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Wine lees valorization: Biorefinery development including production of a generic fermentation feedstock employed for poly(3-hydroxybutyrate) synthesis

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ABSTRACT

This study demonstrates the development of a novel wine lees (WL) based integrated biorefinery for the production of several added-value products. WL were initially fractionated for the production of antioxidants, tartrate and ethanol and the remaining stream was converted into a fermentation nutrient supplement for poly(3-hydroxybutyrate) (PHB) production using the strain *Cupriavidus necator* DSM 7237. Hydrolysis of pretreated WL was carried out using crude enzyme consortia produced via solid state fermentation of *Aspergillus oryzae*. Optimization of hydrolysis was based on the enhancement of total Kjeldahl nitrogen to free amino nitrogen (FAN) conversion yield by evaluating the effect of the initial pH value, temperature, initial proteolytic activity and initial WL concentration. WL hydrolysates and crude glycerol were used as nutrient and carbon sources, respectively, in batch and fed-batch fermentations for the production of PHB. Bacterial growth and PHB production were influenced significantly by the FAN content of the WL derived hydrolysates and by the addition of trace elements. Using an initial FAN concentration of 700 mg L^{-1} and supplementation with trace elements led to the production of 30.1 g L^{-1} of PHB concentration with an intracellular content of 71.3% (w w^{-1}) and a productivity of 0.56 g L^{-1} h^{-1} during fed-batch fermentation.

1. Introduction

Wine making is one of the most important agricultural activities ranging to an approximate worldwide production of 280 million hectoliters (OIV, 2013). Wineries create large volumes of waste and by-product streams, including grape stalk, grape pomace or grape marc and wine lees, which should be recycled, valorized or treated before disposal to prevent environmental pollution. However, several wineries (mainly low-scale wine producers) do not always abide to legislation. This is the main reason that, until recently, waste generation by wineries was constantly increasing, causing environmental problems (Devesa-Rey et al., 2011).

Wine lees (WL), also mentioned as "heavy" or "light" lees (depending on the decanting step), are defined (EEC No. 337/ 79) as "*the residue that forms at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following the filtration or centrifugation of this product*" (Perez-Serradilla & Luque de Castro, 2011). WL represent 2–6% of the total volume of wine produced and it mainly contains ethanol, tartaric acid and yeast cells (Bai, Jin, Li, Chen, & Li, 2008; Naziri, Mantzouridou, & Tsimidou, 2012). Due to the high quantities produced worldwide, lees became an ideal raw material for commercial production of tartaric acid and ethanol (Versari, Castellari, Spinabelli, & Galassi, 2001; Braga, Lencart e Silva, & Alves, 2002). The high polyphenolic content of WL makes unsuitable its utilization as animal feed (Devesa-Rey et al., 2011).

WL have been utilized for the recovery of value-added products. Phenolic compounds were obtained using microwave-assisted extraction (Perez-Serradilla & Luque de Castro, 2011) or supercritical fluids (Wu et al., 2009), while the recovery of squalene from WL using ultrasound assisted extraction was also proposed (Naziri, Nenadis, Mantzouridou, & Tsimidou, 2014; Naziri et al., 2012). The presence of low molecular weight phenolic compounds (released free flavonol aglycones and pyranoanthocyanins), having functional and bioactive properties, was recently reported by Barcia et al. (2014). The WL lipid content, extractability, classification, fatty acid composition and main chemical characteristics have also been determined in order to evaluate the potential use of lees as food or food additive (Gomez, Igartuburu, Pando, Luis, & Mourente, 2004).

Abbreviations: WL, wine lees; PHB, poly(3-hydroxybutyrate); PHA, poly(hydroxyalkanoate); FAN, free amino nitrogen; SFM, sunflower meal; SSF, solid state fermentation; TKN, total Kjeldahl nitrogen.

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The use of WL as nutrient supplement in some fermentations has also been proposed. Bustos, Moldes, Cruz, and Dominguez (2004) used various lees (from red or white wines, distilled or untreated, from the first or second decanting step) as nutrient supplement for lactic acid production (105.5 g L^{-1}) using *Lactobacillus rhamnosus* cultivated on 100–110 g L^{-1} of glucose concentration as carbon source. Rivas, Torrado, Moldes, and Dominguez (2006) employed distilled WL that were used, after the removal of tartaric acid (TA), as nutrient supplement for lactic acid production by *Lactobacillus pentosus* using hemicellulosic vine shoot hydrolysates as carbon source, obtaining high production yields (0.7 g g^{-1}). Salgado, Rodriguez, Cortes, and Dominguez (2010) proposed a process where tartaric acid was recovered and the remaining WL was used as fermentation medium for xylitol production (33.4 g L^{-1}). Perez-Bibbins, Torrado-Agrasar, Perez-Rodriguez, Aguilar-Uscanga, and Dominguez (2014) employed WL (liquid, solid and whole fraction) as nitrogen source for the production of xylitol by *Debaryomyces hansenii*, using commercial xylose as carbon source. However, the aforementioned approaches do not take advantage of the full potential of WL. In a biorefinery approach, WL could be used for the production of ethanol, tartaric acid and antioxidants, while the remaining fraction enriched in yeast cells could be converted into a generic fermentation feedstock.

