Research Article

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Relationship between changes in the total concentration of acetic acid bacteria and major volatile compounds during the acetic acid fermentation of white wine

Silvia Baena-Ruano,[a] Inés M Santos-Dueñas,[a] Juan C Mauricio[b] and Isidoro García-García[a]*

Abstract

BACKGROUND: In the scope of the wine vinegar production, this paper provides comprehensive information about the evolution of some volatile compounds during the biological acetification cycle. These data were compared with the acidity, cell concentration and ethanol concentration. Such information may allow a better understanding of the complex biological processes involved.

RESULTS: The volatile compounds 2-phenylethanol, diethyl succinate (diethyl butanedioate), *meso*-2,3-butanediol (*meso*-butane-2,3-diol), *levo*-2,3-butanediol (*levo*-butane-2,3-diol), methanol and ethyl acetate exhibited no significant changes between the starting wine and produced vinegar, whereas the rest [acetoin (3-hydroxybutan-2-one) excepted] ethyl lactate (ethyl 2-hydroxypropanoate), isoamyl alcohols (3-methylbutan-1-ol and 2-methylbutan-1-ol), isobutanol (2-methylpropan-1-ol), 1-propanol (propan-1-ol), and acetaldehyde were consumed in substantial amounts during the process. Additionally, their specific evolution patterns alongside bacterial cell concentrations, acidity and ethanol concentration are shown.

CONCLUSION: Concentrations of acetic acid bacteria at the end of the acetification cycle were found to vary because of cell lysis, a result of the high acidity and low ethanol concentration of the medium. Variations were similar to those in some volatile compounds, which suggests their involvement in the metabolism of acetic bacteria. The results testify to the usefulness of this pioneering study and suggest that there should be interest in similar, more detailed studies for a better knowledge of the presence of certain volatile compounds and metabolic activity in cells effecting the acetification of wine.

Keywords: wine; acetification; vinegar; volatile compounds; acetic acid bacteria

INTRODUCTION

Wine vinegar is an increasingly appreciated product by virtue of its sensory quality and richness. In fact, today, as much importance is attached to vinegar as to wine in winemaking areas which have traditionally obtained the vinegar as a by-product;[1] this had led to the application of strict analytical and control methods to the acetification process with a view to improving the quality of the end product, and facilitating its characterisation and discrimination.[2,3]

Vinegar owes much of its sensory character to its aroma, which is a combination of the individual contributions of many volatile products.[4–7] The final composition of vinegar in such products depends on the particular raw material used, the way the alcoholic fermentation and subsequent biological oxidation are conducted, and the ageing procedure employed, if any. Many vinegars, some with a designation of origin included, are biologically oxidised in modern industrial fermentation tanks where the culture medium is subjected to substantial aeration. Despite the high efficiency of the aeration process, there is always the risk of some volatile compounds being lost and the quality of the end product diminished as a result. However, using volatile condensers at the gas output and optimising oxygen input to the reactor during the aeration process can help to substantially avoid volatile losses.

Although, as noted earlier, the volatile composition of vinegar is widely variable, it usually includes higher alcohols, esters and some aldehydes and ketones such as acetoin, acetaldehyde, ethyl lactate, 2,3-butanediol, isoamyl alcohols, ethyl acetate, methanol and 2-phenylethanol as major components.[8]

* *Correspondence to: Isidoro García-García, Departamento de Ingeniería Química, Edificio Marie Curie, Facultad de Ciencias, Universidad de Córdoba, Campus Universitario de Rabanales, 14071 Córdoba, Spain. E-mail: isidoro.garcia@uco.es*

a *Departamento de Ingeniería Química, Edificio Marie Curie, Facultad de Ciencias, Universidad de Córdoba, Campus Universitario de Rabanales, 14071 Córdoba, Spain*

b *Departamento de Microbiología, Edificio Severo Ochoa, Facultad de Ciencias, Universidad de Córdoba, Campus Universitario de Rabanales, 14071 Córdoba, Spain*

Acetaldehyde present in vinegar is an intermediate in the catabolism of carbon-containing substrates used by acetic acid bacteria.[9] This aldehyde is formed by oxidation of both ethanol and pyruvate, which in turn can be produced by the Entner–Doudoroff reaction of sugars, if available, or from lactate, especially at low ethanol levels.[10]

Acetoin is produced in substantial amounts during the acetification process. In fact, it is often used as a marker for the biological origin of vinegar since its synthesis is related to cell metabolism in acetic bacteria.[11]

Alcohols are also important to vinegar. Thus, isoamyl alcohols are usually consumed during the acetification of wine.[12] On the other hand, *levo*- and *meso*-2,3-butanediol in wine are reportedly oxidised to acetoin,[5] but were found to change little in content with respect to the starting wine here.

