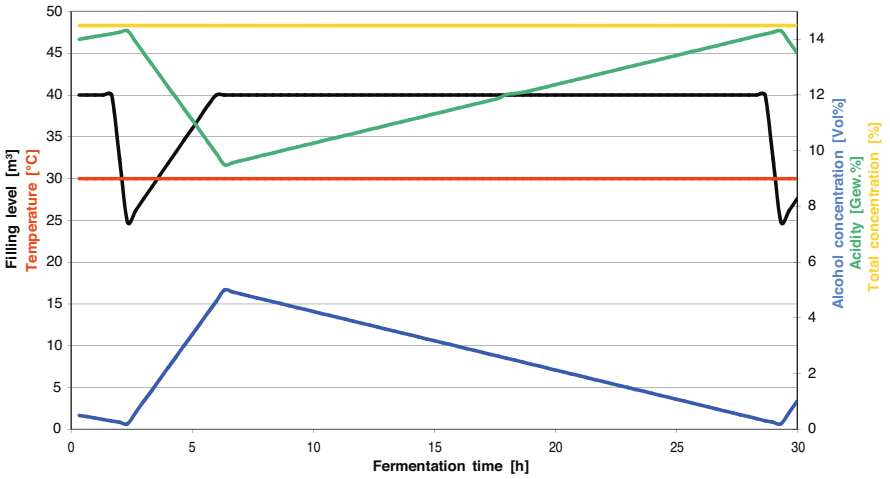


**Figure 9.3** Ethanol, acidity, volume, temperature and total concentration profiles in a semi-batch process for wine vinegar production (courtesy of Cetotec Biotechnologie GmbH)

Continuous fermentation is only possible up to a maximum of 9-10% acetic acid, because the specific growth rate of the bacteria decreases with decreasing ethanol concentration. To obtain high yields, the fermentation must be carried out at low alcohol concentrations.

Some vinegar manufacturers in Italy and Spain uses a special two-stage process, where two fermenters are combined. In the first step, the alcohol is degraded to 2-3%, then a certain quantity of vinegar is transferred into a second fermenter. The first fermenter is then resupplied with new mash. In the second fermenter, the fermentation continues until the alcohol is almost depleted. The whole quantity of finished vinegar is then discharged.

#### 9.2.1.1 Nutrients

Most natural raw materials do not require the addition of extra nutrients. Some grape wines require the addition of ammonium phosphate for an optimal fermentation. Ready-to-use nutrients are commercially available, containing a complex mixture of ammonium and potassium salts, vitamins and trace elements. Essentially, nutrients should be added sparingly in order to exert a selection pressure which leads to a low requirement for nutrients (Ebner et al., 1996b).

### 9.2.2 Storage

During the production process of wine and fruit vinegar, storage is an important factor. During storage, the quality of the vinegar improves considerably, and organic extracts and bacteria precipitate, thus helping further processing.

In the course of acetous fermentation, the pH value of the fermenting mash decreases. Vinegars obtained from natural raw materials therefore show some instability with respect to the solubility of previously dissolved substances. The less the pH changes during fermentation, the longer this lability lasts. A freshly produced cider vinegar, for example, may need several months to become stable. Alcohol vinegar does not show this instability. Vinegar for storage should preferably be undiluted, i.e. it should be the same as when it was discharged from the fermenter. In storage the quality of vinegar always improves, regardless of the type of vinegar or the sort of wine it was made from. The ageing of wine vinegar involves many complicated reactions, as described in detail by Mecca et al. (1979). Vinegar must have a pure aroma which imparts the flavour of the raw material.

### 9.2.3 Filtration

The filtration step following the storage is essential in order to produce a microbiologically stable product. Traditional methods of vinegar clarification by fining and pre-coat filtration are still in use, especially for the treatment of different kinds of wine vinegar. However, newer micro- and ultrafiltration methods are gaining in popularity, especially in the large European and American factories.

#### 9.2.3.1 Traditional Methods

Filters with a diatomaceous earth coating are well suited to most vinegars, whether aged or non-aged, fined or unfined. A filter layer of approximately 1 mm thickness of diatomaceous earth or cellulose is placed on a coat of acid-resistant steel, wood or nylon. The quality of this filter coat determines the flow rate of vinegar filtered through the layer, and the brilliance of the product. During filtration, diatomaceous earth is added continuously to keep the growing filter cake as permeable as possible. As soon as the filtration pressure has increased and the filter capacity has decreased too much, filtration has to be stopped and the filter has to be cleaned and prepared for a new filtration. The main disadvantages of traditional, labour-intensive filtration techniques are the time-consuming method and the disposal of the filter aids. Complete, largely automatic filters with both horizontal and vertical filtering surfaces are now available on the market.

#### 9.2.3.2 Continuous Microfiltration

Cross-flow filtration of vinegar using microporous hollow fibre membranes is nowadays becoming more and more important (Figure 9.4). The raw vinegar is


**Figure 9.4** Model of a cross-flow filtration plant used for continuous microfiltration of vinegar

conducted at high speed diagonally towards the membrane surface, thus preventing the spontaneous formation of a coating. The constant flow ensures a clean surface, which is essential for a high, stable filtration performance. In most cases the microfiltration modules used in vinegar production are equipped with capillary membranes with a pore size of 0.2 µm (Sellmer-Wilsberg and Rodemann, 1998).

Vinegar is a cheap substance; filter costs must therefore be kept low. Consequently the repeated and long-term use of the filter modules is important. The unavoidable formation of a film on the membrane, reducing the filtration rate, must be prevented for as long as possible. Easy, repeated and mainly automatic cleaning of the membrane is also important. Modern cross-flow filters with polysulphone or ceramic membranes are often used for the filtration of various kinds of vinegar.

## 9.3 Microbiology

The ecology and biology of the acetic acid bacteria are relatively well known due to the considerable economic profits and losses that these bacteria cause in industry. Since the first description of acetic acid bacteria, an extensive literature has been accumulated.

The older literature focused on the acetic acid bacteria used in vinegar manufacture and their role as spoilers in beers and wines. The occurrence of acetic acid bacteria in other niches, such as flowers, fruits, beehives, 'tea fungus' and palm wine was not described until much later. In the past 20 years, very many publications from research groups in Japan have appeared, dealing with bacteria from submerged fermentations, immobilized bacteria, and especially with genetic engineering of acetic acid bacteria.

The assignment of the acetic acid bacteria to the genera *Acetobacter*, *Gluconacetobacter* or *Gluconobacter* generally poses little difficulty. The identification at the species level, however, is sometimes more problematic.

### 9.3.1 Summary and Classification

The microorganisms which oxidize ethanol to acetic acid are commonly called acetic acid bacteria. This special primary microbial metabolism at low pH of the surrounding medium differentiates them from all other bacteria. Acetic acid bacteria are polymorphous.

Cells are Gram-negative, ellipsoidal to rod-shaped, straight or slightly curved, 0.6-0.8 µm by 1.0-4.2 µm, occurring singly, in pairs, or in chains. There are nonmotile forms and motile forms with polar or peritrichous flagella. They are obligately aerobic; some produce pigments, some produce cellulose.

Attempts to classify acetic acid bacteria have been summarized by Ebner et al. (1996c). At that time, a major contribution to their taxonomy was made by DNA-rRNA hybridization studies (Gillis and De Ley, 1980). A first conclusion from this work was that *Gluconobacter* and *Acetobacter* were closely related groups, justifying their union in the family *Acetobacteraceae*, clearly distinguishable as a separate branch in Superfamily IV, containing *Rhodopseudomonas*, *Beijerinckia*, *Agrobacterium*, *Rhizobium*, etc.

Before the last edition of *Bergey's Manual*, a set of 32 features was given to differentiate the genera *Gluconobacter*, *Acetobacter* and *Frateuria*; however, not all of these features are necessary for a satisfactory identification (De Ley et al., 1984). The ability to over-oxidize acetate identified the strains from 'high-acid' fermentations as members of the genus *Acetobacter*, and thus separates them from the genera *Gluconobacter* and *Frateuria*. Swings (1992) divided *Acetobacter* into seven species: *A. aceti*, *A. liquefaciens*, *A. pasteurianus*, *A. hansenii*, *A. xylinum*, *A. methanolicus* and *A. diazotrophicus*. In 1997, Yamada et al. proposed a new genus, *Gluconacetobacter* (previously recognized as a subgenus within *Acetobacter*), where several 'old' vinegar-producing *Acetobacter* species are now ascribed, such

as *Gluconacetobacter europaeus* and *Gluconacetobacter xylinus*. The present assessment of acetic acid bacteria taxonomy is reported in the second edition of *Bergey's Manual of Systematic Bacteriology* (Sievers and Swings, 2005), as described in Chapter 3.

### 9.3.2 Required Properties of Industrially Used Strains

If one considers the interest that acetic acid bacteria have aroused in many microbiologists, it is astonishing how little effect the available information about these bacteria has had on vinegar manufacture. This is particularly striking in regard to use of the pure culture approach in the vinegar industry. Henneberg (1926) pointed out the enormous advantages of introducing pure cultures in vinegar manufacture: it would reduce the occurrence of vinegar eels, 'Kahm' yeasts, and the unwanted contaminant *A. xylinus* (now *Ga. xylinus*). It would also permit the selection of suitable strains possessing the required technological and commercial qualities. Shimwell (1954) isolated an *A. aceti* strain as a true 'working' strain. With this strain he produced up to 12% malt, spirit or wine vinegar of excellent quality at rates as fast as in the normal production process. Another strain, thought to be the 'active one' in a quick vinegar process, was isolated from beechwood shavings by Wiame et al. (1959). However the original strain was lost (Suomalainen, 1962), which demonstrates the difficulties in isolating and growing *Acetobacter* strains from industrial vinegar fermentations on solid media.

Considerable success was achieved by propagating bacteria from generators in Japan on a special double-layer agar. The bacteria isolated on this medium have been described as '*Acetobacter polyoxogenes*' (Entani et al., 1985). However, the species *A. polyoxogenes* has not been validly published, and the strain is not available from the Japan Collection of Microorganisms, due to problems of propagation and preservation.

A new species in the genus *Acetobacter*, for which Sievers et al. (1992) proposed the name *Acetobacter europaeus* (now *Ga. europaeus* ), has been isolated and characterized in pure culture from industrial vinegar fermentations in Germany and Switzerland. All investigated strains isolated from submerged fermenters and trickle generators had very low (0-22%) DNA-DNA similarities with the traditional type strains of the genera *Acetobacter* and *Gluconobacter*. Phenotypical differentiation at the species level of the genus *Acetobacter* is rather difficult, since different strains of a single species do not necessarily utilize the same carbon source. A useful and significant criterion for the identification of *Acetobacter europaeus* (now *Ga. europaeus*) is its strong tolerance to acetic acid at a concentration of 4-8% in AE-agar, and the absolute requirement of acetic acid for growth.

Sokollek et al. (1998) isolated two *Acetobacter* strains from red wine and cider vinegar fermentations and described them as new species: *Acetobacter oboediens* sp. nov. and *Acetobacter pomorum* sp. nov. Comparative analysis of the 16S rRNA sequences revealed >99% similarity between the isolated strain LTH 2460 and the type strains of the related species *A. europaeus* (now *Ga. europaeus*) and

*A. xylinus* (now *Ga. xylinus*), and also between strain LTH 2458 and *Acetobacter pasteurianus*. On the other hand, low levels of DNA relatedness (<34%) were determined in DNA-DNA similarity studies. This relatedness below the species level was consistent with specific physiological characteristics, permitting clear identification of these strains within established species of acetic acid bacteria. Based on these results, the species *Acetobacter oboediens* sp. nov. and *Acetobacter pomorum* sp. nov. were proposed, but were then transferred within the genus *Gluconacetobacter* as *Gluconacetobacter oboediens* and *Gluconacetobacter pomorum* (Sievers and Swings, 2005).

Independent of all these problems and difficulties, the vinegar industry's first and foremost interest is to use a strain of acetic acid bacteria which tolerates high concentrations of acetic acid, which requires small amounts of nutrients, which does not over-oxidize the acetic acid formed, and which yields high production rates.

The vinegar industry has always worked with acetic acid bacteria which, in most cases, are not derived from pure cultures. The astonishing fact that common microorganisms used on a large scale for industrial vinegar production have not been properly described and characterized in taxonomic terms can be explained by the difficulty of cultivation of these bacteria on semi-solid media.

Industrial submerged vinegar fermentations are started by inoculation with 'inoculation vinegar', i.e. microbiologically undefined remains from previous fermentations. The lack of defined pure starter cultures is due to the previously mentioned problems in the isolation of the *Acetobacter* strains responsible for high acid production.

Acetic acid fermentation can be carried on for years without interruption or any decrease in efficiency or yield, as long as suitable reaction conditions are chosen to allow an ongoing selection process that favours only those organisms which tolerate high acidity and can survive on a minimum of nutrients. From industrial practice it has long been known that the properties of a newly isolated strain of acetic acid bacteria may change from the very first moment of cultivation in Petri dishes, and that this strain, if cultivated over a number of generations, may show other properties, especially as far as the adaptation to certain concentrations of acetic acid is concerned, but also regarding its phenotypic features. Therefore, in practice, the transformation of acetic acid bacteria from a surface culture to a submerged culture and *vice versa* is highly problematic with regard to the quantitative and qualitative results as long as total concentrations higher than 12% are to be maintained. The mechanism of this extremely high variability, which has not been described so far for other bacteria, is unclear.

## 9.4 Vinegar and Food Law

A legal assessment of vinegar is of great importance in order to define, within the framework of national and international legislation, whether a product is synthetic acid or biologically obtained vinegar, and whether it is a true wine or fruit vinegar, or a blend.

### 9.4.1 Differentiation between Vinegar and Acetic Acid

The problem can be approached from two angles.

- The specific impurities in the acetic acid, which go back to its production process, may serve as a criterion for differentiation. A survey of standard processes for synthetic acetic acid is given by Staege (1981). However, as acetic acid is nowadays produced with a high purity, the secure identification of polluting substances has become more difficult.
- A characterization of the specific compounds in fermentation vinegars is therefore growing in significance. A great variety of qualitative reactions and quantitative determinations are described in the literature.

### 9.4.2 Differentiation between Wine Vinegar and Spirit Vinegar

An accurate and reliable differentiation between true wine vinegars and their blends with spirit vinegar, or more generally between vinegars rich in extract and their blends, is of particular importance.

Generally, a good method is the identification of the specific fruit acids, such as tartaric acid in wine or malic acid in cider vinegar. A formol titration to identify amino acids may also be applied. On the other hand, fruit-specific acids and also amino acids can easily be added. Full analyses are often the best way to decide whether only pure wine has been used, or whether spirit vinegar has been added (Llaguno, 1977). However, and this will generally apply to the methods of differentiation, the limits of confidence for an estimate of the quantity of spirit vinegar added to wine vinegar are rather narrow.

### 9.4.3 Adulteration of Wines

The problem is how to prove the use of low-quality grape pomace wine, pome wine, spoilt vinegar wine, or even synthetic wine. For all these investigations (see Sections 9.5.1.1 and 9.5.1.2) a newly developed method offers very promising results. The authentication of the origin of vinegars can be determined by a modern isotopic method, called SNIF-NMR (site-specific natural isotopic fractionation-nuclear magnetic resonance spectrometry). This method was originally developed at the University of Nantes (Dumoulin, 1993) in France. Eurofins Laboratoires, a research institute in Nantes, carried out a preliminary research project in order to extend and complete the results of the application of the SNIF-NMR method, which is an official EEC analysis for wines, to the field of vinegars. The first results have demonstrated the applicability of the SNIF-NMR method to detecting added synthetic acid to vinegar and, more generally to determining the botanical origin of vinegar (wine, apple, malt, cane or beet alcohol, etc.). With this method it is possible not only to identify the raw materials of the corresponding vinegar but also to ascertain the origin of the grapes, e.g. from the

northern part of Italy. For this purpose a database of authentic vinegars has been compiled by Eurofins.

The detection threshold for adulterations with synthetic acid to vinegar of known and unknown geographical origin is 5% and 10%, respectively. The detection threshold in the case of alcohol vinegar being added to wine vinegar of known and unknown geographical origin is 10% and 20%, respectively.

The present procedure for authentication of vinegars using 2H-NMR spectroscopy and mass spectroscopy (MS) on acetic acid and water is ready to be made official by including it in the Code of Practice.

## 9.5 Analysis of Vinegar

Vinegar is analysed for two purposes: for process control by general routine methods, and to gain a comprehensive knowledge of its chemical constituents.

### 9.5.1 Detection of Alcohol Vinegar in Wine Vinegar

A safe differentiation between true wine vinegars and their blends with spirit vinegar, or generally between vinegars rich in extract and their blends, is of particular importance. Generally, a good method is the identification of the specific fruit acids, such as tartaric acid in wine or malic acid in cider vinegar. A formol titration to identify amino acids may also be applied. On the other hand, it is very easy to add fruit-specific acids and amino acids to any vinegar.

#### 9.5.1.1 Traditional Methods

The following methods have been in use for the differentiation of alcohol from spirit vinegar (for complete details, see Ebner et al., 1996a):

- ratio of acidity to dry residue
- UV-absorption
- chromatography
- determination of potassium.

All these methods are not very specific or, as mentioned above, can be manipulated easily.

#### 9.5.1.2 SNIF-NMR Method

For the detection of alcohol vinegar in wine vinegar, the SNIF-NMR-method is the most specific and precise method (see Section 9.4.3).

The detection threshold of adulteration with alcohol vinegar is:

- 10% of alcohol vinegar into wine vinegar of known geographical origin
- 20% of alcohol vinegar into wine vinegar of unknown geographical origin.

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# Chapter 10
# Traditional Balsamic Vinegar

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## 10.1 Introduction

The term 'balsamic vinegar' is frequently applied to describe sauces, condiments and dressings with particular sweet taste. In Italy there are two types of balsamic vinegar: 'balsamic vinegar of Modena' and 'traditional balsamic vinegar'. The first is a flavoured wine vinegar obtained by blending cooked must and wine vinegar and, in some cases, by adding a small amount of caramel. Traditional balsamic vinegar (TBV) is made in Modena and Reggio Emilia with cooked grape must, through a three-step process: conversion of sugars to ethanol by yeasts; oxidation of ethanol to acetic acid by acetic acid bacteria (AAB); and, finally, at least 12 years of ageing. The final product is a highly dense, dark-brown aged vinegar, having a sweet and sour taste, fruity and complex in flavour.

TBV acquires its special qualities during a long ageing performed with a 'dynamic' technology. Briefly, a set of barrels, generally from five to seven of different sizes and woods, are filled with cooked grape must, previously fermented and oxidized (Solieri et al., 2006). A small part of the vinegar is withdrawn every year from the smallest barrel, which is then refilled with the vinegar from the next sized barrel (Figure 10.1). This procedure, called '*rincalzo*', is done for each barrel, with the last and biggest one receiving new cooked must to replace the vinegar withdrawn, lost by evaporation, and the vinegar transferred from barrel to barrel. Therefore, each barrel contains a blend of differently aged vinegars and the age increases from the largest barrel to the smallest one, depending on barrel volume and amount of vinegar transferred. From a legal point of view, TBV must be aged at least for 12 years.

TBV has recently been acknowledged as a 'protected denomination of origin' product (P.D.O.) by the European Union (Council Regulation (EC) No. 813/2000). It is produced under the supervision of different Consortia according to their Official Production Regulations (Disciplinare di produzione, 2000). Two of these are in Modena – 'Consorzio Tutela Aceto Balsamico Tradizionale di Modena' and

L. Solieri, P. Giudici (eds), *Vinegars of the World*

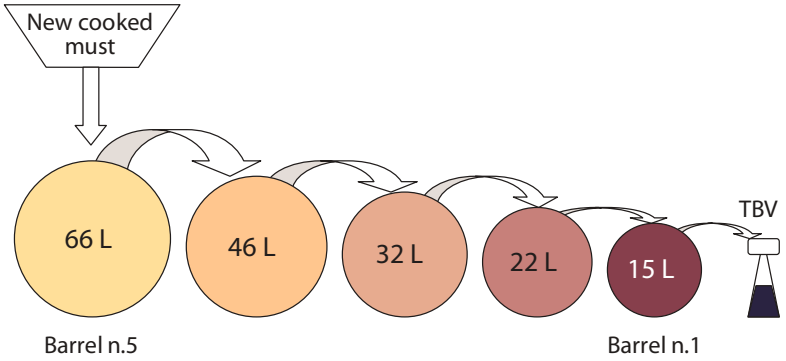


**Figure 10.1** Schematic representation of barrel set for traditional balsamic vinegar (TBV) production and of refilling procedure. The numbers correspond to the barrel capacity

'Consorzio fra produttori di Aceto Balsamico Tradizionale di Modena'. Other two Consortia are in Reggio Emilia – 'Consorzio Produttori Aceto Balsamico Tradizionale di Reggio Emilia' and 'Sindacato fra produttori di Aceto Balsamico Tradizionale di Reggio Emilia'. TBV is marketed in special glass bottles of defined shape and capacities, and the official labels and seals of the DO Consortia are mandatory to ensure its origin (Figure 10.2). DO consortia perform a sensory examination to assess the sensorial properties of products before marketing. On the basis of this analysis, TBV is classified in different quality categories, commanding different prices.


**Figure 10.2** Bottles of traditional balsamic vinegar of Modena (**a** courtesy of Consorzio Tutela Aceto Balsamico Tradizionale di Modena; **b** courtesy of Consorzio fra produttori di Aceto Balsamico Tradizionale di Modena) and of Reggio Emila (**c** courtesy of Consorzio Produttori Aceto Balsamico Tradizionale di Reggio Emilia; **d** courtesy of Sindacato fra produttori di Aceto Balsamico Tradizionale di Reggio Emilia).

## 10.2 Composition

The chemical composition of TBV is very complex and not yet completely known, due to both the great variability of the homemade products and the complex production system including several chemical and biological modifications. The main and best-described compounds are sugars and organic acids. Recently several works have been directed at the detection and identification of the various secondary components, such as polyphenols, end-products of the Maillard reaction and volatile compounds. An overview of the main substances contained in TBV can be found in the following sections.

### 10.2.1 Sugars

The soluble content of grape juice mainly consists of fructose and glucose, which are present in very similar amounts (glucose : fructose ratio, GFR ≈ 1). These sugars play a different role in TBV production and are subject to many chemical and microbiological modifications as a consequence of the must heating, microbial metabolism and ageing processes. Fructose is more sensitive to heat treatment than glucose and is preferentially fermented by osmotolerant non-*Saccharomyces cerevisiae* yeasts during the fermentative phase. On the other hand, glucose is preferentially fermented by *S. cerevisiae* yeast strains (Solieri et al., 2006) and can be directly oxidized to gluconic acid by acetic acid bacteria (AAB).

As result of all these modifications, the total solubles content of highly aged TBV samples is generally very high, mainly due to the natural evaporation of water, as shown in Table 10.1. Sugars have good positive correlations with °Brix values, but represent only a part of the total solutes and show a higher dispersion than °Brix values would suggest (Table 10.1). In the final product both glucose and fructose

**Table 10.1** Chemical characteristics of traditional balsamic vinegar (TBV), expressed as means of 104 samples

| Parameter | Mean | (±SD) |
|---|---|---|
| Soluble solids | 73.86 | (±1.73) |
| Titratable acidity | 6.67 | (±0.88) |
| 'R' ratio | 11.27 | (±1.53) |
| Glucose | 23.60 | (±3.45) |
| Fructose | 21.14 | (±3.37) |
| Tartaric acid | 0.78 | (±0.25) |
| Succinic acid | 0.50 | (±0.70) |
| Acetic acid | 1.88 | (±0.45) |
| Malic acid | 1.04 | (±0.32) |
| Gluconic acid | 1.87 | (±1.27) |
| Lactic acid | 0.12 | (±1.074) |

Amount expressed in g/100 g of TBV, as mean value of 104 samples presented at the annual competition held at Modena in 2005; titratable acidity is expressed as g of acetic acid per 100 g.

**Table 10.2** Soluble solids (°Brix), titratable acidity (TA) and 'R' factor of the best 12 classified traditional balsamic vinegar (TBV) samples in the competitions held in Modena (Palio of S. Giovanni) in 1982, 1996 and 2004*

| Sample | 1982 | | | 1996 | | | 2004 | | |
|---|---|---|---|---|---|---|---|---|---|
| | °Brix | TA | 'R' | °Brix | TA | 'R' | °Brix | TA | 'R' |
| 1 | 61.00 | 11.40 | 5.35 | 73.80 | 7.32 | 10.08 | 74.50 | 6.52 | 11.43 |
| 2 | 61.50 | 7.26 | 8.47 | 75.80 | 8.37 | 9.06 | 77.00 | 6.93 | 11.11 |
| 3 | 64.40 | 9.72 | 6.62 | 76.30 | 8.99 | 8.49 | 71.50 | 6.99 | 10.23 |
| 4 | 61.40 | 9.90 | 6.21 | 70.80 | 8.25 | 8.58 | 72.50 | 7.17 | 10.11 |
| 5 | 63.60 | 8.76 | 7.26 | 73.80 | 7.32 | 10.08 | 72.25 | 8.87 | 8.15 |
| 6 | 63.60 | 7.92 | 8.03 | 73.80 | 7.69 | 9.60 | 74.50 | 7.18 | 10.38 |
| 7 | 65.70 | 9.12 | 7.20 | 71.30 | 9.30 | 7.67 | 75.00 | 6.31 | 11.89 |
| 8 | 70.00 | 8.70 | 8.04 | 75.80 | 8.68 | 8.73 | 73.00 | 5.91 | 12.35 |
| 9 | 63.60 | 8.40 | 7.57 | 71.80 | 8.31 | 8.64 | 73.00 | 7.06 | 10.34 |
| 10 | 57.30 | 9.00 | 6.36 | 72.30 | 6.82 | 10.60 | 74.00 | 6.49 | 11.40 |
| 11 | 61.00 | 8.70 | 7.01 | 71.80 | 6.20 | 11.58 | 74.00 | 6.25 | 11.84 |
| 12 | 59.20 | 11.58 | 5.11 | 73.80 | 8.80 | 8.39 | 74.00 | 8.18 | 9.05 |
| Mean | 62.69 | 9.21 | 6.93 | 73.43 | 8.00 | 9.18 | 73.77 | 6.99 | 10.69 |

* Data kindly provided by Vincenzo Ferrari Amorotti

act as structure promoters, affecting the viscosity of TBV (Falcone et al., 2007) and the GFR is greater than 1 due to the higher reactivity of fructose to the Maillard reaction and the fructophilic metabolism of several TBV yeast species.

Other minor sugars have been evaluated in some TBV samples and their concentration ranges (expressed as $g \cdot kg^{-1}$) are as follows: xylose 0.11-0.39; ribose 0.078-0.429; rhamnose 0.061-0.195; galactose 0.136-0.388; mannose 0.41-1.46; arabinose 0.33-1.00; and sucrose 0.46-6.84 (Cocchi et al., 2006a).

During the last 20 years, the sugar content and, more generally, the soluble solids composition has been greatly changed (Table 10.2). In particular, considering sugar concentration, titratable acidity and their ratio, called the 'R factor', we can observe a considerable increase of soluble solids, with consequent higher 'R factor' values and strong changes in the sensorial properties of TBV, making it sweeter and less pungent. This new taste seems to better meet the preferences of consumers, as well as of professional panel testers, who give the sweetest vinegars the highest scores.

The importance of 'R' values was first established by Gambigliani-Zoccoli (Amorotti, 1999), who described the distribution of the 'R' values in the barrel set and defined the 'R' value range most suitable for each barrel to achieve TBV of high quality.

## 10.2.2 Organic Acids

After glucose and fructose, organic acids are the second main component of TBV. Compared with other wine vinegars, TBV shows very similar values of titratable acidity, but a quite different organic acid composition. While the main organic acid

of a wine vinegar is acetic acid, TBV contains other organic acids, some of which occur in the same amounts as acetic acid, such as tartaric, gluconic, malic and succinic acids (Table 10.1). These compounds play a key role in determining the sensorial properties of the vinegar, since they are less pungent than acetic acid and have a different taste. For example, citric acid possesses sweet and sour sensory notes; succinic acid has a salty-bitter taste; and sour, unripe and irritating/pungent flavours are ascribed to lactic acid, malic acid and acetic acid, respectively. Gluconic acid generally has an acidulant and tangy taste.

Several techniques, including HPLC (high-performance liquid chromatography), gas chromatography (GC), GC/MS (gas chromatography-mass spectrometry) and enzymatic methods, have been used to evaluate the organic acid concentration many different TBV samples (Bertolini et al., 1994; Giudici et al., 1994; Cocchi et al., 2002, 2006b; Sanarico et al., 2003; Masino et al., 2005, 2008). Tartaric acid is present at a low percentage in aged TBV and its concentration decreases noticeably during ageing, probably because of its precipitation as potassic or calcic salts. Succinic and citric acids also occur at low concentrations. Unlike tartaric acid, citric and malic acid concentrations do not undergo a great deal of change during the ageing phase, remaining almost constant in different samples (Cocchi et al., 2002). However, the amount of succinic acid increases in the younger samples and decreases in the older ones, probably because of esterification reactions. Gluconic acid concentration has a wide variability in TBV, depending on ageing and the technological procedure employed. It is generally high in old TBV and has been proposed as quality parameter (Giudici, 1993).

The TBV organic acids have different origins: acetic and gluconic acids are mainly due to the AAB metabolism of ethanol and glucose, respectively; tartaric acid arises from the grape; succinic acid from yeast metabolism; and, finally, D-/L-lactic acids, which are generally present in low amounts, are derived from lactic acid bacteria or yeast metabolism.

### 10.2.3 Molecules with Antioxidant Activity

TBV shows antioxidant activity due to polyphenolic compounds and melanoidins (Piva et al., 2008; Tagliazucchi et al., 2008). Melanoidins and other Maillard reaction products are nitrogenous polymers and co-polymers, which contribute to 45% of the TBV's antioxidant activity. Phenolic compounds are present at 1882.2 ± 53.8 mg/kg TBV and the species most represented in TBV are phenolic acids (37.8 ± 1.7% of the total polyphenols) followed by catechins (36.0 ± 1.8%), polymeric procyanidins (18.8 ± 1.3%) and flavonols (7.4 ± 1.5%). Tannins also contribute significantly to antioxidant activity and are present in amounts ranging from 263 to 307 mg/kg TBV. Piva et al. (2008) reported that thermal treatment determined the degradation of simple phenolics, such as catechins, and the formation of condensed tannins. Considering that uncooked must contains about 260 mg/kg of polyphenols (Antonelli et al., 2004), the increase in the concentration of polyphenols from the uncooked must to the aged vinegar could be due both to a concentration process

and to other mechanisms, such as the extraction of polyphenols from wood (Tagliazucchi et al., 2008).

### 10.2.4 Other Components

The presence of esters, alcohols, aldehydes, ketones and other chemical constituents is well documented, but unfortunately there are no quantitative data for evaluating their influence on the sensorial properties of TBV.

Zeppa et al. (2002) detected 100 volatile compounds during the acetification of three barrel sets: ketones, aldehydes, ethyl esters, and acetates from alcoholic and acetic fermentation, and the furan derivates from the Maillard reaction, such as furfural, 5-methyl-2-furaldehyde, 5-(hydroxymethyl)furan-2-carbaldehyde (HMF), 5-acetoxymethylfurfural and 2-furoic acid, were the most abundant compounds identified.

Volatile compounds have also been evaluated by headspace SPME (solid phase microextraction) (Puglisi et al., 2002). Among these, the following compounds have been detected:

- *alcohol compounds*: ethanol, ethanol, 1-propanol, 1-butanol, 2-methylpropanol, 2-methylbutanol, 3-methylbutanol, phenyl ethyl alcohol
- *carboxylic compounds*: such as acetic acid, hexanoic acid, octanoic acid, 1-undecanoic acid, decanoic acid, 2-methylpropanoic acid, 2-methylbutanoic acid, 3-methylpentanoic acid
- *aldehydes*: mainly acetaldeyde
- *esters*: ethyl or diethyl esters of different carboxylic acids (butanoic acid, hexanoic acid, octanoic acid, decanoic acid, tetradecanoic acid, pentanedioic acid, hexadecanoic acid and hexanedioic acid).

## 10.3 Physical Features

### 10.3.1 Viscosity

There is long-term anecdotal evidence from vinegar producers that both the flow/syrupiness and composition properties (mainly glucose, fructose and acetic acid concentrations) play a key role in determining overall TBV quality. Viscosity is a important parameter for the sensorial quality of vinegars; nevertheless, no procedure has yet been established to determine this objectively. The perceived viscosity of TBV is actually assessed in an empirical manner – i.e. by rotating the TBV in the bottle – but it is wrongly expressed as physical density, which is measured as required by the official sensing procedure used for grading TBV.

Unfortunately, the perceived viscosity of vinegar, as for other liquid foods, depends on its flow behaviour more than on its physical density. From a physical

point of view, the viscosity of a liquid represents a macroscopic measurement of the strength of the inner molecular interactions and can be used to describe the flow behaviour under mechanical stresses. This fundamental property depends on the chemical composition of the fluid and on temperature, irrespective of the experimental conditions imposed.

TBV viscosity and composition are strictly related to the crystallization phenomenon, a physical instability that occurs in highly viscous TBV. Thermodynamic and kinetic forces are involved in the crystallization process. Supersaturation (thermodynamic driving force) is necessary before crystallization can occur. However, not every supersaturated solution will crystallize during a reasonable period of time: an excess of viscosity can retard the mass transfer (kinetic driving forces) and therefore the crystallization, whereas an excess of free water can accelerate the process. This means that TBV, supersaturated with respect to sugars, could crystallize spontaneously at room temperature; while a low mobility of its constituents can determine a kinetically constrained state but not an equilibrium one. One of these mechanisms could be the dominant effect and the assessment of the exact contribution of thermodynamic and kinetic driving forces is a very great challenge for predicting sugar crystallization of TBV (Falcone et al., 2007).

Recently, some rheological research has demonstrated that titratable acidity, °Brix, glucose content, acetic acid, malic acid and D-gluconic acid (in decreasing order) contribute to TBV viscosity (Falcone et al., 2007, 2008). Two rheological parameters were proposed as descriptors of TBV quality: the shear viscosity measured at 500 $s^{-1}$ and 25 °C, and the flow behaviour index. In particular, shear viscosity is a good parameter to distinguish TBV containing small amounts of crystallized sugars not detectable by visual inspection or tasting, and it can be used to 'predict' the susceptibility to sugar crystallization in TBV. Flow behaviour index has been proposed as an objective descriptor of the crystallization extent in TBV.

Apart from sugars, other components, such as high-molecular-weight compounds and melanoidins, can affect viscosity, influencing crystallization in two different ways. As polymers with high molecular weight, melanoidins could act as crystallization/vitrification nuclei; otherwise, they could reduce the macromolecular mobility (diffusion of solutes), increasing the viscosity. In addition, the role of high-molecular-weight compounds in viscosity and crystallization needs further in-depth investigation.

### 10.3.2 Colour

Colour is one of the most important features used by consumers to assess the quality of a food product. The TBV's colour ranges from light to dark brown, and usually the latter is considered a good indicator of ageing. The mechanism involved in colour formation of TBV is not yet fully understood. According to results reported for other foods, melanoidins are mainly responsible for determining the characteristic brown colour (Rivero-Pérez et al., 2002; Mastrocola et al., 2004; Falcone and Giudici, 2008; Piva et al., 2008). Acidic-thermal degradation of sugars, carameliza-

tion and the Maillard reaction are responsible for the formation of melanoidin polymeric compounds in TBV. There is a clear relationship between melanoidin concentration, structure and colour formation: the more concentrated and browned product is, the higher is the melanoidin concentration (Motai, 1976; Martins et al., 2001; Belitz and Grosch, 2004).

### 10.3.3 pH

The pH value of vinegar is of fundamental importance because it affects the dissociation mechanism of acids. TBV is a very concentrated solution, where many different ions and soluble solids act to change the hydrogen ion concentration in any process that involves $H^+$ ions. Because the activity and concentration of these different substances can vary so much in TBV, it is difficult to determine accurate pH values. However, the reading pH values of food samples can be considered to indicate the true $H^+$ concentration within an acceptable margin of error.

Examining more than 100 samples of TBV, Masini and Giudici (1995) reported pH reading values in the range of 2.3-2.8. Even though the reading pH values are strongly influenced by the complex composition of TBV, it is evident that they are lower than in any other vinegar.

The cooking of the must entails a decrease in pH of 0.3 (pH decreases from 3-3.07 to 2.74-2.77) and the ageing process is also characterized by the lowering of pH, due to the concentration of organic acids. In addition, the complex carboxylic acid composition of TBV, characterized by several organic acids with $pK_a$ values lower than that of acetic acid (pK 4.76), such as gluconic acid (pK 3.86), malic acid ($pK_1$ 3.40; $pK_2$ 5.10) and tartaric acid ($pK_1$ 2.98; $pK_2$ 4.34), acts to decrease the pH of TBV. Therefore the low pH values could be considered a distinctive characteristic of TBV related to its production technology and organic acid profile.

## 10.4 Basic Technology

The TBV production process starts from freshly squeezed grape juice and finishes with sensorial evaluation of the aged vinegar. In between, there are a great number of physical, biological and chemical transformations of the grape juice components (Figure 10.3).

Because of its ancient and artisanal mode of production, there are different methods of obtaining TBV. Various different ways of preparing TBV are reported by Saccani and Ferrari Amorotti (1999). For example, a mention of two types of vinegar was reported by Giorgio Gallesio (Saccani, 1998a); and a bizarre ‘alla Modenese’ procedure was described by Abbot Lajosa Mitterparcher, consisting of throwing scorching hot roof tiles into raw Trebbiano must (Saccani, 1998b).