Poly(hydroxyalkanoates) (PHA) are biodegradable polyesters accumulated intracellularly as energy-reserve granules. They could be used in a wide range of applications such as packaging material in the food industry, medicine and agriculture (Philip, Keshavarz, & Roy, 2007) due to their unique properties (biodegradable, thermoplastic material, elastomer, insoluble in water, non-toxic and biocompatible) (Akaraonye, Keshavarz, & Ipsita, 2010). Nonetheless, industrial production of PHA is hindered by high production costs. Hence, research efforts are nowadays focused on the production of PHA from inexpensive raw materials. For this reason, crude glycerol, derived in high quantities as a by-product from the biodiesel industry, has been evaluated as a low-cost carbon source for PHA production (Ashby, Solaiman, & Strahan, 2011; Cavalheiro, de Almeida, Grandfils, & da Fonseca, 2009; Cavalheiro et al., 2012; Hermann-Krauss et al., 2013; Kachrimanidou et al., 2013, 2014; Mothes, Schnorpfeil, & Ackermann, 2007). PHA production in most of these studies was promising – e.g. Cavalheiro et al., 2009 reported the production of 38.1 g L^{-1} of PHB concentration – but the vast majority of studies supplement crude glycerol with commercial inorganic chemicals to supply the necessary nutrients for bacterial growth and maintenance. However, in the forthcoming bio-economy era, chemical and polymer production should be based entirely on renewable resources following a no-waste generation approach with minimum environmental impact.

Koutinas et al. (2014) presented the potential of waste streams from various industrial sectors as renewable feedstocks for chemical and biopolymer production through biorefinery development. Wheat has been utilized as a crude resource for the production of PHB and other co-products (Koutinas, Wang, & Webb, 2007; Xu, Wang, Koutinas, & Webb, 2010).

The main aim of this work was to develop a novel WL-based integrated biorefinery concept (Fig. 1) leading to the production of a nutrient-rich supplement for fermentative production of PHB with simultaneous production of ethanol, antioxidants and tartrate. The process begins with the separation of solid and liquid fractions via filtration. The liquid fraction is used for ethanol separation via distillation, while the remaining liquid is used in the enzymatic hydrolysis of yeast cells. The solids are used for the extraction of antioxidants and tartaric acid, while the remaining solids enriched in yeast cells are hydrolyzed by crude enzyme consortia produced via solid state fermentation (SSF) of *Aspergillus oryzae*. The crude nutrient-rich hydrolysate is supplemented with crude glycerol and is used as the sole renewable resource for the production of PHB (Fig. 1). The material balances of each fractionation process in the proposed WL biorefinery concept and the determination of antioxidants extracted from the solid stream will be presented in a forthcoming study. The present study focuses on WL hydrolysis optimization for the production of fermentation media for PHB production in shake flasks and bench-scale bioreactor. This is the first report implementing WL as nutrient supplement for the production of PHB with high efficiency.

2. Materials and methods

2.1. Microorganisms

SSF for the production of crude enzyme consortia were carried out using an industrial strain of *A. oryzae* (kindly provided by Professor Colin Webb, University of Manchester, UK). The strain was originally isolated and purified by Wang, Law, and Webb (2005) from a soy sauce industry (Amoy Food Ltd., Hong Kong). The fungal strain was maintained in slopes at 4 °C containing 30 g L^{-1} sunflower meal (SFM), 20 g L^{-1} wheat bran and 20 g L^{-1} agar.

The bacterial strain *Cupriavidus necator* DSM 7237 was used for the production of PHB. Inoculum was prepared (using stock cultures stored at 4 °C) in liquid media containing 10 g L^{-1} pure glycerol, 10 g L^{-1} yeast extract and 10 g L^{-1} peptone.

2.2. Raw materials used as fermentation media

SFM and crude glycerol were kindly provided by P. N. Pettas S.A. industry (Patras, Greece) as by-products of the biodiesel production process. The composition of crude glycerol was previously reported by Kachrimanidou et al. (2013, 2014). WL originated from the red wine making of Merlot grape variety, were kindly provided by the winery Ampelou Techni Theodoros Stavrakis (Tyrnavos, Greece).