Esters are also important as regards concentration and their potential influence on vinegar aroma. Wine vinegar usually contains some, such as ethyl lactate, diethyl succinate, ethyl acetate, methyl acetate and isoamyl acetate.[13] Also, isoamyl acetate and ethyl acetate are among the compounds with the highest odour activity value (OAV) in vinegar.[14]

Although the volatile composition of vinegar is widely documented, its changes during biological acetification of wine have seemingly been studied only once.[13] Also, the study in question was quite comprehensive and interesting, the authors only analysed samples at the beginning, middle and end of the acetification cycle, which was inadequate to assess changes in volatile compounds throughout the acetification cycle with a view to identifying potential alterations in their assumed evolution patterns. Recently,[15] changes in volatiles in balsamic and red wine vinegars stored in wooden casks and bottles were studied, but only between the start and end of the process.

No study of the potential relationship between volatiles and microbial concentration changes during the acetification cycle appears to have been conducted to date. Elucidating such a relationship, if it does exist, would be helpful with a view to relating the synthesis and evolution of these compounds with chemical and biological activity in the acetification system.

In this work, we studied the variations in major volatiles in wine vinegar and examined their potential relationship to the total concentration of cells throughout the acetification cycle and to various other important variables.

EXPERIMENTAL

Microorganism

The inoculum used was a mixed culture of acetic bacteria from an industrial fermentation tank in full operation. The total concentration of cells as measured with a method described elsewhere[16] ranged from about 1×10^8 to 3.5×10^8 cells mL^{-1} during the acetification cycle.

Culture medium and fermentation conditions

The raw material used was white wine from the Montilla–Moriles region (Spain) (which is similar to sherry wine) containing 89.7 ± 3.9 g ethanol L^{-1} and having an initial acidity of 4.0 g acetic acid L^{-1}.

The bioreactor employed was operated in a semi-continuous mode (Fig. 1). Thus, once an ethanol concentration of 3.9 g ethanol L^{-1} was reached, a portion of 75% of the total volume of culture medium was unloaded and the reactor replenished at a constant flow-rate of 0.01 L wine min^{-1}. This cycle can be repeated an indefinite number of times.

The reactor was a Frings 8 L fermenter and operated at 31 °C, using an aeration regime of 7.5 L air h^{-1} L^{-1} medium. The fermenter was loaded, unloaded and monitored in an automatic manner according to a programmed sequence. Proper operation in this situation entailed careful on-line measurement of the total ethanol concentration and volume of medium. An online probe Alcosens (Heinrich Frings GmbH & Co. KG, Bonn, Germany) and a differential pressure sensor (Yokogawa Iberia S.A., Madrid, Spain) were used for ethanol and volume determination respectively.[17]

Analysis of volatiles

Major volatile compounds and polyols were quantified on a Model 6890 gas chromatograph from Agilent Technologies (Palo Alto, CA, USA), using the method described by Peinado *et al.*[18] A CP-WAX 57 CB capillary column (60 m long × 0.25 mm i.d., 0.4 µm film thickness) from Varian (Palo Alto, CA) was used, and 0.5 µL aliquots from 10 mL samples previously supplied with 1 mL of 1 g L^{-1} 4-methyl-2-pentanol as internal standard were injected into the instrument. Tartaric acid in the wine was removed by precipitation with 0.2 g of calcium carbonate and centrifugation at 1380 × *g*. Quantification was based on the response factors obtained for standard solutions of each compound. A split ratio of 30:1, an FID, and a temperature program involving an initial temperature of 50 °C (15 min), a 4 °C min^{-1} ramp and a final temperature of 190 °C (35 min) were used. The injector and detector temperatures were 270 and 300 °C, respectively. The flow rate of carrier gas (helium) was initially set at 0.7 mL min^{-1} (16 min) and followed by a 0.2 mL min^{-1} ramp to the final value (1.1 mL min^{-1}), which was held for 52 min.