In recent decades, one procedure has taken the lead and is nowadays considered the standard protocol accepted by officially set rules (Disciplinare di produzione, 2000). The procedure is based on a description written by Francesco Agazzotti in a

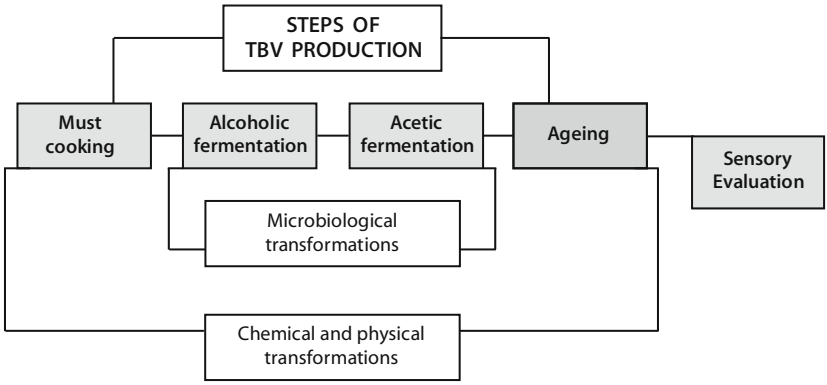


**Figure 10.3** Overview of traditional balsamic vinegar (TBV) production

letter to his friend, Pio Fabriani, dated 1860 (Saccani, 1998b). To our knowledge, this is the first clear description of how to make TBV.

TBV is obtained by a traditional slow process summarized in three practical steps: cooking of must; microbiological transformations (or two-stage fermentation, including alcoholic fermentation of cooked must and subsequent acetification of fermented cooked must); and ageing. Finally, official sensory procedures, defined by the Consortia of Modena and Reggio Emilia, grade TBV and certify its quality before marketing.

### 10.4.1 Must Cooking

Concentrated grape must is used as a sweetener in the food industry, according to European Union (EU) regulations, which define concentrated rectified must as the (non-caramelized) liquid obtained by partial dehydration of the rectified must: the required chemical and physical characteristics are also specified. In TBV production, freshly squeezed juices from local grape varieties (i.e. Trebbiano, Lambrusco, Ancellotta, Sauvignon, Berzemino, Occhio di Gatta and Sgavetta) are concentrated by heating at atmospheric pressure in an open vessel, following the D.O.P. specifications. The must temperature is raised to boiling point, then the must is skimmed to remove dispersed solids and coagulated proteins, and then the temperature is lowered to 80-90 °C and maintained for several hours. The cooking is generally stopped when the grape must concentration reaches 35-60° (°Brix) of soluble solids.

During the cooking of must, the thermal treatment concentrates the volume of grape must, causing non-enzymatic browning reactions. As a consequence, the concentration of many constituents (sugars, organic acids, nitrogenous compounds, metal ions and polyphenols) was observed, together with the increase in neo-formation compounds, such as HMF, melanoidins and other related compounds of the Maillard reaction (Antonelli et al., 2004).

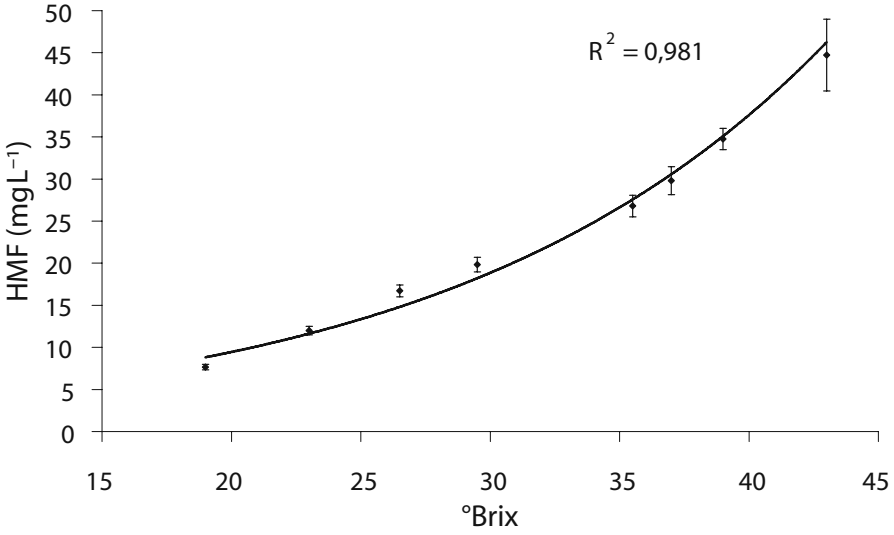


**Figure 10.4** Formation of HMF in grape must at different soluble solids concentrations (reprinted with permission from Muratore et al., 2006, copyright © 2006 American Chemical Society)

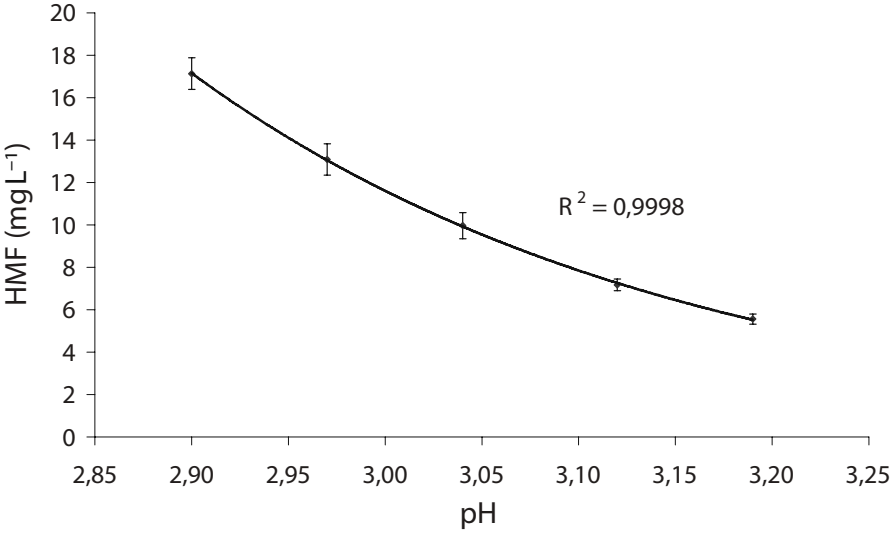


**Figure 10.5** Influence of pH on the formation of HMF (reprinted with permission from Muratore et al., 2006, copyright © 2006 American Chemical Society)

Furanic compounds, and HMF in particular, are supposed to have negative effects on human health and have been extensively studied in recent years. Regarding HMF formation, the Maillard reaction involves hexoses through an acid-catalysed dehydration and cyclization mechanism. Fructose was found to be twice as reactive as glucose in the formation of HMF and brown pigments (Göğüs et al., 1998; Belitz and Grosch, 2004). As sugars are substrates of HMF formation, a proportional relationship should occur between HMF and °Brix. However, Muratore et al. (2006) found that the HMF concentration is correlated with the sugar concentration by an exponential curve (Figure 10.4). This exponential trend has been explained by assuming that the low pH value positively affects the acid-catalysed reaction of hexose degradation, as shown in Figure 10.5. In fact, the cooking of must determines a decrease in pH, and the rate of HMF formation increases at later stages due to this pH reduction (Muratore et al., 2006).

Finally, low water activity ($a_w$) of TBV also enhances HMF formation by changing the equilibrium during the dehydration and cyclization of hexoses. After a slow enolization, the degradation of each molecule of hexose comprises proton-catalysed β-elimination of three molecules of water from the intermediates. As shown in Figure 10.6, low values of $a_w$ could speed up the formation of HMF; the equilibrium of the reaction shifts to the right at low $a_w$ values, due to the easier elimination of water. The importance of $a_w$ in the formation of HMF is also underlined by its high recovery of during the storage of concentrated must.

During must cooking the formation of other furan derivatives occurs, including 2-furfural (FAH), 2-furoic acid (FAC) and 5-acetoxymethyl-2-furaldehyde (AMFA). The former two (FAH and FAC) derive from pentose; the latter (AMFA) from acetylation of HMF (Antonelli et al., 2004).

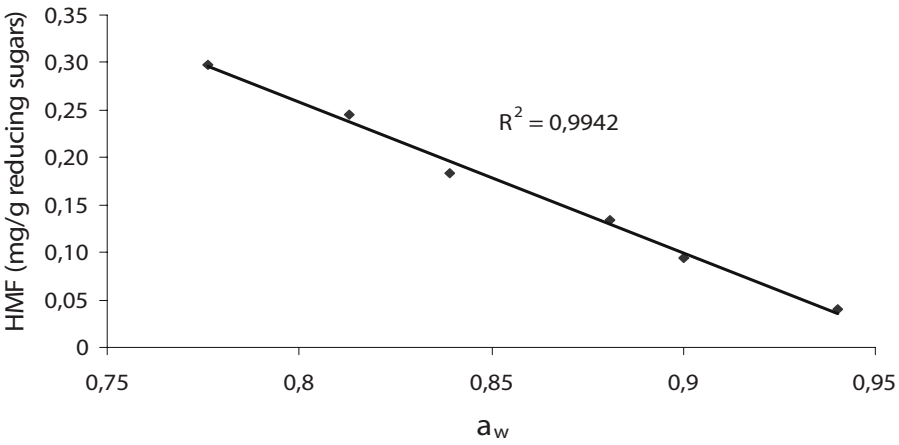


**Figure 10.6** Influence of water activity ($a_w$) on HMF formation in must at 19 °Brix mixed with 0, 5, 10, 15, 20 and 25% NaCl (from Muratore et al., 2006, copyright © 2006 American Chemical Society)

Finally, heat treatment determines the non-enzymatic browning due to the development of melanoidins, which give the musts their typical brown colour and caramel-like odour. Piva et al. (2008) reported that melanoidins increase during the heating concentration, and Falcone and Giudici (2008) found that they also increase during ageing (see Section 10.5.2).

### 10.4.2 Fermentation of Cooked Must

In the past, there was a general assumption that alcoholic and acetic fermentation occur together in the same barrel (Sacchetti, 1970; Bortolotti, 1996; Saccani, 1998b). Nowadays it is an accepted procedure to ferment the must up to 5-7 degrees of alcohol (v/v) and then to start the following steps of acetic fermentation and ageing in different barrel sets (Solieri and Giudici, 2008). The two-step fermentation of TBV was first described and postulated in the 1990s (Giudici, 1990; Giudici et al., 1992), on the basis of the high sensitivity of yeasts to acetic acid in an acid environment. At a pH close to 3.0, 1% of acetic acid (v/v) is enough to inhibit the majority of the yeasts; therefore alcoholic fermentation should always occur before acetic fermentation, following a scalar model.

The yeasts involved in spontaneous fermentation belong to different species and genera, as listed in Table 10.3. The large number of yeast species associated with TBV fermentations reflects the wide variability of TBV samples, produced differently from one farm to another. The composition of cooked must seriously affects

**Table 10.3** Fermentation rate, ethanol production and selective sugar consumption of ten TBV-associated species at increasing sugar concentrations

| Species | Fermentation rate[a] | | | Ethanol[b] | | | Selective sugar consumption[c] | | |
|---|---|---|---|---|---|---|---|---|---|
| | 350 | 400 | 450 | 350 | 400 | 450 | Glu | Fru | Glu/Fru |
| *C. lactis-condensi* | 1.8 | 1.9 | 0.8 | 7.4 | 8.2 | 7.3 | 171.7 | 1.4 | 122.10 |
| *C. stellata* | 1.8 | 1.7 | 0.6 | 7.3 | 7.8 | 6.8 | 151.8 | 9.7 | 15.65 |
| *Z. bailii* | 0.6 | 0.7 | 0.4 | 7.0 | 7.2 | 5.9 | 138.6 | 15.0 | 9.24 |
| *Z. pseudorouxii* | 0.3 | 0.2 | 0.3 | 6.3 | 6.5 | 6.3 | 159.9 | 29.8 | 5.36 |
| *Z. mellis* | 0.4 | 0.4 | 0.3 | 5.7 | 6.0 | 5.7 | 175.6 | 35.8 | 4.90 |
| *Z. bisporus* | 0.5 | 0.4 | 0.3 | 5.8 | 6.1 | 4.6 | 155.8 | 51.1 | 3.05 |
| *Z. rouxii* | 0.4 | 0.4 | 0.4 | 5.3 | 5.7 | 5.3 | 160.6 | 53.2 | 3.02 |
| *H. osmophila* | 1.4 | 1.5 | 0.7 | 5.0 | 5.3 | 4.4 | 98.5 | 133.9 | 0.75 |
| *H. valbyensis* | 1.1 | 0.9 | 0.6 | 5.4 | 5.2 | 4.0 | 101.2 | 127.3 | 0.79 |
| *S. cerevisiae* | 2.0 | 1.9 | 1.0 | 8.6 | 8.2 | 5.9 | 42.3 | 96.4 | 0.44 |

[a] Fermentation rate evaluated as g of $CO_2$ free for 100 ml cooked must after 72 h at 350, 400 and 450 $g \cdot L^{-1}$ sugar concentrations.

[b] Ethanol amount (v/v) evaluated at fermentation and at 350, 400 and 450 $g \cdot L^{-1}$ sugar concentrations.

[c] Residual sugars ($g \cdot L^{-1}$) evaluated at 350 $g \cdot L^{-1}$ starting sugar.

Abbreviations: Glu, glucose; Fru, fructose; C., *Candida*; Z., *Zygosaccharomyces*; H., *Hanseniaspora*; S., *Saccharomyces*.

From Solieri and Giudici, 2005

yeast species, growth rate and species ratio. Two main factors, in particular, inhibit the yeast growth: high sugar concentration from 30% to 50% (w/w) and low pH values, 3 or less, together with an amount of acetic acid higher than 1% (Giudici, 1990).

Many studies suggest that yeast metabolism influences the quality of TBV, even though the mechanisms are not yet fully understood (Landi et al., 2005; Solieri and Giudici, 2008). In particular, yeast sugar uptake affects the GFR of TBV. Glucophilic yeast species, such as *S. cerevisiae*, preferentially use glucose, whilst others, such as *Candida* and *Zygosaccharomyces* spp., preferentially ferment fructose and are defined as fructophilic. These yeasts are generally more osmotolerant than glucophilic yeasts, thus they grow well in highly sugary cooked must, consuming all the fructose and leaving glucose (Table 10.3). The increase in GFR in the cooked must could determine the glucose precipitation as solid crystals (Landi et al., 2005).

TBV fermentation by indigenous yeasts is uncontrolled and frequently slows down or stops before enough ethanol has been produced for the following oxidation by AAB, resulting in a low yield of acetic acid. Therefore technological developments in TBV-making are aimed at increasing awareness of the benefits to be gained from using yeast starter cultures, which can improve the safety, stability and efficiency of alcoholic fermentation, as well as successfully increasing the growth of AAB. Even though starter cultures could positively affect the economic yield of TBV, they are not yet employed (Solieri and Giudici, 2005).

In winemaking, the selection of starter culture is carried out among strains of *S. cerevisiae*, whereas in TBV-making more than one species could be tested, due to the high number of yeast species able to ferment cooked must. Their metabolic profile in cooked must is still unexplored and, consequently, their species/strain-specific influence on TBV quality is poorly known. However, some required traits have been defined to select yeasts for TBV-making (Solieri and Giudici, 2008). For example, glucophilic *S. cerevisiae* strains could be employed to avoid an excess of glucose being involved in crystal precipitation (Solieri and Giudici, 2005, 2008). Clonal selection performed by TBV sample collection, isolation of pure cultures, and their rapid identification and metabolic characterization are the real bottlenecks in setting up a controlled fermentation process.

### 10.4.3 Acetification of Fermented Cooked Must

Ethanol oxidation of fermented cooked must is performed by indigenous AAB. The practice of back-slopping is performed at the beginning of the process to start the acetification in a new barrel set. In brief, freshly made and non-pasteurized wine vinegar, called seed-vinegar, is added to fermented cooked must, to increase the acetic acid content, as well as the number of AAB cells. The amount of acetic acid in fermented must should always be higher than 3%, to completely stop the alcoholic fermentation. If the acetic acid amount is not enough to inhibit the yeast, the AAB grow slowly and the acidification process fails.

The AAB strains most frequently occurring in TBV belong to the following species: *Gluconacetobacter xylinus*, *Acetobacter pasteurianus*, *Acetobacter aceti*,

*Gluconacetoacter europaeus*, *Gluconacetobacter hansenii* and *Acetobacter malorum* (De Vero et al., 2006; Gullo et al., 2006; Gullo and Giudici, 2008). Alcohol, sugar and acetic acid content of fermented cooked must are very important parameters, strongly affecting the growth of AAB in TBV. In general, few AAB species are able to ferment must with a sugar concentration higher than 25% (w/v). As sugar concentration changes according to the dimension of the barrels and their position in the cask set, the biological layer of AAB was observed only in the largest barrels with the lowest sugar content, confirming that the main obstacle to AAB growth is the high sugar concentration of the cooked must. Therefore sugar tolerance has been detected as an important technological trait for selecting AAB starter able to oxidize fermented cooked must, whereas ethanol concentration is a less significant parameter (Gullo et al., 2006). The screening of 48 AAB strains isolated from TBV for their growth at increasing sugar concentrations showed that all of them grew at 20% glucose, but only four at 25% glucose (Gullo and Giudici, 2008). Among the most sugar-tolerant species is *A. malorum* (De Vero et al., 2006), which was first proposed by Cleenwerck et al. (2002) to describe AAB isolated from spoiled apples and able to grow on 30% of glucose.

### 10.4.4 The Ageing Process

Ageing can be defined as the time that vinegar is left in the barrel set, after all the biological transformations have occurred. During this time there is both a concentration of solutes, due to water loss, and several chemical transformations. Even though several studies have been performed to understand the vinegar ageing process, a clear picture of all the physico-chemical phenomena occurring during ageing has yet to emerge.

#### 10.4.4.1 The Barrel Set

The barrel set plays an important role in ageing. It is composed of casks made of different woods, such as oak, mulberry, chestnut, cherry, juniper and acacia. In the past, different woods have been considered important to increase the sensorial properties of TBV but, to our knowledge, there is no scientific evidence to support this. Furthermore, the crude taste of the different woods is considered a defect in TBV, and thus the new barrels have to be 'deflavoured' before use. New barrels are filled with wine vinegar and left to stand for at least 6 months, then the vinegar is discharged and the barrels are ready for TBV ageing.

The role of wood barrels in TBV ageing is mainly due to their gas permeability ($O_2$ and water vapour) and molecular selectivity based on molecular size. The wood acts as a semi-permeable membrane and selects the molecules able to cross the wood as a function of their stereochemical dimensions (Siau, 1984). In general, the strucutre of wood is enough to stop all molecules with a greater dimension than water, such as alcohols, esters and acetic acid (all molecules with sensorial impact). On the other hand, water can freely cross the wood in a vaporized state.

The driving force that moves $H_2O$ from the inside to the outside of the barrel is mainly related to the relative humidity (RH) of the cellar and to the osmotic pressure of the TBV. In general, the lower the RH of the cellar, the faster the rate of mass transfer from the barrel to the air. Conversely, the higher the osmotic pressure of the TBV, the lower the mass transfer. The mass transfer results in an increase of solute concentration inside the TBV barrels.

When the barrels are full of vinegar and closed, the only mass transfer from inside to outside is related to water molecules across the wood. On the other hand, if the barrels are left open, large amounts of molecules with low vapour pressure are lost from the bunghole during the ageing process and the wood does not act as a selective membrane (Giudici et al., 2006). This simple observation does not accord with the common practice of keeping the barrels not completely filled (with a head-space of several centimetres between the upper surface of the liquid and the top of the barrel) and the bunghole open (or covered only with white cotton cloth). Consequently both practices have a negative effect on the TBV because they contribute to the loss of sensorial compounds from the open bunghole. Preliminary results have shown that sealed barrels could improve the sensorial properties of TBV.

## 10.5 The Age of TBV

The commercial value of TBV changes considerably with age and top prices are commanded for very old vinegar (25 years or older). From a legal point of view, TBV must be aged for at least 12 years, but no objective procedure has been set up for assessing age and correctly classifying vinegars. This is a really weak point in the assurance of quality and authenticity of TBV. In addition, the vinegar's age cannot be measured on the basis of barrel set: an old cask could contain young vinegar, because of the partial and continuous vinegar renewal. Several authors have attempted to solve the problem, focusing their efforts on analytical studies (Chiavaro et al., 1988; Plessi et al., 1989; Giudici, 1993; Giudici et al., 1994; Zeppa et al., 2002; Masino et al., 2005; Muratore et al., 2006), chemiometric characterization (Consonni and Gatti, 2004; Cocchi et al., 2006a, 2006b; Durante et al., 2006), and physical measurements (Falcone et al., 2008). Unfortunately, the results have been very far from proving the vinegar's authenticity or differentiating between vinegars of different ages. Nowadays only sensorial evaluation is used to verify the age of TBV. Recently, two new approaches, based on mathematical equations and melanoidin fingerprinting, respectively, have been proposed, and these are briefly summarized in the following sections.

### 10.5.1. Mathematical Model for Calculating TBV Age

In a recent paper, a theoretical model was formulated to calculate the age of TBV (Giudici and Rinaldi, 2007). This approach takes into account the fact that the refilling procedure generates a blend of products with different ages depending on:

(i) the amount of vinegar withdrawn; (ii) the amount of vinegar transferred from barrel to barrel; and (iii) the volume of new cooked must added. The age of the TBV in each individual barrel can be described by a sequence of real numbers depending upon the number of years of the barrel set and volume of vinegar transferred. There is a finite limit for the vinegar age and this upper limit can be formulated through the values of both the volume of vinegar in each barrel ($V_i$) and the volume transferred from barrel to barrel ($R_i$). The numerical $E_i$ sequences are strictly increasing and have a finite limit as $n$ (years) approaches infinity. The limit for each barrel in a hypothetical example of five casks is reported below:

Age limit in barrel number 5 after refilling: $\frac{V_5}{R_5} - 1$

Age limit in barrel number 4 after refilling: $\frac{V_5}{R_5} + \frac{V_4}{R_4} - 1$

Age limit in barrel number 3 after refilling: $\frac{V_5}{R_5} + \frac{V_4}{R_4} + \frac{V_3}{R_3} - 1$

Age limit in barrel number 2 after refilling: $\frac{V_5}{R_5} + \frac{V_4}{R_4} + \frac{V_3}{R_3} + \frac{V_2}{R_2} - 1$

Age limit in barrel number 1 after refilling: $\frac{V_5}{R_5} + \frac{V_4}{R_4} + \frac{V_3}{R_3} + \frac{V_2}{R_2} + \frac{V_1}{R_1} - 1$

Since the age of the withdrawn vinegar at year $n$ is $E_1$ $(n–1)+1$, the limit of the age for the withdrawn vinegar is

$$\frac{V_5}{R_5} + \frac{V_4}{R_4} + \frac{V_3}{R_3} + \frac{V_2}{R_2} + \frac{V_1}{R_1}$$

The model can be repeated if we use a set of $t$ barrels with $t$ being an integer greater or equal to 1. Using similar notations, we conclude in the same manner that for each $i$, with $1 \leq i \leq 1$, the age limit in barrel number $i$ is

$$\sum_{j=1}^{t} \frac{V_j}{R_j} - 1$$

Moreover, the age limit of the withdrawn vinegar is

$$\sum_{j=1}^{t} \frac{V_j}{R_j}$$

The formula proposed by Giudici and Rinaldi (2007) is true, universal and independent of the amount of vinegar lost by evaporation. It allows us to calculate the TBV age in any barrelthe basis of some technological data, i.e. both the fluxes and volumes of vinegar in the barrels. Furthermore, since the added volume is strictly

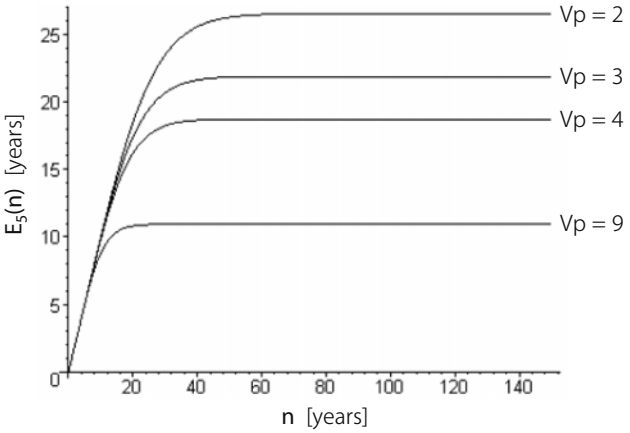


**Figure 10.7** Estimation of vinegar age in the smallest barrel after refilling ($E_5$) at different vinegar withdrawal amounts (2, 3, 4 and 9.0131 litres) (from Giudici and Rinaldi, 2007, copyright © 2007 with permission from Elzevier)

dependent on the volume of TBV withdrawn, it is possible to calculate the ages of TBV at different amounts of vinegar withdrawn (Figure 10.7). This simulation clearly suggests that the volume of TBV withdrawn is the main indicator of TBV age.

From a practical point of view, this mathematical model can define the maximum amount of TBV that each producer can sell as an *X*-year-old product, where *X* is a number of years greater than the minimum legal age of 12 years (Figure 10.8). Any producer could use this model to validate the age of their vinegars and the consumer's interests could therefore be protected.

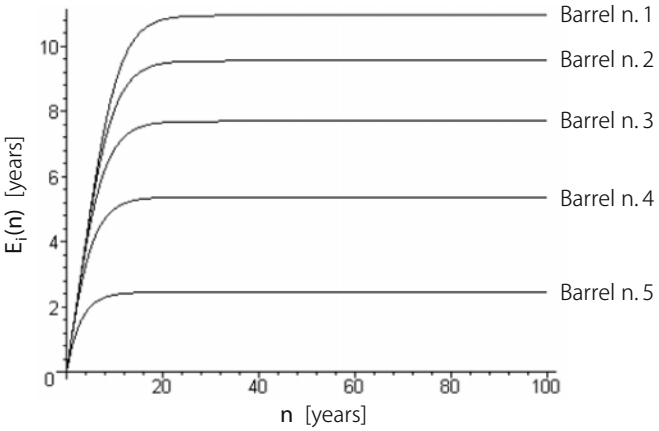


**Figure 10.8** Estimation of vinegar age in five barrels after refilling and with a withdrawn volume of 9.0131 litres. The lowest curve corresponds to the biggest barrel, whereas the upper curve corresponds to the smallest barrel (from Giudici and Rinaldi, 2007, copyright © 2007 with permission from Elzevier)

From a legal point of view, the mathematical model is a very easy tool for defining quantitatively the maximum possible volume of 12-year-old TBV for any producer, as well as for predicting the age of reference vinegar samples before performing the analytical assessment of their composition.

### 10.5.2 Molecular Weight Distribution as an Ageing Marker

Melanoidins are polymeric compounds, produced from acidic-thermal degradation of sugars, caramelization and the Maillard reaction. They affect the physical properties of TBV, including colligative properties, refractive index, density, viscosity, specific heat capacity, and glass-transition temperature. Recently, a high-perform-

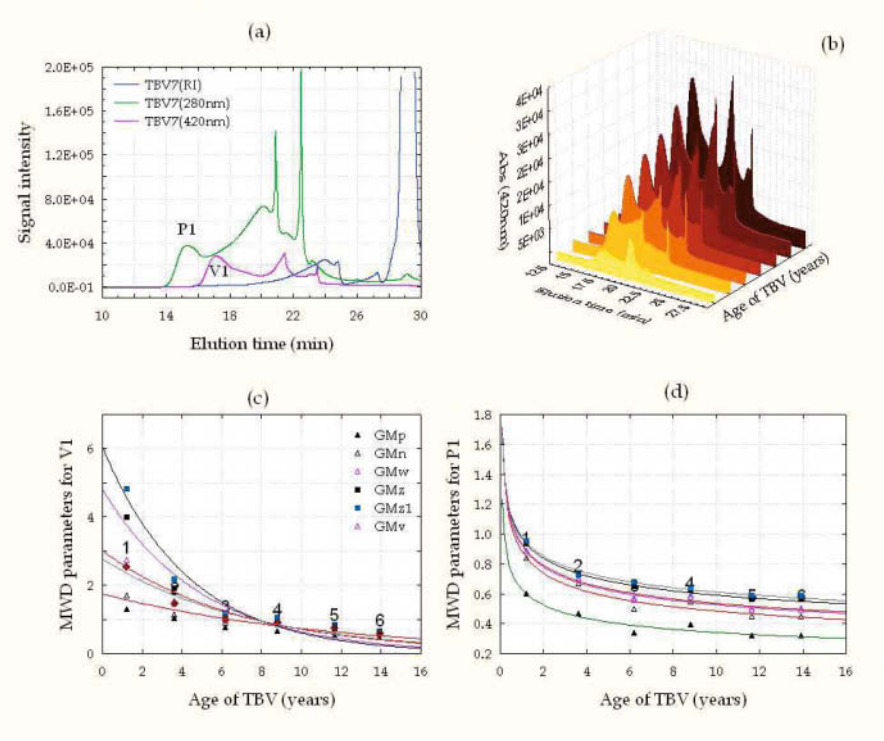


**Figure 10.9** Size-exclusion chromatography (SEC) elution pattern showing: **a** the molecular weight distribution of a 14-year-old TBV sample as detected by refractive index (RI) and ultraviolet-visible (UV/VIS) spectroscopy; **b** UV-SEC elution profiles of TBV samples as detected at 280 nm over 14 years of ageing (peaks represent all melanoidin populations containing coloured chromophores); **c** time-course of the MWD parameters for a coloured melanoidin population detected at 420 nm; **d** an uncoloured melanoidin population detected at 280 nm

ance size-exclusion chromatographic (HPSEC) procedure has been developed to study molecular weight (MW) and molecular weight distribution (MWD) in both the cooked grape must and TBV samples with increasing age (Falcone and Giudici, 2008). The authors defined the melanoidin distributions by determining MWD parameters, and showed that polymerization and depolymerization reactions take place during TBV ageing, affecting structure, composition and distribution of molecular weight constituents as well as the end-properties of vinegar, including colour and viscosity-related functionalities. These findings suggest that the SEC-elution profiles represent a 'structural fingerprinting' of the non-equilibrium status of TBV upon ageing (Figure 10.9a,b). Some MWD parameters for melanoidin populations were observed to be age-related properties, which reach an upper asymptotic limit (Figure 10.9c,d), and therefore these parameters have been proposed as objective descriptors of TBV age.

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# Chapter 11
# Jerez Vinegar

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## 11.1 Introduction

### 11.1.1 Geography

Wine vinegar is an enological product that is greatly appreciated by the consumer; different varieties are available, depending on the raw material and elaboration process used. Jerez vinegar (JV) is one of the most renowned products of this type in the world (Palacios et al., 2002). In recent years, vinegar has become a product of primary importance in the Jerez-Xérès-Sherry, Manzanilla de Sanlúcar and Vinagre de Jerez Denomination of Origin (DO) regions in southwestern Spain. Vinegar is now a highly prized, high-quality product on a par with the wines and brandies typical of the region.

Vinegars can be produced from a variety of different sugar raw materials (white and red wine, cider, malted barley, honey, pure alcohol, etc.) and by different methods. In the Denomination of Origin regions, Jerez vinegar is exclusively produced from Sherry wines by following traditional methods of ageing (Consejería de Agricultura y Pesca, 1995, 2000).

Directly linked to the wine Designations of Origin Jerez-Xérès-Sherry and Manzanilla-Sanlucar de Barrameda, with which it shares a Regulating Council, the first Regulation for the geographical designation Vinagre de Jerez was published in 1995 (Consejería de Agricultura y Pesca, 1995). Its area of production, the region of Jerez, expands over eight municipalities of the province of Cadiz (Jerez de la Frontera, El Puerto de Santa Maria, Sanlúcar de Barrameda, Chiclana, Chipiona, Puerto Real, Rota and Trebujena) and one in Seville (Lebrija). Nevertheless, ageing in wood is restricted to Jerez, Sanlúcar and El Puerto, municipalities where there are currently 48 wineries.

It can be said that Jerez vinegar exists since the corresponding Sherry wine exists. The first written references to Jerez vinegar (JV) come from Columela in the first century (AD). The prevailing climatic conditions, together with certain

L. Solieri, P. Giudici (eds), *Vinegars of the World*


**Figure 11.1** Denomination of Origin label for Jerez vinegar

elements of the traditional methods of production of Sherry wines, often led to spontaneous high volatile acidities. Because of the action of acetic bacteria, the wines ended up by becoming vinegars. Spoiled wines were then isolated in independent cellars, and aged following the traditional system employed for wines. The special characteristics of this method, type of barrels, and the microclimate of the warehouses, caused a series of transformations in the vinegars, conferring on them special organoleptic characteristics.

Therefore, the accidental acetification of wines resulted in high-quality vinegars that are marketed as DO (protected Denomination of Origin) Jerez vinegar. The long enological tradition in the regions around Jerez de la Frontera, Spain, has led to the development of vinegar of premium quality, which has subsequently become widely appreciated in overseas markets. Production of such vinegar has reached levels of more than 4 million litres per year. The special characteristics of this vinegar, along with its positive image, led to the protected Denomination of Origin in 1995 (Consejeria de Agricultura y Pesca, 1995). Only wines made from Palomino, Pedro Ximénez and Muscat (Moscatel) grapes, grown in vineyards registered in the 'Jerez-Xérès-Sherry' Denomination are permitted to be used to produce Jerez vinegar. The vinegar can be made from fortified wines or young, unfortified wines. The former are usually wines from Sherry cellars which, for one reason or another, have reached a high level of acetic acidity. JV is marketed in glass bottles of different capacities, but the official label of the DO Council is mandatory in order to ensure its origin (Figure 11.1).

### 11.1.2 Elaboration

Traditionally, Sherry vinegars are elaborated in oak casks, with the bacteria culture being placed on the surface of the wine substrate. Thus, the availability of oxygen

to the bacteria is limited. This implies that long periods of time are required in order to obtain a high degree of acetic acidity (Tesfaye et al., 2002a). As a consequence, ageing also occurs, and excellent organoleptic properties are acquired. However, the volume of production is limited by the long period of time needed for the vinegar to acquire the desired properties.

According the special Regulations for Sherry vinegars, wines can be acetified either by surface culture in wooden barrels (Orléans method) or by submerged cultures in stainless steel vessels. In the first method, the acetic acid bacteria are placed at the air-liquid interface in direct contact with atmospheric air (oxygen) (Tesfaye et al., 2002b). Barrels (normally of 500 litres capacity) are filled to two-thirds of their total capacity. Acetification and ageing are accomplished simultaneously. For the second method (submerged culture), the bacteria are placed in the wine-vinegar mixture. Large fermenters are used and strong aeration is applied until the desired degree of acidity (7 °AA, where °AA represents acetic acid degrees) is reached, usually within 24-36 hours. Once the vinegar is obtained, it must be aged in oak (or less frequently chestnut) barrels in order to acquire the required quality characteristics (Figure 11.2).

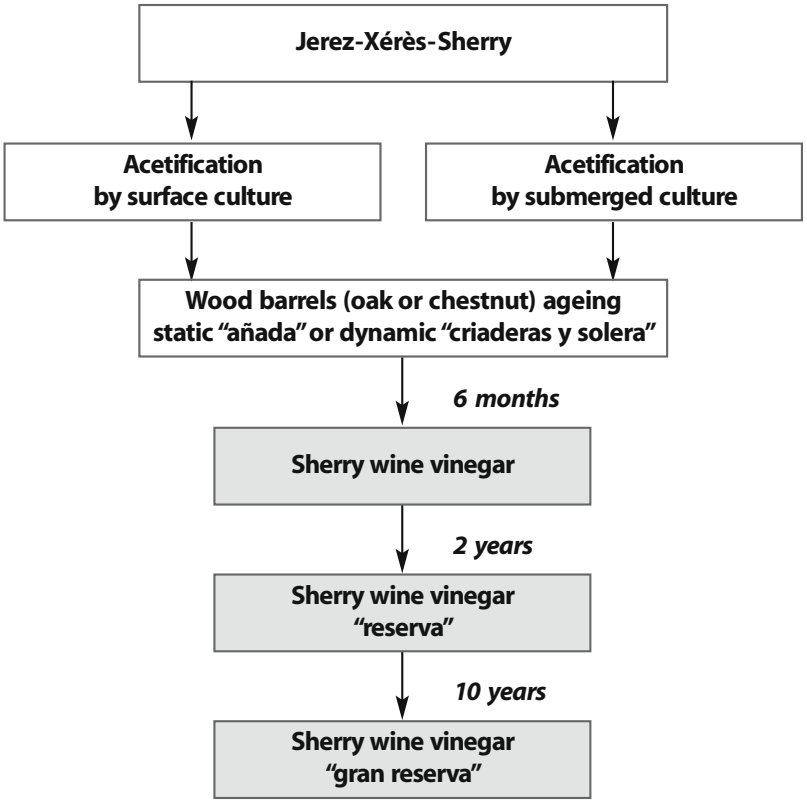


**Figure 11.2** Schematic representation of Jerez wine vinegar elaboration

The microbiology of Sherry wine has been studied in depth, and this has shown that the production of Sherry wine comprises two separate and distinct processes, carried out in succession: first, the alcoholic fermentation of grape must by yeast to produce white wine, and second, the biological ageing (using the solera system) of the wine under a velum (flor) produced by yeast, the so-called 'flor yeast', on the surface of the wine (Esteve-Zarzoso et al., 2001). Physiological and molecular characterization of the yeasts involved in the velum of Sherry wines has reported the presence of different *Saccharomyces cerevisiae* 'races', mainly *S. cerevisiae* var. *beticus*, *S. cerevisiae* var. *cheresiensis*, *S. cerevisiae* var. *montuliensis* and *S. cerevisiae* var. *rouxii* (now renamed *Zygosaccharomyces rouxii*), which are able to grow aerobically in wine (Martínez et al., 1997; Castrejón et al., 2003). The most significant metabolic change occurring in biological ageing is the production of a large amount acetaldehyde, which has an important organoleptic contribution, together with a substantial glycerol and acetic acid consumption and a moderate ethanol metabolism that, in the absence of glucose, is used by the yeasts as a carbon source. There is also a simultaneous consumption of all the amino acids, especially of proline (Moreno-Arribas and Polo, 2005).