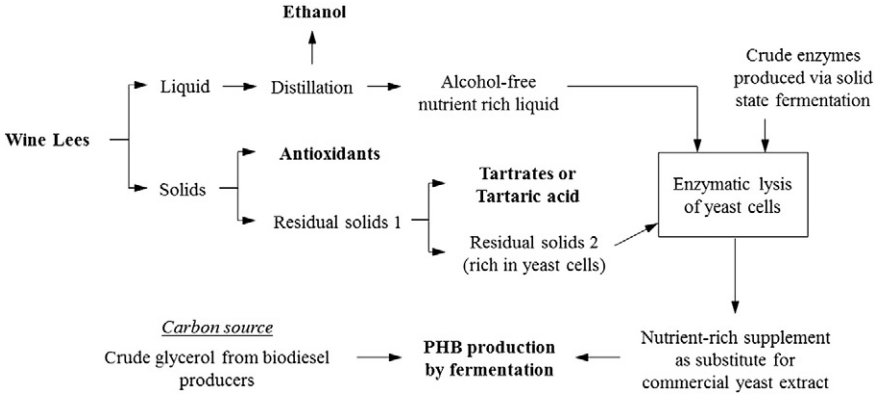

Fig. 1. Process flow sheet of WL-based biorefinery concept.

2.3. Solid state fermentation

SSFs were conducted in Erlenmeyer flasks (250 mL) that contained 5 g of SFM. All flasks were sterilized at 121 °C for 20 min prior to inoculation with a fungal spore suspension (2×10^6 spores mL^{-1}). The moisture content was adjusted at 65% (w w^{-1}, on a dry basis, db). All SSFs were incubated at 30 °C for 48 h.

2.4. WL fractionation

WL were treated to isolate value-added products, i.e. ethanol, phenolic compounds and tartrate. As it is shown in Fig. 1, the first step was the centrifugation of WL (15 min, 12,000 *g*, 10 °C) so as to separate the two phases for further processing. The supernatant was distilled generating an alcohol-free liquid, which was used as the liquid phase for hydrolysis of WL residual solids. The sediment (solids) was used for the separation of phenolic compounds according to the method described by Chira, Schmauch, Saucier, Fabre, and Teissedre (2009), which includes sequential extraction with acetone:H_2O (80:20, v/v) and methanol:H_2O (60:40, v/v) mixtures, followed by centrifugation (15 min, 12,000 *g*, 10 °C). Thereafter, the WL obtained (residual solids 1) were dissolved in 3.15 L H_2O per kg dry weight and treated with 0.361 L HCl per kg dry weight for 10 min to solubilize tartaric acid, as described by Salgado et al. (2010). Finally, after centrifugation (15 min, 12,000 *g*, 10 °C), the remaining WL solids (residual solids 2) were utilized for the hydrolysis process.

2.5. Production of WL hydrolysates

After the end of SSF, the fermented solids were suspended in the alcohol-free liquid, macerated using a kitchen blender and filtered via vacuum filtration in order to obtain the crude enzyme consortia extract, which was subsequently used in WL hydrolysis experiments. The enzyme-rich extract was added in 1 L Duran bottles that contained varying quantities of pretreated WL solids (residual solids 2) depending on the experiment. The Duran bottles were placed in a water bath at varying temperatures depending on the experiment. Four sets of experiments were carried out to evaluate the effect of temperature (35–55 °C), pH value (4.5–6.5), initial solid concentration (50–400 g L^{-1}) and initial proteolytic activity (12 and 24 U mL^{-1}), during the hydrolysis process of WL. Mixing of the suspension was achieved with magnetic stirrers. Samples were collected at random intervals and the solids were separated via centrifugation (10 min, 3000 *g*). The supernatant was used for the analysis of free amino nitrogen (FAN) and inorganic phosphorus (IP). Hydrolysis yield was expressed as the percentage of total Kjeldahl nitrogen (TKN) to FAN conversion.

After the end of hydrolysis, remaining solids were removed by vacuum filtration and WL hydrolysates were filter-sterilized using a 0.2 μm filter unit (Polycap ™ AS, Whatman Ltd.). The pH value of WL hydrolysates was adjusted to the optimum range (6.7–6.9) for *C. necator* growth with 5 M KOH.

2.6. Bacterial bioconversions

Bacterial fermentations were conducted at 30 °C and initial pH value in the range of 6.7–6.9. Shake flask fermentations were carried out in 250 mL Erlenmeyer flasks (50 mL broth volume) placed in a rotary shaker and agitated at 180 rpm, using 1 mL of pre-culture medium as inoculum. Crude glycerol initial concentration was ~25 g L^{-1}. WL hydrolysates with an initial FAN concentration of 200–500 mg L^{-1} were used as crude nutrient supplements.