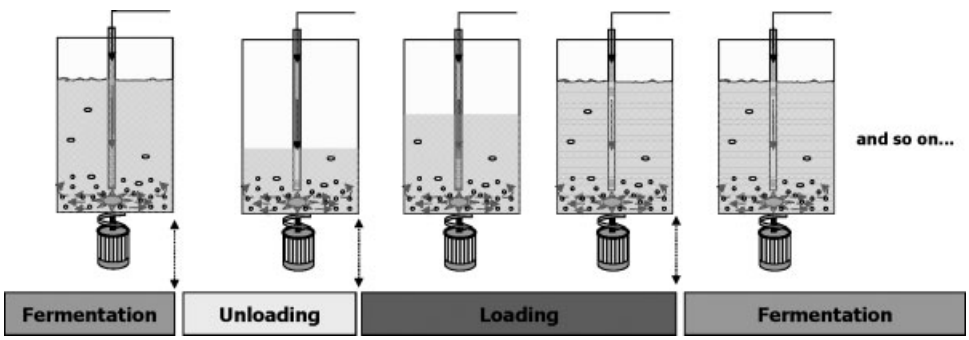

Figure 1. Stages of a semi-continuous acetification process.

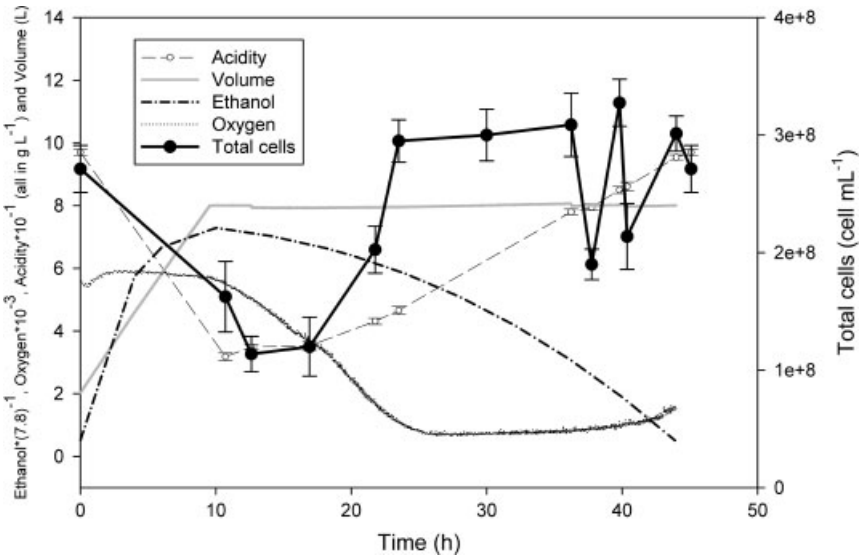

Figure 2. Variation of the concentrations of ethanol and oxygen, as well as the acidity and volume of the medium, in relation to the total number of cells during the acetification cycle. Bars represent standard deviations. The corresponding mean standard deviations for ethanol, oxygen and volume were about 3%, 2% and 2%, respectively.