The subsequent acetification process encompasses the activity of acetic acid bacteria from the genera *Acetobacter* and *Gluconobacter* (Caro et al., 1998). Up until now, no studies have been conducted to identify the main species involved in making traditional Sherry vinegar. In the special case of surface culture, despite its different behaviour, this appears to be similar to development by yeast during biological ageing beneath the yeast film of Fino- and Manzanilla-type sherries (Palacios et al., 2002). During the biological phase, the total acidity increases progressively, mainly in the first ageing stages. In any case, special qualities are acquired during ageing, which is essentially the same ageing process that is used for Sherry wine. The ageing systems in use are the dynamic criaderas and solera systems, and the static methods (Figure 11.3). The criaderas and solera systems consist of a series of casks arranged in sequence whose number may vary from three to eight (Figure 11.3). The substrate wine is introduced at the step at the top of the system and the final product is withdrawn from the step at the bottom, which is the most aged; the transferred volume will never exceed one-third of the total volume. Barrels in stage 1 are immediately filled with vinegar from stage 2, which, in turn, are filled with the contents of the barrels in step 3. The product is in this way homogenized throughout the whole system. This is the dynamic solera system, and is very different from the static method in which vinegar is aged in a single barrel (Figure 11.3). This dynamic ageing system provides optimum conditions for the development of the acetic acid bacteria. In each series of casks, and via the system of 'running the scales', the product that was formerly wine acquires an increasing acidity, concentration and complexity. This dynamic system (Alonso et al., 2004) has the added advantage of an improvement in the transfer of oxygen, which increases oxygen concentration as compared with the classical Orléans process.

The traditional barrel employed for ageing Jerez vinegars is a 500-litre American oak Sherry butt which has previously contained Sherry wine. However, the Regulations of the DO state that other oak or chestnut containers, with a capac-

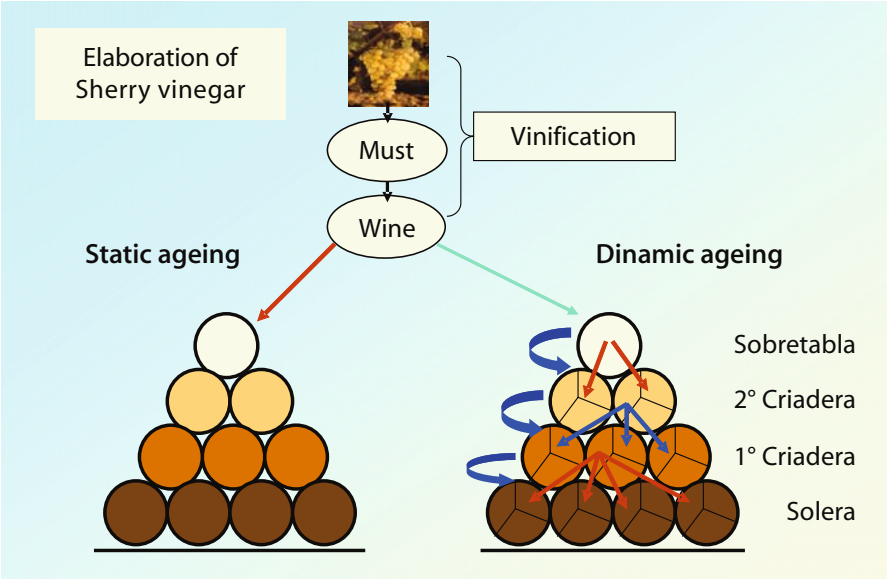


**Figure 11.3** Dynamic and static ageing of Jerez vinegar

ity of less than 1000 litres, can also be used. The Regulations distinguish three types of vinegar, depending on their degree of ageing. Hence, 'Vinagre de Jerez' has to be aged for at least 6 months. 'Vinagre de Jerez Reserva' requires at least 2 years of ageing. However, the Region's winegrowers often age their vinegars for much longer periods, up to 20 or even 30 years. For this special case there is a third category named 'Vinagre de Jerez Gran Reserva' (more than 10 years old). Due to the fact that Jerez vinegar production uses traditional methods, the Regulation allows for up to 3% vol. of residual alcohol and a minimum content of 1.3 $g \cdot L^{-1}$ dry extract per degree of acetic acidity. The minimum acidity of Jerez vinegar is 70 $g \cdot L^{-1}$ (7 °AA).

The ageing of JV usually implies exposure to relatively high temperature (in summer up to 30-35 °C) and high humidity (more than 70%), which favours the development of acetic acid bacteria. At the same time, flavour and aroma are developed, leading to the formation of the characteristic bouquet of these vinegars.

## 11.2 Authentication

In the past few years, scientific interest in the issue of authentication of regional products, characterized by a small production area and by particular production procedures, has been steadily growing. This interest has been mainly determined by the need to support authenticity and traceability by more objective analytical methodologies, with respect to paper certification.

The final quality of Sherry vinegar is determined by the raw material (wine) used as substrate, the acetification process, and the ageing in wooden barrels (Morales at al., 2001b). The analytical characteristics of Sherry vinegar include: total acidity (as acetic acid) at least 70 $g \cdot L^{-1}$ (7 °AA), total dry extract at least 1.30 $g \cdot L^{-1}$, and total ash content between 2 and 7 $g \cdot L^{-1}$. These figures have been established under the DO Regulations (Consejería de Agricultura y Pesca, 1995, 2000) and are mandatory for JV. For vinegars that have been aged for long periods of time ('Vinagre de Jerez Gran Reserva' superior quality), acetic degree can reach values of up to 10.5 °AA due to concentration by water losses through wood pores.

There is considerable interest in differentiating between wine vinegars made by quick acetification and those made by traditional methods in which surface culture is involved, since the price commanded by the latter is much higher. Good results have been achieved using different analytical parameters as follows: acidity, total extract, ash content, glycerol, alcohol and sulphates (Guerrero et al., 1994); in addition, eight mineral elements (As, Ca, Cu, Fe, K, Mg and Zn) (Guerrero et al., 1997) were found to be useful for this purpose when commercial samples were considered.

On the other hand, Benito et al. (1999) demonstrated that it is possible to characterize the vinegars obtained from wine of certified Denomination of Origin Rioja and Jerez according to analytical parameters such as acidity, dry extract, ash, pH, chlorides, organic acids, proline, 3-hydroxy-2-butanone and glycerol.

## 11.3 Chemical Composition

### 11.3.1 Amino Acids

It has generally been observed that the content of total amino acids increases throughout the biological activity phase and, in fact, doubles in concentration from the first stage to the solera stage. This increase could be a consequence of bacterial metabolism and evaporation losses and is produced during year 2 of the process (Palacios et al., 2002). The most marked increases in amino acids occur for proline and arginine. This effect is the opposite of that observed in the biological ageing of Sherry wine (Botella et al., 1976; Martínez et al., 1993) and in the submerged acetification process of different types of wines (Valero et al., 2005; Callejón et al., 2008), where a significant consumption of amino acids occurs.

### 11.3.2 Organic Acids

Organic acids are compounds of interest for characterizing all products derived from grapes. The level and nature of the organic acids present in any given vinegar can provide information both about its origin and, to a lesser extent, about the techniques of processing and ageing to which it has been subjected. The organic acids in wine vinegars comprise volatile (acetic, propionic, etc.) and non-volatile (tartar-

**Table 11.1** Concentration of organic acids in different Jerez vinegars produced by the dynamic solera system

| Organic acids ($g \cdot L^{-1}$) | SV1 | SV2 | SV3 | SV4 | SV5 | SV6 | SV7 | SV8 |
|---|---|---|---|---|---|---|---|---|
| Citric acid | – | 63 | 183 | 106 | 71 | 74 | – | 62 |
| Lactic acid | 312 | 278 | 556 | 55 | 88 | 320 | 300 | 210 |
| Malic acid | 80 | 170 | 94 | 264 | 101 | 230 | 248 | 240 |
| Tartaric acid | 1.52 | 2.56 | 2.32 | 3.71 | 2.56 | 2.9 | 1.65 | 2.57 |
| Acetic acid | 88.0 | 55.7 | 103 | 99.7 | 87.2 | 76.8 | 69.4 | 86.3 |

–, value under quantification limit.

From: Morales et al., 1998; Morales, 1999

ic, citric, malic, succinic, etc.) compounds. The acid that identifies the vinegar is acetic acid, the amount of which can vary depending on the carbohydrate substrate used; acetic acid is the only organic acid present in vinegars derived from pure alcohol (Castro et al., 2002).

The type and content of the non-volatile organic acids seems to depend on the type of vinegar analysed. In cider vinegars, malic acid is the most abundant organic acid; whereas, in malt vinegars, lactic and malic acids are the most abundant. Wine vinegars are characterized by their tartaric acid content, but contain relatively little malic acid, the amount of which depends on the origin of the wine and on the enological techniques to which it has been subjected. Because this acid is converted into lactic acid during malolactic fermentation, the ratio of the amounts of these two acids in the vinegar can be indicative of the extent to which this key fermentative process had developed in the particular base wine. The lactic acid content can, in turn, be reduced during the acetic fermentation (Morales et al., 2001a). The amounts of citric and succinic acids, formed during the alcoholic fermentation, can sometimes be reduced by the presence of microorganisms which can transform these compounds into acetic acid. In a study of the effects of the procedures used for ageing, Morales et al. (1999) found clear differences between the amounts of organic acids and aromatic compounds in JV aged traditionally in barrels of American oak and those produced in a laboratory fermenter. Because of their unusual and specific production process (acetification and subsequent ageing in wooden casks that have previously held wine), JV is different in composition from vinegars produced by other methods. Hence, the composition of Sherry wine vinegars is similar to that of the more oxidized Sherry wines (Olorosos and Amontillados) typical of the region (Castro et al., 2002).

Apart from acetic acid, tartaric acid is the most abundant organic acid in Sherry vinegar, due to its grape origin. Figures for this acid fluctuate between 1 and 4 $g \cdot L^{-1}$ for JV (Morales et al., 1998). Other acids present in significant amounts are: citric, succinic, malic and lactic acids (Table 11.1).

### 11.3.3 Aromatic Compounds

The sensory quality of vinegar is mainly determined by its aroma. The flavour of vinegar depends on the raw materials, the constituents formed during fermenta-

tion, and those originated during ageing. Final aroma of JV is the result of the contribution of some hundreds of volatile compounds. Among these, carbonyl compounds, ethers, acetals, esters, alcohols, lactones, phenols and acids can be found. Some of them can be found in high concentrations, whilst other are present in small quantities, but their low perception threshold makes their presence relevant to the final overall sensation.

This product has a specific raw material, Sherry wine. Although the major grape variety employed to obtain this wine, Palomino, is quite poor in aroma, the resulting wine has a characteristic aromatic composition due to its special elaboration. Sherry wines develop their aromatic profile especially during biological ageing under the so-called 'flor yeast'. Although a number of aromatic compounds come from the raw materials, during acetification some of the volatile compounds from wine substrate may suffer important transformations. The acetic acid bacteria can metabolize high alcohols, in a similar way to ethanol, producing an increase in acid concentrations.

Ageing in wood also contributes to the increase in aromatic complexity. During ageing several phenomena may take place, as follows:

- loss of water through the pores of the wood casks and, consequently, concentration of chemical compounds
- extraction of some compounds from wood
- condensation reactions
- chemical oxidation.

The major aromatic compound in JV is ethyl acetate; this compound has a peculiar aroma, similar to the odour of glue (Blanch et al., 1992). Ethyl acetate is formed by the condensation of ethanol and acetic acid with the loss of one water molecule. This esterification reaction seems to be favoured in the slow acetification surface process, where high amounts of ethanol in conjunction with acetic acid are present. Since JV can contain a maximum of 3% (v/v) of residual ethanol (Consejería de Agricultura y Pesca, 2000), the formation of ethyl acetate is specially favoured.

Acetoin is a characteristic product of acetification, its concentration is very high in traditional vinegars such as JV. Values of 1000 mg $\cdot$ $L^{-1}$, or even higher, have been reported in JV (Morales et al., 2002). Acetoin can be formed through several pathways. One of them is the condensation of two molecules of acetaldehyde, an intermediate product of ethanol oxidation to acetic acid. In addition, acetic acid bacteria can synthesize acetoin from piruvate and acetaldehyde; this reaction is carried out by acetoin synthase (Parés and Juárez, 1997). Finally, acetoin can be formed by oxidation of 2-3-butanediol, a polyol commonly found in Sherry wines submitted to biological ageing (Moreno et al., 2005).

Another compound found at high concentrations (100-1000 g $\cdot$ $L^{-1}$) in JV is isovaleric acid, which is probably formed by oxidation of 3-methyl-1-butanol. A consumption of isoamylic alcohols (3-methyl-1-butanol and 2-methyl-1-butanol) has been observed by several authors (Nieto et al., 1993; Morales et al., 2001a) during the acetification of wines.

Acetaldehyde is a very volatile compound and an intermediate product in the transformation of ethanol to acetic acid by chemical as well as biological oxidation. Therefore, its content tends to decrease during acetification. Moreover, open acetification systems usually lead to substantial losses in acetaldehyde due to its volatility. Sherry wines with biological ageing contain acetaldehyde in significant amounts. This compound can, in turn, be produced in vinegar through the oxidation of lactic acid to piruvic acid and subsequent transformation to acetaldehyde. Acetic acid bacteria consume lactic acid during acetification, especially when the content of ethanol is low (Morales et al., 2001a). However, it is considered to be a key aroma constituent of Sherry vinegar because its substrates are rich in acetaldehyde (Moreno et al., 2005).

Esters contribute to the bouquet and flavour of vinegar. Ethylic esters may be hydrolysed during acetification, yielding ethanol for the bacteria. On the other hand, condensation reactions occur, mainly during the ageing process, increasing acetic esters such as isoamyl acetate and 2-phenylethanol acetate. JV also shows a high concentration of ethyl formate (Morales et al., 2001b). Also, diacetyl, a volatile compound that gives vinegar a buttery flavour, increases along with ageing, and can be considered as an indicator of maturation of vinegar (Del Signore, 2001). In JV it is present at levels in the range 10-50 mg $\cdot$ $L^{-1}$ (Morales et al., 2002), and these values are increased in most aged samples ('Reserva' and 'Gran Reserva'). Values commonly found for aromatic compounds in a set of JV samples are shown in Table 11.2.

**Table 11.2** Range of concentration of major volatile compounds found in Jerez vinegar (n = 13)

| Compound | (mg $\cdot$ $L^{-1}$) | Compound | (mg $\cdot$ $L^{-1}$) |
|---|---|---|---|
| acetaldehyde | 3.10 - 70.1 | acetoin | 192 - 821 |
| ethyl formate | 137 - 16,704 | γ-butirolactone | 16.5 - 54.6 |
| ethyl acetate | 60.0 - 1451 | 2,3-butanediol | 76.5 - 355 |
| isoamyl acetate | 9.18 - 21.6 | methyl acetate | 3.74 - 15.6 |
| 2-methyl-1-butanol | 0.82 - 11.5 | 2-phenylethanol | 4.62 - 24.9 |
| 3-methyl-1-butanol | 3.07 - 27.3 | propyl acetate | 9.25 - 110 |

From Morales, 1999

Volatile compounds play a major role in the differentiation of vinegar samples, according to their ageing period in wood (Natera et al., 2003). The toasting of the wood, which results in a breakdown of cellulose and hemicellulose, may release certain substances into the wood. The extraction of compounds from wood during ageing has been broadly studied in wine and spirits.

This phenomenon was related to the ethanol content of the final beverage. Nevertheless, an experimental ageing of wine vinegar with oak chips demonstrated a noteworthy release of these compounds in acid media such as vinegar (Morales et al., 2004). Thus, aromatic wood compounds present in vinegar, such as furfuraldehyde, vanillin, eugenol and whisky lactones, may be extracted during the vinegar ageing process.

Studies carried out on the contribution of oak to the olfactory characteristics of wine have shown that these are mainly influenced by compounds such as furfural, guaiacol, whisky lactone, eugenol, vanillin and syringaldehyde. More specifically, furfural (2-furancarboxaldehyde) originates from the degradation of monosaccharides produced by the partial hydrolysis of hemicellulose. It contributes to the character of dried fruits, and particularly that of burned almonds. Eugenol (2-methoxy-4-(2-propenyl) phenol), a volatile phenol, is produced by the breakdown of lignin during wood toasting and contributes the characteristic flavours of spices, cloves and smoke. Vanillin (4-hydroxy-3-methoxybenzaldehyde) emanates from lignin degradation and can be synthetically produced from eugenol or guaiacol. It influences wine aroma directly and pleasantly by attributing a character of vanilla. Syringaldehyde (hydroxy-3,4-dimethoxybenzaldehyde) is formed by lignin breakdown during wood toasting and is related to the vanilla character of wine.

Volatile compounds of JV have been studied by several authors; the simplest analysis is carried out by gas chromatography coupled with a flame ionization detector (GC-FID) with direct injection. This method has been used to analyse the major volatiles of up to 25 compounds (Morales et al., 2002). Some other extraction techniques, such as solid phase extraction (SPE), solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE), are needed if we wish to determine the minor volatile components. The two last techniques, SPME and SBSE, couple gas chromatography with mass spectrometry detection analysis and allow the detection of analytes at very low levels (micrograms or even nanograms) (Blanch et al., 1992, Natera et al., 2002; Guerrero et al., 2006).

Although several studies dealing with the volatile compounds of vinegars have been carried out in an attempt to understand this complexity, it is still not known which of them have the highest sensory impact; that is, which are the key odorants of vinegars. The contribution of each volatile compound to the aroma of wine vinegar can be evaluated qualitatively by means of its associate descriptors, and quantitatively by the mean of its odour activity value (OAV). Ascertaining which are the key odorants in JV is a challenge for future research on this field.

### 11.3.4 Polyphenols

Phenolic compounds have been extensively studied in grape-derived products. They are relevant constituents in wine and wine vinegar and are located in the solid parts of the grape (skins, seeds, and stalks). Considered as secondary metabolites in the Plant Kingdom, they have a strong influence on the organoleptic properties (colour, flavour and astringency) of any product of plant origin. More recently, they have been the focus of extensive studies related to their antioxidant and healthy properties. It is now widely accepted that antioxidants play a crucial role in the prevention of many diseases, thanks to their capacity for capturing, deactivating or repairing the damage caused by the free radicals that are implicated in such diseases. Fruits and vegetables, and all the foods and drinks derived from these, are rich in polyphenolic compounds, which have been demon-

strated to be powerful antioxidants (Alonso et al., 2004: Davalos et al., 2005). Hence these protective health effects derived from the consumption of such foods have been attributed to their polyphenol content. Phenols are also of major interest in the chemotaxonomic differentiation of vegetal species, as they are widespread in the Plant Kingdom.

Phenols are present in wine vinegar due to their natural occurrence in grapes or as a result of extraction from wood during ageing (García-Parrilla et al., 1997; Alonso et al., 2004). Seeds and grape skins account for a very large part of the total phenolic content of the grape (mainly phenolic flavan-3-ols and associated oligomeric proanthocyanidins in seeds and anthocyanins and stilbenes in skins). The flesh is poorer in phenolic compounds, with phenolic acid being its main constituent.

Oak wood is mainly composed of three large insoluble polymers – cellulose, hemicellulose, and lignin. These large polymers can yield other compounds of lower molecular weight, such as volatile and non-volatile acids, aldehydes, furans, sugars, steroids, terpenes, volatile phenols and lactones, which can be extracted in wine or hydroalcoholic solvents. Generally the aromatic aldehydes and ellagitannins are extracted during ageing in wooden barrels.

Phenolic compounds in wine vinegar can be determined by liquid chromatography (LC) and diodearray detection (Carrero Gálvez et al., 1994; García-Parrilla et al., 1994; 1996). By using this method, more than 20 compounds can be determined in JV, including compounds with different polarities and molecular weights. The advantage of this method is that the qualitative and quantitative determination of a number of phenolic compounds is possible in a single run, and it is therefore an efficient method for monomers and dimers. However, the main difficulty is that a large number of compounds remain to be identified due to the lack of pure standards. Identification is carried out by comparing retention times with those of commercially available standards and by spiking samples with standard solutions.

The major phenolic components of Sherry wines are caftaric acid and its *p*-coumaroyl analogue. For the vinegars in process of production (not aged), caftaric acid, the major phenolic compound found in recently prepared musts, is also significant (Alonso et al., 2004). Gallic acid and tyrosol are also major components. In laboratory and industrial experiments, it was demonstrated that phenolic compounds do not change significantly during acetification (García-Parrilla et al., 1998; Morales et al., 2001a).

By the other hand, ageing in wood assumes important changes in the phenolic composition of JV (García-Parrilla et al., 1999; Tesfaye et al., 2002a). In general, gallic acid is concentrated during ageing; the aromatic aldehydes and their derivatives, produced by alcoholysis of the lignin of the wood, are present at higher levels in aged vinegars. Hence, compounds such as vanillin, *p*-hydroxybenzaldehyde and protocatechualdehyde are present at higher levels in the samples aged in oak casks. In Table 11.3, the figures for 'Vinagre de Jerez Reserva' are displayed. Gallic acid is one of the major compounds, which increases during the course of ageing (García-Parrilla et al., 1999). This fact is explained by the hydrolysis of gallic tannins from wood. Especially remarkable are the mean levels of aldehydes (*p*-hydroxybenzaldehyde, protocatechualdehyde and syringaldehyde) found for JV.

**Table 11.3** Content ($mg \cdot L^{-1}$) of phenolic compounds in 'Vinagre de Jerez Reserva' samples obtained by the static method

| Phenolic compounds | S1 | S2 | S3 | S4 | S5 |
|---|---|---|---|---|---|
| gallic acid | 689 | 665 | 300 | 314 | 271 |
| 5-(hydroxymethyl)furan-2-carbaldehyde | 337 | 373 | 242 | 263 | 1230 |
| caffeoltartaric acid | 90 | 86 | 76 | 85 | nd |
| protocatechaldeyde | 33 | 39 | 28 | nd | 43 |
| coumaroyltartaric acid glycoside | nd | nd | nd | nd | nd |
| coumaroyltartaric acid | nd | nd | nd | nd | nd |
| 2-furaldehyde | 154 | 156 | 10 | nd | 252 |
| *p*-hydroxybenzoic acid | nd | nd | nd | 16 | nd |
| tyrosol | nd | nd | nd | nd | nd |
| *p*-hydroxybenzaldehyde | 1 | 10 | nd | 4 | 41 |
| caffeic acid | 2 | 3 | 5 | 5 | nd |
| gallic ethyl ester | 85 | 116 | 8 | 4 | 49 |
| vanillin | 9 | nd | nd | nd | 17 |
| syringaldehyde | 23 | 37 | nd | nd | 5 |
| *p*-coumaric ethyl ester | 10 | 29 | 7 | 8 | 8 |
| isoquercitrin | nd | nd | nd | nd | nd |
| caffeic ethyl ester | 1 | 2 | 1 | 1 | nd |

nd: not detected.

From García-Parrilla et al., 1999

5-(hydroxymethyl)furan-2-carbaldehyde in vinegar has traditionally been attributed to the employment of cooked must or to the addition of caramel. Some authors have found a clear increase in this compound during ageing in wood (García-Parrilla et al., 1997, 1999; García-Moreno and Barroso, 2002), which implies that its presence could not be considered exclusively as an indicator of this addition. Two facts, the ageing in wood and the process of production (with a slow heating process), should explain the high content of 5-(hydroxymethyl)furan-2-carbaldehyde and 2-furaldehyde found in Sherry vinegar.

## 11.4 Sensory Analysis

The quality of a given food can be evaluated by taking into account different aspects such as nutritional value, food safety and sensory properties. In the case of vinegar it is strongly determined by sensory properties, as it is used mainly as a condiment. Sensory analysis is a valuable tool by which the organoleptic properties of food and beverages are analysed through our senses. However, one of the main difficulties of tasting this product is the strong contribution of acetic acid to the overall sensation. As its content is at least 5% or 6% (w/v) of acetic acid, other sensations can be masked or hardly perceived (Tesfaye et al., 2002c).

Generally speaking there are two models for vinegar sensory analysis. The first one consists in preparing vinegar in the approximate way that it is normally consumed. Vinegar is diluted up to 1% acetic acid with 1.5 g of sodium chloride in 100 mL of water, and 25 g of lettuce is suspended in each 30-mL aliquot

(González-Viñas et al., 1996). This model is helpful to establish preferences among different vinegar samples and is suitable for a large number of non-trained assessors taking part in the study. The second model encompasses testing the undiluted vinegar as it is, using wine glasses. This model is the usual procedure for performing sensory analysis in Sherry vinegar cellars. It was found that the procedures used for treating the vinegar sample before sensory analysis tend to distort the aroma or to attenuate differences between samples (Gerbi et al., 1997; Tesfaye et al., 2002c). Even though there are no standardized tasting glasses, a standard wine glass (standard 3591; ISO, 1977) can be used with slight modification. To avoid the influence of colour, cups have to be opaque and covered with a lid to avoid the loss of volatiles. Tasting ought to be performed within intervals of one minute between tastings. Cups are inclined 45 ° with respect to the nose to capture the aroma properly.

Sensory analysis requires a well-trained testing panel who are able to perceive concrete and adequate attributes and are capable of differentiating between vinegar samples with a high degree of certainty. A limited number of vinegar samples are examined at each tasting session, in order not to excessively tire the assessors.

To select the members of the sensory panel, specific techniques can be used. The so-called taste acuity test (standard 3972; ISO, 1991) is useful for classifying a group of people with regard to their capacity to recognize the basic tastes. Its aim is to detect the most acutely sensitive individuals from the initial group. It is performed using previously established threshold concentrations (standard 3972; ISO, 1991).

Once the members are selected, they are trained in accordance with the desired objectives. Concerning the literature, there is no consensus on the duration of the training period. Certain authors recommend 10-20 hours (Stone and Sidel, 1993; Drake et al., 1996) and others suggest more than 100 hours (Meilgaard et al., 1991; Heisserer and Chambers, 1993). Tesfaye et al. (2002c) recommended that training should be based on ranking solutions of aromatic compounds usually present in vinegar prepared at different concentrations. These were: acetic acid (0, 4, 6, 7, 8 and 10%), ethyl acetate (0, 0.5, 1.5, 2.0, 2.5 and 3.0 $g \cdot L^{-1}$), and wood extract obtained by macerating a hydroalcoholic solution in oak wood chips. Both ethyl acetate and wood extract solutions were prepared in a 7% acetic acid solution in order to perceive a similar sensation as in vinegar itself. Ethyl acetate dissolutions were prepared on the basis of already reported data for Sherry wine vinegars (Troncoso and Guzmán 1987, 1988; Blanch et al., 1992).

Regarding to the sensory tests employed, a wide range of tests can be applicable. Discriminatory tests include a triangular test (especially useful when differences are not pronounced) (standard 4120; ISO, 1983a) and paired comparison tests (standard 5495; ISO, 1983b), which are useful to establish differences and preferences among different vinegars.

Descriptive analysis is used, as it usually offers a wide range of information on the aromatic profile of the samples (standard 6564; ISO, 1987). Sensory profiles of wine vinegars have been built on the basis of attributes freely chosen by panellists taking part in the trials (Nieto et al., 1993). These authors selected the following

attributes: colour, odour quality, woody aroma, nutty odour, herb odour, fruity odour, odour intensity, flavour quality, woody taste, nutty taste, herb taste, fruity taste, mouth feel and overall impression.

Graphical representations of the sensorial profiles of different types of wine vinegar (Sherry wine vinegar, aromatized vinegars, and table wine vinegars) were different enough to distinguish these vinegars from each other. Wine vinegar sensorial studies have been designed in different ways: (i) using vinegars from different raw materials (Gerbi et al., 1997); (ii) on the basis of the various elaboration process employed (González-Viñas et al., 1996); and (iii) on the basis of different ageing periods, with the purpose of assessing the influence of ageing on the sensorial quality of Sherry wine vinegars and also of contributing to their sensorial description (Tesfaye et al., 2002c). The estimated intensity for each attribute was recorded by the assessors drawing a mark on a 10-cm unstructured scale, and ranks were expressed in a continuous numeric digit sequence which can be expressed with a spider chart.

A typical representation of an aromatic profile for Sherry wine vinegar during its ageing period (0, 6, 12, 18 and 24 months) is shown in Figure 11.4. From the spider chart it can be observed that, during ageing, pungent sensation, and woody and ethyl acetate aromas increase, whereas wine character is not affected to any large extent. Woody odour and odour intensity were the most relevant descriptors to

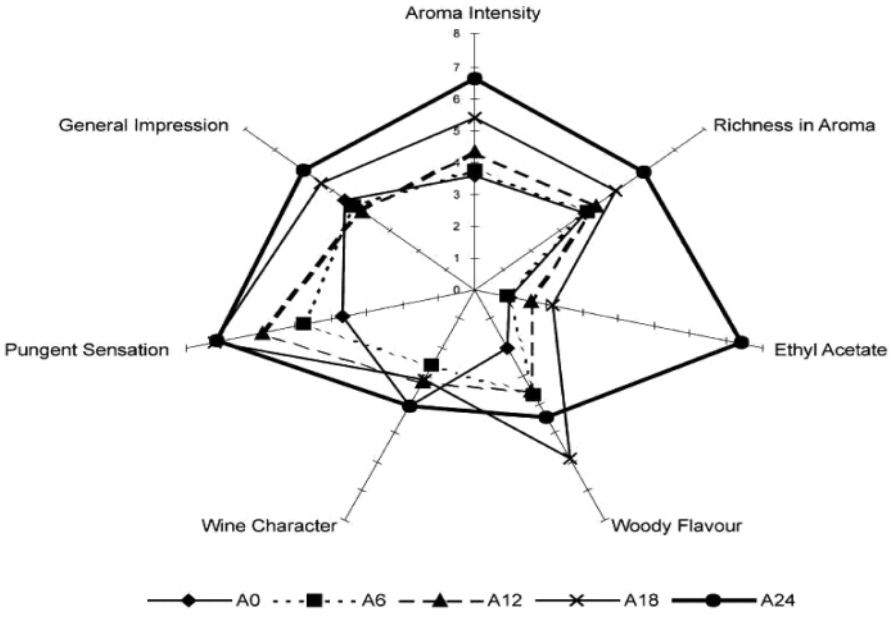


**Figure 11.4** Typical 'spider chart' representation of aromatic profile of Jerez wine vinegar during ageing (0, 6, 12, 18 and 24 months). For the list of attributes found in the literature, see Tesfaye et al., 2002c

establish statistical significant differences among Sherry wine vinegars and table wine vinegars. Indeed, the acceptance of Sherry wine vinegar by consumers is largely based on its woody flavour (González-Viñas et al., 1996).

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# Chapter 12
# Cider Vinegar: Microbiology, Technology and Quality

**V.K. Joshi and Somesh Sharma**

## 12.1 Introduction

Apple vinegar or cider vinegar (CV) is made with apple juice or concentrated apple juice (CAJ) through a double fermentation: alcoholic and acetic. Cider vinegar is extensively used in several countries including Austria, the UK, the USA and Switzerland (Ebner, 1982; Lea, 1988).

The minimum legal strength for cider vinegar varies from country-to-country and in USA it is 4% acetic acid (w/v). Cider vinegar is classified into low-strength and high-strength depending on the chemical composition of the cider used. *Low-strength cider vinegar* refers to vinegar that is produced from cider with a solute concentration (acid % w/v plus alcohol % v/v) of less than 8-9%. *High-strength cider vinegar* is made from cider with more than 9% and up to 13% solute concentration.

Natural apple cider vinegar is made from fresh, crushed, organically grown apples and is allowed to mature in wooden barrels.

In the traditional method, both alcoholic and acetic fermentation of apple juice is carried out in the same barrel by naturally occurring yeasts and acetic acid bacteria (AAB). The barrel is placed in a warm, damp place and the bung-hole of the barrel is covered with a piece of cloth to keep out the dust and flies. It takes about 5-6 months to complete the whole alcoholic and acetous fermentation to form CV from apple juice. The main drawbacks of this process are that alcoholic fermentation is often incomplete and slow, and also that acetic fermentation has low yields, resulting in a CV of poor quality.

Nowadays, cider vinegar is made mainly by submerged culture, as for wine vinegar; a complete description of submerged culture is reported in Chapter 6.

Cider vinegar is popular in folk medicine and is suggested as a remedy to various diseases, from obesity and overweight to arthritis, but also for asthma, coughs, diarrhoea, colitis, eczema, hair loss, and many other conditions.

More conventional uses of cider vinegar are as a flavouring agent and as a food preservative.

L. Solieri, P. Giudici (eds), *Vinegars of the World*

## 12.2 Microbiology of Cider Vinegar

Different microorganisms play an important role during CV production. Like that of grapes, the microflora of apples consists of yeasts (*Kloeckera apiculata*, *Metschnikowia pulcherrima*, *Candida* spp. and *Pichia* spp.), lactic acid bacteria (*Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* spp.), and acetic acid bacteria (*Acetobacter* and *Gluconobacter*) (Fleet, 1998). Yeasts carry out the glycolytic conversion of sugars to alcohol by the EMP pathway, which gives a practical yield of alcohol of around 50% of the sugar weight initially present. The wild yeast microflora, which are present on fruit or come from the surfaces of the processing equipment, perform the first phase of alcoholic fermentation, but *Saccharomyces cerevisiae* and *Saccharomyces uvarum* (or *Saccharomyces bayanus*) are the most important species because of their special traits of great ethanol tolerance and high fermentation rate.

Acetic acid bacteria (AAB) are responsible for the oxidation of ethanol to acetic acid. Nowadays, they are classified into the family *Acetobacteraceae* as a branch of the acidophilic bacteria in the α-subdivision of the *Proteobacteria* (De Ley et al., 1984; Sievers et al., 1994). Genotyping relationships among AAB, based on 16S rRNA gene sequences and ubiquinone systems, have revealed that the currently recognized AAB are classified into ten genera; more details on current classification are reported in Chapter 3. AAB species are widely distributed in nature in plant material rich in sugar, and include *Acetobacter pasteurianus*, *Acetobacter oboediens*, *Acetobacter pomorum*, *Gluconacetobacter hansenii*, *Gluconacetobacter europaeus* and *Gluconacetobacter xylinus* (Kittelmann et al., 1989; Sievers et al., 1992; Swings, 1992; Sokollek et al., 1998). In particular, *Ga. europaeus* has been described as the dominant species in industrial submerged-culture vinegar manufacturing in central Europe, whereas *Ga. xylinus* has been frequently recovered from traditional processes.

Many vinegar fermentations are carried out by mixed and wild AAB cultures, not microbiologically defined, that are generally called 'seed vinegar'. Experimental methods for starter preparation have been described and applied to the cultivation and preservation of isolates from vinegar fermentation at laboratory scale (Sokollek and Hammes, 1997; Sokollek et al., 1998). Although species belonging to the genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* are resistant to acetic acid concentrations, they differ in several metabolic aspects. For instance, *Acetobacter* and *Gluconacetobacter* spp. are able to carry out the overoxidation of acetic acid to $CO_2$ and water from ethanol, whereas *Gluconobacter* spp. are not able to do this, due to their non-functional α-ketoglutarate dehydrogenase and succinate dehydrogenase of the tricarboxylic acid cycle.

## 12.3 Cider Vinegar Production

Processing from apples to CV can be summarized in three steps: (i) raw material preparation; (ii) alcoholic fermentation of apple juice to produce cider; (iii) acetous

fermentation of cider to vinegar. A complete overview of CV production is depicted in Figure 12.1.

### 12.3.1 Raw Materials and Preparation

The amount of sugars and acids in fresh apples can vary significantly according to cultivar, production region, season, and maturity of apples. There are about 5000 cultivars of apple grown all over the world. The European apple cider cultivars at the proper ripened stage have total soluble solids (TSS) ranging from 10 to 15 °Brix (average 9-11 °Brix), with a titratable acidity of 0.12-0.31%, expressed as malic acid. The tannin content, which is responsible for the astringency, ranges from 37 to 233 mg · 100 $mL^{-1}$, while pectins (0.25-0.75%) are mostly responsible for the body or viscosity of apple juice. The unripe fruits give juices with less total soluble solids and aroma, a higher content of starch and acids, and a bitter or astringent (green apple) flavour. Juice made with overly mature apples have lower yields due to the difficulty in extraction procedure, and exhibit a sweeter, but flatter, flavour. The pH ranges from 3.0 to 3.8, depending upon the concentration of malic acid (3-5 g · $L^{-1}$). The raw material can differ depending upon manufacturer (i.e. if other kinds of vinegar are made in the same factory) and the processed apple products (i.e. apple juice, fermented apple cider or CAJ). Culled, undersized, substandard or unmarketable surplus apples are improperly used. On the contrary, the required high quality of apple juice is the result of using sorted, clean apples and a well-designed and equipped facility operated according to good management practices.

Mechanical treatment in a stainless steel mill and the action of pectolytic enzymes are used to break down the cellular structure and increase the yield of juice that is extracted by pressing the grated apples. In making traditional cider, a system with hydraulic, roller or pneumatic pressure is used to rupture and compress the cells until the recoverable juice is separated from cellular solids called *pomace*. However, the processing of cores and peels results in an increase of non-sugar solids including procyanidin which, later on, causes problems in the vinegar by increasing haze and colour formation. Usually apple juice has TSS (total soluble solids) between 10 and 11 °Brix (Lea, 1988) and contains around 11% by weight of solids; at least 90% of the solids are carbohydrates, mainly dextrose, fructose and sucrose (Bureau International du Travail, 1990). Uncontrolled microbial growth is the main cause of shortened shelf-life in apple juice because the organic acids of apples do not inhibit the growth of yeasts, moulds and acid tolerant bacteria.