Bioreactor fermentations were performed in a 1-L bioreactor (New Brunswick Scientific Co, USA) using a 10% (v/v) pre-culture medium as inoculum. Fermentation temperature was maintained at 30 °C, the aeration rate was maintained at a flow rate of 1 vvm and the pH value was regulated in the range of 6.7–6.9 using 5 M NaOH and 10% (v/v) H_2SO_4 solutions. The agitation speed was controlled in the range of 200–500 rpm in order to control the dissolved oxygen (DO) concentration in the bioreactor at 20% of saturation. Concentrated crude glycerol (75% v/v) solution was used as feeding media in fed-batch bioreactor fermentations, while the trace element solution contained: 1.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.15 g L^{-1} $FeCl_3 \cdot 6H_2O$, 0.02 g L^{-1} $ZnSO_4$, 0.06 g L^{-1} $MnSO_4$, and 0.15 g L^{-1} $CaCl_2 \cdot 2H_2O$. All bacterial fermentations were carried out in duplicate.

The WL hydrolysates that were used in all fermentations were produced after hydrolysis of WL solids (50–100 g L^{-1}) at 40 °C, using initial proteolytic activity of 6.4 U mL^{-1}.

The supernatant of the fermentation samples (obtained after centrifugation at 3000 *g* for 10 min) was analyzed for FAN, phosphorus, and glycerol determination. The sediment was washed with distilled water, centrifuged again and the remaining solids were suspended in acetone and transferred in pre-weighed 14 mL McCartney universal bottles for the determination of residual cell mass (RCM) and PHB concentration.

2.7. Analytical methods

Glycerol concentration was determined by High Performance Liquid Chromatography (HPLC) equipped with an Aminex HPX-87H column, coupled to a differential refractometer (RI). PHB concentration was determined by GC-FID using the method described by Riis and Mai (1988), with benzoic acid as internal standard. The operating conditions of HPLC and GC as well as the analytical methods employed for the determination of TKN, FAN and IP concentrations were described in a previous publication (Kachrimanidou et al., 2013). The total dry mass (TDM) was analyzed by drying the sediment from each fermentation sample at 50 °C until constant mass. The RCM was determined by subtracting the PHB concentration measured by GC from the TDM. All analyses were performed in triplicate.

3. Results and discussion

3.1. Optimization of WL hydrolysis

The present study investigates a new approach of WL valorization aiming to the production of fermentation feedstock with simultaneous separation of value-added products.

Organic acids have traditionally been applied to a wide variety of foods and are currently the most commonly used food preservatives (Theron & Lues, 2011). In particular, tartaric acid alone or mixed with other organic acids exhibits great antibacterial and antifungal properties (Eswaranandam, Hettiarachchy, & Johnson, 2004; Shokri, 2011). Taste masking technologies in oral pharmaceuticals are another industrial field that tartaric acid finds application as a taste improver (Sohi, Sultana, & Khar, 2004). Still, one of the most common tartaric acid applications is its usage as an acidity regulator (E334) in various foodstuffs (e.g. bakery, candies, jams, juices and the wine industry itself) (Council Directive No. 95/2/EC).

The high amounts of phenolic compounds that remain in winery by-products streams and the positive correlation between the antioxidant activity and the total phenolic compounds present in winery waste streams have already been previously reported (Alonso, Guillιn, Barroso, Puertas, & Garcνa, 2002). These phenolic compounds, well-known as natural antioxidants, are reported to be effective as anti-inflammatory, anti-carcinogenic, anti-mutagenic, anti-ulcer, anti-atherogenic, anti-microbial agents and as the inhibitors of human low density lipoprotein oxidation (Folts, 2002; Shrikhande, 2000).

Ethanol is an important product for either the food sector or as platform molecule with significant importance for sustainable chemical production (Sun & Wang, 2014).

From the aforementioned facts it becomes clear that any attempt to valorize WL should take into account, at least, those three major

important ingredients. Hence, our approach was initially focused on the extraction of these value-added products, prior to any further treatment of the remaining WL solids. In the frame of a holistic biorefinery concept, the remaining WL solids were subsequently hydrolyzed in order to produce nutrient rich fermentation media. The effect of initial pH value, temperature, initial proteolytic activity and initial solid concentration during the enzymatic hydrolysis of WL was evaluated and optimized.