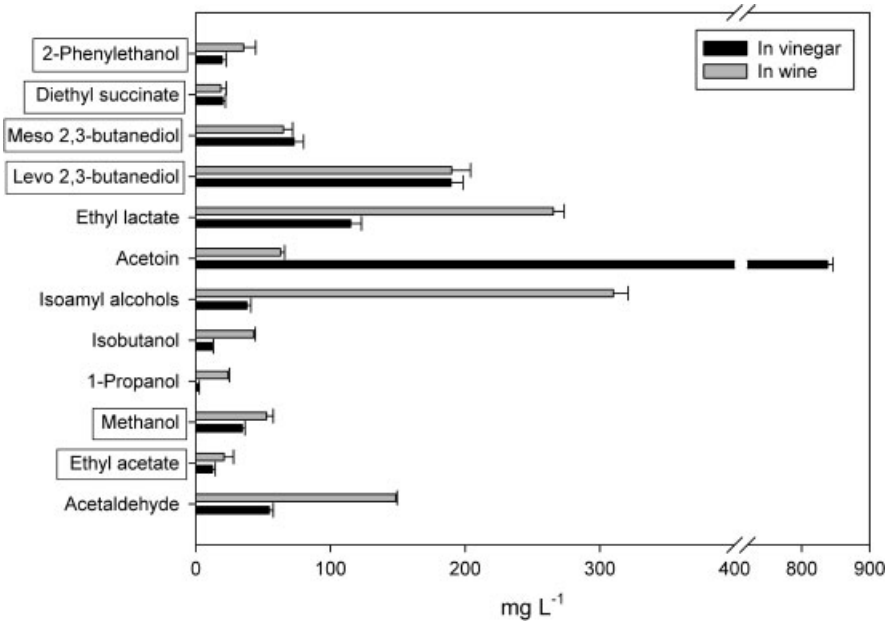

Figure 3. Contents in volatiles of the starting wine and the resulting vinegar. Bars represent standard deviations.

RESULTS AND DISCUSSION

Figures 2 to 8 show the experimental data. The results shown in this work are the means for four cycles. Bars represent standard deviations.

Figure 2 shows the variation of the main system variables during the acetification cycle. As can be seen, the volume and concentration of ethanol in the medium increased with time during the first 10 h (loading stage); by contrast, the concentration of bacterial cells and the acidity of the medium decreased markedly by effect of dilution over the same period. The ethanol and acidity values at the end of the loading phase are the result of both the addition of wine as well as the bacteria activity. In fact, a global mass balance can shows that, approximately, 10 g L^{-1} of acetic acid has been produced during this stage. Acetic bacteria were subjected to a high stress as a result of abrupt changes in their environment during the loading stage, which was followed by an adaptation lag stage that lasted 8 h. Then, bacterial cells entered an exponential growth stage which lasted about 4 h. The last stage of the vinegar production cycle was especially interesting on account of the marked changes undergone by the bacterial cells. In a previous study[19] on the variation of amino acids concentrations during the vinegar production cycle, such changes were found to be due to cell lysis phenomena. In fact, each anecdotal decrease in cell concentration was accompanied by a simultaneous anecdotal increase in the contents of many amino acids present that was ascribed to cellular autolysis. There was also evidence of the opposite changes (i.e. an increase in cell concentration concomitant with a decrease in amino acid levels).

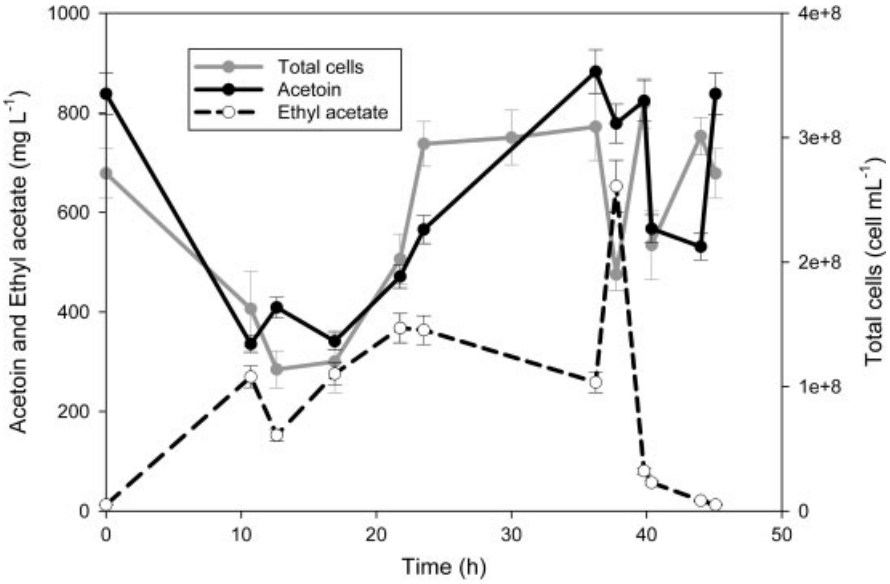

Figure 4. Variation of the concentrations of acetoin and ethyl acetate in relation to the total number of cells during the acetification cycle. Bars represent standard deviations.