In some countries, CV can also be made from concentrated apple juice (CAJ), which has as much as 60-72% of TSS. The principal methods applicable to apple juice concentration include evaporation, reverse osmosis, and freezing of clarified apple juice (Sharma and Joshi, 2005). In this case, CAJ is diluted with water or apple juice to the desired concentration (10-13% of TSS) before performing the alcoholic fermentation. The water used for preparing mashes/juices must be bacteriologically clean, clear, colourless, odourless and without any sediment or suspended particles. In extreme cases, it must then be demineralized by the addition of

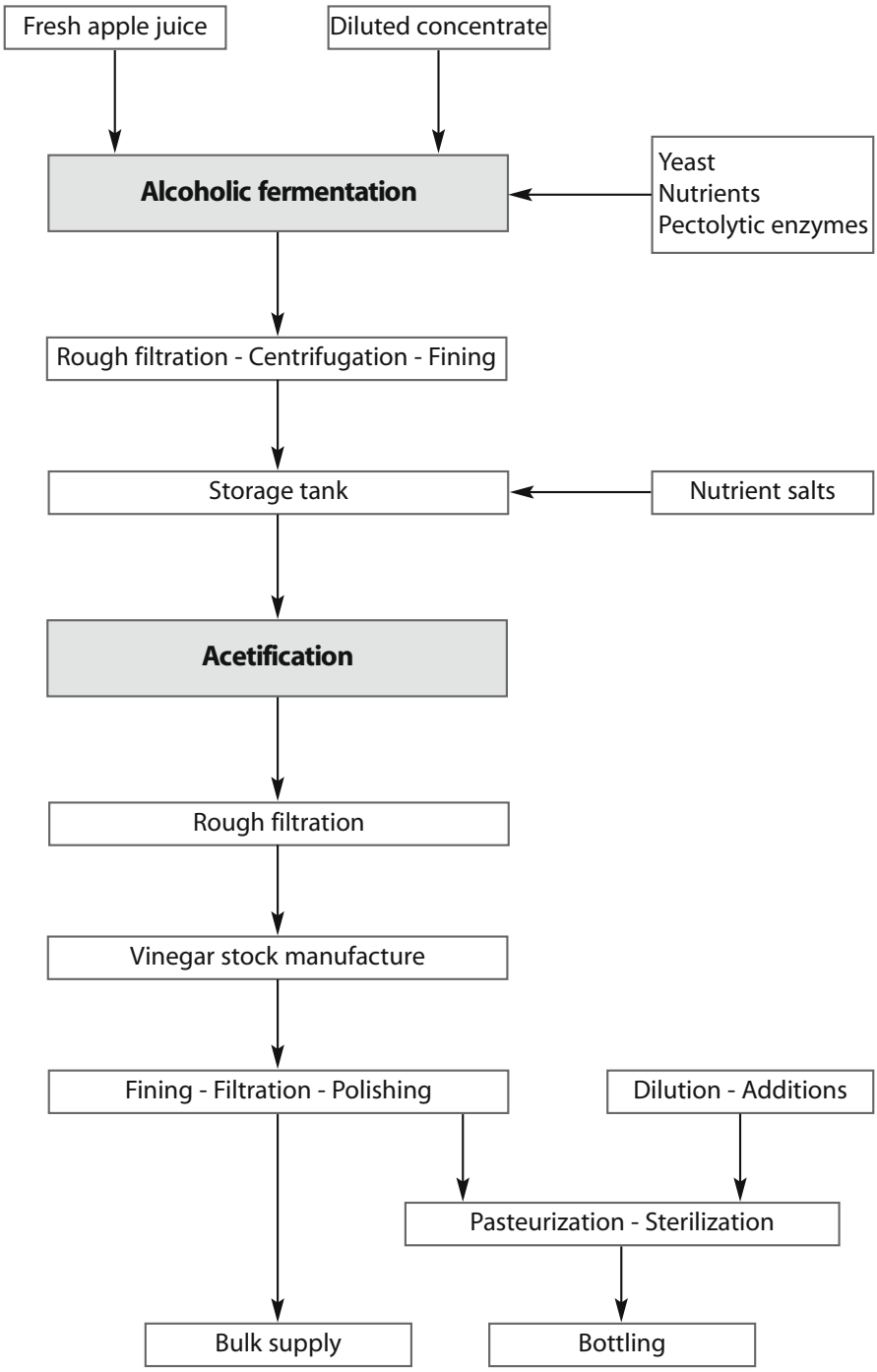


**Figure 12.1** Flow chart for cider vinegar production

the required minerals. When CV is made entirely from CAJ, ammonium phosphate and thiamine are added at concentrations of 150 ppm and 0.15 ppm, respectively before mash fermentation.

### 12.3.2 Alcoholic Fermentation

Alcoholic fermentation of apple juice is mainly carried out by *Saccharomyces cerevisiae* and can be performed either by pure culture inoculation or by natural spontaneous fermentation. Nowadays, it has almost become a routine to use dried wine yeast, which is added to the mash at a concentration of 150 ppm without any prepropagation. Usually, $SO_2$ is not added to cider used for vinegar production, even when recommended (Webb, 1983). Mash fermentation is not generally temperature-controlled, but in cold climates, when the fermentation slows due to low temperature, the mash is warmed using heat exchangers.

In spontaneous fermentation of mash, the following succession of yeast species is observed: *Kloeckera/Hanseniaspora uvarum* predominate at the beginning, while *Saccharomyces cerevisiae* dominates at the end of fermentation, and *Dekkera/Brettanomyces* are the main species during the maturation phase (Morrissey et al., 2004). The final alcohol content of cider, as well as the length of fermentation, is a function both of the TSS of the mash and the yeast strains employed. In particular, the alcohol content differs a lot between different countries: i.e. in Ireland the alcohol content can reach 9-10% v/v, while in North America the cider has 5-6.5% alcohol and the length of fermentation is close to one week (Jeong et al., 1999; Seo et al., 2001).

At the end of fermentation, yeasts and fruit pulp settle to form a compact mass at the bottom of the tank from which the fermented liquid is separated. However, manufacturers normally prefer to remove yeast and pulp from the cider by centrifugation. The clear liquid is stored in vessels for the successive conversion into vinegar by acetic acid fermentation.

### 12.3.3 Acetous Fermentation

Cider with a high alcohol content is adjusted to 7-8% v/v by adding water, and is then mixed with CV from a previous cider acidification, with the purpose of inoculating AAB and in order to avoid the growth of undesirable microorganisms. Usually, one part of 'mother of vinegar' is added to five parts of cider. A strong improvement in the ethanol oxidation rate is achieved with the use of specific AAB (Mendonca et al., 2002; Kocher et al., 2003; Kocher et al., 2007).

From a technological point of view, the acetification of the cider can be achieved either by traditional slow methods, such as the Orléans process, or quick submerged methods, based on different kinds of acetators, that increase acetification rate and acetic acid yield using semi-continuous or continuous processes (Joshi and Thakur, 2000).

### 12.3.4 Maturation/Ageing

CV is stored and matured in wooden or stainless steel tanks. In the past there was the widespread practice of maturing the vinegar for at least a year, but nowadays vinegar is often matured for only 1 or 2 months at the most, due to higher production costs and the expense of storage. In some plants, it may undergo a rough filtration at this stage.

Many changes occur in cider vinegar during storage. Its harsh flavour changes to a more pleasant aroma and bouquet, probably due to the oxidation of vinegar caused by air entering through the pores of the wood. Acetic acid may also react with residual alcohol to form ethyl acetate, which has a fruity flavour.

Moreover, the colour of CV is modified during the ageing step. Specifically, the colour, which intensifies during milling and pressing due to polyphenol oxidase activity, a constitutive enzyme of apples, fades during the fermentation and maturation phases, probably due to the polymeration of procyanidins and other polyphenols. It is a general practice to use polyvinylpolypyrrolidone and carbon to reduce the colour by removing oxidized and polymerized procyanidins. The colour of CV made by CAJ is thought to be due to Maillard reactions, whereas the oxidation of polyphenols is mainly involved in determining the colour of CV made from fresh apple juice (Lea, 1988). Unfortunately, our present knowledge does not allow us to predict the final colour of CV at bottling stage simply on the basis of its analytical parameters.

### 12.3.5 Clarification

Various technological practices are used to achieve stable and clear vinegar. Self-clarification by sedimentation depends upon acidity and age. CV of low acidity normally does not clear even after long storage, whereas high-strength vinegars usually do so within a couple of months.

Fining reduces the total load of suspended material (mainly the debris of AAB cells) and may help to reduce the polyphenol levels. This is considered to be an alternative practice to rough filtration. In general, the fining procedure consists of the addition of gelatin, bentonite and/or liquid silicon dioxide, following the two main protocols described below: (i) 260 g gelatin and 400 g bentonite are added to each 1000 L of CV, and the suspension is stirred and left to settle for at least 1 week before racking; or (ii) liquid silicon dioxide (5 L at 30% solution per 5000 L of CV) followed by gelatin (1 kg per 5000 L) are added to CV and left to settle. A final filtration could be necessary to completely remove suspended materials and AAB cells.

### 12.3.6 Ultrafiltration

Ultrafiltration has recently been promoted to replace normal filtration and sterilization procedures. CV is pumped continuously and partly recirculated through the

membrane with a typical cut-off point of 50,000 molecular weight. The permeate consists of clarified stable vinegar, whereas yeast and bacterial cells, as well as high-molecular-weight compounds are retained. To minimize the risk of bacterial contamination, ultrafiltration is done immediately before bottling.

Ultrafiltration does not prevent the formation of non-microbiological post-bottling haze, since haze precursor molecules (procyanidins) have molecular weights ranging from 500 to 2500 and can, therefore, easily pass through even the smallest of ultrafiltration membranes.

### 12.3.7 Pasteurization and Bottling

After ageing and clarification, cider vinegar is pasteurized to avoid any spoilage.

CV can be flash-pasteurized by passing it through a tubular heat exchanger at 66 °C for a short time and, whilst still hot, poured into glass bottles, or cooled before being put into flasks or plastic bottles.

Bottled vinegar can also be pasteurized by immersing the bottles in hot water until the vinegar inside the container attains a temperature of 60 °C.

A particular chemical pasteurization, the 'silver process', is done by passing the CV through silver-bearing sand, to reach a silver ion concentration (about 2 ppm) sufficient to sterilize it.

### 12.3.8 Additives

Chemicals can be added to CV to prevent browning, non-microbiological haze formation, and bacterial growth. Iron, copper, oxygen, procyanidin and oxidized ascorbic acid are the main factors that promote post-bottling haze formation and vinegar darkening. Therefore, these compounds should be reduced to the lowest possible level.

The main additives used in cider vinegar stabilization are the following:

- $SO_2$ is the most effective antimicrobial agent, especially at low pH. Moreover, it inhibits enzymatic polyphenol polymerization and has antioxidant properties.
- Ascorbic acid is an antioxidant which is less effective than $SO_2$ unless used in very high amounts (>250 ppm). However, the breakdown products of ascorbic acid (dehydroascorbic acid and diketogulonic acid) are carbonyls which have been found to be potent pro-oxidants, encouraging browning reactions and haze formation unless excess ascorbic acid is present. The addition of small amounts of ascorbic acid (<100 ppm) to CV are probably worse than useless.
- Pectin and arabic gum can be added to stabilize CV against haze formation.
- Citric acid forms a stable complex with metals, such as iron and copper, which catalyse the oxidative polymerization of polyphenols.
- Potassium ferrocyanide is used as a fining agent (blue fining) to remove iron from vinegars.

## 12.4 Composition of Cider Vinegar

The composition of CV is strongly influenced by raw materials, technology and cultural tradition; for this reason it is difficult to compile a comprehensive table of the composition of all the CV produced in the world. In Table 12.1 some data are reported about the main constituents of CV.

**Table 12.1** Physico-chemical characteristics and composition of cider vinegar

| **Physico-chemical characteristics** | **Value** |
|---|---|
| Relative density | 1.013 - 1.024 |
| Total acid as acetic (% w/v) | 3.3 - 9.0 |
| Non-volatile acids as malic (% w/v) | 0.03 - 0.4 |
| Total solids (% w/v) | 1.3 - 5.5 |
| Total ash (% w/v) | 0.2 - 0.5 |
| Alkalinity of ash (0.01 M acid per mL of vinegar) | 2.2 - 5.6 |
| **Composition (% w/v)** | **Value** |
| Non-sugar solids | 1.2 - 2.9 |
| Total sugar | 0.15 - 0.7 |
| Alcohol | 0.03 - 2.0 |
| Protein | 0.03 |
| Glycerol | 0.23 - 0.46 |
| Sorbitol | 0.11 - 0.64 |

Adapted from Lea, 1988

A flavouring profile of cider vinegar comprises many volatile and non-volatile compounds. As many as 61 compounds have been identified in one or more samples of six commercial vinegars from both the submerged and trickling (quick vinegar) processes (De Ley, 1959), and at least 20 more were also present in cider vinegar but remained unidentified. Aurand et al. (1966) identified 19 components from three natural cider vinegars. The data on the minor components of CV are relatively sparse and there are no published comparisons on the differences arising from different fermentation and acetification procedures, as well as from different raw materials. Differences in the apples employed can have an important effect on the compositional analysis, particularly for non-volatile compounds. Also the process affects the cider vinegar flavour: slow acetification with the Orléans method produces a high-quality vinegar much higher in esters than that produced by the submerged culture process (Carr, 1982; Webb, 1983).

On the basis of the available literature, the major volatile constituents of cider vinegars, other than acetic acid, are acetaldehyde, ethyl formate, ethyl acetate, ethanol, isobutanol, 2-methylbutanol, 2-butanol, isobutyl acetate, acetoin, isopentanol and 2-phenylethanol, although other carbonyl esters and alcohols have also been found (Suomalainen and Kangasperko, 1963; Kahn et al., 1972). Diethyl succinate, β-phenethyl formate and β-phenethyl alcohol have been found to be essen-

tial for giving cider vinegar flavour and aroma (Ebner, 1982). Acetaldehyde, acetal, ethyl acetate and fusel alcohols in high concentrations arise from yeast metabolism. Other trace components arise from oxidative AAB catabolism on compounds present in apple juice, such as gluconic acid from glucose and acetoin from DL-lactate (De Ley, 1959). Acetoin is formed also by the microbial activity of lactic acid bacteria and yeasts during alcoholic fermentation.

### 12.4.1 Specification of Cider Vinegar

The analytical specifications of cider vinegar are usually rather simple. They include total acidity (by titration), alcohol content (by distillation), iron, copper and heavy metal contents, appearance or turbidity and colour. The values of the specifications for cider vinegar established by the Vinegar Institute in the USA are listed in Table 12.2.

**Table 12.2** Specifications for cider vinegar

| Characteristics | Limit | Test method |
|---|---|---|
| Acid content | 4.0 g/100 mL minimum expressed as acetic acid | AOAC 30.071 |
| Colour | Light to medium amber as per reference sample | AOAC 30.062 |
| Trace metals | | |
| Copper | 5.0 ppm max | AOAC 30.035 |
| Iron | 10.0 ppm max | AOAC 30.079 |
| Heavy metal | 1.0 ppm max | AOAC 30.058 |
| Alcohol content | 0.5% by volume, max | AOAC 30.078 |

Adapted from Lea, 1988

In addition, the Vinegar Institute stipulates the following manufacturing requirements: cider vinegar shall be made by the alcoholic and subsequent acetous fermentation of the juice of apples or apple concentrate. Each shipment is guaranteed as of the date of delivery, not to be adulterated nor misbranded within the meaning of the Federal Food, Drug and Cosmetic Act. It shall be manufactured in accordance with the federal guidelines for good manufacturing practices.

Furthermore, cider vinegars may be distinguished from synthetic acetic acid solutions by their relatively higher content of sorbitol and by the presence of unique phenolic compounds, such as phloridzin, which are chromatographically detectable. A gas chromatographic method is described for the analysis of arabitol, mannitol, sorbitol and inositol in cider vinegar (Santa-Maria et al., 1985).

The absence of malic acid is not considered to be proof that a vinegar is not derived from cider, since lactic acid bacteria convert malic acid to lactic acid, which can in turn be oxidized by acetic acid bacteria. Finally, carbon isotope analysis techniques can even distinguish between the fruit-derived acetic acid and acetic acid derived from other material, as well as between natural acetic acid and synthetic acetic acid.

## 12.5 Defects in Cider Vinegar

The major defects in cider vinegar are the following:

- *Growth of 'mother of vinegar'*: this is the name used to indicate the cellulosic film produced by *Gluconacetobacter xylinus*.
- *Vinegar eels*: nematodes of the species *Turbatrix aceti* can grow at the top of the fermentation liquid and storage vessels. These can appear at any stage in the manufacture of vinegar, but more frequently during storage or maturation of vinegar. The presence of vinegar eels has both advantages and disadvantages. The advantage lies in the fact that they scavenge dead or dying *Acetobacter* cells and keep the acetification clean and active. Their harmful effects include reduction in the total bacterial count and lowering of the acidity of vinegar. Vinegar eels are controlled by raising the temperature of the infested vinegar to 40-45 °C to kill the eels but not the *Acetobacter* cells, or by adding $SO_2$. Their removal can also be done by filtration and pasteurization.
- *Non-microbial hazes*: polyphenols, very abundant in apple juice, cider and cider vinegar, are the chief cause of haze in cider vinegar. Generally, most of the polyphenols polymerize during the maturation of cider vinegar and the sediment falls to the bottom of the vat. Thus, the product remains stable. However, if the cider vinegar is not completely mature, the polyphenols continue to non-oxidatively polymerize in the bottle and, when the bottle is opened and oxygen is admitted, polymerization occurs rapidly and sediment is formed at the bottom. Non-microbial hazes can be controlled by the addition of preservative (mainly $SO_2$) or by increasing the maturation time, as well as by using a fining agent, such as gelatin, kieselsol or bentonite.

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# Chapter 13
# Vinegars from Tropical Africa

**Ángel González and Luc De Vuyst**

## 13.1 Introduction

Vinegar is one of the most ancient fermented beverages consumed by humans (Conner and Allgeier, 1976). In Africa, traces of vinegar were found in vessels of the old Egyptians. Leftovers of vinegar were detectable in vessels from 6000 years before Christ. This Egyptian product was called *hequa*. It was brewed from barley and, after some time, the wort was acidified. Later, vinegar was found to be a good food preservative and it was also used as a curing drink.

According to data from the Vinegar Institute (www.versatilevinegar.org), in 2005, the world consumption of vinegar was estimated at to be worth almost US$ 220 million. However, levels of African vinegar production are difficult to estimate, for several reasons (Ndoye, 2007). First, there is the black market existing in most African countries. Second, up until recent years most of the vinegar used was obtained mainly through dilution of acetic acid of petrochemical origin.

Nowadays, in many African countries, the production of vinegar is being regulated by the government, as new laws come into force. These laws fix the necessary qualities and conditions of manufacture for commercial products that carry the name 'vinegar'. These laws and regulations emphasize that vinegar destined for human use should be produced in a safe and biotechnological way, i.e. through a double fermentation (an alcoholic fermentation followed by an acetic one, the so-called acetification) of agricultural products. Acetic acid of chemical origin may not be used for products intended for human consumption in order to avoid potential health risks.

Although new vinegar products are being introduced into the market worldwide, as exemplified for the period 2003-2005 in Figure 13.1, overall numbers are highest in the Western world. Of particular interest is the slight increase in Europe and North America and the substantial growth in the Asia-Pacific region in 2004 as well as in Latin America in 2005. In the Middle East and Africa, which have consistently lower overall figures, a slight decrease occurred in 2004 and 2005. However, biotechnological vinegar production seems to be important not only

L. Solieri, P. Giudici (eds), *Vinegars of the World*

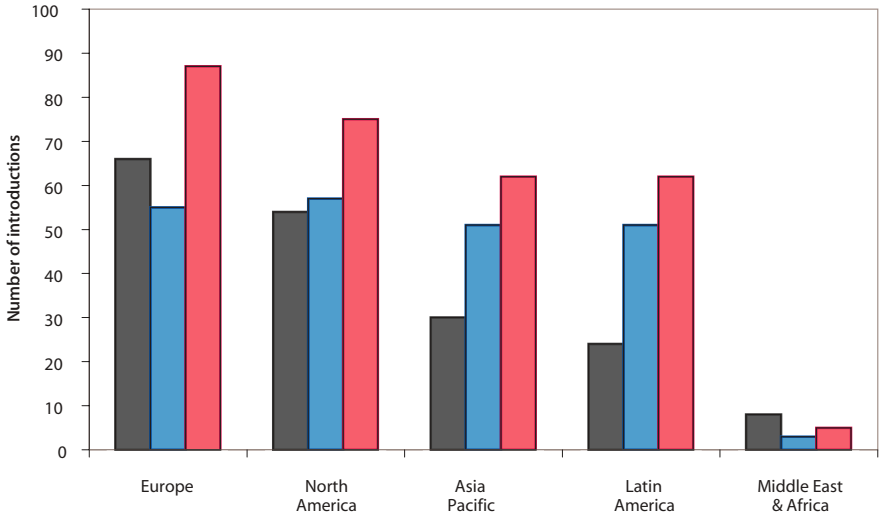


**Figure 13.1** Global vinegar introductions of new products by region. Number of introductions means new products introduced into the market in years 2003 (gray), 2004 (blue), and 2005 (red). Based on data from the Vinegar Institute (http://www.versatilevinegar.org)

from a health viewpoint but also for the economy of developing countries and its impact on industrial processes that may replace traditional, small-scale and family-based production.

In African countries, vinegar is nowadays largely used as a food flavouring and condiment or as a food preservative. Most of the vinegar consumed in Africa is imported from Europe. In Africa, there has never been a wide tradition of artisanal vinegar production. However, in recent years this has been changing, because the few existing small-scale food companies are incapable of absorbing all the agricultural products that are produced in excess, such as cereals and tropical fruits, which are abundant in wide regions of sub-Saharan Africa. They do, however, represent a valuable raw material for vinegar production and do not demand large investments in infrastructure by local factories. This practice has the added value of utilization of local agricultural products, most of them being waste products yielding no economic gain; this therefore gives an economic boost to the region where the vinegar is produced.

The problems encountered by large-scale vinegar making in Africa are mainly caused by high ambient temperatures (above 30 °C) in most of the regions, the high cost of the necessary cooling of the bioreactors to carry out acetification to keep the fermentation temperature constant, and the large amounts of water needed for that purpose that are not always readily available. Another problem is the lack of infrastructure such as cooled storage chambers as well as good transportation systems.

The vinegar produced in Africa comes from very different and varied sources, such as coconut water, palm wine, bamboo wine, grape wine (mainly from some

Mediterranean countries and South Africa), some tropical fruits (mangoes, dates and bananas) and cereals (e.g. sorghum). Most of these products are widely produced in Africa and are important for the local economy and the survival of large numbers of families. All these products are widely used for various purposes, and therefore the production of vinegar represents an additional, secondary purpose; however, the manufacture of vinegar has been growing in importance during recent years.

## 13.2 The Main Vinegars Produced in Africa

### 13.2.1 Coconut Water Vinegar

Coconut trees are the most extensively grown nut trees in the world and provide people with the raw materials for basic needs such as food, drinks, furniture, etc. In 1996, according to the Asian and Pacific Coconut Community (APCC, 1996), 460,000 ha of coconut trees were cultivated in Africa, with an overall production of $2.19 \cdot 10^9$ coconuts. Tanzania was the largest producer of coconuts in Africa, with a cultivated area of 310,000 ha, followed by Ghana with 40,000 ha, Madagascar with 33,000 ha, and Ivory Coast with 32,000 ha.

Coconut-based technologies require very simple, locally available, materials and equipment and are quite easy to perform (Banzon and Velasco, 1982). This makes the coconut a very appropriate primary product for the bioconversion of coconut water into vinegar in rural areas, since no sophisticated apparatus or other equipment is required. Coconut water is considered as a waste material in copra making or in desiccated coconut factories. Vinegar production gives an added value to its use and also provides an extra source of income for the coconut farming communities (Punchihewa, 1997).

The estimated cost of vinegar production from coconut water (without taking into account the integrated process of vinegar production and other coconut products), including all expenses, is around US $776 to generate an income estimated to be around $1090 (calculation based on the use of 4368 nuts), resulting in a net income of $314 (Batugal and Ramanatha Rao, 1998).

Coconut water vinegar is processed by allowing filtered coconut water to undergo an alcoholic fermentation and a subsequent acetification at ambient temperature. The first step is filtering the coconut water through a filter cloth. The sugar content of the coconut water is adjusted to 162 $g \cdot L^{-1}$ by the addition of refined sugar (normal sugar content of coconut water is around 1% w/v). The liquid is then boiled for its pasteurization and, once cooled, it is inoculated with active dry yeast for an alcoholic fermentation taking 5-7 days. If the use of active dry yeast is not possible, a spontaneous alcoholic fermentation is performed without the pasteurization step. After fermentation, the alcoholic water is transferred to other containers, usually made of plastic or stainless steel, and ‘mother of vinegar’ or a starter culture of acetic acid bacteria (AAB) is added. The container is not fully filled to allow a

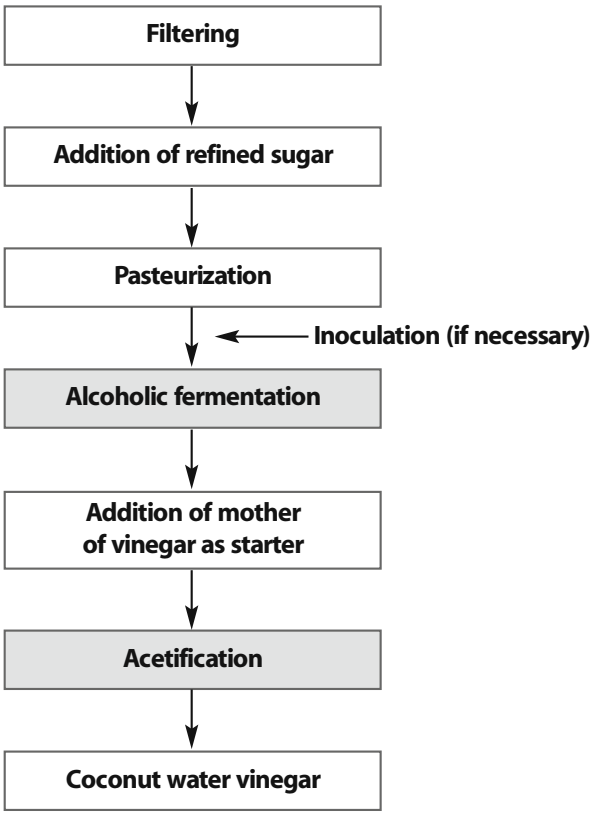


**Figure 13.2** Flow diagram of coconut water vinegar production

headspace for aeration, as AAB are strictly aerobic microorganisms, thus ensuring a higher effectiveness of the process. The mixture is stirred and covered with a cloth. The acetification process takes around 7 days (Punchihewa, 1997) (Figure 13.2). The end product contains between 3% and 4% (v/v) acetic acid and is an indispensable commodity in many households (Sanchez, 1990).

### 13.2.2 Mango Vinegar

Nowadays the mango tree (*Mangifera indica*) is appreciated in many countries for its fruit and its shade, although it is a recent introduction in Africa (Rey et al., 2006). The mango tree originates from India and was first reported in Africa in 1824, in Senegal. Its expansion became significant in the first half of the 20th century, being introduced in most of the French-speaking West African countries (Rey et al., 2006).

The total world production of mango was over 24 million tonnes (t) in 1999 (FAOSTAT, 2000). The global production is concentrated mainly in Asia, and more precisely in India, which produced 12 million Mt in 1999. In recent years, mangoes

have become well established as a fresh fruit and processed fruit products are widely available in the global market. The world demand for mango is increasing and the fruit is becoming very popular. An increase in mango production in non-traditional mango-producing areas is seen and this includes parts of Asia, West Africa, Australia, South America and Central America (Rey et al., 2006).

Mango is most usually eaten raw. However, production volumes of this fruit are high and large quantities are often wasted (Akubor, 1996). Those wasted mangoes can be used for the production of jelly, wine and vinegar, which have an important added value as by-products.

Vinegar is produced from mango pulp by alcoholic fermentation as a first step and acetification as a second step. The pulp is usually diluted in water (in a pulp : water ratio of 1 : 5) and filtered through a filter cloth to obtain the juice (Garg et al., 1995). Mango juice normally contains 3.6% (w/v) of total sugars. Therefore, more sugar is added to the filtered juice up to approximately 200 $g \cdot L^{-1}$ to obtain sufficient conversion of sugar into ethanol. As is done with coconut water vinegar, the juice is pasteurized when the fermentation is carried out by the addition of a starter culture of AAB. The alcoholic fermentation lasts for 14 days, resulting in around 8% (v/v) of ethanol (Akubor, 1996). If the fermentation continues for up to 3 weeks the ethanol concentration can increase up to 9% (v/v), probably due to the additional conversion of malic acid into ethanol (Kunkee and Amerine, 1970; Akubor, 1996). The mango wine is then transferred to other containers for the acetification process. 'Mother of vinegar' is added as a starter. Another technique applied for this second process is the use of immobilized cells of AAB on wood shavings (Garg et al., 1995). In the latter study the immobilized microorganism was *Acetobacter aceti*, which is – together with *Acetobacter pasteurianus* – the most common AAB found in acetification processes. *Acetobacter lovaniensis* has also been isolated from mangoes (Lisdiyanti et al., 2001), and this could also be a potential starter for mango vinegar production. The final mango vinegar has an acidity of around 5.3% (v/v) of acetic acid.

### 13.2.3 Sorghum Vinegar

Sorghum is the fifth most important cereal for human consumption, after rice, wheat, maize and barley. Although Africa is one of the lowest producers of cereals globally (e.g. 28.2% of the global production of sorghum; FAO, 1995), it constitutes the main food grain for people who live in semi-arid regions. The largest group of producers are small-scale subsistence farmers (Bennett et al., 1990).

The structure of sorghum is similar to that of other cereals. The major components of the grain are the pericarp (outer covering), the testa (between pericarp and endosperm, if present), the endosperm and the embryo (Hulse et al., 1980).

Grain sorghum is normally grown in regions which are too dry or too hot for successful maize production. This cereal is adapted to drier climates due to several factors: (i) sorghum exhibits a low transportation rate (amount of water, in kg, required to produce 1 kg of plant material) compared with other cereals (141 kg for

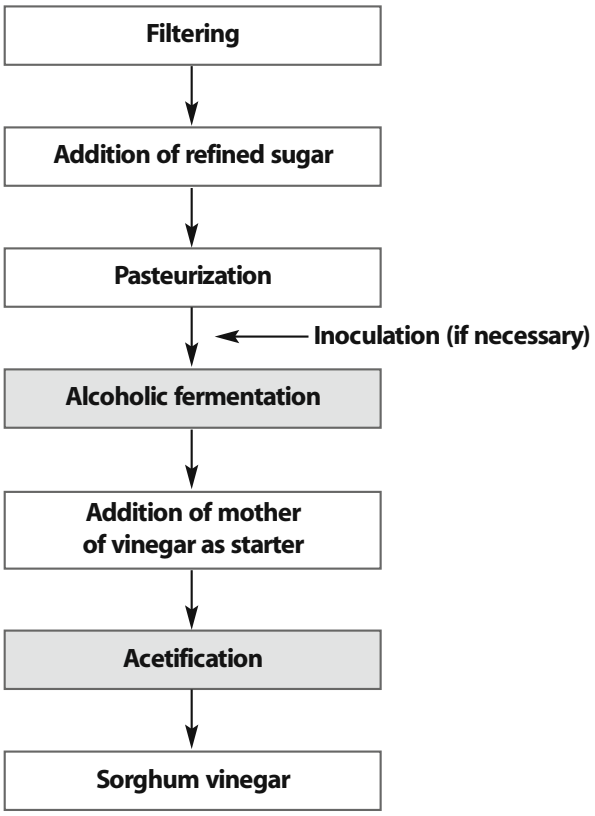


**Figure 13.3** Flow diagram of sorghum vinegar production

sorghum, 170 kg for maize, and 241 kg for wheat); (ii) sorghum can withstand temperatures above 38 °C; and (iii) sorghum can tolerate a wide range of soil textures and pH values (Bennett et al., 1990).

The main food products from sorghum are porridges, fermented and unfermented breads, lactic and alcoholic beers and beverages, malted flours for brewing, etc. Some pre-fermentation processing is necessary, as is the case for cereals in general, such as drying or steeping of the grains. These treatments are performed to prevent the growth of moulds or germination of the grains. In developing countries the grains are normally sun-dried, but they can also be dried by using forced warm air (McFarlane et al., 1995).

To obtain sorghum vinegar the process is quite similar to those mentioned above, but in this case there is a previous step of grinding the grains to allow extraction of the sugars (Figure 13.3). The main source of sugars for their bioconversion into ethanol is starch, but residues from sugar extraction can also be used for vinegar production. Water and a yeast starter culture are added to the ground grains to start the alcoholic fermentation. Once finished, the alcoholic product is transferred to other vessels, where 'mother of vinegar' is added for acetification.

Little research has been done on sorghum vinegar, because its production is mainly performed on a small scale by small local farmers. Most of the research is focused on the cultivation of sorghum and on the production of sorghum dough.

### 13.2.4 Palm Vinegar

The palm tree is very abundant in tropical regions. The palm family is one of the most economically important widespread tree families and supplies many needs for human survival, such as edible fruits, sugars and building materials, and also has commercial uses for oil, wax, starch, fibre and vegetable ivory production (Hoyt, 1990). Cut spathes (flowering sheaths) of some palms, such as coconut or palmyra, yield toddy or sugary sap that can be converted into alcoholic beverages, such as wine and vinegar (Mozingo, 1989).

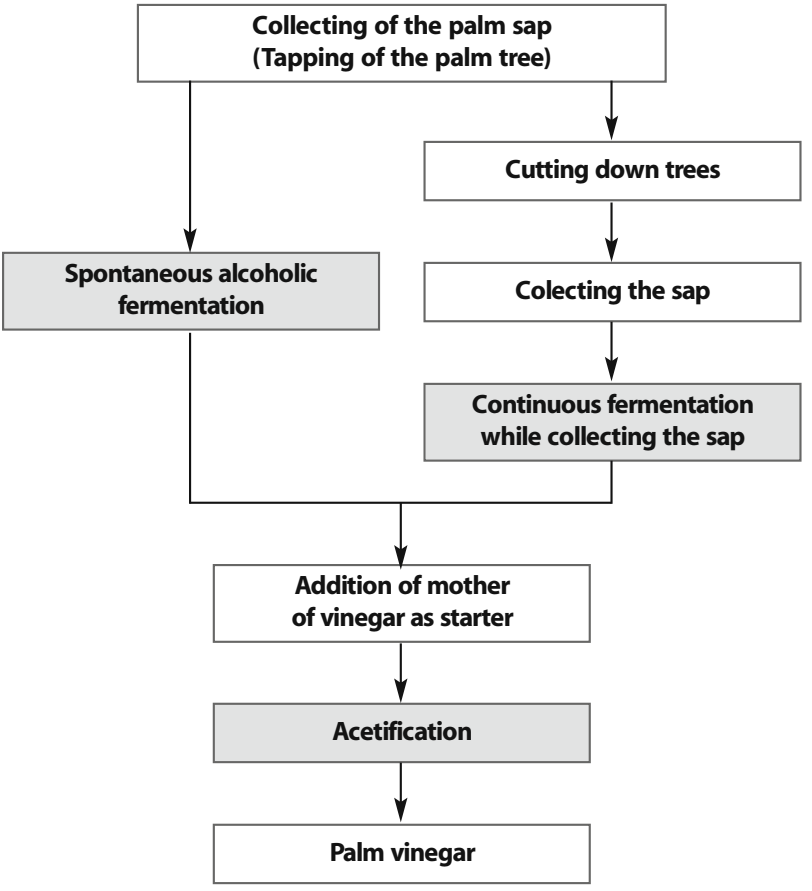


**Figure 13.4** Flow diagram of palm wine vinegar production

The palms most frequently tapped for sap are raphia palms (*Raphia hookeri* and *Raphia vinifera*) and the oil palm (*Elaeis guineese*). The sugar content of the palm sap is between 10% and 14% (w/v), making it an ideal substrate for yeast growth.

The first step in palm vinegar production is the collection of the sap by tapping the palm tree. This is made by making an incision into the core part of the plant and collecting the sap in containers. This process can take 1 or 2 days. There is a second system, widely used in Ghana, in which the sap is obtained from felled trees (Amoa-Awua et al., 2006). This technique involves first cutting down the trees, and leaving the felled trees for 2 weeks before tapping of the sap. The sap is collected during a period of up to 8 weeks (Hartley, 1984). In this second method the sap is spontaneously fermented by yeasts, mainly *Saccharomyces cerevisiae* (Amoa-Awua et al., 2006), in the sap collectors during tapping, representing a method of continuous production. The alcoholic fermentation of the sap is usually spontaneous and starts a few hours after sap collection. It takes around 2 days to complete the fermentation, reaching ethanol concentrations of above 7% (v/v) (Amoa-Awua et al., 2006). The second fermentation or acetification also takes place spontaneously. Spontaneous acetification of palm wine is one of the major problems of wine producers. Palm wines have to be consumed within a few days, otherwise the acetic acid levels become unacceptable (Amoa-Awua et al., 2006). The acetification process takes about 4 days to be completed and the acetic acid concentrations are around 4% (v/v) (Battcock and Azam-Ali, 1998) (Figure 13.4).

## 13.2.5 Other African Vinegars

### 13.2.5.1 Wine Vinegar

Wine vinegar is one of the most common vinegars in the Mediterranean countries (Tesfaye et al., 2002). In Africa, both red and white grapes are cultivated in countries such as Algeria, Morocco and South Africa. Vinegar production from both red and white grapes follows similar steps as for other vinegars described above. First, an alcoholic fermentation takes place, followed by acetification of the wine by AAB.

Depending on the quality of the wine vinegars, they are aged in wooden barrels for several months or years.

### 13.2.5.2 Honey Vinegar

Honey has traditionally been used in many African countries for the production of honey beer, which still is a very common beverage in non-Islamic African regions (Krell, 1996). Also, honey wine is produced in Africa, but the alcoholic fermentation lasts for months or even years before it is finished, due to the high sugar concentration of honey. Yeasts commonly carrying the alcoholic fermentation are osmophiles, such as *Zygosaccharomyces*, which are normally found in media with more than 50% (w/v) of sugars (Krell, 1996). Honey vinegar is produced from honey wine by acetification with AAB. Its production, however, is hardly feasible

economically, mainly due to the time required. It is commonly produced for home consumption only (Krell, 1996).