3.1.1. Effect of pH value on WL hydrolysis

It is well known that the pH value is of great importance in enzymatic reactions, as it can affect their activity by changing the charges on an amino acid residue which is functional in substrate binding or catalysis (Sharma & Tripathi, 2013). The effect of pH value on WL hydrolysis yield was evaluated by conducting experiments at pH values in the range of 4.5–6.5. Comparable TKN to FAN conversion yields were achieved at pH values in the range of 4.5–5.5, while hydrolysis yield was gradually decreased at pH values from 5.5 to 6.5 (Fig. 2). The pH value of 5.5 was determined as the optimum for WL hydrolysis, as an overall TKN to FAN conversion yield of 33% was obtained. To our knowledge, there is no literature regarding WL hydrolysis, although several studies report the usage of commercial or crude enzymes that have been applied in hydrolyses of various by-products. In particular, hydrolysis of sunflower meal by crude enzymes produced by the same strain of *A. oryzae* employed in this study showed that the highest FAN production was achieved when the pH value was uncontrolled (Kachrimanidou et al., 2013).

The differences reported in literature-cited publications regarding the optimum pH value of proteolytic enzymes could be explained by the action of different types of proteases. Kumura, Ishido, and Shimazaki (2011) fractionated a crude enzyme complex produced by *A. oryzae* on a medium containing a whey protein isolate and revealed the presence of acid as well as alkaline proteases. Further investigation of the degradation profile of whey protein hydrolysis by these fungal proteases showed that a diversity of proteases emerged depending on the optimum pH value, temperature and cleavage sites of protein.

3.1.2. Effect of temperature and initial enzyme activity on WL hydrolysis

The effects of temperature (in the range of 35–55 °C) and initial enzyme activity (12 and 24 U mL^{-1}) on WL hydrolysis were simultaneously investigated conducting experiments using 100 g L^{-1} initial solid concentration and pH value of 5.5. As it is depicted in Fig. 3, the hydrolysis yield was increased to a maximum at 40 °C and then abruptly declined with further increase of temperature, indicating

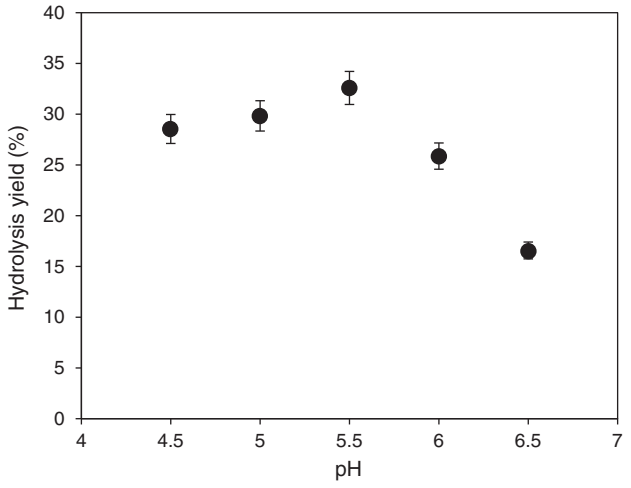

Fig. 2. Effect of pH value on hydrolysis yield (percentage of TKN to FAN conversion) of WL using an initial solid concentration of 100 g L^{-1} after 24 h reaction at 40 °C using 12 U mL^{-1} of initial proteolytic activity.

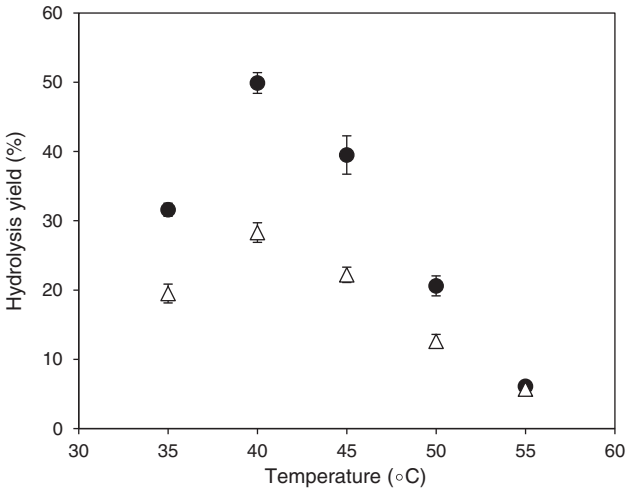

Fig. 3. Effect of temperature (35–55 °C) and initial proteolytic activity – (△) 12 U mL^{-1} and (●) 24 U mL^{-1} – on hydrolysis yield (percentage of TKN to FAN conversion) of WL using an initial solid concentration of 100 g L^{-1} after 24 h of reaction.

that temperatures higher than 40 °C increase the deactivation of proteases leading to a significant decrease of FAN production. The optimum temperature observed in the present study complies with the optimum temperature that was reported in the case of rapeseed meal hydrolysis using crude enzymes produced by the same strain of *A. oryzae* (Wang et al., 2010). However, many differences regarding the optimum temperature of proteases are reported in the literature. This occurs because hydrolysis of crude renewable resources is strongly dependent on the concerted action of various enzymes (Kachrimanidou et al., 2013). Wang et al. (2009) reported that the maximum activity of proteolytic enzymes produced by the same strain of *A. oryzae* was observed at 55 °C. However, temperatures higher than 45 °C led to lower protein hydrolysis when crude enzymes from the same fungal strain of *A. oryzae* were used in SFM hydrolysis experiments (Kachrimanidou et al., 2013).