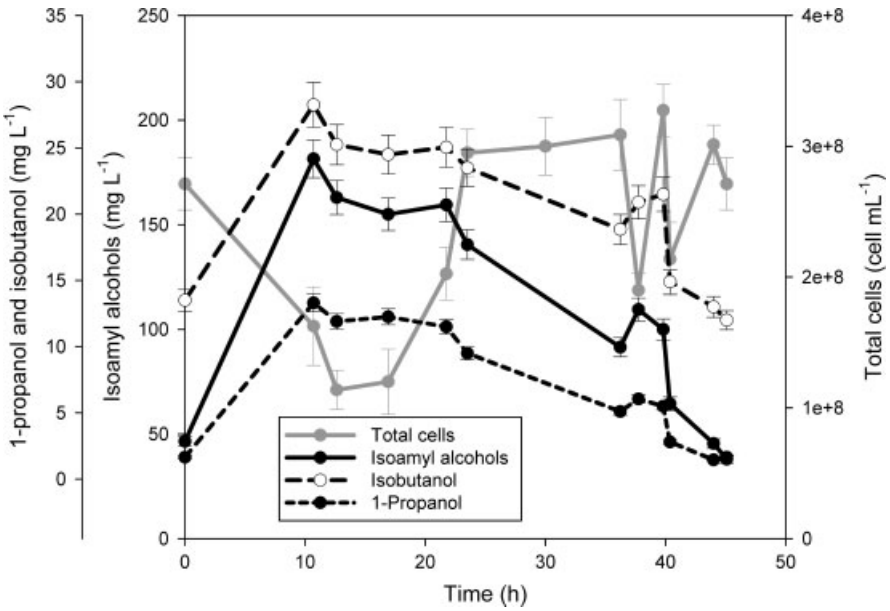

Figure 5. Variation of the concentrations of isoamyl alcohols, isobutanol and 1-propanol in relation to the total number of cells during the acetification cycle. Bars represent standard deviations.

These facts may be indicative or even provide evidence for a complex response of bacterial cells to stressing conditions in order to ensure survival of their population via 'programmed cell death', which allows cells to live on lysis products from dead cells.

The previous results led us to examine changes in various compounds with a strong impact on the sensory properties of vinegar.[5] Fig. 3 shows their concentrations in the starting wine and end product (vinegar), as well as the intervening changes. The compounds inside the box exhibited no significant changes, whereas those outside it, acetoin excepted, were consumed in substantial amounts during the process. Acetoin is known to be involved in the biological oxidation of ethanol by acetic bacteria. Therefore, it is present in virtually all types of vinegar, albeit at widely variable concentrations. Thus, its content in pineapple vinegar is typically in the region of 2 mg L^{-1},[20] whereas that in vinegar from sherry wines[21] or cider[22,23] can be as high as 1000 mg L^{-1} or even higher. Acetoin can form in various ways[5,11] including the condensation of two acetaldehyde molecules, the reaction between pyruvate and acetaldehyde, and the oxidation of 2,3-butanediol.

Figure 4 shows the variation of the acetoin concentration alongside that of the bacterial cell concentration. As can be seen, both evolved virtually in parallel throughout the process. Because of its low level in the starting wine, the acetoin concentration decreased by effect of dilution during the reactor loading stage. Worthy of special note were the oscillations observed at the end of the process, consistent with changes in the total cell concentration which, as noted earlier, may be a result of cell lysis

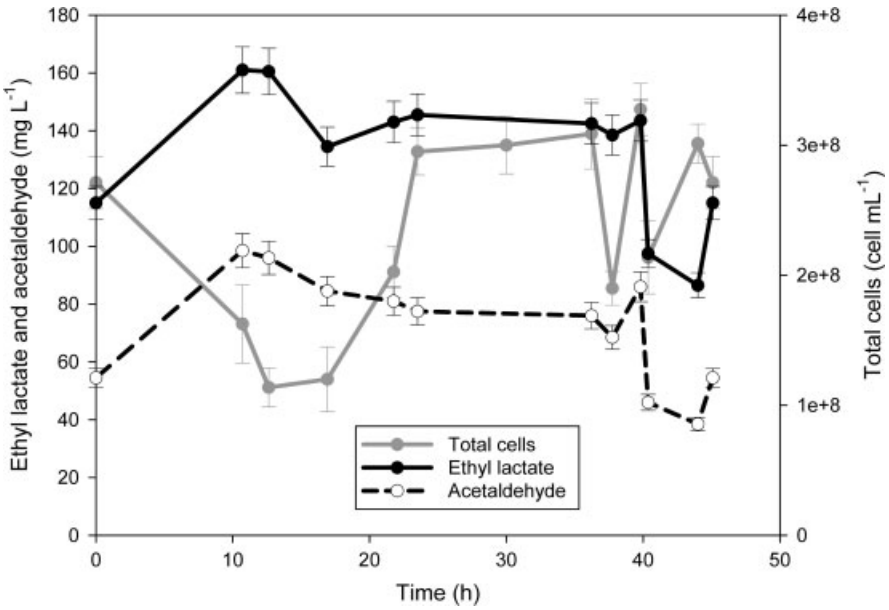

Figure 6. Variation of the concentrations of ethyl lactate and acetaldehyde in relation to the total number of cells during the acetification cycle. Bars represent standard deviations.