#### 13.2.5.3 Banana Vinegar

Bananas are a widespread fruit in Africa and are usually consumed raw. As bananas have a short shelf-life, there is a rapid rate of deterioration of this fruit (Akubor et al., 2003). Fermenting banana juice is therefore considered to be an attractive way of utilizing wasted and over-ripe bananas to produce derived products with economic benefits. The main fermented products of these fruits are beer, wine and vinegar. The first step in the production of banana vinegar is to wash, peel, and slice the fruits. Slices are blended and boiled with water and then filtered (Akubor et al., 2003). Prior to the alcoholic fermentation, sugars are usually added. The next steps of the vinegar production process are as for other vinegars, as described above.

#### 13.2.5.4 Date Vinegar

The lack of acidity of dates, together with the fact that most date production is in Muslim countries where alcoholic beverage consumption is prohibited, makes them a small market for the production of date wine and, therefore, date vinegar (Barreveld, 1993). For the production of date vinegar, water is added to the dates and the mixture is boiled in the case of a non-spontaneous alcoholic fermentation (Ali and Dirar, 1984). The acetification of the date wine is carried out by adding 'mother of vinegar' to the wine. The final vinegar common reaches values of acetic acid of 4-5% (v/v).

#### 13.2.5.5 Bamboo Vinegar

Bamboos grow naturally in all the continents except for Europe and Antarctica (Liese and Hamburg, 1987). They have played an essential role for people living in tropical countries in many different ways (e.g. buildings, decorations, processed products, etc.) (Sharma, 1980; Zhou, 1983). Bamboo vinegar is obtained, as in the case of palm vinegar, from the sap of the plant. The process of obtaining sap and vinegar is, therefore, very similar to that one used in the production of palm vinegar. The main difference is that the alcoholic fermentation of bamboo sap is extremely fast. It only takes between 5 and 12 hours to obtain bamboo wine. The speed of the fermentation confers an added value to bamboo vinegar production.

## 13.3 Problems and Solutions for Vinegar Production in Africa

In most cases, vinegar production is not an industrial process in Africa. Its future commercial development in this part of the world is dependent on socio-economic conditions and various technological and biological factors. Possible solutions and future perspectives to overcome the problems hindering efficient, large-scale vinegar production in Africa are described below.

### 13.3.1 Socio-Economic Factors

African countries have no tradition for the production of vinegar, although there are several types of vinegar that have been made in Africa for a long time by traditional methods. However, most of this production is for self-consumption and there are no markets for the distribution of these products. Most of the vinegar consumed until recently was either imported or illegally diluted, chemically produced, acetic acid. The progressive introduction of clear and severe regulatory laws for vinegar production, as well as a growing awareness of the damage caused to health by the consumption of diluted chemical acetic acid, is helping to develop a social consciousness of the benefits of vinegar produced through fermentation, both for human health and to supplement family incomes.

The development of agriculture in Africa is creating significant quantities of surplus food products that small farms are not able to absorb. These waste foods are an excellent raw material for the production of vinegar, and economic investment in factories for such processes has been low. The availability of a wide variety of foods for biotransformation into vinegar is advantageous for the farmers (fitting in well with their main agricultural activities) and helps in the conservation of natural resources. However, the large number of different raw materials used makes it impossible to introduce common techniques and processes to the producers, and this increases the cost of vinegar production. The possibility of standardization of the production of such vinegars, as well as stimulating economic development, could be very important when specialized products from particular raw materials are produced. Specialized, added-value products represent an attractive niche market, both because of the economic benefits and also, in particular, from a gastronomical point of view. This happens, for instance, in Italy with traditional balsamic vinegar (TBV) production, and in Spain with Sherry vinegar production, conferring added value and premium quality to these products.

There is also a lack of any official development programmes offered by governments or any cooperation between private vinegar producers, which would enable cost savings to be made and would make investment a more attractive proposition. Nevertheless, vinegar is an important ingredient in a variety of commercial products, such as ketchup, mayonnaise, sauces and mustards. Therefore, local vinegar production, particularly if it uses agricultural waste products, should be encouraged.

### 13.3.2 Technological and Biological Factors

There are several technological factors to take into account for vinegar production in African countries, such as room temperature, AAB used, amount of water, etc.

Temperature is the main limiting factor for vinegar production in Africa. A normal temperature for the acetification process is 30 °C and the optimal temperature for growth of AAB is 25-30 °C (Holt et al., 1994). However, the ambient temperature in most parts of Africa is usually around 30-40 °C. The fermentation temperature for vinegar production falls within a narrow range; a temperature increase of

2-3 °C causes a serious deterioration in both the acetification ratio and efficiency (Adachi et al., 2003). In submerged cultures, large amounts of heat are generated, so that cooling costs become rather higher (Adachi et al., 2003). The need for cooling of the bioreactors (which is normally performed with ordinary water) increases the overall economic cost of vinegar production. The shortage of water in many regions of Africa should also be considered. Finally, the storage of the finished products, as well as of the raw materials, suffers from high ambient temperatures, as there are no suitable cool storage rooms or good transport networks available.

Although the optimal temperature for growth of AAB is below the ambient temperature of many regions of Africa, there are some recent studies dealing with the isolation and exploitation of thermotolerant AAB that can tolerate higher temperatures of 37-40 °C (Saeki et al., 1997; Moonmangmee et al., 2000; Ndoye et al., 2006). The use of strains of AAB that can work optimally at 37-40 °C can also decrease the expenses for cooling.

Saeki et al. (1997) isolated thermotolerant *Acetobacter* species able to produce acetic acid at temperatures up to 40 °C. The acetification efficiency with thermotolerant strains at 38-40 °C was almost the same as that of mesophilic strains at 30 °C. Vinegar production at higher temperatures was successful with both submerged and static cultures. These thermotolerant AAB also displayed good acetic acid and ethanol tolerance (Saeki et al., 1997). Ndoye et al. (2006) investigated the possibility of isolating thermotolerant AAB from mangoes and cereals that were produced in excess in Senegal and Burkina Faso, respectively, in order to find appropriate strains from African sources to be exploited for vinegar production in that part of the world. As a result, they isolated two interesting strains: one strain of *Acetobacter pasteurianus* and one strain of a novel *Acetobacter* species, named *Acetobacter senegalensis* (Ndoye et al., 2007a). Both strains were found to be successful for use in vinegar production at high temperatures.

A second problem with AAB is the conservation of the cells to be used as starter cultures. Sokollek et al. (1998) and Ndoye et al. (2007b) preserved AAB successfully by dehydration. The continuous availability of viable starter cultures of AAB will have a great impact on vinegar production in developing countries. Reliable starter cultures can accelerate the fermentation process and confer an added value to vinegar production using autochthonous AAB. In turn, this may increase our knowledge of these bacteria and improve control over the fermentation process, as well as preventing contamination with unwanted substances, and will therefore enhance the production of standardized, natural, high-quality and safe vinegars for human consumption.

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# Chapter 14
# Taiwan Fruit Vinegar

**Andi Shau-mei Ou and Rei-Chu Chang**

## 14.1 Introduction

Vinegar has been used as a seasoning in cooking since ancient times. However, recent research has shown that, in addition to its well-known anti-bacterial activity, vinegar (when consumed as a drink) confers considerable health benefits, including lowering blood pressure, acting as an anti-oxidant, alleviating the effects of diabetes, preventing cardiovascular diseases, providing refreshment after exercise, etc. (Nishidai et al., 2000; Ogawa et al., 2000; Fushimi et al., 2001, 2002; Kondo et al., 2001; Shimoji et al., 2002; Sugiyama et al., 2003; Johnston et al., 2004). Consumers are now beginning to appreciate the health benefits of drinking vinegar. Therefore, in addition to the traditional use of vinegar products such as rice vinegar, wine vinegar and cider vinegar as a food flavouring, there is a growing demand for fruit vinegar products that are sold as a health food. The emergence of these new products has resulted in name changes from wine or cider vinegar to grape or apple vinegar, with these being categorized as fruit vinegars. Thus, there are two types of fruit vinegar products on the market: one with a high content of acetic acid which is either used as a seasoning or is diluted with 4-8 times as much water as a health drink, and another that is ready to drink as a beverage.

There are only a few reports in the literature on research into fruit vinegar products made from fruits other than grapes or apples. Most research has studied the acetic acid yield from fermentation using fruit peel and other wastes as the raw materials (Richardson, 1967; Anon., 1973; Adams, 1978; Grewal et al., 1988), and there has been little research work on the quality aspects of fruit vinegars. Koizumi et al. (1987) evaluated the general composition, amino acids and organic acids of some high-priced special vinegars in Japan, and found that they were not of high quality. This tells us that, if fruit vinegar products are good for health and for drinking, much work needs to be done in figuring out the important criteria for manufacturing and marketing healthy and palatable fruit vinegar products. In general, many consumers, especially the younger generation (under 20 years old), do not like fruit vinegar products. This chapter discusses the definition of fruit vinegar and

L. Solieri, P. Giudici (eds), *Vinegars of the World*

**Table 14.1** The Chinese National Standard (CNS) quality standards for edible vinegar (Chinese National Standard, 2005)

| Variety \ Composition | | **Acidity** (%, calculated as acetic acid) | **Salt-excluded soluble solids** (%) | **Non-volatile acidity** (%, calculated as acetic acid) | **Total nitrogen(%)** |
|---|---|---|---|---|---|
| Brewing vinegar | Grain vinegar | Not less than 4.2 | Not less than 1.3 | – | – |
| | Fruit vinegar | Not less than 4.5 | Not less than 1.2 | – | – |
| Other brewing vinegar | | Not less than 4.0 | Not less than 1.2 | – | – |
| High-acidity vinegar | | Not less than 9.0 | Not less than 1.5 | – | – |
| Condiment vinegar | | Not less than 1.0 | Not less than 6.0 | – | – |
| Artificial vinegar | | Not less than 4.0 | Not less than 1.2 | Not more than 1.0 | Not more than 0.2 |

**Table 14.2** The regulation for composition of vinegar by FAO/WHO (Joint FAO/WHO Food Standards Programme, 2000)

| | **Wine vinegar** | **Vinegars other than wine vinegar** |
|---|---|---|
| Total acid content | Total acid not less than 60 $g \cdot L^{-1}$ (calculated as acetic acid) and not more than the amount detainable through the use of biological fermentation | Total acid not less than 50 $g \cdot L^{-1}$ (calculated as acetic acid) and not more than the amount detainable through the use of biological fermentation |
| Residual alcohol content | Residual alcohol (v/v) not more than 0.5% | Residual alcohol (v/v) not more than 1% |
| Soluble solids (exclusive of added sugars or salt) | Soluble solids not less than 1.3 $g \cdot L^{-1}$, 1% acetic acid | Soluble solids not less than 2.0 $g \cdot L^{-1}$, 1% acetic acid |

describes the types of fruit vinegar products available in Taiwan. It then examines the manufacture of fruit vinegar using pineapple as the raw material, and shows the results of analysis using near infrared spectroscopy to determine its physico-chemical properties.

## 14.2 Definitions of Fruit Vinegar for Taiwan and the FAO/WHO

Edible vinegar is classified into brewing vinegar and artificial vinegar according to the Chinese National Standard (CNS) definitions CNS14834 and N5239 (Chinese National Standard, 2005). The difference between the two vinegars is due to the addition of glacial acetic acid (or acetic acid). The definition of a fruit vinegar, such as cider, wine or orange vinegar, according to the CNS brewing vinegar standards, is that it must have been fermented from at least one fruit, and each litre of raw material must contain more than 300 g of fruit juice. The quality specification for edible vinegar by the CNS mainly concerns the acidity and salt-excluded soluble solids, as shown in Table 14.1. The acidity of grain vinegar and fruit vinegar is above 4.2 % and 4.5%, respectively. The contents of salt-excluded soluble solids in grain vinegar and fruit vinegar must be above 1.3 % and 1.2%, respectively.

As defined by FAO/WHO, vinegar is a liquid, fit for human consumption, produced exclusively from suitable products containing starch and/or sugars by the process of double fermentation; first alcoholic and then acetous: and fruit vinegar is a vinegar obtained by acetous fermentation from wine of fruit (Joint FAO/WHO Food Standards Programme, 2000). Their composition specification not only includes the acidity and salt/sugar-excluded soluble solids, but also includes the residual alcohol content, food additives and food impurity (Table 14.2). Regarding the name of a vinegar, it is named 'X vinegar' if the raw material used is X only. If there is more than one raw material, it is named either X or Y vinegar depending on whether X or Y is the predominant material.

To compare the two above-mentioned specifications, it must be remembered that the FAO/WHO guidelines refer to soluble solids in the fruit vinegar exclusive of sugar and salt added during the manufacturing process, while the Taiwanese CNS standard excludes salt but does not mention the sugar content, or consider the influence that added sugars may have on soluble solids calculated for different types of vinegar. This is because, in the past, edible vinegars used for seasoning were made exclusively from grains with no added sugar. It is obvious that the regulations for edible vinegar in Taiwan need to be updated, especially for the various types of fruit vinegar as well as the sugar-added vinegars that are already available.

## 14.3 Fruit Vinegar in Taiwan

Taiwan is located in the subtropical and tropical zones and its climate is very suitable for growing fruit, so a wide variety of fruits are available in all seasons. Traditionally, commercially produced vinegar is made from rice or unpolished rice.

However, because of a lack of the appropriate fermentation technology and facilities for fruit vinegar production in Taiwan, many fruit vinegars are not made by alcoholic and acetic fermentations. Many of them are simply made by adding fruit or fruit juice and sugar into the rice vinegar and leaving it for more than 3 months. The rice vinegar is used as a solvent to extract the nutrients and aroma of the fruit. There are only a few fruit vinegar products obtained from real alcoholic and acetous fermentation. These are made either by going through a natural fermentation process or by using manufacturing methods and microorganisms that have been kept secret.

In 2002-2003, we surveyed the fruit vinegar products sold in Taiwan to examine their label information and physico-chemical properties (Chang et al., 2005). In total, 66 fruit vinegar products, comprising 12 mei (also known as Japanese apricot), 17 cider, 3 mulberry, 4 lemon, 8 blended, 15 wine, 2 orange and 5 other (starfruit, blueberry, pineapple, grapefruit and passion fruit) vinegars were collected, as listed in Table 14.3. Among these, 8 cider and 14 wine vinegar were imported. For the domestic vinegars, apart from the wine vinegar which is used solely as a seasoning, all the other products can be diluted with 4-8 parts of water before drinking as a beverage.

Two samples, acquired from organic food stores, had no label except for the product name. Of the 64 samples with labels, 29 had nutrients listed on the label, while 35 did not. Production methods were classified into five categories, according to information on the labels. They were brewed from juice by alcoholic and vinegar fermentation (F); brewed from juice and alcohol (FA); F mixed with grain vinegar (FG); juice mixed with grain vinegar (JG); and F mixed with juice (FJ). From Table 14.3, it can be seen that 28 samples were F, while 26 were JG. Those categorized as FG, FA and FJ had 6, 3 and 1 samples, respectively. Domestic products, such as mei, cider, mulberry, lemon and blended vinegars, were mostly made with juice mixed with rice vinegar or sorghum vinegar, while most imported products were produced by alcoholic and acetous fermentation. The average price of wine vinegars, per 100 mL, was the highest of all the fruit vinegars.

Total sugar content of vinegar, with no extra sugar added, was less than 3%, while those with sugar added ranged from 8% to 64%. Most imported cider and wine vinegars had no sugar added, with the acidity being about 5-7%. Most domestic products with sugar added had an average acidity of less than 3%. Besides acetic acid, the major organic acids found in fruit vinegars were malic, lactic and citric acids. Mulberry vinegar was found to be higher in lactic and succinic acids than the others. Red wine vinegars were rich in tartaric, malic and lactic acids.

Twenty-six out of 44 Taiwan local fruit vinegar samples belonged to this type, while only nine samples were made purely from fruit juice fermentation. In addition, for healthy drinking and to increase palatability and consumer acceptance, sugar is normally added to balance the sourness of acetic acid produced. By so doing, the acidity of resultant vinegar usually cannot meet the regulations of either CNS or FAO/WHO. In addition, the proportion of salt-excluded soluble solids becomes higher than it should be due to the addition of sugar. This clearly shows that the regulation by CNS is re-adjustable. The soluble solids should be measured by exclusion of salt and sugar, rather than salt only.

**Table 14.3** Label analysis and classification of fruit vinegars available in the Taiwan marketplace

| | Domestic | | | | | | | | Imported | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Samples** | **Total** | **Mei** | **Cider** | **Mulberry** | **Orange** | **Lemon** | **Blended** | **Others** | **Total** | **Cider** | **Wine** |
| No. of samples | 44 | 12 | 9 | 3 | 2 | 4 | 8 | 6 | 22 | 8 | 14 |
| Classification[a] | | | | | | | | | | | |
| F | 9 | 2 | 3 | 2 | – | – | 2 | | 19 | 6 | 13 |
| JG | 26 | 5 | 4 | 1 | 2 | 4 | 6 | 4 | – | – | – |
| FG | 6 | 3 | 2 | – | – | – | – | 1 | – | – | – |
| FA | – | – | – | – | – | – | – | – | 3 | 2 | 1 |
| FJ | 1 | 1 | – | – | – | – | – | – | – | – | – |
| Unknown | 2 | 1 | – | – | – | – | – | 1 | – | – | – |
| Labelled | 42 | 11 | 9 | 3 | 2 | 4 | 8 | 5 | 22 | 8 | 14 |
| Without NL[b] | 16 | 6 | 1 | 3 | 0 | 0 | 4 | 2 | 19 | 5 | 14 |
| With NL[b] | 26 | 5 | 8 | 0 | 2 | 4 | 4 | 3 | 3 | 3 | 0 |
| Incorrectly labelled[c] | 14 | 2 | 5 | – | 2 | 2 | 2 | 1 | 2 | 2 | – |

[a] F, vinegar brewed from juice by alcoholic and vinegar fermentation. FA, vinegar brewed from juice and alcohol. FG, F mixed with grain vinegar. JG, juice mixed with grain vinegar. FJ, F mixed with juice.

[b] NL, nutrient label.

[c] Total sugar contents are >20% greater than the amount stated on the label.

In terms of the names for fruit vinegars, according to the regulation of CNS, edible vinegar is classified by the raw material used at the alcoholic fermentation stage; while by FAO/WHO regulations it is classified by the raw material used at the acetous fermentation stage, such as wine vinegar or cider vinegar. Any other substance added in the product should have some suitable descriptor on the label. According to the classification standards by both CNS and FAO/WHO, most of the fruit vinegar products available on the Taiwan market should have the name of either rice or sorghum vinegar.

Furthermore, since the consumer treats fruit vinegar as a health drink, the daily consumption of fruit vinegar should not be overlooked. The inspection of labels for accuracy of information needs to be taken seriously in order to protect the consumer. Our survey revealed that 61.9% of domestic fruit vinegar products had nutrition information on the label, but only 13.6% of imported ones had nutrition labelling. In addition, the total sugar content in 55.2% of labelled products was found to be 20% higher than the values stated. These results show that the quality of fruit vinegar sold in the Taiwan market is not well controlled.

## 14.4 The Brewing of Pineapple Vinegar

The flavour and mouth-feel of edible vinegar are influenced by the acetic acid bacteria strains used (Lin and Chen, 2002), base wine for brewing (Ciani, 1998), brewing method (Lin and Chen, 2002) and storage (Okumura, 1995; Tesfaye et al., 2002). Along with the increase in standard of living, people gradually now care more about the natural characteristics and health benefits of foods than they did in the past, and the demand for high-quality foods is increasing. Manufacturing a good product with genuine high quality is the only way to attain a substantial market share. Pineapple is a major economic crop in Taiwan. As well as canned pineapple or pineapple juice, pineapple vinegar is also available in the Taiwan market. However, it is mainly produced by mixing pineapple juice with grain vinegar. Only a few come from brewing, and the quality needs to be improved before they are widely accepted by the consumer. The following sections describe the complete procedure for the brewing of high-quality pineapple vinegar in terms of the raw materials used, the brewing of pineapple base wine, the selection of acetic acid bacteria, and the effects of additional nutrients and storage on the quality of pineapple vinegar.

### 14.4.1 Raw Materials and Brewing of Pineapple Base Wine

High-quality raw materials are essential for manufacturing high-quality processed foods. Pineapple vinegar is no exception. The ‘Smooth Cayenne’ cultivar of pineapple was selected as the raw material because of its unique flavour. After cutting off the head, tail and peel, the fruit was further cut and blended into pulp. To every kilogram of pulp was added 0.2 mL Pectinex Ultra SP-L (Novo Nordisk

Ferment Ltd., Japan), and the pulp was then left for 30 minutes at room temperature to obtain better juice yield and clarity. The pineapple juice was centrifuged and heated at 60-70 °C for 10 min to get rid of spoilage bacteria. After cooling, the juice was stored at –20 °C before use (Chang, 2007).

For brewing the pineapple base wine to be used for acetous fermentation, the optimum fermentation conditions for brewing pineapple wine, as established in our laboratory (Wen, 2001), were used. After thawing, sugar was added to the pineapple juice to increase the total soluble solids (TTS) to 26 °Brix and pH was adjusted to 3.5. The 50 mL starter (*Saccharomyces* spp.) was inoculated into 830 mL juice and the fermentation took place at 12-14 °C. The end-point of brewing was chosen as the point when the TTS no longer changed. After brewing, the wine was centrifuged (15,400 × g) for 30 min, ready for further acetous fermentation.

### 14.4.2 Selection of Acetic Acid Bacteria

Pineapple base wines with different pH and alcohol concentrations were used as materials to compare the volatile compounds and sensory quality of fermented pineapple vinegar in order to select the most suitable acetic acid bacteria (AAB) for brewing pineapple vinegar. Five different AAB strains: *Acetobacter aceti* BCRC 12324, *A. aceti* BCRC 14156, *A. aceti* BCRC 11569, *A. aceti* 3012 and *Acetobacter* sp., were investigated (Chang et al., 2006).

For examining their ethanol tolerance and fermentation efficiency (FE, percentage of acetic acid produced from alcohol), these five AAB were inoculated into the base wine with ethanol content ranges of 3-9% and the brewing of pineapple vinegar took place at 30 °C. The results showed that the first three AAB had a FE of up to 90%, which was much higher than the other two AAB when the ethanol content is around 5-7% (Table 14.4).

Using pineapple base wine with 6% ethanol content as the substrate, these five AAB were inoculated for acetous fermentation. The major volatiles, including 3-methyl-1-butanyl acetate, 2-phenylethyl acetate, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-phenylethanol, 3-methyl butanoic acid, hexanoic acid, octanoic acid, *n*-decanoic acid and benzaldehyde (Table 14.5), were found in the headspace of the pineapple vinegar bottle.

After being diluted with 6% acetic acid solution to a concentration of 50 ppm, the ten volatiles listed above were smelt by 25-35 panelists and the odour characteristics were identified. Esters and alcohols were recognized as a ripened-fruit aroma or floral aroma while the acids were more related to oily, aged or mouldy odours. These results are in agreement with those of Lin (1994) in wines and Charles et al. (1992) in red wine vinegar. The volatiles of esters and alcohols in the pineapple vinegar brewed by *A. aceti* BCRC 12324, *A. aceti* BCRC 14156 and *A. aceti* BCRC 11569 were much higher than those by *A. aceti* 3012 and *Acetobacter* sp., and the acids with an unpleasant smell were much lower. Therefore, combining the FE and sensory data, it was shown that AAB of *A. aceti* BCRC 11569 and *A. aceti* BCRC 14156 are suitable for brewing pineapple vinegar.

**Table 14.4** Effects of different alcohol contents in pineapple wine on acetic acid production and fermentation efficiency by five acetic acid bacteria[a]

| | | Alcohol (%, v/v) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Strain** | **Time (weeks)[b]** | **3.0** | **4.0** | **5.0** | **6.0** | **7.0** | **8.0** | **9.0** |
| *A. aceti* BCRC 12324 | 3.0 | | 3.09 (74.03b) | 4.08 (78.20ab) | 4.93 (78.74ab) | 6.07 (83.10a) | 5.25 (62.89c) | 3.33 (35.46d) |
| *A. aceti* BCRC 14156 | 2.5 | | | 4.78 (91.62b) | 5.94 (94.87a) | 6.69 (91.59b) | 7.35 (88.05c) | 8.17 (87.00c) |
| *A. aceti* BCRC 11569 | 2.5 | | | 4.69 (89.89a) | 5.50 (87.85a) | 6.59 (90.22a) | 6.62 (79.30b) | 8.20 (87.31a) |
| *A. aceti* 3012 | 4.0 | | 4.29 (102.78a) | 5.13 (98.32ab) | 5.68 (90.72b) | 6.72 (92.00b) | 5.35 (64.09c) | |
| *Acetobacter* sp. | 3.0 | 2.08 (66.44ab) | | 3.08 (59.03b) | 5.08 (81.14a) | 5.88 (80.05a) | 6.59 (78.94a) | 6.72 (71.56ab) |

[a] The data (n=2) are expressed as the difference in acidity after and before acetous fermentation, and the values in parentheses are the fermentation efficiency. The means in each row bearing different letters are significantly different at $P<0.05$.

[b] Fermentation period.

**Table 14.5** Analysis of volatile compounds (ppm) in pineapple vinegar fermented by five acetic acid bacteria[a]

| Volatile compounds | *A. aceti* BCRC 12324 | *A. aceti* BCRC 14156 | *A. aceti* BCRC 11569 | *A. aceti* 3012 | *Acetobacter* sp. | Descriptor[b] |
|---|---|---|---|---|---|---|
| **Esters** | **9.20** | **10.79** | **12.70** | **5.40** | **4.53** | |
| 2-propenyl acetate | 0.04a | 0.11a | 0.18a | 0.03a | 0.08a | |
| 3-methyl-1-butyl acetate | 5.90ab | 6.38ab | 11.54a | 1.71b | 1.29b | Banana and apple-like |
| 2-phenylethyl acetate | 3.26b | 4.30b | 5.48a | 3.66b | 3.16b | Rose-like |
| **Alcohols** | **37.70** | **37.97** | **36.76** | **19.43** | **16.88** | |
| 3-methyl-1-butanol | 20.64a | 20.02a | 22.01a | 6.35b | 4.93b | Banana-like |
| 2-methyl-1-butanol | 1.46ab | 1.68a | 1.82a | 1.04b | 1.23b | |
| 2-phenylethanol | 15.60ab | 16.27ab | 16.93a | 12.04bc | 10.72c | Rose-like |
| **Acids** | **57.24** | **52.34** | **56.78** | **78.16** | **72.60** | |
| propanoic acid | 3.40a | 1.42b | 1.67b | 2.03b | 3.84a | |
| 2-methylpropanoic acid | 2.46a | 2.64a | 3.52a | 3.22a | 2.99a | |
| 3-methyl butanoic acid | 12.94b | 13.43b | 13.12b | 34.30a | 27.86a | Stink of feet |
| hexanoic acid | 3.11a | 3.06a | 3.29a | 3.74a | 3.04a | Slightly rancid |
| octanoic acid | 26.50a | 22.26a | 23.98a | 25.94a | 24.28a | Rancid and musty |
| *n*-decanoic acid | 8.83a | 9.53a | 11.20a | 8.93a | 10.59a | Rancid and stuffy closet |
| **Others** | | | | | | |
| benzaldehyde | 137.67a | 122.55a | 52.85b | 80.52b | 54.28b | Almond |
| 3-methylpentane | – | – | 0.61a | 0.04a | – | |
| **Sum** | **241.81** | **223.65** | **159.70** | **183.55** | **148.29** | |

[a] The means (n=3) with different letters for each compound differ significantly at $P<0.05$.

[b] Descriptor for each compound with concentration of 50 ppm in 6% acetic acid.

–, undetectable. BCRC, Bioresource Collection and Research Centre.

In addition, a further comparison on the quality of pineapple vinegar fermented by these two AAB, *A. aceti* BCRC 11569 and *A. aceti* BCRC 14156, was carried out. These two starters with five different ratios of 1:0, 2:1, 1:1, 1:2 and 0:1 were used for brewing the pineapple vinegar. The results showed that the pineapple vinegar fermented by the starter of *A. aceti* BCRC 14156 alone (0:1) was the best, due to its low content of acids and significantly high values of ethyl acetate and benzaldehyde, with a little ester and almond aroma, respectively. It is concluded the *A. aceti* BCRC 14156 is better than *A. aceti* BCRC 11569 for pineapple vinegar fermentation.

### 14.4.3 The Effect of Pineapple Juice with and without Peel on the Quality of Pineapple Vinegar

The pineapple juice used for brewing pineapple vinegar was obtained by the juice-making process. It is of interest to know whether the peel makes any contribution to the flavour of pineapple vinegar.

In order to examine the effect of pineapple juice with or without peel on the quality of vinegar, the following three different juice treatments were tested (Chang, 2007).

**Table 14.6** The analysis of volatile compounds in different pineapple vinegars[a]

| Volatile compounds (ppm) | PVA | PVB | PVC |
|---|---|---|---|
| **Esters** | **113.76** | **151.57** | **51.67** |
| ethyl acetate | 94.15a | 112.53a | 25.42b |
| 2-propenyl acetate | 1.03b | 2.28a | 2.76a |
| 3-methyl-1-butyl acetate | 13.58b | 26.01a | 14.79b |
| 2-phenylethyl acetate | 5.00b | 10.75a | 8.70ab |
| **Alcohols** | **32.83** | **63.70** | **41.41** |
| 3-methyl-1-butanol | 22.51b | 40.94a | 21.67b |
| 2-phenylethanol | 10.32b | 22.76a | 19.74a |
| **Acids** | **26.27** | **43.14** | **46.30** |
| propanoic acid | 0.31a | 0.39a | 0.04a |
| 2-methylpropanoic acid | 1.65a | 2.01a | 1.08b |
| 3-methyl butanoic acid | 4.38a | 4.01b | 3.53c |
| hexanoic acid | 1.91b | 5.08a | 5.36a |
| octanoic acid | 11.94b | 25.31a | 27.80a |
| *n*-decanoic acid | 6.08b | 6.34b | 8.49a |
| **Others** | | | |
| benzaldehyde | 95.44a | 43.85b | 56.52ab |
| acetoin | 2.10a | 1.07b | 1.97a |
| 3-methylpentane | – | 0.36a | 0.31a |
| **Sum** | **270.04** | **303.69** | **198.18** |

[a] Means (n=3) with different letters for each compound differ significantly at $P<0.05$.

–, undetectable.

**Table 14.7** The sensory evaluation of different pineapple vinegars[a]

| | Aroma | | | | Flavor | | | |
|---|---|---|---|---|---|---|---|---|
| | Fruity | Wine | Pungency | Ester | Sour | Wine | Pungency | Overall mouth-feel |
| PVA | 2.87c | 2.46a | 2.00a | 3.35a | 2.81a | 2.84a | 2.39a | 2.92c |
| PVB | 3.35b | 2.50a | 1.77b | 2.46b | 2.34a | 2.31ab | 1.89b | 3.19b |
| PVC | 4.31a | 2.08b | 1.96a | 2.00b | 3.27a | 2.11b | 2.73a | 3.46a |

[a] Means (n=12) of 5-point scaling: weak (1), moderate (3), strong (5). Means in each column with different letters are significantly different at $P<0.05$.

- *Pineapple base wine A (PWA)*: Pineapple juice obtained without peel was used and sugar was added to increase the total soluble solids (TTS) to 26 °Brix. After adjusting the pH to 3.5, the 50 mL starter (*Saccharomyces* sp.) was inoculated into 830 mL juice and fermentation took place at 12-14 °C. The end-point of brewing was when the TTS no longer changed. After brewing, the wine was centrifuged (15,400 × g) for 30 min, ready for further acetous fermentation.
- *Pineapple base wine B (PWB)*: Pineapple juice obtained without peel was used and no sugar was added. The following steps were as for PWA.
- *Pineapple base wine C (PWC)*: Pineapple juice obtained with peel was used and again no sugar added. The following steps were all the same as for PWA.

The alcohol contents of the three pineapple base wines (PWA, PWB and PWC) were 15.35%, 7.53% and 6.25%, respectively. Using the three pineapple base wines for acetous fermentation, the first step was to adjust the ethanol content down to 6% (v/v). Then 10% starter of *A. aceti* BCRC 14156 was added and brewing process took place at 30 °C until the acidity no longer increased. After centrifugation, the pineapple vinegars (PVA, PVB and PVC) were produced.

From the results of the volatile analysis (Table 14.6) and sensory test (Table 14.7), it was found that the PVA (no peel and high in sugar) was higher in benzaldehyde but lower in esters, alcohols and acids than PVB. The sensory characteristics of PVA, such as fruity aroma and mouth-feel, were much weaker than that of PVB (no peel and low sugar) and PVC (with peel and low sugar); only pungency was stronger.

On the other hand, there were many more volatiles, such as 3-methyl-1-butyl acetate, 3-methyl-1-butanol, 2-propenyl acetate, 2-phenylethyl acetate, 2-phenylethanol, hexanoic acid, octanoic acid and *n*-decanoic acid, found in PVB and PVC. Although the concentration of the first two volatiles (3-methyl-1-butyl acetate and 3-methyl-1-butanol), was lower in PVC than in PVB, the fruity aroma was much higher in PVC than in PVB and PVA.

The peel of pineapple might contain some undetectable trace volatile compounds which conferred a significant fruity aroma to the finished vinegar. Although the concentrations of most volatile compounds of PVC were no higher than those of PVB, the fruity aroma of PVC was significantly higher than PVB and PVA.

### 14.4.4 The Effect of Additional Nutrients on the Quality of Pineapple Vinegar

There are several studies showing the beneficial effects of nutrients added during the brewing of vinegar (Nanba and Kato, 1985a, 1985b; Lai, 1989). Nanba and Kato (1985b) reported that total amino acid content decreased gradually, and glutamic acid, aspartic acid and serine contents decreased considerably in the early stages of acetic acid fermentation. Our study showed that the final acidity of pineapple vinegar was raised by adding 0.05% ammonium sulphate, 0.2% peptone, 0.1% L-proline, 0.1% L-aspartic acid or 0.5% yeast extract in PWA before vinegar fermentation. As the protein content was only 0.14-0.21%, these five added nutrients could be supplied as a nitrogen source and would stimulate the acetous fermentation.

After L-aspartic acid addition, the pineapple vinegar was higher in fruitiness and mouth-feel and less pungent than the control without L-aspartic acid. Furthermore, the influence of the addition of 0.1% L-aspartic acid on the volatile compounds and sensory quality of PVA, PVB and PVC were investigated. The analysis of volatile compounds revealed that there was no significant difference for most volatile compounds in PVB and PVC with or without L-aspartic acid added. For PVA with no L-aspartic acid added, the volatile compounds such as 2-propenyl acetate, 3-methyl-1-butyl acetate, 2-phenylethyl acetate, 3-methyl-1-butanol, 2-phenylethanol, 3-methyl butanoic acid and octanoic acid were significantly lower in concentration than for PVB and PVC. With L-aspartic acid addition, the above-mentioned volatile compounds in PVA were substantially higher than those of the control, and there was no obvious difference from PVB and PVC, except that the concentrations of 3-methyl-1-butyl acetate and 2-phenylethanol were still lower. In terms of the results of sensory evaluation, this indicated that sensory characteristics of PVB and PVC, which were more flavourful, did not change significantly after L-aspartic acid added, but that PVA was more fruity-scented than the control.

### 14.4.5 The Effects of Storage on the Quality of Pineapple Vinegar

After brewing, the pineapple vinegar (PVA, PVB and PVC) was subjected to the storage test of 8-12 months at room temperature. The appearance of all the vinegars gradually became darker due to the Maillard reaction or the polymerization reaction of polyphenols. During the first 6 months storage of PVA, owing to the existence of some previously undetected secondary products of acetic acid bacteria in the pineapple vinegar, the acetic acid and these secondary products were able to participate in further reactions in the pineapple vinegar. As a result, alcohol volatile compounds such as 3-methyl-1-butanol and 2-phenylethanol continued to undergo esterification with acetic acid, which in turn led to an increase in the concentration of esters such as 3-methyl-1-butyl acetate and 2-phenylethyl acetate in the headspace of the pineapple vinegar (Table 14.8). This result is in agreement with the change in phenylethyl acetate of sherry wine vinegar during the early

**Table 14.8** Changes in volatile compounds (ppm) in pineapple vinegars with different base wine during storage[a]

| Vinegar | PVA | | | PVB | | | PVC | |
|---|---|---|---|---|---|---|---|---|
| **Storage time (months)** | **0** | **6** | **12** | **0** | **6** | **12** | **0** | **8** |
| **Esters** | **16.45** | **26.61** | **26.91** | **44.74** | **77.34** | **25.64** | **153.22** | **120.90** |
| ethyl acetate | 2.51a | 2.64a | 2.40a | 8.59a | 4.93b | 2.64c | 127.07a | 83.68b |
| 2-propenyl acetate | 0.20a | 0.59a | 0.96a | 1.18a | 4.44a | 1.81a | 2.76a | 3.50a |
| 3-methyl-1-butyl acetate | 3.26b | 8.50a | 10.05a | 12.55b | 49.04a | 9.87b | 14.79a | 9.93b |
| 2-phenylethyl acetate | 10.48a | 14.88a | 13.50a | 22.42a | 18.97ab | 11.32b | 8.70b | 23.79a |
| **Alcohols** | **22.93** | **29.25** | **25.03** | **60.83** | **40.45** | **28.88** | **41.41** | **41.46** |
| 3-methyl-1-butanol | 7.95b | 15.29a | 13.29a | 26.09a | 24.07a | 15.29b | 21.67a | 20.19a |
| 2-phenylethanol | 14.98a | 13.96a | 11.74a | 34.74a | 16.38b | 13.59b | 19.74a | 21.27a |
| **Acids** | **32.45** | **30.94** | **25.58** | **84.42** | **42.05** | **42.18** | **46.31** | **40.04** |
| propanoic acid | 0.96ab | – | 1.80a | 2.30a | – | 1.59b | 0.04 | – |
| 2-methylpropanoic acid | 2.51a | – | 0.96b | 3.87a | – | 2.14b | 1.08b | 8.80a |
| 3-methyl butanoic acid | 5.53c | 12.55a | 10.00b | 10.62b | 16.23a | 9.95b | 3.53a | 2.52b |
| hexanoic acid | 1.94 | – | – | 6.17 | – | – | 5.37 | – |
| octanoic acid | 13.99a | 12.77a | 9.38a | 44.41a | 19.41b | 14.99b | 27.80a | 25.00a |
| *n*-decanoic acid | 7.52a | 5.62ab | 3.44b | 15.05a | 6.41b | 13.51a | 8.49a | 3.72b |
| **Others** | | | | | | | | |
| benzaldehyde | 19.49b | 38.31a | 34.11a | 8.95c | 93.35a | 55.72b | 56.52b | 163.21a |
| acetoin | 1.38a | 1.29a | 1.70a | 2.22a | 0.97b | 1.21b | 1.97a | 2.43a |
| 3-methylpentane | – | 0.16 | – | 0.55a | 0.37a | – | – | – |
| **SUM** | **92.70** | **127.56** | **113.33** | **201.71** | **254.53** | **153.63** | **299.53** | **268.04** |

[a] Means (n=3) with different letters for the same vinegar differ significantly at $P<0.05$ during storage.