Regarding the initial proteolytic activity that was used during the WL hydrolysis experiments, as it can be observed in Fig. 3, a two-fold increase of the initial proteolytic activity (24 U mL^{-1}) resulted to an almost proportional increase of hydrolysis yield.

3.1.3. Effect of initial solid concentration on WL hydrolysis

The evaluation of the effect of initial solid concentration on WL hydrolysis was performed at pH value 5.5 and at 40 °C using an initial enzyme activity of 12 U mL^{-1}. Fig. 4 shows that the hydrolysis yield was maintained above 30% at initial solid concentrations up to 100 g L^{-1}, while it decreased significantly using initial solid concentrations higher than 100 g L^{-1} (e.g. 8% at 400 g L^{-1} initial solid concentration). Similar trend has been reported in the case of SFM hydrolysis (Kachrimanidou et al., 2013). More specifically, the highest protein hydrolysis yield was around 40%, which was decreased at initial SFM concentrations higher than 100 g L^{-1}. Higher hydrolysis yield (43.4%) has been observed in the case of 100 g L^{-1} defatted peanut flour hydrolysis by crude protease extract produced by a fungal strain of *A. oryzae* (Su, Ren, Yang, Cui, & Zhao, 2011).

Fig. 5 presents the production of FAN during hydrolysis of 50 and 100 g L^{-1} WL using initial proteolytic activity of 12 and 24 U mL^{-1}. It is evident that increased initial enzyme activity results to higher FAN production. It is worth noting that FAN production was almost 50% higher during the hydrolysis of 100 g L^{-1} of WL, using 2-fold higher initial proteolytic activity (24 U mL^{-1}). The FAN concentration produced was equivalent to a 24 g L^{-1} liquid medium of yeast extract, which contains around 50 mg FAN per g of yeast extract. In the same manner, a maximum FAN concentration of 1500 mg L^{-1} was achieved

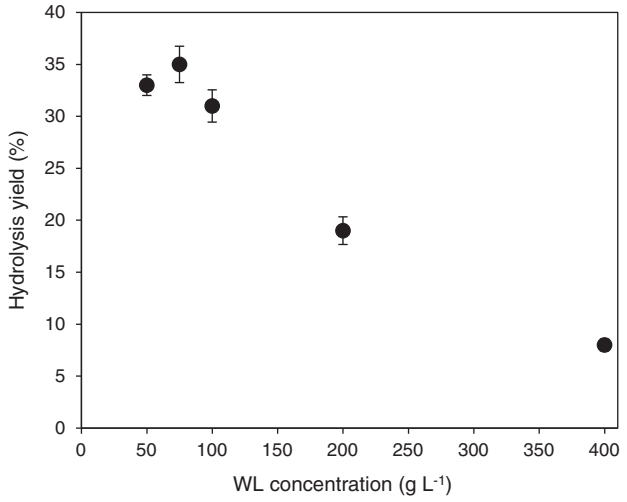

Fig. 4. Effect of initial WL solid concentration (50–400 g L^{-1}) on hydrolysis yield (percentage of TKN to FAN conversion) after 24 h of reaction at 40 °C, pH value 5.5 and 12 U mL^{-1} of initial proteolytic activity.

during the hydrolysis of 90 g L^{-1} of initial concentration of SFM (Kachrimanidou et al., 2013). Hydrolysis of rapeseed meal resulted in the production of 2087.2 mg L^{-1} of FAN concentration, while the TKN to FAN conversion yield was 54% (Wang et al., 2010).

Yeast cell lysis is usually achieved using a mixture of various enzymes, including glucanases, proteases, mannanases and chitinases, which act synergistically in order to achieve efficient cell wall lysis (Salazar & Asenjo, 2007). Scott and Schekman (1980) reported the necessity for synergy of glucanases and proteases. Hence, WL hydrolysis should have been also carried out by a crude enzyme consortium. The fungal strain of *A. oryzae* used in this study can produce a variety of enzymes, including proteases, phosphatases, lipases, glucanases, amylases, and invertases among others (Kachrimanidou et al., 2013).