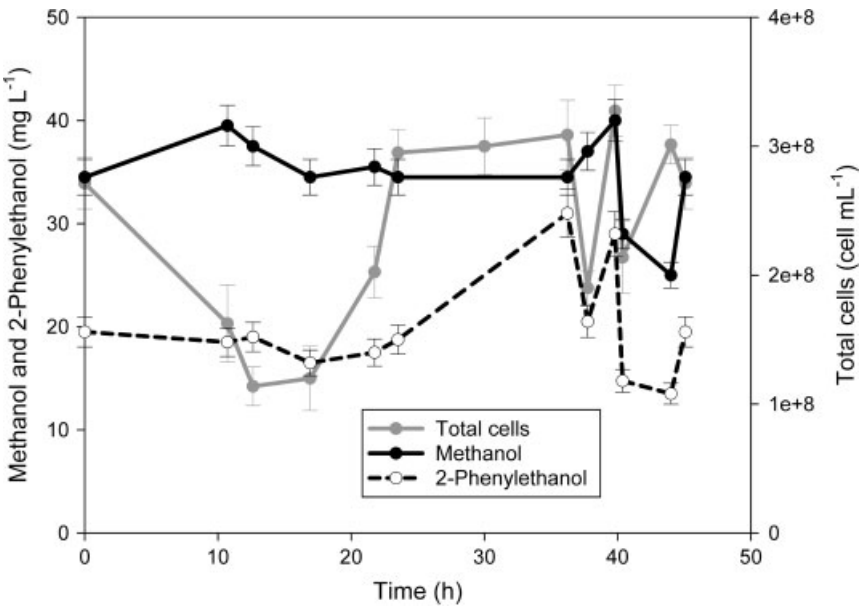

Figure 7. Variation of the concentrations of methanol and 2-phenylethanol in relation to the total number of cells during the acetification cycle. Bars represent standard deviations.

caused by an increased acidity and a reduced nutrient availability in the medium. The concomitance of these oscillations in the acetoin and cell concentrations provides further evidence for a relationship of the synthesis and changes in acetoin to biological activity in the system.

Figure 4 also shows the variation of the ethyl acetate concentrations. Although the final content of the vinegar in this compound was roughly the same as in the starting wine, this does not exclude potential changes in its concentration during the process. In fact, as can clearly be seen from the figure, this compound exhibited strong changes and an early evolution pattern differing markedly from that for acetoin. Judging by the high concentrations of acetic acid and ethanol present in the medium, the ester was most likely formed by esterification outside bacterial cells. As fresh wine was added to the fermenter during the loading stage, the medium was supplied with additional ethanol that reacted with acetic acid accumulating in it to give the ester. Once loading was finished and ethanol started to be consumed by acetic bacteria, the reverse reaction (hydrolysis of ethyl acetate) gradually prevailed and led to a decrease in the ester concentration. Although the esterification reaction must occur outside cells, oscillations in cell concentrations had a marked effect on the ethyl acetate concentration (for example, the release of acetate to the medium by effect of cell disruption can displace equilibria to the ester formation). Despite the subsequent oscillations in cell concentrations, the increasing scarcity of ethanol in the medium led to a more limited esterifica-