**Table 14.9** Changes in sensory quality of pineapple vinegars fermented from different base pineapple wine during storage[a]

| Vinegars | Storage (months) | Aroma | | | | Flavour | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fruity | Wine | Pungency | Ester | Sour | Wine | Pungency | Overall mouth-feel |
| PVA | 0 | 2.83a | 2.03a | 2.46a | 2.25a | 3.00a | 1.38b | 2.88a | 2.13b |
| | 6 | 2.88a | 2.53a | 1.88ab | 2.12a | 2.54b | 2.23a | 2.13b | 2.54ab |
| | 12 | 2.39a | 2.42a | 1.48[b] | 2.65a | 2.59b | 2.59a | 2.50ab | 3.07a |
| PVB | 0 | 3.50a | 1.63b | 1.75a | 2.42a | 2.67a | 1.34b | 2.26a | 2.79b |
| | 6 | 3.33a | 2.56a | 2.17a | 2.38a | 2.39b | 2.27a | 2.11a | 3.50a |
| | 12 | 2.42b | 3.15a | 2.27a | 2.54a | 2.42b | 2.79a | 2.05a | 3.15ab |
| PVC | 0 | 4.31a | 2.08b | 1.96b | 2.00a | 3.27a | 2.11a | 2.73a | 3.46a |
| | 8 | 2.56b | 3.00a | 2.60a | 2.20a | 2.85a | 2.33a | 2.52a | 2.24b |

[a] Means (n=13) of 5-point scaling: weak (1), moderate (3), strong (5). Means with different letters for the same vinegar differ significantly at $P<0.05$ during storage.

stages of storage (Morales et al., 2002; Palacio et al., 2002). Meanwhile, compounds such as 3-methyl-1-butanol and 2-phenylethanol would be further synthesized because the secondary products from acetic acid bacteria would continue to react, and thus the concentration of these two compounds would not decrease even though both are consumed by the esterification process; the same result was also found in the above-mentioned studies (Morales et al., 2002; Palacio et al., 2002). However, the changes in concentration of these compounds were not significant for PVA after 6 months storage. The fruity aroma of PVA was slightly reduced after 1 year of storage, while the wine flavour and overall mouth-feel were significantly enhanced over time (Table 14.9).

In the first 6-month storage period, the volatile compounds in the headspace of PVB, such as 2-propenyl acetate, 3-methyl-1-butyl acetate, 2-phenylethyl acetate, 3-methyl-1-butanoic acid and benzaldehyde, continued to increase, while the volatile compounds of high concentration before storage, such as 2-phenylethanol and octanoic acid, decreased instead of increasing further, because the concentration in the headspace of the pineapple vinegar was already saturated (Table 14.8). After 1 year of storage, the decrease in the volatile compounds of PVB was clearly pronounced. After 6 months storage, the fruity aroma of PVB did not show any obvious difference from that before storage, but the fruity aroma clearly became weaker after 1 year of storage. Similar to PVA, the aroma and flavour of wine, as well as the overall mouth-feel of the PVB was significantly enhanced over time (Table 14.9).

The fruity aroma, which was originally strong in PVC, became noticeably weaker after 8 months of storage. One reason might be that the concentration of 3-methyl-1-butyl acetate had dropped significantly (Table 14.8), or there were undetected changes in the minuscule amount of volatile compounds in the pineapple peel that helped increase the fruity odour in vinegar. Consequently, the scent of the PVC showed no discernible difference from that of the PVA or PVB after 8 month storages, and the overall mouth-feel of the PVC also weakened substantially due to the considerable attenuation of its fruity aroma (Table 14.9).

Okumura (1995) and Tesfaye et al. (2002) have reported that overall impression or aroma intensity of sherry wine vinegar, rice or grain vinegar were clearly increased with ageing (or storage). However, fruity volatile compounds with high volatility would be gradually lost from the cap of the storage bottle over time, and this would cause the reduction in fruity odour and overall mouth-feel of PVC, which originally had the most fruit scent and good overall mouth-feel. To prevent the loss of fruity aroma during storage, the pineapple vinegar should be stored at low temperature or consumed within a short period of time.

On the other hand, the pineapple base wine brewed from peeled pineapple with sugar added to 26°Brix (PWA) had a higher alcohol content (15-16%, v/v), and could be used for vinegar fermentation after 2-3 times dilution. Although this would result in vinegar with less fruity aroma and overall mouth-feel, the addition of 0.1% L-aspartic acid in PWA before the vinegar fermentation could enhance the fruity aroma in the final product.

## 14.5 Monitoring the Changes in Physico-Chemical Properties During Brewing of Pineapple Wine and Vinegar Using Near Infrared Spectroscopy

Traditionally, food analysis has been laborious and time-consuming, but recently, near infrared spectroscopy (NIRS) has become the preferred tool for analysing foods, especially major quality-related components, in the food industry. NIRS is not only fast, easy, and does not require the use of chemicals, but is also highly precise. Because the edible vinegar available in Taiwan is not regulated adequately, this study aims to establish NIRS calibration models for the soluble solids, total sugar content, alcohol content, acidity and acetic acid content in vinegar. In addition, changes in the brewing methods for pineapple wine or vinegar could be monitored and the results could be used for quality control (Chang, 2007).

A total of 130 samples were randomly selected from pineapple juice, the fermentation intermediate and products of pineapple wine and vinegar. All samples were divided into two parts – one for physico-chemical analyses and the other for scanning with a near infrared spectrometer at wavelengths between 400 and 2500 nm. For NIRS, ten samples were first selected randomly as the unknown set. After eliminating samples with variations that were too large or with an analytical value of zero from the remaining 120 samples, the rest of the samples were divided into the calibration set and the validation set in a ratio of 3:1. The calibration set was used to establish the calibration models, and the validation set was used to check the closeness between the NIRS predicted and experimental values. Finally, the unknown set was used to compare the difference between the experimental values and NIRS predicted values using a paired Student's *t*-test.

Table 14.10 shows the calibration values of soluble solids, total sugar content, alcohol content, acidity and acetic acid content for the calibration set as established by the NIRS. The explanation ability ($R^2$) of the five models was 0.975, 0.951, 0.972, 0.983 and 0.948, respectively. Subsequently, the spectra of the validation set were placed into the five calibration models to evaluate the predictability of the calibration curves. The outcome indicated that the correlation coefficient ($r$) between the NIRS predicted values and the experimental values for soluble solids, total sugar content, alcohol content, acidity and acetic acid were 0.987, 0.983, 0.985, 0.986 and 0.965, respectively. RPD (residual predictive deviation) – the ratio of standard deviation (SD) of the experimental data to the SEP (standard error of prediction) – was the statistical standard used to check the validation of the calibration models. It has been suggested that RPD > 3.0 indicates that the calibration model should perform well for quantitative analyses (Williams and Sobering, 1996). The RPD values for these five calibration models were 5.93, 5.47, 5.25, 4.50 and 3.20, respectively. This clearly indicates these calibration models should be applicable in actual situations. Finally, the ten randomly selected samples of unknown set were placed into the above calibration models, and the results showed there was no significant difference between the NIRS predicted values and the experimental values of soluble solids, total sugar content, alcohol content, acidity and acetic acid.

**Table 14.10** Statistical descriptors for NIRS calibration and validation sets for physico-chemical properties of vinegar samples[a]

| | Calibration set | | | | Validation set | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Descriptors** | ***N*** | **Range** | **SEC** | $R^2$ | ***N*** | **Range** | **SEP** | ***r*** | **RPD** |
| Soluble solids (°Brix) | 82 | 3.1-28.0 | 0.913 | 0.975 | 28 | 3.0-22.0 | 0.920 | 0.987 | 5.93 |
| Total sugars ($g \cdot 100\ mL^{-1}$) | 81 | 0.30-29.55 | 1.577 | 0.951 | 27 | 0.10-17.63 | 0.679 | 0.983 | 5.47 |
| Ethanol (%, v/v) | 72 | 0.04-17.17 | 0.947 | 0.972 | 26 | 0.02-16.25 | 0.970 | 0.985 | 5.25 |
| Acidity (%, w/v) | 81 | 0.25-7.16 | 0.260 | 0.983 | 27 | 0.39-6.32 | 0.424 | 0.986 | 4.50 |
| Acetic acid ($mg \cdot 100\ mL^{-1}$) | 59 | 0.04-76.17 | 5.931 | 0.948 | 18 | 0.14-47.30 | 6.139 | 0.965 | 3.20 |

[a] N, sample number. SEC, standard error of calibration. SEP, standard error of prediction. RPD, the ratio of the standard deviation (SD) of the sample to the SEP.

It can be observed from the outcome that the five calibration models for soluble solids, total sugar content, alcohol content, acidity and acetic acid could be used for monitoring the changes in these five constituents during fermentation of pineapple wine and vinegar and the products as well. It is concluded that the NIRS technique is a suitable tool for monitoring the quality of pineapple wine and vinegar during the fermentation processes.

## 14.6 Conclusions

Human beings have been consuming vinegar as a seasoning for thousands of years. Recently, with the appearance of reports showing that the consumption of vinegar is associated with health benefits, many fruit vinegar products have become available in the Taiwan market. Most Taiwanese people understand the term 'fruit vinegar' to mean rice vinegar that has been infused with fruit and sugar, and this is obviously different from the definition of fruit vinegar given by CNS and FAO/WHO. Therefore, there is a need to distinguish this kind of product, which usually has large amounts of sugar added, from the healthier brewed fruit vinegar. In addition, there is an urgent need to revise or update the regulations for edible vinegar products and to improve the technique for manufacturing healthy and palatable fruit vinegar products. We believe that these finding are not only applicable to Taiwan but to other places as well.

Our study also showed that the quality of fruit vinegar is greatly affected by the raw material of the fruit with regard to variety, processing with or without peel, the acetic acid bacteria chosen, the addition of nutrients, and storage time and temperature. The effects of the above-mentioned factors on the quality of the end-product should be evaluated not only by the fermentation efficiency, but also by sensory tests.

For acetous fermentation, we found that *A. aceti* BCRC 14156 was the best strain for brewing pineapple vinegar because it resulted not only in higher in fermentation efficiency, but also in higher volatile compounds with floral and fruity aromas. Because the alcohol tolerance of these acetic acid bacteria is around 5-7%, the pineapple base wine (PWA) with 15% alcohol was diluted before brewing vinegar. This resulted in lower ester, alcohol and acid contents and in a weaker fruity aroma and mouth-feel. However, with the addition of nutrients such as L-aspartic acid, the volatile compounds in PVA were substantially increased, so that there was then no obvious difference between PVA and the other two samples: PVB and PVC. On the other hand, the pineapple base wine with peel and no added sugar (PWC) was directly brewed without dilution. With many more constituents from the peel, its favourable sensory characteristics were clearly derived. Therefore, we conclude that using the pineapple base wine from the juice with peel but no added sugar is the best option. Another observed benefit is that the peeling step is thus eliminated, saving labour and time. Although storage test results indicated that PVC was less stable than the other vinegars, its quality should be improved when it is stored at low temperature instead of room temperature.

Besides the sensory evaluation for examining the sensory quality, monitoring the important physico-chemical properties such as the contents of ethanol, acetic acid, sugar, volatiles and acidity, pH, etc. in all the steps during alcoholic and acetous fermentation and storage is an important task for manufacturing a genuinely high-quality product. Our work also indicates that using NIRS is a feasible way of monitoring changes occurring at all stages of the brewing and storage of pineapple vinegar.

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# Chapter 15
# Cereal Vinegars Made by Solid-State Fermentation in China

**Fusheng Chen, Li Li, Jiong Qu and Chunxu Chen**

## 15.1 Introduction

In China there is a proverb saying that, in daily life, the seven indispensable substances are firewood, rice, edible oil, salt, sauce, vinegar and tea. From this proverb, we can see the vinegar has a very important position in Chinese daily life. In the historical literature, we find that vinegar originated more than 3000 years ago in China. It is reported that the first written mention of vinegar was in 1058 BC in a book named *Zhou Li* about the rites of the Zhou Dynasty, and a professional workshop for vinegar making appeared in the Chunqiu Dynasty (770-476 BC) (Zhao, 2004; Hu, 2005; Zhao and Li, 2005; Shen, 2007). At that time, vinegar was so costly that only the rich noblemen could afford it. Vinegar first became popular with ordinary people in the Donghan Dynasty (25-220 AD) (Shen, 2007). Up until the Northern and Southern Dynasties (420-581 AD), a book named *Qi Ming Yao Shu*, about the essential techniques of farming, written by Sixie Jia, recorded in detail 23 different methods for brewing vinegars (Zhao, 2004; Hu, 2005).

Besides a seasoning, vinegar is regarded as a herbal medicine in China. In the book, *Compendium of Materia Medica*, written by Shizhen Li in 1857, the medicinal functions of vinegar were described as dissipating blood stasis, treating the diseases of jaundice and yellow sweat, improving appetite and nourishing the liver (Qin and Wu, 2001; Zhao, 2004; Hu, 2005). In the *Dictionary of Chinese Medicine*, it was also stated that vinegar had a curative effect for acute and chronic hepatitis (Qin and Wu, 2001).

There are many legends in China about the origins of vinegar. For example, Zhenjiang aromatic vinegar, one of the famous China-style vinegars, was invented by Heita, the son of Dukang, who was considered to be the inventor of rice wine in China (Shen, 2007). One day, after Heita had prepared rice wine, he put it aside. Twenty-one days later, when he remembered it, the wine had become a sour liquid. As soon as he tasted it, he was aware that the sour rice wine could be used as a seasoning with a concordance of sourness and sweetness. Gradually, the sour rice wine was used in cooking and was named vinegar.

L. Solieri, P. Giudici (eds), *Vinegars of the World*

Nowadays, in Chinese markets, there are more than 20 types of homemade vinegars, most of which are brewed with starchy materials such as rice, sorghum, corn (maize), barley and wheat, even though there are now also some fruit vinegars and fruit vinegar drinks which have been available since the early 1990s (Liu et al., 2004; Ma, 2005). Traditional Chinese vinegars are therefore also called cereal vinegars. Among these, the most famous Chinese vinegars are Shanxi aged vinegar, Zhenjiang aromatic vinegar, Sichuan bran vinegar and Fujian *Monascus* vinegar, respectively using sorghum, sticky rice, wheat bran and red yeast rice as the main raw materials or the starter (Chen, 1999a; Huang and Cai, 1998; Liu et al., 2004). These vinegars are also highly prized as four famous China-style vinegars due to their unique flavour, long production history, massive yields and characteristic fermentation processes, and are described in detail in Section 15.5. Besides their raw materials, Chinese vinegars can be classified into different groups according to their colour, their special flavour and their production processes; such as black (brown) vinegar, red vinegar, white vinegar, smoky vinegar, herbal vinegar, and so on.

In this chapter, we describe the general process, the raw materials, and the main microorganisms for brewing cereal vinegars, as well as four famous China-style vinegars. We also cover the analysis of cereal vinegars.

## 15.2 Solid-State Fermentation Process for Cereal Vinegars

Chinese cereal vinegars may be brewed either by a solid-state fermentation (SSF) process or a liquid-state fermentation (LSF) process, although most of them, especially the traditional Chinese vinegars, are produced by a SSF process (Chen, 1999a; Huang and Cai, 1998; Liu et al., 2004). SSF refers to the growth of the microorganisms on moist solid substrates without or in the near absence of free-flowing water (Liu et al., 2004). SSF has been widely used since ancient times in the oriental food industry in China to produce vinegar, as well as distilled spirits, rice wine, soy sauce, fermented soy bean curd, and other local fermented foods. In recent years, SSF has shown much promise in the development of several bioprocessing end-products, such as industrial enzymes and metabolites (Wang and Yang, 2006). Compared with LSF, also called 'submerged fermentation', SSF is considered to be a more suitable and useful technique for the production of food and industrial goods, due to its low technology and energy requirements, its use of cheap unrefined agricultural products as substrates, its moderate capital investment and operating costs, high productivity in a low reactor volume, and less stringent aseptic processing methods (Liu et al., 2004).

### 15.2.1 SSF Flowchart for Cereal Vinegars

Although SSF is complex, uncertain and empirical for the different sorts of fermented foods, its general scheme in cereal vinegar preparation includes the following five stages.

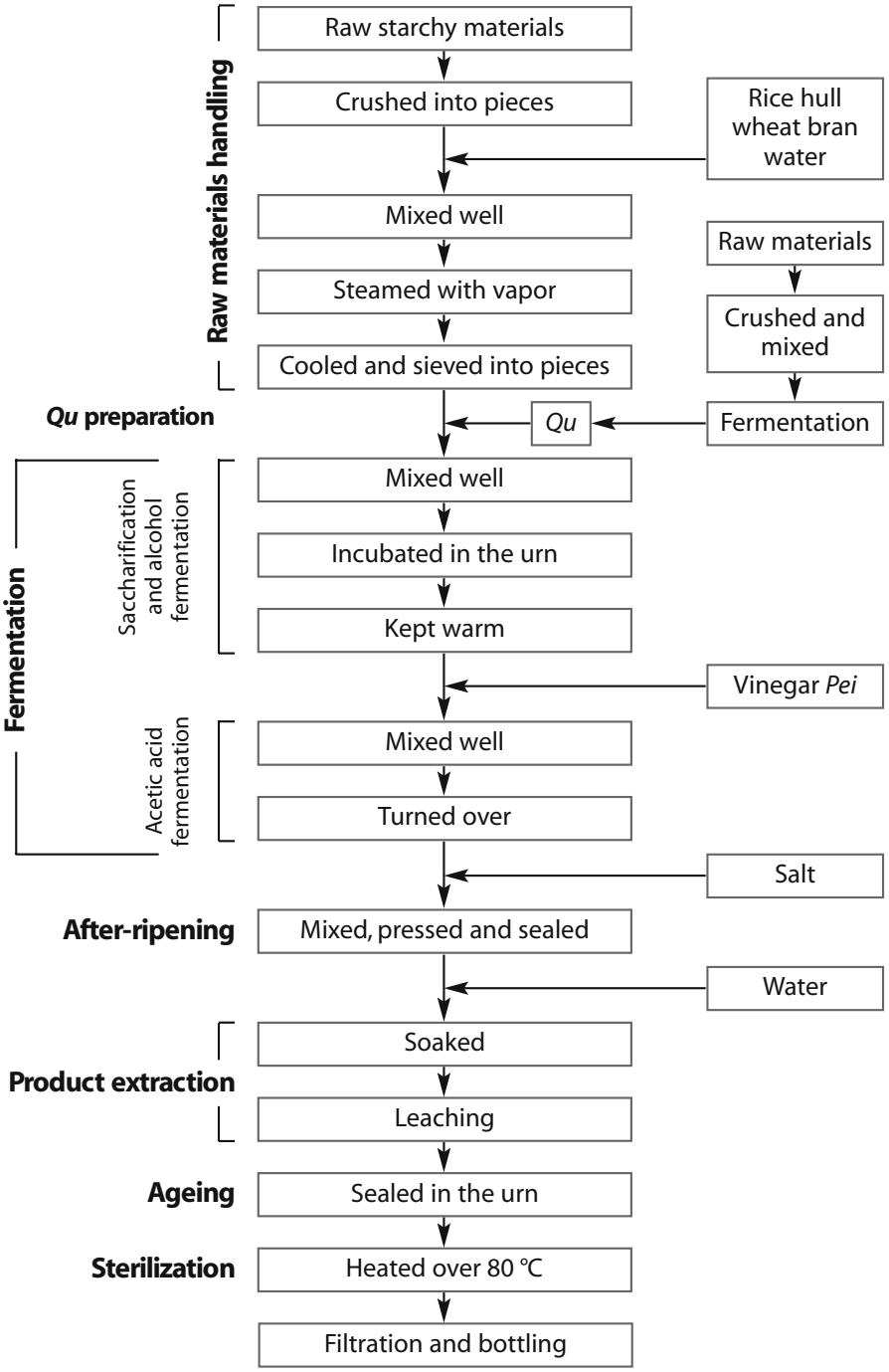


**Figure 15.1** Flowchart of a general SSF process for cereal vinegars

- Starter (called *Qu* in Chinese) preparation, during which the dominant microorganisms are enriched by choosing the raw materials and controlling the cultural conditions.
- Saccharification and alcohol fermentation, during which the dominant microorganisms, such as moulds and yeasts from the starter, grow in fermentation substrates to hydrolyse starch into sugar and convert sugar to ethanol.
- Acetic acid fermentation, during which ethanol is fermented into acetic acid by acetic acid bacteria.
- Leaching vinegar, during which the final products are dissolved from fermented materials (called *Pei* in Chinese).
- After-ripening or ageing, during which the flavour components are formed (Huang and Cai, 1998; Chen, 1999a; Liu et al., 2004).

Figure 15.1 shows a generalized flowchart for cereal vinegar production by the SSF process.

### 15.2.2 Qu Preparation

*Qu* (*koji* in Japanese) is the starter used in SSF, which is made from grains such as rice, sorghum, wheat, barley, and beans such as peas and soy beans, in a loose form or various pressed forms. Microorganisms from the raw materials, water and

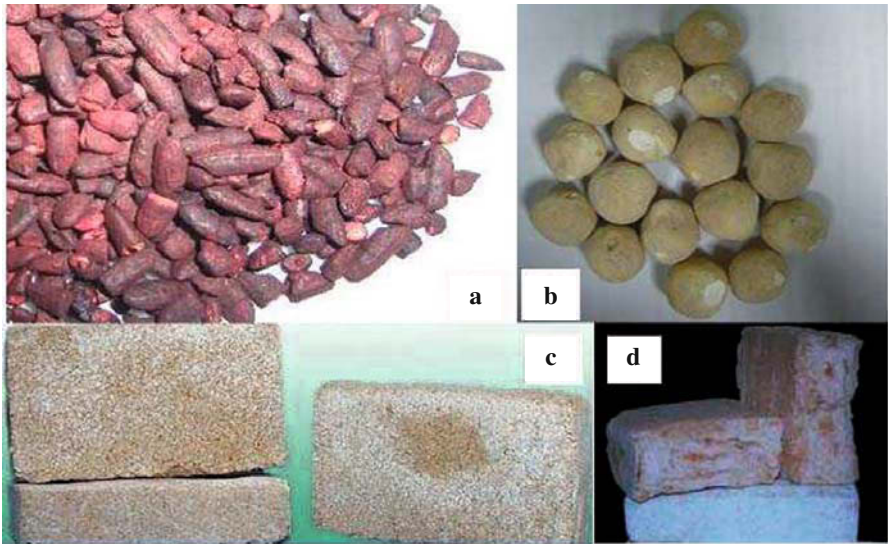


**Figure 15.2** Some kinds of Qu in China: **a** Hong Qu, also called red yeast rice; **b** Xiao Qu, made of rice and rice chaff in an egg-shaped form; **c** Da Qu, made of barley, wheat and peas, looks like a brick; **d** Da Qu, with a red core, used especially for Shanxi aged vinegar production

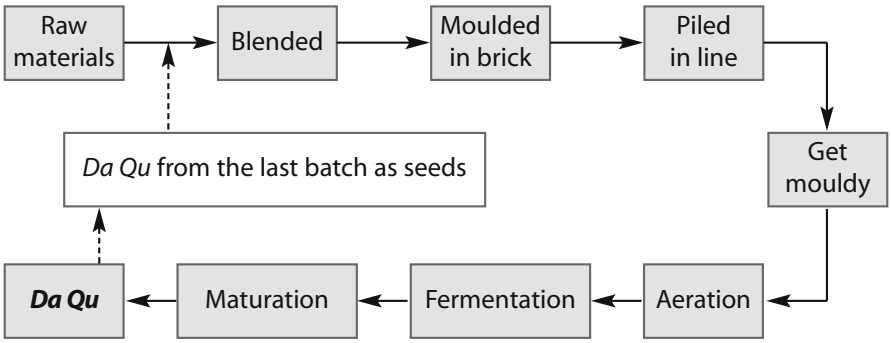


**Figure 15.3** Typical flowchart for Da Qu preparation

atmosphere, including moulds, yeasts and bacteria, grow on/in it spontaneously. The dominant microorganisms can be enriched by controlling the temperature, moisture and air content in a Qu preparation house (called *Qu Fang* in Chinese). The main microorganisms in Qu are moulds of the genera *Aspergillus*, *Rhizopus* and *Monascus*, yeasts of the genus *Saccharomyces*, as well as lactic acid bacteria. Besides microorganisms, Qu contains more than 50 types of enzymes produced by microbial cells, including α-amylase, glucoamylase, acid protease and lipase (Steinkraus, 2004).

There are several kinds of Qu in China, namely *Mai Qu*, *Xiao Qu*, *Da Qu*, *Hong Qu* and *Fu Qu* (Huang and Cai, 1998; Chen, 1999a); some of them are shown in Figure 15.2. Mai Qu, originating in the north of China, is made with wheat in brick-shaped form and its main microbe is *Aspergillus* spp. Xiao Qu is produced in the south of China and is made with rice and rice chaff in an egg-shaped form and its main microbe is *Rhizopus* spp. Da Qu is derived from Mai Qu and also looks like a brick. It is made of barley, wheat and peas, and the dominant microbes are *Mucor* spp. and *Rhizopus* spp. Hong Qu, also called red yeast rice or red fermented rice, is made of rice in a loose form and the main microorganism is *Monascus* spp. Fu Qu, which has just been developed recently, is made of wheat bran by inoculating a pure culture of *Aspergillus* spp.

Figure 15.3 outlines a typical flowchart for Da Qu preparation.

### 15.2.3 Raw Materials Handling

Normally, the starchy raw materials, such as corn (maize), wheat and barley, are crushed into pieces to expand the area in contact with microorganisms and to utilize the components of the raw materials effectively. After being soaked in water for about 12 h in summer, or 24 h in winter, the materials are steamed with vapour to turn the starch granules into a sol-shaped form to make the starch hydrolyse more easily. In addition, the raw materials are partly sterilized during steaming (Chen, 1999a).

### 15.2.4 Saccharification and Alcohol Fermentation

After the steamed materials have been cooled and sieved into pieces (Figure 15.1), they are mixed with Qu powder, and put into a ceramic urn with a rice hull or rice straw cover to keep warm. Starch is immediately hydrolysed by enzymes and microbes from Qu to produce saccharified mash. Simultaneously, as alcohol fermentation starts, the temperature in the urn is increased. In order to keep the temperature at about 28 °C, the mixture of raw materials and Qu – also called *Pei* in Chinese – is transferred into another empty urn once a day to decrease the temperature and, meanwhile, to improve the air content and reduce the evaporation of ethanol in Pei. Normally, after 2-3 days, the temperature gradually decreases. The simultaneous reactions of saccharification and alcohol production take about 5 days (Huang and Cai, 1998; Chen, 1999a).

### 15.2.5 Acetic Acid Fermentation

After alcohol fermentation, vinegar 'seeds', which come from the last batch of vinegar Pei, are added into the urn and mixed well with some coarse rice hull, which is used to increase the porosity for oxygen uptake and heat discharge. With sufficient oxygen, acetic acid bacteria grow and metabolize rapidly, producing a large amount of acetic acid, which inhibits mould and yeast growth at 1-2% content. During this phase, the temperature in the urn should be stabilized at 38-40 °C by turning over and pressing the vinegar Pei to reduce oxygen supply and decrease the rate of ethanol consumption and heat production. Normally, vinegar Pei is turned over once a day, and this stage lasts at least 12 days (Huang and Cai, 1998; Chen, 1999a).

### 15.2.6 Adding Salt

Salt has the ability to strongly inhibit acetic acid bacteria at more than 1% concentration (Miao and Ma, 2006). So, at the end of acetic acid fermentation, salt at 2-5% of vinegar Pei weight is added into the urn and mixed with vinegar Pei to prevent the oxidization of acetic acid to carbon dioxide and the production of off-flavours (Chen, 1999a). Moreover, salt can modify the flavour and taste of the finished cereal vinegars.

### 15.2.7 After-ripening

After adding salt, the vinegar Pei should be kept in the urn for several days for esterification and further oxidization of ethanol to acetic acid (Shen, 2007). This step is named after-ripening, which promotes the aroma and colour of the vinegar.

### 15.2.8 Leaching Vinegar

Leaching vinegar is the progress of adding water to vinegar Pei to dissolve out any soluble components such as acids, amino acids, sugar and other metabolites. The leached solution is called 'fresh vinegar'. Normally, vinegar Pei should be leached three times (Chen, 1999a; Shen, 2007). The fresh vinegar leached for the first time is called first-level vinegar; at the second time, it is named second-level vinegar; and at the last time, it is called third-level vinegar. In practice, the second-level vinegar is used to soak vinegar Pei to obtain the first-level vinegar, the remaining solid is soaked with the third-level vinegar to get the second-level vinegar and, after that, water is used to immerse the residue to get the third-level vinegar. Figure 15.4 shows a typical flowchart for leaching vinegar.

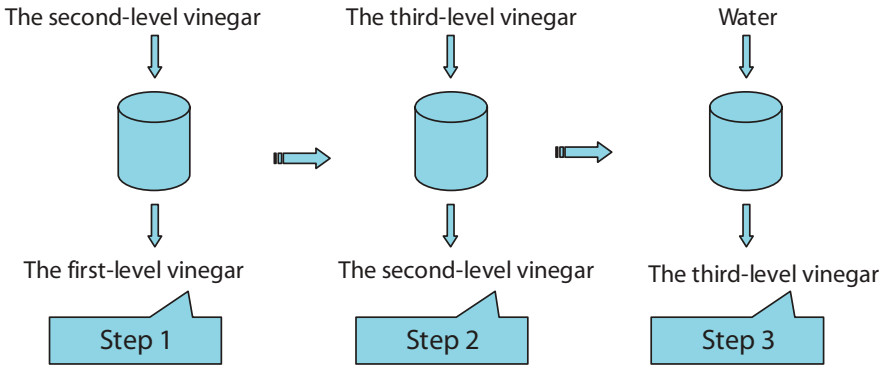


**Figure 15.4** Typical flowchart for leaching vinegar

### 15.2.9 Ageing

Ageing is a kind of storage and ripening progress, during which many flavour substances of vinegar can be formed by chemical reactions. There are two general ways in which the cereal vinegars can be aged: one is to age the vinegar in sealed or open ceramic urns for several months or years; the other is to age the vinegar Pei, which is deposited for several months after adding salt and before leaching vinegar, and subsequently to age the fresh vinegar (Shen, 2007). After ageing, vinegar will take on a bright colour and lustre and a scented mellow flavour.

### 15.2.10 Sterilization and Packaging of Finished Products

After the concentrations of acetic acid and other components in the aged vinegar have been adjusted, the vinegar should be heated to over 80 °C for sterilization before filtering and packaging (Chen, 1999a).

## 15.3 Raw Materials of Cereal Vinegars

### 15.3.1 Main Raw Materials

The main raw materials of cereal vinegars are grains, such as rice in the south of China, and sorghum in the north of China.

Recently, other starchy materials, such as barley, corn (maize), wheat, potato, sweet potato, cassava and so on, have been used as the main materials of vinegar (Huang and Cai, 1998; Chen, 1999a) (Table 15.1).

**Table 15.1** Composition of raw materials employed in cereal vinegar production (%)

| Materials | Carbohydrate | Protein | Lipid | Moisture |
|---|---|---|---|---|
| Sticky rice | 69-73 | 5-8 | 2.4-3.2 | 13-15 |
| Sorghum | 62-68 | 8-15 | 3-5 | 10-14 |
| Barley | 58-65 | 12-18 | 1.8-3.7 | 10-12 |
| Wheat | 66.4 | 13 | 1.5 | 14.4 |
| Corn (maize) | 62-70 | 8-16 | 3-5.9 | 11-19 |
| Potato | 68.5 | 3.8 | – | 12.8 |
| Sweet potato | 65-75 | 6 | 0.5 | 12 |
| Cassava | 67-72 | 3-9.5 | 0.9-1.3 | 14 |

–, not detected.

Adapted from: Huang and Cai, 1998; Chen, 1999b

### 15.3.2 Supplementary and Filling Materials

Besides the main materials, many supplementary materials, including rice bran, wheat bran, soybean meal and peas are included in cereal vinegar production (Huang and Cai, 1998; Chen, 1999a). They do not only contain a certain amount of carbohydrates, but also proteins and minerals, which can provide nutrients for the microorganisms during brewing and increase the amino acid and mineral contents of the vinegar. Additionally, supplementary materials also play important roles in absorbing water, loosening vinegar Pei and storing air. In the SSF process of vinegar production, some filling materials, such as rice hulls, sorghum hulls, cornstalks, corn cores and so on, are used to loosen vinegar Pei and improve air content, which is good for the acetic acid bacteria in aerobic fermentation.

### 15.3.3 Other Materials

Other materials, including salt, sugar, parched rice and some spices, are added to the cereal vinegar to adjust the colour, improve the flavour, and modify the taste (Huang and Cai, 1998; Chen, 1999a). Salt is a very important additive for cereal vinegar. When the vinegar Pei is fermented to a certain extent, salt is put into the

urn to restrain the acetic acid bacteria and prevent decomposition of acetic acid. Salt also plays a role in reconciling vinegar flavour and inhibiting pathogenic bacteria growth (Miao and Ma, 2006).

Normally, sugar such as sucrose is added to the vinegar to increase sweetness and thickness. Some spices, such as ginger, garlic, cloves and fennel, are included in order to produce characteristically flavoured vinegars (Li et al., 2004). For Zhenjiang aromatic vinegar, the parched rice is used to improve colour and modify flavour (Shen, 2007).

## 15.4 Main Microorganisms

The microorganisms involved in cereal vinegar production by SSF include moulds, yeasts and bacteria. Nowadays, some manufacturers use pure cultures of microbes to brew vinegar in China, but the traditional method of vinegar brewing still uses spontaneous and mixed culture fermentation in the majority of vinegar factories (Huang and Cai, 1998; Chen, 1999a; Liu et al., 2004).

### 15.4.1 Moulds

Qu is the main source of moulds to brew cereal vinegars. The dominant moulds depend on the type of Qu (Huang and Cai, 1998; Chen, 1999a). For example, in Da Qu for brewing Shanxi aged vinegar, *Mucor* spp. and *Absidia* spp. are the main moulds, while *Rhizopus* spp. and *Aspergillus* spp. are present to a lesser extent, and *Monascus* spp. is in the minority (Wu, 2004) (Table 15.2).

Moulds can secrete an abundance of enzymes such as amylase, glucoamylase, protease, lipase, tanninase and so on, which play important roles in hydrolysing macromolecule substances, like starch and protein, to small molecular substances like dextrin, glucose, peptides and amino acids, which provide nutritional sources for yeasts during the ethanol fermentation (Figure 15.1) (Huang and Cai, 1998; Chen, 1999a).

**Table 15.2** Amount of the main mould in Da Qu[a] for Shanxi aged vinegar, expressed as cfu/g Da Qu

| Sampling parts of Da Qu | *Mucor* spp. | *Absidia* spp. | *Rhizopus* spp. | *Aspergillus* spp. | *Monascus* spp. |
|---|---|---|---|---|---|
| On the surfaces | $2.23\times10^8$ | $2.10\times10^8$ | $4.02\times10^7$ | $7.32\times10^7$ | ng |
| At edges and corners | $3.72\times10^8$ | $2.84\times10^8$ | $8.24\times10^7$ | $1.00\times10^7$ | ng |
| In the core | $1.41\times10^8$ | $3.27\times10^7$ | $1.28\times10^7$ | $8.00\times10^6$ | $3.10\times10^6$ |

[a] Da Qu refers to the starter prepared with barley, wheat and peas, which looks like a brick. When brewing cereal vinegar, it should be milled into powder and mixed with the raw materials.

ng, not grown.

Adapted from Wu, 2004

### 15.4.2 Yeasts

*Saccharomyces cerevisiae*, *Hansenula anomala* and *Candida berkhout* are the main yeasts in the alcohol fermentation during vinegar brewing (Mao, 1998; Chen, 1999a; Wu, 2004). Sugar turns into alcohol and carbon dioxide mainly by the yeast glycolysis pathway. After the alcoholic fermentation, yeast cells remaining in the vinegar Pei can undergo autolysis, which provides nutritional material to support the growth of acetic acid bacteria.

### 15.4.3 Bacteria

Acetic acid bacteria are the main bacteria at the stage of acetic acid fermentation, which oxidizes ethanol into acetic acid. Several species of acetic acid bacteria are present in SSF of cereal vinegars, such as *Acetobacter pasteurianus*, *A. aceti*, *A. liquefaciens*, *A. rancens*, *A. hansenii*, *A. xylinum* and *Gluconobacter* spp. (Mao, 1998; Hu and Hao, 2004; see chapter 3 for the up to date AAB classification).

Lactic acid bacteria, mainly belonging to the genus *Lactobacillus*, contribute a great deal to the content of lactic acid in the vinegar, which can moderate the irritating sour smell and promote a soft taste of the vinegar. Wu (2004) reported that 70-90% of non-volatile acids in some Chinese traditional vinegars was lactic acid.