3.2. Fermentations for PHB production

There are a few studies dealing with PHB production using agro-industrial hydrolysates as nitrogen source (Kachrimanidou et al., 2014; Koller et al., 2005a, 2005b; Obruca, Benesova, Oborna, & Marova, 2014; Page & Cornish, 1993) but this is the first report focusing on PHB production from WL hydrolysates.

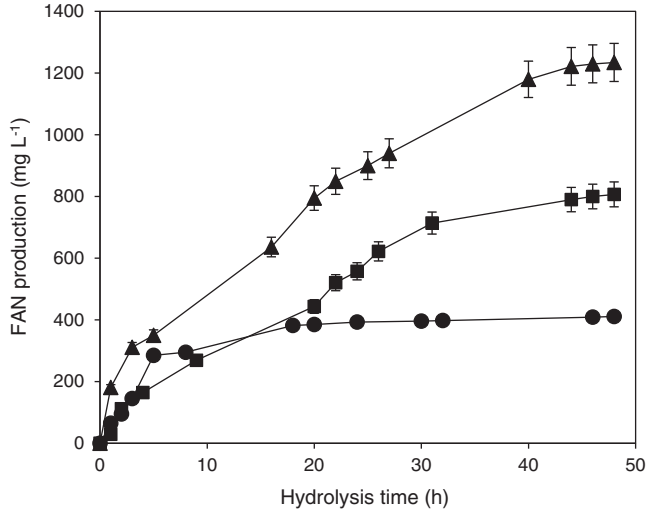

Fig. 5. FAN production during hydrolysis of 50 g L^{-1} and 100 g L^{-1} WL using 12 U mL^{-1} and 24 U mL^{-1} of initial proteolytic activity performed at 40 °C and pH value of 5.5. (●) 50 g L^{-1} WL and 12 U mL^{-1} proteolytic activity; (■) 100 g L^{-1} WL and 12 U mL^{-1} of proteolytic activity; (▲)100 g L^{-1} WL and 24 U mL^{-1} of proteolytic activity.

Table 1
Production of microbial mass and PHB during shake flask fermentations using crude glycerol and WL hydrolysates as carbon and nitrogen source respectively.

Initial glycerol (g L^{-1})	Initial FAN (mg L^{-1})	TDM (g L^{-1})	RCM (g L^{-1})	PHB (g L^{-1})	PHB content (%)
25	200	7.6	2.5	5.1	67
25	300	11.1	5.3	5.8	52
25	400	11.9	6.2	5.7	48
25	500	10.5	5.5	5.0	47

Bacterial shake flask fermentations using *C. necator* DSM 7237 were carried out using crude glycerol from a biodiesel production plant and WL hydrolysates with different initial FAN concentrations (ranging from 200 to 500 mg L^{-1}). Table 1 shows that the use of high FAN concentrations resulted to lower PHB content. A similar result has been also observed by Koutinas et al. (2007) during fermentation of *C. necator* on wheat hydrolysates. The maximum PHB concentration (5.8 g L^{-1}) was achieved when initial FAN concentrations of 300 and 400 mg L^{-1} were used. The highest PHB content (67%, w w^{-1}) was achieved on FAN concentration of 200 mg L^{-1}. Shake flask experiments showed that WL hydrolysates can be successfully utilized as nutrient supplement for fermentative production of PHB.

Fermentations were also carried out in fed-batch bioreactor mode, aiming to optimize the addition of WL hydrolysate at the beginning of fermentation. The initial glycerol concentration was maintained constant in all fermentations and optimization was focused on the initial FAN content. WL hydrolysates with different FAN concentrations were produced via enzymatic hydrolysis and tested in bacterial fermentations regarding their effect on PHB accumulation. Table 2 presents the results from five bioreactor fermentations operated in fed-batch mode under varying initial FAN concentrations, with or without addition of trace elements. All presented parameters correspond to the fermentation time when the maximum PHB concentration was obtained, while the initial glycerol concentration was around 25 g L^{-1} in all fermentations.

PHB production, in the absence of minerals, reached a maximum concentration of 10.7 g L^{-1} with a productivity of 0.23 g L^{-1} h^{-1} when an initial FAN concentration of 700 mg L^{-1} was used (Fig. 6). This fermentation can be divided into two distinct phases. The first phase, where microbial proliferation takes place, lasts for approximately 8–9 h until IP is completely depleted from the fermentation broth. The second phase that begins after complete IP consumption is associated with PHB accumulation. The same trend is also observed in Fig. 7, indicating that PHB accumulation occurs due to phosphorus limitation. The results presented in Fig. 7 demonstrate that the addition of trace elements resulted to a significant increase of PHB accumulation. Addition of trace elements led to a final PHB concentration of 30.1 g L^{-1}, an intracellular PHB content of 71.3% (w w^{-1}) and a productivity of

Table 2
Effect of different FAN concentrations on *C. necator* fed-batch bioreactor fermentation using WL hydrolysates and crude glycerol (~25 g L^{-1}) as nitrogen and carbon source, respectively.