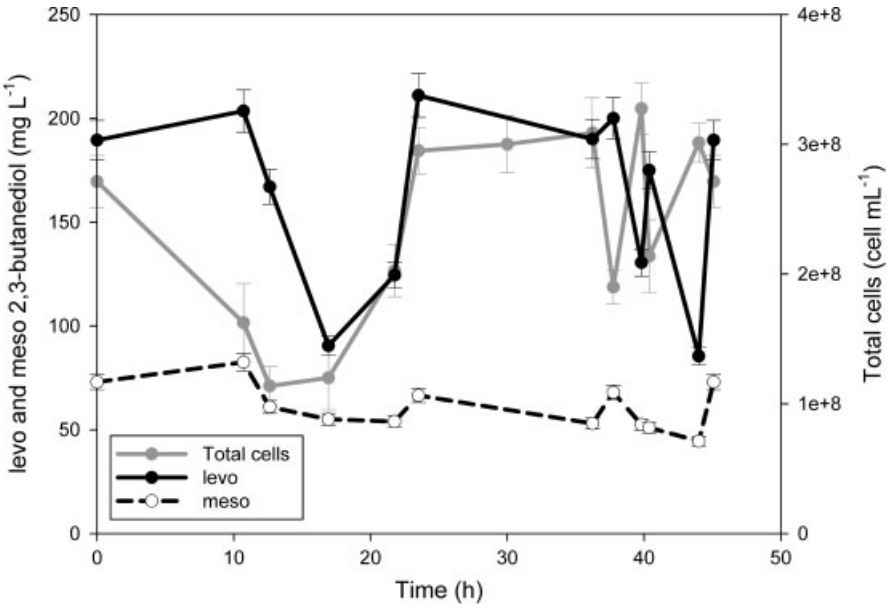

Figure 8. Variation of the concentration of 2,3-butanediol in relation to the total number of cells during the acetification cycle. Bars represent standard deviations.

tion reaction and prevented further ester concentration rises by effect of cell lysis.

The four alcohols of Fig. 5 exhibited an increase in concentration at the start of the cycle as a result of their being supplied during the loading process. Subsequently, the alcohols started to decrease in parallel with the increase in acidity of the medium, which suggests that esterification with acetic acid was the main factor governing their evolution. However, the small, transient increase at the start of the oscillations in cell concentrations may also indicate that the three alcohols are involved in the metabolism of acetic bacteria.

As can be seen from Fig. 6, there was a close relationship between cell, lactate and acetaldehyde concentrations. This is unsurprising if one considers the significance of acetaldehyde as an intermediate in the oxidative metabolism of ethanol and various other compounds in acetic bacteria.[9] Also, there is the well-known ability of acetic bacteria in using lactate to produce acetate via pyruvate first and acetaldehyde then.

The other volatile compounds (Figs 7 and 8) exhibited no significant differences in concentration between the starting wine and the vinegar. Again, there were anecdotal changes in concentration at the end of the production cycle, which suggests the involvement of these compounds in the metabolism of acetic bacteria.

CONCLUSION

In summary, this paper provides for the first time comprehensive information about the evolution of some volatile compounds during the biological acetification cycle. Such information may allow a better understanding of the complex biological processes involved. Thus, the oscillations in cell concentrations observed at the end of the cycle, which can be ascribed to cell adaptation and survival mechanisms, coincided with similar oscillations in the concentrations of the target compounds. Their apparent relationship may be of use to identify the specific compounds involved in the metabolism of acetic bacteria. To our knowledge, no study of this type has been conducted to date.

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REFERENCES

1 Durán-Guerrero E, *Control de los procesos de elaboración, calidad y trazabilidad del Vinagre de Jerez*. PhD thesis. Puerto Real, Cádiz (2008).
2 Callejon RM, Morales ML, Troncoso AM and Ferreira ACS, Targeting key aromatic substances on the typical aroma of sherry vinegar. *J Agric Food Chem* **56**:6631–6639 (2008).
3 Tesfaye W, Morales ML, Garcia-Parrilla MC and Troncoso AM, Improvement of wine vinegar elaboration and quality analysis: instrumental and human sensory evaluation. *Food Rev Int* **25**:142–156 (2009).
4 Blanch GP, Tabera J, Sanz J, Herraiz M and Reglero G, Volatile composition of vinegars – simultaneous distillation extraction and gas-chromatographic mass-spectrometric analysis. *J Agric Food Chem* **40**:1046–1049 (1992).
5 Tesfaye W, Morales ML, García-Parrilla MC and Troncoso AM, Jerez Vinegar, in *Vinegars of the World*, ed. by Solieri L and Giudici P. Springer-Verlag Italia, Milan, pp. 180–195 (2009).
6 Troncoso AM, Evaluación fisicoquímica y sensorial de vinagres, in *Second Symposium on R+D+I for Vinegar Production*, ed. by García-García I. Servicio Publicaciones de la Universidad de Córdoba, Córdoba, pp. 195–199 (2006).
7 Duran-Guerrero E, Marin RN, Mejias RC and Barroso CG, Stir bar sorptive extraction of volatile compounds in vinegar: validation study and comparison with solid phase microextraction. *J Chromatogr A* **1167**:18–26 (2007).
8 Callejon RM, Torija MJ, Mas A, Morales ML and Troncoso AM, Changes of volatile compounds in wine vinegars during their elaboration in barrels made from different woods. *Food Chem* **120**:561–571 (2010).
9 Bidan P, Divies C and Cachon R, Microorganismos de alteración de los vinos, in *Enología: Fundamentos Científicos y Tecnológicos*, ed. by Flanzy C. A Madrid Vicente Ediciones and Ediciones Mundi-Prensa, Madrid, pp. 344–356 (2000).
10 Morales ML, Gonzalez GA, Casas JA and Troncoso AM, Multivariate analysis of commercial and laboratory produced sherry wine