*Bacillus* spp. is a group of aerobic microorganisms which can occur in cereal vinegar. They produce organic acids, such as citric acid, succinic acid and malic acid, through the tricarboxylic acid cycle pathway (Wu, 2004). These organic acids can modify the irritating sour taste caused by a high concentration of acetic acid and create a softer taste. Moreover, the high activity of protease produced by *Bacillus* spp. can digest proteins into amino acids, which play an important role in the taste and colour of the vinegar (Wu, 2004).

## 15.5 Four Famous China-Style Vinegars

Chinese vinegars have specific local features. Every region has its own vinegar factories depending on particular raw materials, local climate and a specific production process (Liu et al., 2004; Zhao and Li, 2005). Consequently, each type of vinegar has its own taste, flavour and market. In China, there are more than 20 types of homemade vinegar on the market (Liu et al., 2004). Among these vinegars, Shanxi aged vinegar, Zhenjiang aromatic vinegar, Sichuan bran vinegar and Fujian *Monascus* vinegar are well-known as four famous traditional Chinese vinegars, whose geographical distribution is shown in Figure 15.5.

### 15.5.1 Shanxi Aged Vinegar

Although vinegars in Shanxi province can be traced to 479 BC, the traditional brewing technique for Shanxi aged vinegar was created in 1368 in the workshop called

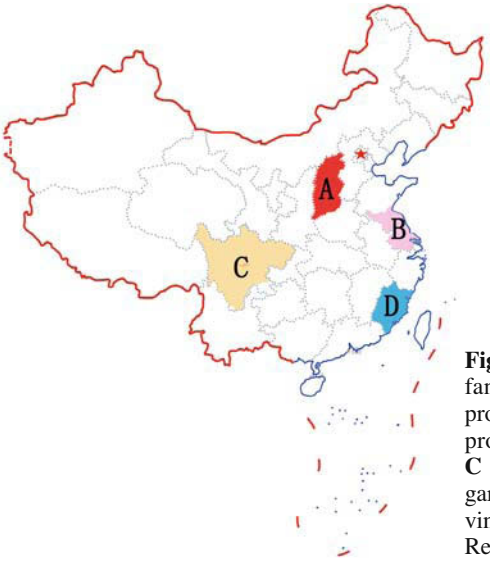


**Figure 15.5** Original locations of four famous China-style vinegars: **A** Shanxi province (Shanxi aged vinegar); **B** Jiangsu province (Zhenjiang aromatic vinegar); **C** Sichuan province (Baoning herbal vinegar); **D** Fujian province (Fujian *Monascus* vinegar); ★ Beijing, the capital of People's Republic of China

*Mei He Ju* in Qingxu county (Wang and Chen, 2004; Hu, 2005;Yan et al., 2006). Nowadays, the traditional brewing technique has been developed successfully by the Shanxi Aged Vinegar Group Co., which is the authority for Shanxi aged vinegar.

Unlike Zhenjiang aromatic vinegar (see Section 15.5.2), the raw materials for brewing Shanxi aged vinegar are sorghum, wheat bran, barley and pea. Among these grain materials, barley and pea are mainly used to make Da Qu with a red core (Figure 15.2), which is a source of enzymes and microbes specially tailored for Shanxi aged vinegar (Wang, 1999; Wang and Chen, 2004). During brewing, the ratio of Da Qu to sorghum, the main raw material, is up to 0.6:1, while the dosage of Xiao Qu or Mai Qu for other Chinese vinegars is usually less than one-tenth of the raw materials (Huang and Cai, 1998; Yan and Yan, 2003). The large amount of Da Qu used in the vinegar brewing process contributes a great deal to the complex flavours and nutrient components of Shanxi aged vinegar. Therefore the quality of Da Qu has an important effect on the quality of the vinegar. It is often said that Da Qu is the soul of Shanxi aged vinegar.

Besides the raw materials, the brewing process of Shanxi aged vinegar has some distinct characteristics (Huang and Cai, 1998; Yan and Yan, 2003; Wang and Chen, 2004). For example, after acetic acid fermentation (Figure 15.1), the vinegar Pei is taken out and put into another urn once a day , and its temperature is gradually heated to 80-90 °C on the third day by a moderate fire, and then decreased to about 50 °C on the sixth day. As a result, the vinegar Pei turns from yellow to brown in colour, and takes on a distinctive smoky flavour. This special process can greatly improve the quality of Shanxi aged vinegar with regard to colour, fragrance, lustre and taste.

Another important step in Shanxi aged vinegar production is the ageing. As mentioned above (see Section 15.2.9), there are two traditional vinegar ageing methods

a b c

**Figure 15.6** Two ageing methods for the traditional vinegar production in China: **a** fresh vinegar is put into open urns in an open room for at least 1 year; **b** ageing vinegar Pei by mixing mature vinegar Pei with salt and covering with plastic sheet to keep for 1-3 months before vinegar leaching; **c** ageing the vinegar – after leaching, the fresh vinegar is sealed in ceramic urns for several months or years

in China. The first is just to age the vinegar; the other is to age both vinegar Pei and vinegar. Shanxi aged vinegar belongs to the first category, while Zhenjiang aromatic vinegar belongs to the second (Figure 15.6). The fresh vinegar is put into an open urn in an open room for at least one year. In summer, exposure of the urn to the sun causes intense vaporization, whilst in winter, ice in the urn is removed. As a result, the volume of vinegar decreases to one-third of its original amount, the concentrations of soluble solid and non-volatile acids increase, esterification of acids and alcohols occurs greatly, while the content of volatile acids reduces (Yan and Yan, 2003; Wang and Chen, 2004). Finally, Shanxi aged vinegar with an excellent flavour, good mouth-feel and mild acidity, is obtained.

### 15.5.2 Zhenjiang Aromatic Vinegar

Zhenjiang aromatic vinegar can be traced back as far as 1400 years ago in the city of Zhenjiang, located in the eastern coastal province of Jiangsu. Nowadays, the most famous corporation producing Zhenjiang aromatic vinegar is Jiangsu Hengshun Vinegar Industry Co., which was established in 1840 (Shen, 2007).

In the brewing process, the traditional brewing technique for Zhenjiang aromatic vinegar embodies the quintessence of brewing Chinese spirit (Bao, 2000). Both Xiao Qu and Mai Qu are used for brewing this kind of vinegar, whereas only one kind of Qu is applied in the brewing of other vinegars. During the saccharification and alcohol fermentation, Xiao Qu powder, about 0.4% of the rice weight, is added and mixed with the steamed rice, and the mixture is kept for about 48 h at about 30 °C. Then Da Qu powder, about 6% of the rice weight, and water, 1.5 times the weight of the rice, are poured into the urn and mixed. After that, the mixture solution (called *Lao* in Chinese ) is kept for about 5 days at approximately 28 °C for the saccharification and alcohol fermentation.

In Xiao Qu, which is made with rice, the main microbes are *Rhizopus* spp. and yeasts, while in Mai Qu, which is made of wheat, barley and pea, the dominant microbes are *Aspergillus* spp. The combined use of Xiao Qu and Mai Qu con-

tributes a large amount to the flavour substances of the vinegar, including amino acids and organic acids, and makes the saccharification and alcohol fermentation more efficient, due to the mix of microbes and hydrolytic enzymes from both sorts of Qu.

In Zhenjiang aromatic vinegar production, a multilayer SSF is employed (Huang and Cai, 1998; Liu et al., 2004; Shen, 2007). A vinegar Pei mixture of wheat bran, rice hull, alcoholic solution (obtained from the saccharification and alcohol fermentation), and vinegar seed from last batch of vinegar Pei, is poured into an urn to half-fill it, and is kept warm with a rice straw cover for 2-3 days in summer or 5-6 days in winter. When the temperature in the top layer of the mixture reaches 45-46 °C, some rice hull is added and mixed. After that, once a day, another batch of rice hull is put into the urn and mixed with the vinegar Pei for about 10 days until the vinegar Pei fills the urn. Finally, the whole vinegar Pei is turned over into another urn and kept for about 7 days to obtain the mature vinegar Pei, which is then mixed with salt and covered with a plastic sheet to store for 1-3 months to age before leaching the vinegar (Figure 15.6).

Another distinctive feature of brewing Zhenjiang aromatic vinegar is the use of parched rice as a colourant to increase the vinegar's colour and lustre (Huang and Cai, 1998; Shen, 2007). Non-glutinous rice is parched in a heated wok until the colour of the rice is coal black. Then water is added to the parched rice and it is cooked for 15 min; the filtrate is added to the vinegar to modify its colour and flavour during the leaching phase.

### 15.5.3 Sichuan Bran Vinegar

In Sichuan province, vinegars are mainly brewed with wheat bran, and Baoning herbal vinegar is the most famous one (Huang and Cai, 1998). According to historical records, Baoning herbal vinegar originated in Langzhong City in 1618, where the history of vinegar brewing is longer than 2000 years (Chen, 1999b). Up until now, Sichuan Baoning Vinegar Co., has been the only company authorized to produce the traditional Baoning herbal vinegar (Chen, 1999b; Liu, 2003).

Sichuan bran vinegars, and especially Baoning herbal vinegar, are the only medicinal vinegars among the four famous China-style vinegars, and this is due to the use of 60 kinds of Chinese traditional herbs, such as eucommia bark (*Eucommia ulmoide*), villous amomum fruit (*Amomum villosum*) and licorice roots (*Glycyrrhiza glabra*), in Qu preparation. Some compounds from the medicinal herbs, such as polyphenol and flavone, can affect the microbial composition in Qu preparation through the inhibition of some bacteria such as lactic acid bacteria, while promoting some fungi, such as *Rhizopus* spp. and *Aspergillus* spp., and also make an important contribution to the special flavour of the vinegar (Shang, 2003).

As a kind of bran vinegar, the ratio of wheat bran to rice used for brewing is high – up to 25:1 – while it is only about 2:1 for Zhenjiang aromatic vinegar (Huang and Cai, 1998; Shang, 2003). As no rice hull is added, wheat bran also serves as a fill-

ing material. After inoculating with herbal Qu, the mixed materials are put loosely into a wooden trough 2.4 m long, 1.25 m wide and 0.7 m deep, rather than into an urn (Huang and Cai, 1998). In the trough, saccharification, alcohol fermentation and acetification take place in sequence. The air content and temperature in vinegar Pei are controlled by turning. Then the mature vinegar Pei in the wooden troughs is pressed into the urns and covered with a 3 cm-thick layer of salt on the surface to age for at least 1 year.

### 15.5.4 Fujian *Monascus* Vinegar

Fujian province is well known for red yeast rice produced by *Monascus* spp. The application of red yeast rice in vinegar was prevalent in Yongchun county during the early years of the North Song Dynasty (960-1125 AD), according to ancient records. Thus, Fujian *Monascus* vinegar is also referred as Yongchun *Monascus* vinegar, which gets its name from the red yeast rice and the producing area. In the past, Fujian *Monascus* vinegar was brewed in the traditional way, with the method being handed down from generation to generation. In 1955, the first vinegar plant, the Yongchun brewing plant, was established, which contributed a great deal to the industrial production of Fujian *Monascus* vinegar.

Red yeast rice, especially *Gutian Hong Qu*, is used as the saccharifying agent, owing to the large quantities of hydrolytic enzymes, such as α-amylase, β-amylase, glucoamylase, protease and lipase, produced by *Monascus* spp. during Fujian *Monascus* vinegar brewing (Huang and Cai, 1998; Yuan, 2000; Bao, 2001). Sesame and white sugar are used to improve the flavour of the vinegar.

Unlike other traditional Chinese vinegars brewed by SSF, Fujian *Monascus* vinegar is brewed from sticky rice by LSF (Huang and Cai, 1998; Yan, 2004). Neither wheat bran nor rice hull is used during brewing Fujian *Monascus* vinegar. At the end of the alcohol fermentation, red rice wine with a high ethanol and amino acid concentration is put into the first urn at half volume with 1-year-old vinegar, and mixed well. One year later, a half volume of vinegar from the first urn is poured into the second urn with a half volume of 2-year-old vinegar. One year later, a half volume of vinegar from the second urn is poured into the third urn with a half volume of 3-year-old vinegar, and kept for one more year. Therefore, the Fujian *Monascus* vinegar is at least 3 years old when it is taken out as a commercial product (Huang and Cai, 1998).

## 15.6 Analysis of Cereal Vinegars

The Chinese government has been concerned about the safety and quality of foods, including vinegars, of which more than 3.2 million litres are consumed every day in China. The hygienic standards for the vinegars in China have been revised four times since the first national hygienic standard of vinegar (GBn5-1977) was published in 1977. Tables 15.3 and 15.4 show the recent national hygiene standards for

vinegar (GB2719-2003), in which the contents of free mineral acids, arsenic, lead, aflatoxin $B_1$ and the number of total plate colony, coliform group and pathogens such as *Salmonella* spp., *Shigella* spp. and *Staphylococcus aureus* are strictly limited. Methods of analysis for the hygiene standard of vinegar were also published in GB/T 5009.41-2003.

**Table 15.3** Physical and chemical indexes required by Vinegar Hygiene Standard GB2719-2003

| Item | Index |
|---|---|
| Free mineral acids | Not to be found |
| Total arsenic (counted with As), mg/L | ≤ 0.5 |
| Lead, mg/L | ≤ 1 |
| Aflatoxin $B_1$, μg/L | ≤ 5 |

**Table 15.4** Microbial indexes required by Vinegar Hygiene Standard GB2719-2003

| Item | Index |
|---|---|
| Total plate colony (cfu/mL) | ≤ 10,000 |
| Coliform group (MPN/100 mL) | ≤ 3 |
| Pathogenic bacteria (*Salmonella* spp., *Shigella* spp. and *Staphylococcus aureus*) | Not to be found |

Besides the hygiene standards, the quality standards of fermented vinegar have also recently been modified in GB18187-2000. According to the standards, the quality of fermented vinegar should be analysed for colour, fragrance, taste and body, as well as for the contents of total acids, non-volatile acids and soluble solids. The parameters for the sensorial properties and physico-chemical indexes of fermented vinegar are listed in Tables 15.5 and 15.6.

With regard to studies on qualities and flavours of cereal vinegars, Zhang et al. (2006, 2008) have recently established a gas-sensing fingerprint database of Chinese vinegars through the use of an 'electronic nose' which they used to analyse 17 different commercial Chinese vinegars. Liu et al. (2005) analysed the flavour

**Table 15.5** Organoleptic properties of fermented vinegar required by GB18187-2000

| | Requirement | |
|---|---|---|
| **Item** | **SSF vinegar** | **LSF vinegar** |
| Colour and lustre | Amber or reddish brown | Depending on special requirements of the products |
| Fragrance | The unique aroma of solid-state fermentation vinegar | Depending on special requirements of the products |
| Taste | Softly sour, long aftertaste, no foreign odour | Softly sour, no foreign odour |
| Body state | Clarification | |

**Table 15.6** Physical and chemical indexes of fermented vinegar, according to GB18187-2000

| Item | Index | |
|---|---|---|
| | SSF vinegar | LSF vinegar |
| Total acids (counted with acetic acid, g/100 mL) | 3.50 | 3.50 |
| Non-volatile acids (counted with lactic acid, g/100 mL) | 0.50 | –[a] |
| Soluble solids without salt (g/100 mL) | 1.00 | 0.50 |

[a] Contents of non-volatile acids in LSF vinegar are without requirements.

compounds of cereal vinegars by HS-SPME-GC-MS and found more than 130 kinds of trace ingredients, including alcohols, aldehydes and phenols; the particular compound and their proportions being dependent on the kind of vinegar analysed.

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# Chapter 16
# Other Tropical Fruit Vinegars

**Richard O. Igbinadolor**

## 16.1 Introduction

The African continent has many different indigenous cereals, shrubs and fruit-bearing trees.

A number of these fruit trees, especially some of the exotic ones, have been domesticated and some are grown under agroforestry farming systems. The fruits produced by many of these indigenous trees are edible and can ripen within a very short period of time, which usually leads to an overabundance of the fruit at the time of harvest. Some of these are consumed fresh, but large quantities are wasted during peak harvest periods. This rapid post-harvest deterioration is due to the high temperature and humidity, poor handling, poor storage procedures, and microbial infections.

Fermentation is a relatively efficient and low-energy preservation process which increases the fruit's shelf life and decreases the need for refrigeration or other food preservation technologies. Indigenous fermented beverages and vinegars prepared from fruits are common in many parts of African continent. Nevertheless, the history of vinegar fermentation in Africa is obscure, because there are no documents and oral history is not precise. The absence of a written culture in most African countries makes the origin difficult to trace.

Vinegar can be made from any non-toxic raw material that provides a juice or solution containing fermentable sugars. Ideally, sugars should be present at levels sufficient to produce vinegar with an acetic acid content according to local standards.

Theoretically, 1 g of glucose will produce 0.67 g of acetic acid but, as this figure is never achieved in practice, at least 2% (w/v) sugar is required for every 1% (w/v) acetic acid in the final product. In most cases the raw material used in vinegar production contains sufficient nutrients to support the growth and metabolism of acetic acid bacteria (AAB). However, low-sugar juices can be supplemented with exogenous sugars or concentrated by evaporation or reverse osmosis.

L. Solieri, P. Giudici (eds), *Vinegars of the World*

## 16.2 Cocoa Vinegar

### 16.2.1 Cocoa Sweatings

Cocoa mucilage (sweatings) is a pale yellowish liquid, which is a waste by-product of the cocoa industry. It is derived from the breakdown product of the mucilage (pulp) surrounding the fresh cocoa beans of the tree *Theobroma cacao* and constitutes about 10% of the weight of the cocoa fruit (Adams et al., 1982).
Cocoa sweatings are a by-product obtained of the traditional cocoa fermentation, obtained by the activity of pectolytic enzymes which are secreted by some microorganisms involved in the fermentation process (Ansah and Dzogbefia, 1990). It is rich in soluble sugars and pectin, as well as having chemico-physical characteristics suitable for producing soft drinks, alcoholic drinks, vinegar, pectin, toffee, etc. (Opeke, 2005). The chemical composition of cocoa mucilage is shown in Table 16.1. Cocoa mucilage is free of alkaloids and other toxic substances. Children usually like collecting the mucilage for drinking due to its high sugar content before fermentation sets in.

**Table 16.1** Chemical composition of cocoa mucilage

| Composition | Percentage |
|---|---|
| Water | 79.2-84.2 |
| Dry substances | 15.8-20.8 |
| Non-volatile acids | 0.77-1.50 |
| Volatile acids | 0.02-0.04 |
| Glucose | 11.60-5.32 |
| Sucrose | 0.11-0.90 |
| Pectin | 5.00-6.90 |
| Starch | – |
| Protein | 0.42-0.50 |
| Ash | 0.40-0.50 |

### 16.2.2 Alcoholic Fermentation

The initial pH of the pulp is approximately 3.6 due to its high citric acid content; this favours the growth of yeasts, together with a low level of oxygen. Different yeast species contaminate cocoa pulp, mainly *Kloeckera apiculata*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Pichia fermentans*, *Lodderomyces elongisporus* and *Candida bombi*. During the fermentation process, *Saccharomyces cerevisiae* is the most dominant yeast species due to its ability to tolerate high ethanol concentrations. Therefore *Saccharomyces cerevisiae* is the yeast most frequently used in fermentation of most fruit juices (Prashant and Rajendra, 1989). The succession of different yeast species active during alcoholic fermentation of cocoa pulp is summarized in Table 16.2.

**Table 16.2** Frequencies (%) of yeast species isolated during cocoa fermentation

| Time (h) | Species | Frequency (%) |
|---|---|---|
| 0 | *Saccharomyces cerevisiae* | 33.3 |
| | *Kloeckera apiculata* | 13.3 |
| | *Kluyveromyces marxianus* | 13.5 |
| | *Pichia fermentans* | 13.3 |
| | *Lodderomyces elongisporus* | 13.3 |
| | *Candida bombi* | 13.3 |
| 12 | *Saccharomyces cerevisiae* | 35.3 |
| | *Kloeckera apiculata* | 30.0 |
| | *Kluyveromyces marxianus* | 17.0 |
| | *Pichia fermentans* | 5.9 |
| | *Candida bombi* | 5.9 |
| | *Candida rugapelliculosa* | 5.9 |
| 24 | *Saccharomyces cerevisiae* | 22.7 |
| | *Candida bombi* | 20.0 |
| | *Candida rugopelliculosa* | 20.0 |
| | *Kluyveromyces marxianus* | 9.0 |
| | *Kloeckera apiculata* | 5.0 |
| | *Lodderomyces elongisporus* | 5.0 |
| | *Torulaspora pretoriensis* | 5.0 |
| 48 | *Saccharomyces cerevisiae* | 38.0 |
| | *Candida bombi* | 16.0 |
| | *Candida rugopelliculosa* | 16.0 |
| | *Candida pelliculosa* | 10.0 |
| | *Candida rugosa* | 10.0 |
| | *Torulaspora pretoriensis* | 10.0 |
| 72 | *Candida rugopelliculosa* | 20.0 |
| | *Candida pelliculosa* | 20.0 |
| | *Candida rugosa* | 20.0 |
| | *Saccharomyces cerevisiae* | 10.0 |
| | *Torulaspora pretoriensis* | 10.0 |

From Schwan et al., 1995

Alcoholic fermentation of cocoa mucilage is carried out in relatively simple vessels, such as open vats of wood or concrete, or earthenware pots, and without any form of temperature control, particularly when small vessels are used and the ambient temperature is suitable for yeast growth (25-30 °C). No starter cultures are generally used.

The progress of fermentation can be monitored visually by observing the rate of carbon dioxide evolution, but more reliably by determining specific gravity of alcohol content in the fermenting mucilage. The alcoholic fermentation generally ends within 48-72 hours.

Values of specific gravity, titratable acidity, and soluble solids during cocoa alcoholic fermentation are reported in Table 16.3.

**Table 16.3** Chemico-physical parameters during alcoholic fermentation of cocoa mucilage (from Akinwale, 2000)

| Time (days) | Specific gravity[a] | Titratable acidity[b] | Total soluble solids[a] (%) | pH |
|---|---|---|---|---|
| 0 | 1.080 | 0.110 | 20.00 | 3.80 |
| 2 | 1.069 | 0.250 | 10.00 | 3.20 |
| 4 | 1.048 | 0.345 | 15.50 | 3.08 |
| 6 | 1.019 | 0.585 | 8.50 | 3.10 |
| 8 | 1.019 | 0.585 | 8.50 | 3.25 |
| 10 | 1.004 | 0.685 | 7.25 | 3.30 |
| 12 | 0.993 | 0.715 | 5.25 | 3.15 |
| 14 | 0.990 | 0.945 | 5.50 | 3.35 |

[a] Evaluated at 20 °C

[b] Calculated as % tartaric acid in 100 mL of cocoa wine.

Ethanol is the main product of alcoholic fermentation of the hexoses present in pulp mucilage. The transformation of a hexose by *S. cerevisiae* can be represented chemically by the Gay-Lussac equation:

$$\underset{\text{fermentable hexose (180 g)}}{C_6H_{12}O_6} \rightarrow \underset{\text{ethanol (92 g)}}{2C_2H_5OH} + \underset{\text{carbon dioxide (88 g)}}{2CO_2}$$

Other than to produce ethanol, little sugar can be used during alcoholic fermentation to increase yeast cell biomass, or to produce secondary by-products such as glycerol and succinic acid. This leads to a decrease in the yield of ethanol: a theoretical yield would be 95%, whereas a good practical yield is approximately 90%.

### 16.2.3 Acetification

When fermentation is complete, the cocoa alcoholic beverage may be centrifuged to remove yeast cells and is then mixed with a proportion of suitable 'seed' vinegar or 'mother of vinegar', which is generally a portion of previous successful acetification. AAB involved in vinegar production belonging mainly to the genera *Acetobacter* and *Gluconacetobacter* (Sievers and Swings, 2005). There have been no studies attempting to elucidate the AAB species involved in cocoa acetification.

## 16.3 Palm Wine and Vinegar

Palm wine is an alcoholic beverage traditionally produced from the sugary sap of various palms (tribe *Cocoineae*, family *Palmae*) throughout the tropics (Table 16.4). In Nigeria it is obtained from the sap of the raphia palm, *Raphia hookeri* and *Raphia vinifera*, and the oil palm tree *Elaeis guineensis* (Okafor, 1975).

**Table 16.4** Composition of palm sap

| Composition | Percentage |
|---|---|
| Sucrose | 11.0 |
| Glucose | 0.95 |
| Fructose | 1.0 |
| Raffinose | 0.8 |
| Ammonia | – |
| Vitamin C (mg/100 mL) | – |
| Vitamin $B_{12}$ | – |

From Okafor, 1975

Palm wine can be used as an alcoholic beverage or as alcoholic intermediate to produce palm wine vinegar.

### 16.3.1 Palm Sap

Palm sap is a sweet whitish liquid that gradually turns milky as a result of the growth of microorganisms which contaminate the sap as it oozes out of the tree, causing a spontaneous fermentation.

The methods of procuring the unfermented sap from palm trees vary according to the tree and the locality. One method includes the felling of the oil palm tree (*Elaeis guineensis*) and the collecting of the sap from a cut on the stem or by cutting the terminal bud. Tapping from a mature felled palm tree results in a different composition of palm sap compared with that obtained from living trees, and the palm wine produced, called 'down wine', differs from other palm wines because of its high contents of ethanol, methanol and propanol (Ayernor and Matthews, 1971). This type of palm wine is not highly appreciated in Nigeria because of its high alcohol content and because it may result in the gradual elimination of the palm tree population (Okafor, 1987). In another method of tapping, an incision is made at the base of the immature male inflorescence after removing the bracts. It is left to dry for 2 days after which the hole is reopened and the sap is collected in a gourd. The hole is reopened twice daily for 2-3 weeks, during which time the sap is tapped. This is the most acceptable method as it spares the life of the trees and produces a wine that commands a high price. Alternatively, the sap may be tapped through a hole under the terminal bud after clearing the tree. The sap of the *Raphia* palm is obtained by cutting the terminal bud.

The sap yield can vary depending upon the season of tapping and the type of palm. Up to 3 litres per day for 14-21 days of tapping the oil palm and 2-11 litres per day from the *Raphia* palm have been reported (Uraih and Izuagbe, 1990).

### 16.3.2 Microbiological Transformation

As the sap drips from the tapping hole, it is contaminated by microorganisms from the bark of the male inflorescence. Due to this contamination, distribution of the products to distant places is difficult, as it ferments rapidly, losing its sweet taste and becoming sour and milky-white within 24 hours and becoming unacceptable to the consumer (Uraih and Izuagbe, 1990).

The microorganisms associated with palm wine fermentation have been studied by various authors (Uraih and Izuagbe, 1990). The yeasts belong mainly to the genera *Saccharomyces*, *Schizosaccharomyces*, *Candida*, *Kloechera*, *Pichia* and *Endomycopsis*, whereas species of the genera *Lactobacillus*, *Acetobacter*, *Micrococcus*, *Serratia*, *Leuconostoc*, *Sarcina*, *Streptococcus*, *Zymomonas*, *Bacillus*, *Brevibacterium*, *Pediococus* and *Klebsiella* are the bacteria most frequently isolated. Moulds, such as *Penicillium* spp. and *Aspergillus* spp., have occasionally been found in palm wine. One of the most striking features of the micro-

biology of palm wine is the variability in microbial composition due to different tapping sites. Saps obtained from different palm trees spaced 2-4.5 m apart have been reported to exhibit great differences in the types of microorganisms present (Okafor, 1978). However, there are no systematic studies on the occurrence of these microorganisms in palm wine obtained using saps from different tapping sites.

**Table 16.5** Changes in the taste, alcoholic content, sugar and acid content during spontaneous oxidation of palm wine (modified from Chinnarasa, 1968)

| | Time (h)[a] | | | |
|---|---|---|---|---|
| **Characteristics** | **0** | **3** | **6-12** | **24** |
| Taste | Fresh, sweet | Sweet | Slightly sour | Very sour |
| Alcohol (%) | 3.78 | 4.84 | 6.32 | 6.70 |
| Sucrose (%) | 6.80 | 4.18 | 1.48 | 0.35 |
| Acetic acid (%) | 0.49 | 0.54 | 0.57 | 0.69 |

[a] Hours after collection at 28-30 °C.

Modified from Chinnarasa, 1968

Fermentation of the sugars and other nutrients present in the juice by the indigenous microflora of the palm trees leads to alcohol and organic acid production (Table 16.5). According to his preliminary studies on spontaneous fermentation of palm sap, Bassir (1962) reported that the fermentation of the fresh palm sap into palm wine occurs in two stages. The first stage involves the production of organic acids by bacteria, whereas the second stage is triggered at pH 6.8 and ends at pH 4.4, and involves alcohol and organic acid production. There has been some criticism of this theory because it assumes that yeasts were solely responsible for the inversion of sucrose present in the sap, whereas it is known that several bacteria can also cause it. Yeasts and micrococci also seem to occur consistently in palm wine (Faparusi and Bassir, 1971; Okafor, 1975). Moreover, several studies on the succession of microorganisms in palm wine have consistently reported the development of *Acetobacter* after about 3 days; by this time alcohol is present in reasonable quantities (Faparusi and Bassir, 1972; Faparusi, 1973; Okafor, 1975). Since both bacteria and yeasts have been found at various stages of the fermentation, the fermentation of palm wine may well be due to the combined activities of those two groups of microorganisms. Recently Amoa-Awua et al. (2007) have reported that a concurrent alcoholic, lactic acid and acetic acid fermentation occurred during the tapping of palm wine from felled oil palm trees. Yeasts, mainly *S. cerevisiae*, started to grow immediately after tapping and alcohol concentrations became high in the product after the third day. Lactic acid bacteria, mainly *Lb. plantarum* and *Lc. mesenteriodes*, were responsible for a rapid acidification of the product during the first 24 hours of tapping, whilst the AAB belonging mainly to *Acetobacter* and *Gluconacetobacter* species became pronounced after the build-up in alcohol concentrations on the third day.

Palm wine usually possesses three desirable characteristics – fresh sugary taste, whitish coloration and vigorous effervescence. It has a variable alcohol content of

0.5-7.1% (v/v). The pH value at the time of consumption is usually between 3 and 5. A litre of palm wine provides approximately 300 calories, 0.5-2.0 g of protein and considerable amounts of vitamins (Okafor, 1987). The major components of the palm wine are sugars, alcohols, organic acids and protein. Although it is produced mainly in rural areas by tappers in Nigeria, the bulk of the beverage is consumed in urban areas.

Although no systematic studies have been carried out on AAB occurring in palm vinegar, it is very probable that species of the genus *Acetobacter* are responsible for ethanol oxidation of palm wine to acetic acid.

### 16.3.3 Ogogoro

This distilled beverage is produced from palm wine in Nigeria and some other West African countries, such as Ghana, where it is referred as *akpeteshi* (Okafor, 1987). It is a colourless liquid with an alcohol content of 26.8-39.9% (w/v). It is consumed mainly by low-paid workers due to its relatively cheap price, but it is also used for traditional ceremonies and as a solvent in various medicinal concoctions.

The traditional method of *ogogoro* production involves the pooling of palm saps in a metal drum where they are thoroughly mixed and allowed to ferment for 24 hours with occasional stirring. The fermentation usually takes place between 25 and 30 °C at a pH between 4.0 and 4.5. The fermented sap is then distilled over a fire and the vapour is condensed. The first distillate is usually discarded and successive distillates are collected and often re-distilled to obtain a product with a higher alcohol content.

## 16.4 Cashew Vinegar

### 16.4.1 Cashew

The cashew tree (*Anacardium occidentale*) is a medium-sized fruit-bearing tree, widespread throughout the tropics, having a high productivity and growing on poor soils due to its high drought resistance. The fruit consists of a kidney-shaped nut and a pseudoapple with a brilliant yellow or red skin colour. The cashew apple is five to ten times as heavy as the nut when ripe and is found to contain 85% juice with 10% sugar (Table 16.6), most of which is invert sugar (Ohler, 1979).

The cashew fruit is a climacteric fruit and shows a prominent increase in respiration coincident with ripening. It reaches its respiratory peak very fast due to a large amount of ethylene being produced concomitant with the evolution of aromatic volatiles, and thus also has a short storage life. Togun (1977) estimated that more than 3000 tonnes of cashew apples are wasted annually in cashew plantations in Oyo State alone. The Federal Government of Nigeria has recognized the great potential of cashew as an important commodity crop and source of industrial mate-

rials, and added cashew utilization and production to the mandate of the Cocoa Research Institute of Nigeria (CRIN) in 1971 (Cashew Coup and CRIN, 1999).

**Table 16.6** Chemico-physical parameters of cashew juice

| Parameter | Value |
|---|---|
| Specific gravity | 1.030 |
| Total reducing sugar (g/l) | 788 |
| Sugar (%) | 8.19 |
| Total titratable acidity (%) | 0.36 |
| Soluble solid (°Brix) | 4.4 |
| Extract (w/v) (%) | 8.04 |
| Refractive index | 1.3395 |
| pH | 3.8 |
| Amino acid | 4.238 |
| Total nitrogen (%) | 0.039 |
| Crude protein (%) | 0.2438 |
| Potassium (g/l) | 1.53 |
| Calcium (mg/l) | 68 |
| Sodium (mg/l) | 105 |
| Magnesium (mg/l) | 16.0 |
| Zinc (mg/l) | 22 |
| Ash (w/v) (%) | 0.455 |
| Potential alcohol | 5.0 |

From Osho, 1995

## 16.4.2 Alcoholic Fermentation, Acetification and Clarification

Cashew juice is obtained by removing the nut from the apples, cutting the fruits into small pieces and squeezing them by hand or with a machine. The juice is often clarified by filtering through a sieve. A large number of different yeasts can colonize the cashew juice due to the somewhat low pH (3.8-4.0). Therefore it is often pasteurized and fermentation is started by inoculating a desired yeast starter, such as *Saccharomyces cerevisiae*. After fermentation, back-slopping with seed vinegar harvested from the previous batch is carried out to produce cashew vinegar.

The clarification can be effected by filtration or by fining. Generally filtration is preferred, as it reduces the bacterial population and removes vinegar eels if present (Cruess, 1958; Frazier and Westhoff, 1978; Adams et al., 1982). In the fining method, clarifying agents such as casein, gelatin, bentonite, sodium alginate and isinglass are mixed with the cashew vinegar and the mixture is allowed to stand until clear vinegar appears (Prescott and Dunn, 1959). Pasteurization of the clarified cashew vinegar should also be necessary. The bottles should be completely filled and tightly capped or corked with treated corks to prevent the entry of air. The temperature and time of pasteurization vary according to microbial contamination and conditions of filling. Generally speaking, a temperature of 60-66 °C for about 30 minutes is sufficient for adequate cashew vinegar pasteurization.

## 16.5 Other Tropical Alcoholic Fermented Beverages with Potential for Vinegar Production

### 16.5.1 Burukutu

Sorghum beer is a popular alcoholic beverage in sub-Saharan Africa. It is known as *burukutu* in the northern Guinea savanna region of Nigeria, the Benin Republic and Ghana, and by various other names in other parts of West Africa. It is brewed from Guinea corn (*Sorghum bicolor*), the prevalent grain in this area (Odunfa and Oyewole, 1985; Kayodé et al., 2007).

As in the conventional lager beer process, the method for preparing burukutu consists of three phases: malting, mashing and fermentation. However, depending on geographical location, variations may occur in the process. The procedure described here is based on a report by Faparusi et al. (1973). Sorghum grains are steeped in water overnight. The grains are then put into a basket and the water drained off. The grains are spread on mats in a bed about 6 cm thick and covered with banana leaves to allow the grains to germinate. In this phase (malting period), the grains are watered on alternate days and periodically turned over. The purpose of malting is to effect the hydrolysis of starch in the sorghum to fermentable sugars. Germination starts within 24 hours after steeping and continues until the plumule (portion of the young shoot above the cotyledons) attains a certain length; this is usually within 4-5 days. The malted grains are spread out in the sun to dry for 1-2 days. The dried malt is ground in a disc mill, and then mixed with *garri* (a starch powder produced from the tuber of the cassava plant, *Manihot utilissima*) and water. The resulting mixture (garri-malt-water), which is in a ratio of roughly 1:2:6 by volume, is stirred and allowed to ferment for 2 days. At the end of the fermentation the mixture is boiled for about 4 hours and then left to mature for another 2 days. The resulting drink is a cloudy liquid with a sour taste.

The sorghum malt contains mainly yeasts and moulds. In the fermenting mixture the yeasts isolated belonged to the species *Saccharomyces cerevisiae*, whereas the main bacterial species are *Leuconostoc mesenteroides* and *Lactobacillus* spp. The acidity of the fermenting mixture falls from about pH 6.4 to about pH 4.2 within 24 hours and drops to 3.7 after 48 hours. At the end of the 2 day maturing stage, the dominant microorganisms are *Acetobacter* spp. and *Candida* spp. (Faparusi et al., 1973). The yeasts and bacteria are killed by boiling. Unlike some other alcoholic beverages, the specific characteristic of burukutu is a vinegary taste and sharp smell. There is up to 0.4-0.6% of acetic acid in fully matured burukutu beer.

### 16.5.2 Plantain Drink

Apart from cereals, another source of sugars for producing alcoholic and vinegary beverages are bananas and plantains, which can be subjected to spontaneous fermentation to produce a plantain beer called *agadagidi*. Due to hot and humid cli-

matic conditions in southern Nigeria and Cameroon, plantains and bananas can be affected by quick over-ripening and bacterial spoilage. These overripe bananas are peeled, sliced and soaked in containers to ferment. The most frequently isolated microorganisms are *Saccharomyces*, *Leuconostoc* and *Streptococcus*. *Bacillus* and *Micrococcus* species also occur occasionally (Sanni and Oso, 1988; Sanni, 1989). The nutrient content of the beverage produced is very high (Ketiku and Scott-Emuakpor, 1975; Sanni and Oso, 1988).