FAN (mg L^{-1})	*t* (h)	TDM (g L^{-1})	RCM (g L^{-1})	PHB (g L^{-1})	PHB content (%)	Productivity (g L^{-1} h^{-1})
300	41	5.3	3.4	1.9	35.8	0.05
500	47	10.6	6.5	4.1	38.7	0.09
700	46	25.8	15.1	10.7	41.5	0.23
700[a]	54	42.2	12.1	30.1	71.3	0.56
950[a]	70	26.3	9.8	16.5	62.7	0.24

[a] With the addition of trace elements.

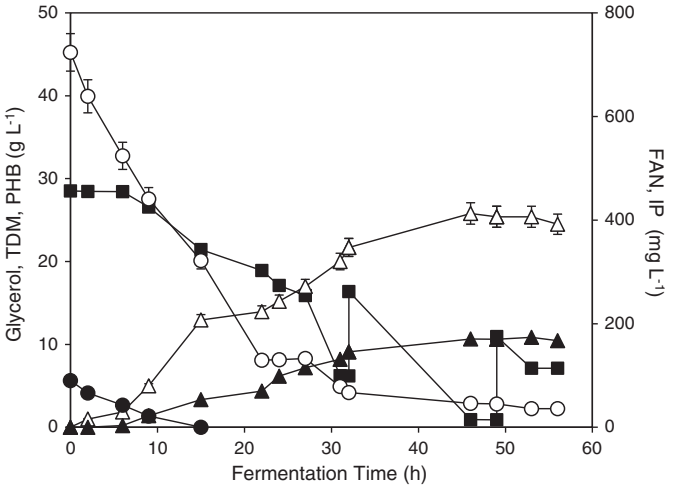

Fig. 6. Consumption of (○) FAN, (■) glycerol and (●) IP as well as production of (△) TDM and (▲) PHB during fed-batch bioreactor fermentation of *C. necator* on WL hydrolysate and crude glycerol without addition of minerals in the medium.

0.56 g L^{-1} h^{-1}. The intracellular PHB content and productivity achieved in this study are among the highest reported in the literature when glycerol and renewable resources are used as the sole fermentation feedstocks. The highest intracellular content of PHAs (75.4% co-polymer) produced by biodiesel industry by-products was recently reported by Hermann-Krauss et al. (2013). The highest productivity using crude glycerol as carbon source (but with commercial nutrients as nitrogen source) was reported by Cavalheiro et al. (2009).

4. Conclusions

Food supply chain wastes could be used as raw material for the production of fuels, chemicals, biopolymers, food additives and value-added products. Biorefining of food supply chain waste could lead to the development of sustainable processes. This study showed that wine lees could be used for the production of nutrient-rich fermentation supplements for highly efficient PHB production after the extraction of value-added components with diversified market outlets. Synergistic utilization of renewable resources, as presented in this study with the utilization of crude glycerol and wine lees derived hydrolysates, could lead to highly efficient bioprocesses.

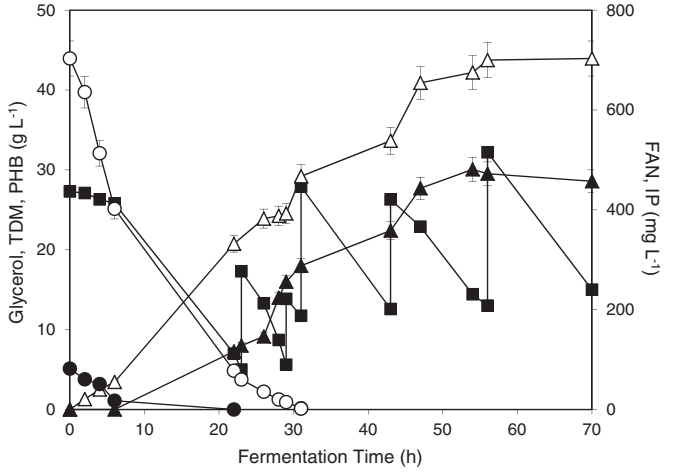

Fig. 7. Consumption of (○) FAN, (■) glycerol and (●) IP as well as (△) TDM and (▲) PHB during fed-batch bioreactor fermentation of *C. necator* on WL hydrolysate and crude glycerol with addition of minerals in the medium.

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