vinegars: influence of acetification and aging. *Eur Food Res Technol* **212**:676–682 (2001).
11 Polo MC and Sánchez-Luengo AA, Las bacterias acéticas, in *El Vinagre de Vino*, ed. by Llaguno C and Polo MC. Consejo Superior de Investigaciones Científicas, Madrid, pp. 25–47 (1991).
12 Nieto J, González-Viñas MA, Barba P, Martín-Álvarez PJ, Aldave L, García-Romero E and Cabezudo MD, Recent progress in wine vinegar R&D and some indicators for the future, in *Food Flavour, Ingredients and Composition*, ed. by Charalombous G. Elsevier Science, New York, pp. 469–499 (1993).
13 Morales M, Tesfaye W, Garcia-Parrilla MC, Casas JA and Troncoso AM, Sherry wine vinegar: physicochemical changes during the acetification process. *J Sci Food Agric* **81**:611–619 (2001).
14 Callejon RM, Morales ML, Ferreira ACS and Troncoso AM, Defining the typical aroma of sherry vinegar: sensory and chemical approach. *J Agric Food Chem* **56**:8086–8095 (2008).
15 Callejon RM, Tesfaye W, Torija MJ, Mas A, Troncoso AM and Morales ML, Volatile compounds in red wine vinegars obtained by submerged and surface acetification in different woods. *Food Chem* **113**:1252–1259 (2009).
16 Baena-Ruano S, Jimenez-Ot C, Santos-Duenas I, Cantero-Moreno D, Barja F and Garcia-Garcia I, Rapid method for total, viable and non-viable acetic acid bacteria determination during acetification process. *Process Biochem* **41**:1160–1164 (2006).
17 García-García I, Santos-Dueñas I, Jiménez-Ot C, Jiménez-Hornero J and Bonilla-Venceslada J, Vinegar Engineering, in *Vinegars of the World*, ed. by Solieri L and Giudici P. Springer-Verlag Italia, Milan, pp. 97–120 (2009).
18 Peinado R, Moreno J, Munoz D, Medina M and Moreno J, Gas chromatographic quantification of major volatile compounds and polyols in wine by direct injection. *J Agric Food Chem* **52**:6389–6393 (2004).
19 Maestre O, Santos-Duenas I, Peinado R, Jimenez-Ot C, Garcia-Garcia I and Mauricio JC, Changes in amino acid composition during wine vinegar production in a fully automatic pilot acetator. *Process Biochem* **43**:803–807 (2008).
20 Ou ASM and Chang RC, Taiwan fruit vinegar, in *Vinegars of the World*, ed. by Solieri L and Giudici P. Springer-Verlag Italia, Milan, pp. 223–242 (2009).
21 Morales ML, Tesfaye W, Garcia-Parrilla MC, Casas JA and Troncoso AM, Evolution of the aroma profile of sherry wine vinegars during an experimental aging in wood. *J Agric Food Chem* **50**:3173–3178 (2002).
22 Llaguno C, Definición y tipos de vinagre, in *El Vinagre de Vino*, ed. by Llaguno C and Polo MC. Consejo Superior de Investigaciones Científicas, Madrid, pp. 133–145 (1991).
23 Valero E, Berlanga T, Roldan P, Jimenez C, Garcia I and Mauricio JC, Free amino acids and volatile compounds in vinegars obtained from different types of substrate. *J Sci Food Agric* **85**:603–608 (2005).