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# Chapter 17
# Whey Vinegar

**Javier Parrondo, Luis A. Garcia and Mario Diaz**

## 17.1 Introduction

Vinegar can be produced by acetic fermentation of any alcoholic beverage (Mollenhauer, 1986), but is usually produced by fermentation of wine or cider. Vinegars can also be produced from other non-conventional sources containing sugars such as fermented whey or whey permeate, which are by-products of the dairy industry.

Whey or milk plasma is the liquid remaining after milk has been curdled and strained; it is a by-product of the manufacture of cheese and has several commercial uses, like the production of 'ricotta' and 'gjetost' cheeses and many other products for human consumption. It is used as an additive in many processed foods, including bread, crackers and commercial pastry products. In addition, whey is used for feeding animals. Whey proteins mainly consist of α-lactalbumin and β-lactoglobulin. Depending on the method of manufacture, whey may also contain glycomacropeptides (GMP).

The whey proteins separated from this mixture are often sold as a nutritional supplement. Such supplements are especially popular among bodybuilders. Liquid whey contains lactose, vitamins and minerals along with traces of fat. Nilsson et al. (2007) discovered that whey appears to stimulate insulin release. They also found that whey supplements can help regulate and reduce spikes in blood sugar levels among people with type 2 diabetes by increasing insulin secretion.

In 2003, the European Union (data from 15 countries) produced 126 million tonnes (Mt) of milk (European Commission, 2005). The dairies turned out approximately 3.3 Mt of processed milk (pasteurized, UHT, etc.), 40 Mt of fresh milk products (except cream), 7.3 Mt of cheese, 0.5 Mt of processed cheese, 1.9 Mt of cream, and 0.8 Mt of milk powder.

Led by consumer demand, dairies are turning increasingly to the production of cheese and fresh products, such as yoghurts and dairy desserts. During the last 10 years there has been a 30% increase in the production of cheese. Around 80% of

L. Solieri, P. Giudici (eds), *Vinegars of the World*

**Table 17.1** Typical composition of acid whey, sweet whey and whey permeate

| | Milk | | Sweet whey | | Acid whey | | Whey permeate | |
|---|---|---|---|---|---|---|---|---|
| **Composition (%)** | Milk | Dry matter (12.4%) | Sweet whey | Dry matter (7.0%) | Acid whey | Dry matter (6.5%) | Whey permeate | Dry matter (5.7%) |
| Water | 87.6 | – | 93.0 | – | 93.5 | – | 94.3 | – |
| Fats | 3.4 | 27.5 | 0.4 | 1.5 | 0.1 | 1.5 | 0.0 | Traces |
| Casein | 2.6 | 21.0 | Traces | Traces | Traces | Traces | Traces | Traces |
| Soluble nitrogen | 0.7 | 5.6 | 0.9 | 13.0 | 0.7 | 10.5 | 0.2 | 2.3 |
| Lactose | 4.7 | 38.0 | 5.0 | 71.0 | 4.5 | 69.0 | 4.9 | 86.2 |
| Salts (ashes) | 0.9 | 7.3 | 0.6 | 8.6 | 0.7 | 10.5 | 0.5 | 8.8 |
| Lactic acid | – | – | 0.1 | 1.5 | 0.6 | 0.9 | 0.2 | 2.6 |

From Linden and Lorient, 1999

cheese production comes from French, German, Italian, Dutch and Danish dairies. The 7.3 Mt of cheese produced yields around 66 Mt of whey as a by-product (to make 1 kg of cheese, 9 kg of whey is produced).

The USA's milk production was around 77 Mt in 2003 (National Agricultural Statistics Service, 2004) of which around one-third was used for cheese manufacture, producing 3.9 Mt cheese (around 25% of the world's cheese) and 23 Mt of liquid whey.

The production of cheese in the European Union and the USA was approximately 11.2 Mt in 2003 and represents around 70% of the world's cheese production. This amount of cheese leads to the production of around 100 million tonnes of whey (around 150 Mt worldwide) and hence 4.2 million tonnes of lactose. The development of new uses for lactose is therefore of great interest, as it always has been, because of the high chemical oxygen demand (COD) of the liquid whey, ranging from 60,000 to 80,000 $mg \cdot L^{-1}$.

There are three types of whey: sweet whey, acid whey and whey permeate. Acid whey results from the manufacture of fresh curd or soft cheese or lactic or acid casein. Sweet whey is produced during the coagulation of non-acid milk, by means of rennin. And, finally, whey permeate is the result of sweet whey ultrafiltration during cheese manufacture for the recovery of proteins. The average composition of these three kinds of whey is given in Table 17.1. This can vary, because it depends on the original milk and also on the cheese manufacturing process.

Whey presents a fairly high salinity (between 7% and 10% of dry matter) and, as a consequence, it has limited use as an additive in the elaboration of foods to enhance their protein content. This limitation means that it is important to discover and develop new strategies for its use, methods for the recovery of its valuable constituents, and/or treatment of whey effluents.

The industry has developed many uses for whey lactose and for whey itself in order to solve the disposal problem. However, because of the increasing volumes of cheese production and whey protein concentrate, the problem is still unsolved, particularly because solutions for high volumes are required. Large amounts of whey and whey permeate need a market. Estimates made in 1992 place the surplus in the USA at 13-17 million tonnes of whey (American Dairy Products Institute, 1994).

Currently, the most common uses for whey and whey permeate, however, are often aiming at keeping the surplus out of the sewers, rather than at producing a highly desirable product. The ultimate goal should be to turn whey lactose into a profit-generating feedstock for high-value-added products. Examples include chemical or enzymatic lactose hydrolysis and the production of chemical derivatives (Yang and Silva, 1995) such as lactitol, lactulose, lactosyl urea, lactobionic acid and gluconic acid.

Whey, whey permeate – the by-product of enhanced cheese manufacture – and lactose can also be used directly as substrates for microorganism growth to obtain other valuable products, such as protein (Yang and Silva, 1995; Ghaly and Kamal, 2004), enzymes, yeast extract, ethanol, glycerol, organic acids and oils (Yang and Silva, 1995). Among these uses, ethanol production is of particular interest (Tyagi, 1984; Rosa et al., 1988; Parrondo et al., 2000a).

## 17.2 Production

The production of vinegar from whey consists of two fermentations: an alcoholic fermentation of whey substrate followed by an acetic fermentation. The alcoholic fermentation of whey or whey supplemented with lactose can be carried out with *Kluyveromyces marxianus* under micro-aerobic conditions. This fermentation is a critical step in the production of vinegar because whey has nutritional deficiencies that make yeast growth difficult.

The alcoholic product obtained by fermentation of whey supplemented with lactose with *K. marxianus* is employed as substrate in the acetic fermentation, which is an aerobic biotransformation of the ethanol contained in the product to acetic acid. The process should be carried out using *Gluconacetobacter liquefaciens* or *Acetobacter pasteurianus* strains owing to their better growth in ethanol. The details of these fermentations are discussed more in depth in the following sections.

### 17.2.1 Alcoholic Fermentation

The alcoholic fermentation of whey is performed using a lactose-fermenting yeast, typically *K. marxianus*. This fermentation is a critical step, as difficulties in yeast growth increase the risk of bacterial contamination, diminishing alcoholic yield and changing organoleptic properties.

Before starting the fermentation process, the medium has to be sterilized in order to avoid contamination. The medium can be sterilized by employing a cross-flow microfiltration technique or using an autoclave. When the latter method is used, the pH of the whey has to be adjusted to 7.3 to prevent the precipitation of most of the proteins. Because of this obstacle, treatment using microfiltration (0.45 μm filters) is usually preferred.

The yeast inoculum is prepared from a pure culture of *K. marxianus* stored in glycerol at –20ºC or lyophilized. Cultures can also be stored for shorter periods of time in TYED (triptone 2 g, yeast extract 1 g, D-glucose 2 g per litre) plus 2% w/v agar slopes. The amount of yeast needed to seed the batch fermentations is obtained in several steps, keeping a relationship of 10 volumes of starter culture per 100 volumes of fresh medium to increase biomass growth rates and to avoid contamination risk.

First, a colony is transferred from a slope to a small amount of synthetic medium previously sterilized (10 mL TYED medium in a 50 mL flask) and propagated aerobically for 24 hours at 30 °C; during this time the yeast reaches its steady state (Ghaly et al., 2003) and hence has to be transferred to a fresh medium (100 mL TYED in a 500 mL flask) and propagated for another 24 hours in the same conditions to increase biomass amount. Afterwards, this culture is used as an inoculum, using 10 volumes per 100 volumes of fresh whey, giving an initial yeast concentration around 0.5 $g \cdot L^{-1}$, and cultured in aerobic conditions for 24 hours. This step should be repeated until the required amount of yeast is obtained.

### 17.2.1.1 Kinetics of Yeast Growth, Lactose Consumption and Ethanol Production

Sweet whey, acid whey or whey permeate, whose compositions are shown in Table 17.1, can be used as substrates in the alcoholic fermentation. These wheys can be supplemented with lactose (lactose is also obtained from whey) to increase its initial concentration and subsequently the level of ethanol produced via alcoholic fermentation. The final alcoholic product, obtained using lactose concentrations around 120 $g \cdot L^{-1}$, shows ethanol concentrations ranging from 40 to 60 $g \cdot L^{-1}$.

The specific growth rates at 30 °C of the yeast *K. marxianus* (μ), biomass/lactose yields ($Y_{X/S}$), specific lactose consumption rates ($q_S$), ethanol/ lactose yields ($Y_{P/S}$), and specific ethanol production rates ($q_P$) for lactose concentrations ranging from 45 to 130 $g \cdot L^{-1}$ are shown in Table 17.2. The increase in initial lactose concentration decreases specific biomass growth and lactose consumption rates.

**Table 17.2** Effect of lactose concentrations on kinetic parameters of yeast growth, lactose consumption and ethanol production

| Lactose ($g \cdot L^{-1}$) | μ ($h^{-1}$) | $Y_{X/S}$ ($g \cdot g^{-1}$) | $q_S$ ($h^{-1}$) | $m$ ($h^{-1}$) | $Y_{P/S}$ ($g \cdot g^{-1}$) | $q_P$ ($h^{-1}$) |
|---|---|---|---|---|---|---|
| 45 | 0.22 | 0.093 | 2.4 | – | 0.51 | 0.11 |
| 90 | 0.18 | 0.087 | 2.1 | 0.061 | 0.51 | 0.09 |
| 135 | 0.16 | 0.093 | 1.8 | 0.12 | 0.45 | 0.07 |
| 180 | 0.16 | 0.086 | 1.9 | 0.13 | 0.45 | 0.07 |

μ: specific growth rates at 30 °C of *K. marxianus*. $Y_{X/S}$: biomass/lactose yields (g biomass/g lactose). $q_S$: specific lactose consumption rate. $m$: cellular maintenance coefficient. $Y_{P/S}$: ethanol/lactose yields (g ethanol/g lactose). $q_P$: specific ethanol production rate.

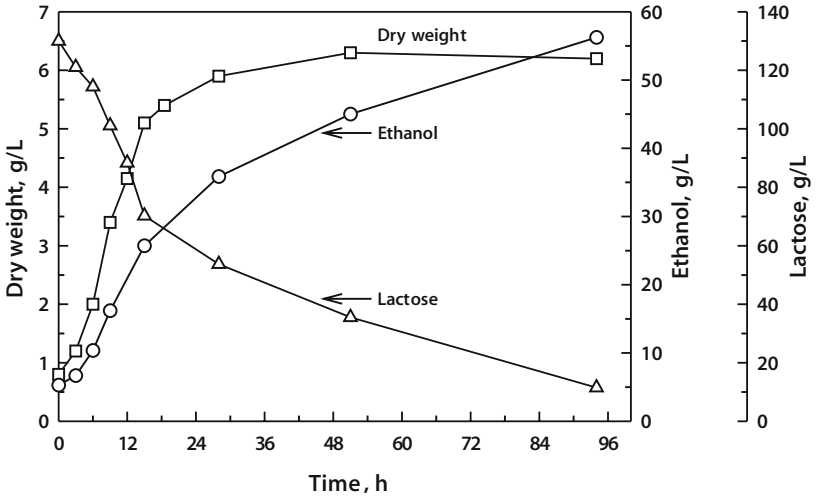


**Figure 17.1** Alcoholic fermentation of whey supplemented with lactose at 30 °C under micro-aerobic conditions

Biomass/lactose yield remains almost constant, having a value around 0.1 grams of biomass per gram of lactose consumed.

The ethanol/lactose yield decreases slightly when the initial lactose concentration is raised; Due to the decrease in yeast specific growth rate, the increase in lactose concentration decreases ethanol productivity ($q_P$). The initial concentration must be selected to achieve a final concentration of ethanol that is as high as possible, with higher productivity and biomass growth. The evolution of ethanol, lactose and biomass during an alcoholic fermentation on whey with an initial lactose concentration of 135 $g \cdot L^{-1}$ is shown in Figure 17.1 (Parrondo et al., 2000a). Using this concentration of sugar, a final concentration of ethanol around 55 $g \cdot L^{-1}$ is reached within 48 hours.

Temperature is also an important parameter affecting alcoholic fermentation performance, as can be seen in Table 17.3. An increase in temperature from 15 to 40ºC doubles the specific growth rate but decreases biomass/lactose yield. Ethanol/lactose yield has a maximum at 30 °C, yielding an efficiency in the biotransformation of 90%. Specific productivity also increases with temperature despite the slight decrease in ethanol/lactose yield observed at higher temperatures. These results suggest that ethanol fermentation from whey occurs well at temperatures around 30ºC.

**Table 17.3** Effect of fermentation temperature on kinetic parameters of yeast growth, lactose consumption and ethanol production

| **Temperature** (°C) | $\mu$ ($h^{-1}$) | $Y_{X/S}$ ($g \cdot g^{-1}$) | $q_S$ ($h^{-1}$) | $Y_{P/S}$ ($g \cdot g^{-1}$) | $q_P$ ($h^{-1}$) |
|---|---|---|---|---|---|
| 15 | 0.10 | 0.11 | 0.9 | 0.40 | 0.35 |
| 20 | 0.12 | 0.12 | 1.1 | 0.43 | 0.46 |
| 30 | 0.17 | 0.09 | 1.9 | 0.48 | 0.91 |
| 35 | 0.17 | 0.09 | 1.9 | 0.47 | 0.90 |
| 40 | 0.19 | 0.08 | 2.3 | 0.46 | 1.06 |

μ: specific growth rates of *K. marxianus* at 45 $g \cdot L^{-1}$ lactose concentration. $Y_{X/S}$: biomass/lactose yield (g biomass/g lactose). $q_S$: specific lactose consumption rate. $m$: cellular maintenance coefficient. $Y_{P/S}$: ethanol/lactose yields (g ethanol/g lactose). $q_P$: specific ethanol production rate.

Other operating parameters, such as initial pH, have an effect on ethanol production, as reported by several authors (Parrondo et al., 2000a; Kargi and Ozmihei, 2006). The best results are obtained by using whey with an initial pH between 5 and 6. Higher pH values are not recommended because of the risk of spoilage by lactic acid bacteria. This risk is diminished due to the fast decrease in the pH of the medium during the initial stages of the fermentation, reaching a final pH value around 4.

### 17.2.1.2 Growth Limitations

The supply of oxygen must be considered during the alcoholic fermentation in order to improve yeast growth, ethanol productivity, and to diminish the risk of bacterial contamination. It is widely recognized that, although alcoholic fermentation is an anaerobic biotransformation, the yeast requires small amounts of oxygen to grow and to produce essential constituents (Tyagi, 1984; Andreasen and Stier,

1956). Some yeast strains are not able to grow for more than five generations under fully anaerobic conditions (Jones et al., 1981). During alcoholic fermentation, small amounts of oxygen promote yeast growth without diminishing ethanol/sugar yield. The presence of oxygen allows the biosynthesis of unsaturated lipids (Mercer, 1984), which are essential for the proper working of the plasmatic membrane (Kirsop, 1974). The optimum amount of oxygen required depends on the strain used, the composition of the broth and the volume of the fermenter. The use of sterol supplements in the formulation of the broth diminishes the requirement for oxygen because the membrane's essential constituents are supplied in the feed (Andreasen and Stier, 1956). In whey fermentations at pilot-plant scale, an aeration stage is required to allow the fermentation to occur at a sufficient rate and, as a consequence, avoid the risk of contamination by lactic acid bacteria.

The effect of scaling-up on yeast growth and ethanol production is shown in Figure 17.2. The results are for fermentations of whey aerated during the first 6 hours. As can be seen in Figure 17.2, lower yeast growth is observed at higher fer-

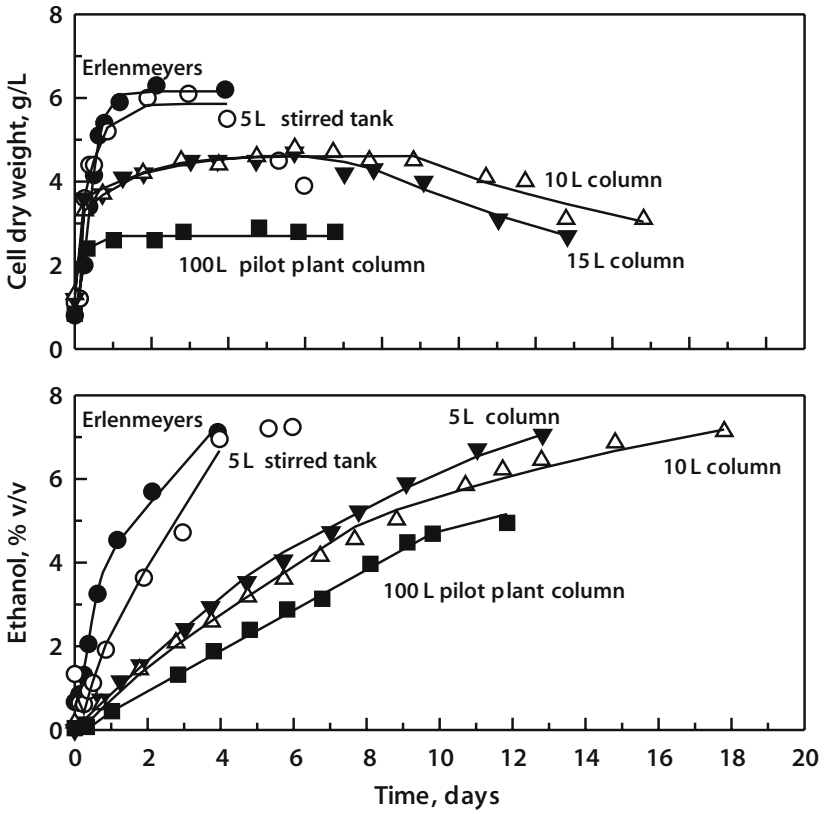


**Figure 17.2** Effect of scaling-up on fermentations of whey at 30 °C under micro-aerobic conditions

mentation volumes. A low biomass concentration in the fermenter slows down ethanol production and diminishes ethanol yield because of the increase in maintenance requirements; the fermentation in the columns lasts for more than 10 days.

#### 17.2.1.3 Secondary Metabolism

The volatile composition is responsible for the aromatic properties of food and, for this reason, must be taken into account. In addition to ethanol (the main product of an alcoholic fermentation), a large number of other 'higher' alcohols or fusel alcohols are formed during fermentation (Mangas et al., 1996), as well as esters and ketones. These compounds, when present in high concentrations, modify the organoleptic properties of the beverage.

It is well recognized that the fusel alcohols are products of amino acid and protein synthesis in yeast (Derrick and Large, 1993) and thus the protein concentration and the amino acid composition of the culture medium are of great significance for the formation of higher alcohols during fermentation. Other environmental conditions, such as aeration, also promote the production of these compounds (Ingraham et al., 1961). Due to their role in determining flavour, they have been extensively studied in the most popular beverages: cider, beer and wine.

1-propanol is formed from the carbon skeleton corresponding to the amino acid threonine by means of transamination. After ethanol, the main product in alcoholic fermentation, this 'higher' alcohol is the simplest alcohol formed during yeast growth. The concentrations of 1-propanol over time for a fermentation of whey at

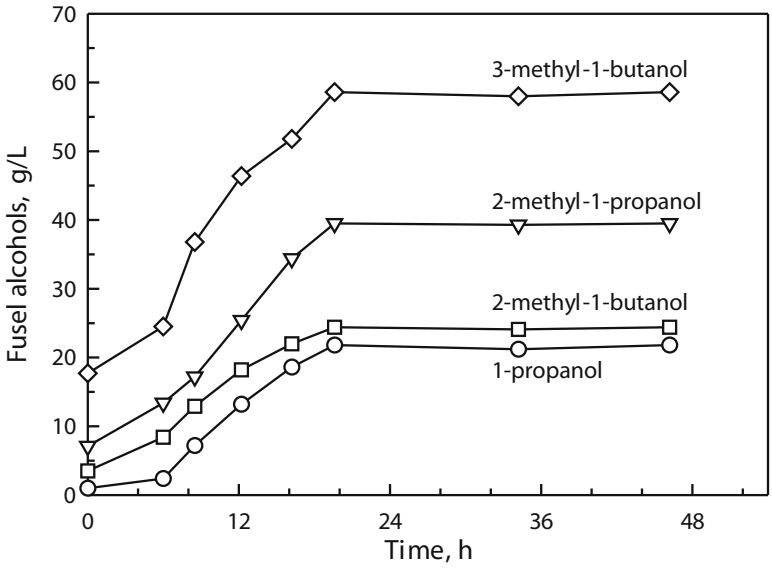


**Figure 17.3** Production of fusel alcohols during alcoholic fermentation of whey at 30 °C (from Parrondo et al., 2000b)

30 °C are depicted in Figure 17.3 (Parrondo et al., 2000b). Final concentrations of around 20 mg · $L^{-1}$ were found.

2-methyl-1-propanol originates from the amino acid valine. Like the amyl and isoamyl alcohols, it gives a sharp aroma to beverages. Final concentrations in fermented whey, as can be seen in Figure 17.3, are below 50 mg · $L^{-1}$ and, hence, negative effects in the organoleptic properties are not expected.

2-methyl-1-butanol (related to isoleucine) and 3-methyl-1-butanol (originating from the amino acid leucine) concentrations are also given in Figure 17.3. These follow the same pattern observed in the previously discussed alcohols, but present slightly higher concentrations than the other secondary alcohols.

The total concentration of fusel alcohols is below 200 mg · $L^{-1}$ (Parrondo et al., 2000b), being an acceptable value for an alcoholic beverage. These values are similar to those reported for cider or wine. There are, however, some differences: lower amounts of some alcohols and the absence of some compounds, such as 2,3-butanediol, ethyl lactate and methanol, that are detected in wine and cider (Mangas et al., 1996) but remain below detection level in whey products. From results reported in literature, we can conclude that the volatile composition of fermented whey is similar to that of other fermented beverages.

## 17.2.2 Acetic Acid Fermentation

The alcoholic product obtained by fermentation of supplemented whey can be used as the substrate for an acetic acid 'fermentation' for the production of vinegar. Throughout this chapter we use the term 'fermentation' in a broad sense to refer to a production process using microorganisms even though it may be aerobic, anaerobic or micro-aerobic. The biotransformation of the ethanol contained in the alcoholic product is performed by acetic acid bacteria. There are several acetic acid bacteria with different capabilities, as will be explained in the next section.

### 17.2.2.1 Acetic Acid Bacteria

Acetic acid bacteria belong to a group of aerobic Gram-negative bacilli, with the ability of grow at low pH, that are able of oxidize alcohols to organic acids (Krieg and Holt, 1984). The acetic acid bacteria involved in vinegar fermentation belong mainly to three genera: *Acetobacter*, *Gluconobacter* and *Gluconacetobacter*.

In industry they are used in the production of acetic acid (vinegar) from alcoholic beverages (mainly wine or cider). The difference between the three genera is the ability of *Acetobacter* strains to oxidize acetic acid to carbon dioxide and water under aerobic conditions; in *Gluconacetobacter* species, over-oxidation of acetate depends upon acetate concentration in the medium; *Gluconobacter* species cannot metabolize acetic acid.

It is desirable to use pure cultures in the acetic acid fermentation to increase the yield of the main product and to avoid undesirable reactions that change the organoleptic properties of the vinegar obtained. The acetic acid bacteria can be iso-

lated from cider or wine vinegars. The protocol of isolation and characterization of the strains is explained in the following paragraphs.

The active acetic acid bacteria that are contained in the vinegar are cultured in Petri dishes with yeast glucose agar medium (glucose 20 g, yeast extract 10 g, Bromocresol green 20 mg, agar powder 15 g and distilled water 1 L). Bromocresol green is an acid-base indicator used to detect the presence of acid-producing colonies. There are other media for the growth and isolation of acetic bacteria based on ethanol, but the growth of the bacteria in these media is very slow (15 days or more). It is preferable to use non-selective media in the isolation of strains in order to avoid the presence of contaminating strains that do not grow in that medium.

To assess whether the bacteria isolated are acetic acid bacteria suitable for the production of vinegar, several assays must be performed. This identification can be performed using genetic assays (PCR) but it may be sufficient to do a partial identification using simple assays. Firstly, the bacteria must be Gram-negative (Prescott et al., 1999) and must consume ethanol and produce acetic acid when growing in an ethanol medium (20 g ethanol, 1 g yeast extract, and 100 mL distilled water). Acetic acid-producing strains belong to the family *Acetobacteriaceae* (Krieg and Holt, 1984). The differences between vinegar-producing species are shown in Table 17.4.

In industry, the vinegar is produced mainly with *Acetobacter pasteurianus* strains. Biomass concentration can be followed during fermentations by plate counts or by measuring dry weight. In the latter case, special care must be taken because some *A. pasteurianus* bacteria produce extracellular cellulose. The production of cellulose increases in old cultures (not related to growth metabolites) and is transferred to the fermentation broth by the starter culture.

**Table 17.4** Differences between the some acetic acid bacteria species

| **Characteristics** | ***A. aceti*** | ***A. pasteurianus*** | ***Ga. liquefaciens*** | ***Ga. hansenii*** |
|---|---|---|---|---|
| Catalase | + | + | + | + |
| Ketogenesis from glycerol | + | – | + | + |
| Production from D-glucose of: | | | | |
| 5-ketogluconate | + | – | d | d |
| 2-ketogluconate | + | d | + | + |
| D-gluconate | + | d | + | + |
| Ethanol growth | d | d | + | – |
| Production of γ-pyrones | – | – | d | – |
| Brown pigment | – | – | + | – |
| G+C, % mol | 56.2-57.2 | 51.8-53 | 62-65 | 58-63 |

d, 11-89% of the strains positive.

Modified from Krieg and Holt, 1984; Sievers and Swings, 2005

### 17.2.2.2 Production of Vinegar

The alcoholic product obtained by fermentation of whey supplemented with lactose with *K. marxianus*, is employed as the substrate in the acetic fermentation. The process can be carried out using one of the species of the genera *Acetobacter* or *Gluconoacetobacter*; it seems preferable to use strains *of Ga. liquefac*iens *or A.*

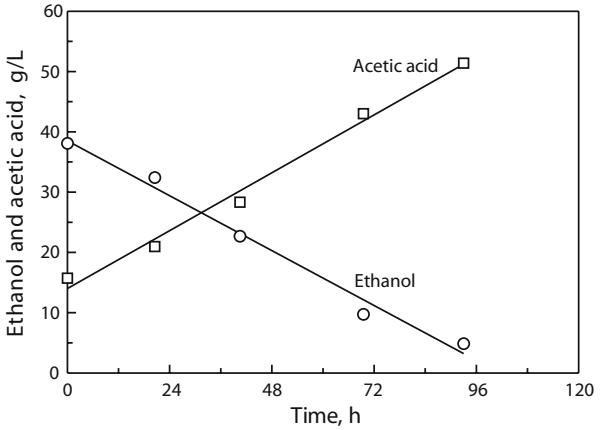


**Figure 17.4** Acetic acid and ethanol evolution during acetic fermentation of whey at 30 °C

*pasteuria*nus because they grow better in ethanol. The vinegar obtained in the previous batch fermentation can be used as a starter culture. This vinegar is used as an inoculum without any separation stage in a proportion of 1 volume of starter culture per 2 volumes of alcoholic product (with a lower amount of starter culture the fermentation is too slow). A high amount of inoculum assures fast fermentations and avoids the culture becoming contaminated during the process (Tesfaye et al., 2000). Whey has the nutrients required for the growth of acetic acid bacteria and hence it is not necessary to add any other nutrients.

The evolution of ethanol and acetic acid concentrations in the whey during fermentation at laboratory scale are shown in Figure 17.4. Ethanol is completely metabolized in 4 days, reaching an acetic acid concentration of 4.9% (v/v). The rate of acetic acid production is 0.04 acetic degrees per hour. Agitation increases the rate of biotransformation of ethanol into acetic acid (between two- and fivefold) because of the increase in oxygen transfer rate (Blanch and Clark, 1996). The oxygen is the limiting substrate in this process (the oxidation of ethanol into acetic acid is an aerobic biotransformation) and hence the strategies designed to improve the transference of oxygen increase the performance of the overall process.

### 17.2.2.3 Kinetic Modelling

The bacteria growth rate is related to the oxygen transfer rate, assuming pseudo-stationary conditions and oxygen mass-transfer limitations, by means of the following expression (Blanch and Clark, 1996):

$$r_X = Y_{X/O_2}\left(-r_{O_2}\right) = Y_{X/O_2}k_L a'\left(C^* - C\right)$$

where $r_X$ is microorganism growth rate ($g \cdot L^{-1} \cdot h^{-1}$), $Y_{X/O_2}$ is biomass/oxygen yield (g biomass/g oxygen), $r_{O_2}$ is oxygen consumption rate ($g \cdot L^{-1} \cdot h^{-1}$), $k_L a'$ is the oxy-

gen transfer coefficient, $C^*$ is the oxygen saturation concentration, and $C$ is the actual concentration of oxygen in the broth.

The saturation constant for oxygen for *Acetobacter* strains is less than 1% of saturation concentration and hence, when the bacteria is not growing at maximum rate, the concentration of oxygen in the broth is usually much lower than the saturation value, giving rise the following simplified equation:

$$r_X = Y_{X/O_2} C^* k_L a'$$

This equation establishes that bacteria growth rate is proportional to oxygen transfer coefficient.

Acetic acid is a primary metabolite (final product of ethanol catabolism) and hence its production must be related to bacteria growth (Czuba, 1988), according to the following equation:

$$r_P = Y_{P/X}\left(-r_X\right) = Y_{P/X} Y_{X/O_2} C^* k_L a'$$

where $r_P$ is the production rate of acetic acid ($g \cdot L^{-1} \cdot h^{-1}$) and $Y_{P/X}$ is the acetic acid (product)/biomass yield. The experimental results (Parrondo et al., 2003) show proportionality between acetic acid production and oxygen transfer coefficient (see Figure 17.4).

Ethanol consumption is related to acetic acid production by means of product/substrate yield:

$$-r_S = \frac{r_P}{Y_{P/X}} = \frac{Y_{P/X} Y_{X/O_2} C^* k_L a'}{Y_{P/X}}$$

where $r_S$ is ethanol consumption rate and $Y_{P/S}$ is acetic acid/ethanol yield.

Oxygen transfer rate coefficient ($k_L a'$) depends on hydrodynamic conditions, such as stirring, reactor filling level and rheological properties. If these variables do not change during the biotransformation, acetic acid production rate and ethanol consumption rate are constant (see Figure 17.4). This simple kinetic model can initially be used for scaling-up and for the analysis of fermentation results.

Acetic acid fermentations using *A. pasteurianus* strains must be stopped before total consumption of substrate to avoid the oxidation of the acetic acid produced to $CO_2$ and water. Concentrations of ethanol of 5 $g \cdot L^{-1}$ or more are required under aerobic conditions, to inhibit the oxidation of acetic acid by the bacteria. The model proposed here can be used to estimate the time required to stop the fermentation.

## 17.3 Operation

Laboratory results show that vinegar can be produced from whey supplemented with food lactose. The global process comprises two fermentations: an alcoholic fermentation and an acetic fermentation. In Figure 17.5 a proposal of a process to produce vinegar from whey is plotted.

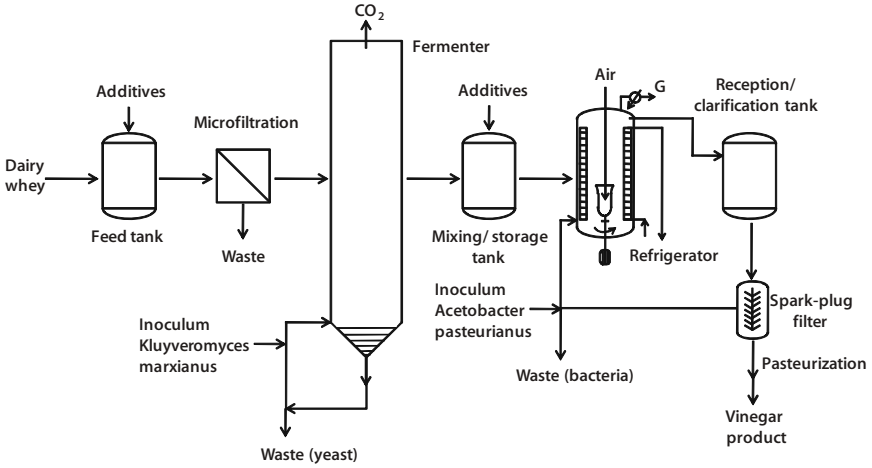


**Figure 17.5** Flow diagram of an industrial process for the production of vinegar from whey

The broth can be prepared using liquid sweet whey or powder whey. This whey is supplemented with lactose up to a concentration around 135 $g \cdot L^{-1}$ to increase ethanol production during alcoholic fermentation. The alcoholic fermentation of whey lactose is carried out using a *K. marxianus* strain; this fermentation is a critical step in the process due to the slow growth of yeasts in whey and hence, to avoid contamination with other microorganisms, the broth should be autoclaved or microfiltered. Alcoholic fermentation is carried out in a bubble-type fermenter using a controlled amount of microfiltered air (0.45 μm).

Yeasts are removed at the end of the alcoholic fermentation and stored. They can be used as starter culture, but only two or three times in order to avoid the bacterial contamination during alcoholic fermentation. New starter cultures should be prepared from pure cultures stored in glycerol or slopes.

Vinegar is produced by aerobic biotransformation of the alcoholic product previously obtained. The alcoholic beverage is fermented without sterilization and should be inoculated using a vinegar inoculum in a proportion of 1:2 to increase the efficiency of the process. The acetic acid fermentation lasts 4-7 days. The vinegar is then filtered to remove the bacteria, and stored or bottled.

As shown in Table 17.5, the volatile composition of whey vinegar is similar to that of its alcoholic precursor. However, there are differences, such as the presence

**Table 17.5** Main volatile compounds in vinegar produced from whey

| **Compound** | **Concentration** ($mg \cdot L^{-1}$) |
|---|---|
| Ethyl acetate | 13.4 ± 0.4 |
| 2-methyl-1-propanol | 33.9 ± 0.1 |
| 2-methyl-1-butanol | 9.0 ± 0.1 |
| 3-methyl-1-butanol | 40.8 ± 0.2 |

of some amounts of ethyl acetate, lower amounts of isoamyl alcohols, and the absence of 1-propanol, but these differences are tiny and the organoleptic properties remain the same.

Acetic acid can also be produced by direct biotransformation of the lactose contained in the whey using co-cultures of heterofermentative and homoacetogen bacteria. Several authors have reported the production of acetate from whey using these anaerobic co-cultures with higher yields than those obtained by means of alcoholic fermentation followed by an acetic one (Huang and Yang, 1998; Talabardon et al., 2000a, 2000b; Collet et al., 2003).

When *Clostridium thermolacticum* is co-cultured with *Moorella thermoautotrophica*, lactose is first mainly converted to lactic acid, and then to acetic acid, with a zero residual lactic acid concentration and an overall yield of acetate around 80%; under such conditions, only 13% of the fermented lactose was converted to ethanol by *C. thermolacticum*. The problem is that bacteria belonging to the genus *Clostridium* are pathogens and cannot be used in the production either of foods or of additives to be used in the elaboration of foods. The acetic acid produced using this approach can only be exploited in the industrial field.

## 17.4 Conclusions

The increasing amounts of whey that are being produced worldwide as a by-product of the dairy industry need to be treated to avoid a negative impact on the environment. Whey is currently used for the production of infant formula milks, chemical or enzymatic lactose hydrolysis and for the production of chemical derivatives and single cell proteins, but alternative uses are required due to the increasing amounts being produced.

In this chapter, we analysed the production of vinegar from whey by means of two fermentations: an alcoholic fermentation followed by an acetic one. The alcoholic fermentation of whey (or whey supplemented with lactose, if higher concentrations are desired) is carried out with a lactose-fermenting yeast: *K. marxianus*. This fermentation is a critical step in the production of vinegar because whey has nutrient deficiencies that make yeast growth difficult.

The addition of oxygen during alcoholic fermentation is an important factor that affects cell growth and hence ethanol productivity; a good yeast growth also diminishes the risk of bacterial contamination. Oxygen increases some anabolic reactions, allowing the biosynthesis of unsaturated lipids that are essential for the proper working of the plasmatic membrane.

The optimum amount of oxygen required depends on the strain used, the composition of the broth and the volume of the fermenter. The optimum amount also depends on the use of sterol supplements in the formulation of the broth. In fermentations of whey without supplementation at pilot-plant or industrial scales, aeration stages are required in order to allow the fermentation to occur at a sufficient rate and to avoid contamination risk. The aeration must be optimized for the scale being used.

The alcoholic product arising from fermentation of whey supplemented with lactose by *K. marxianus* is employed as a substrate in the acetic fermentation, aerobic biotransformation of the alcoholic product. The process should be carried out using *Gluconacetobacter liquefaciens* or *Acetobacter pasteurianus* strains because of their better growth in ethanol. Results reported in the literature suggest that the process is controlled by the oxygen transfer rate. Acetic acid fermentation lasts 4-7 days and the final product, using whey supplemented with lactose, has an acetic concentration around 5% (v/v). The vinegar obtained is filtered to remove the bacteria and then stored or bottled.

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# Subject Index