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Evaluation of by-products from the biodiesel industry as fermentation feedstock for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) production by *Cupriavidus necator*

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HIGHLIGHTS

- Valorisation of biodiesel industry by-products for PHA production.
- Replacement of commercial carbon sources and nutrient supplements.
- Replacement of precursors for co-polymer production.
- Influence of salt impurities concentration on PHA production.
- Analysis of thermophysical properties of the produced PHAs.

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ABSTRACT

Utilization of by-products from oilseed-based biodiesel production (crude glycerol, rapeseed meal hydrolysates) for microbial polyhydroxyalkanoate (PHA) production could lead to the replacement of expensive carbon sources, nutrient supplements and precursors for co-polymer production. Batch fermentations in shake flasks with varying amounts of free amino nitrogen led to the production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)) with a 2.8–8% 3HV content. Fed-batch fermentations in shake flasks led to the production of 10.9 g/L P(3HB-*co*-3HV) and a 55.6% P(3HB-*co*-3HV) content. NaCl concentrations between 2 and 6 g/L gradually became inhibitory to bacterial growth and PHA formation, whereas in the case of K_2SO_4, the inhibitory effect was observed only at concentrations higher than 20 g/L. Differential scanning calorimetry (DSC), thermogravimetric analysis (TGA) and nuclear magnetic resonance (^{13}C NMR) demonstrated that the incorporation of 3HV into the obtained P(3HB-*co*-3HV) lowered glass transition temperature, crystallinity and melting point as compared to polyhydroxybutyrate. Integrating PHA production in existing oilseed-based biodiesel plants could enhance the viability and sustainability of this first generation biorefinery.

1. Introduction

Most processes for biodiesel production generate significant quantities of by-products. For instance, the utilization of oilseeds as raw materials leads to the production of a protein-rich oilseed meal and a crude glycerol stream. Valorisation of oilseed meals and crude glycerol into various products including chemicals, biodegradable polymers, value-added ingredients (e.g. extracts with antioxidant properties), food and feed would improve the economics of biodiesel production. Crude glycerol is a platform chemical that could be converted into different chemicals through chemical synthesis or fermentation (Koutinas et al., 2007).

Polyhydroxyalkanoates (PHAs) are a family of biodegradable polymers produced as intracellular energy-reserve granules during fermentation by more than 300 microorganisms including *Cupriavidus necator* (Lee, 1996; Choi et al., 1998). The microbial production of PHAs by *C. necator* is mainly based on the limitation of a nutritional element such as N, P, Mg, K, O, or S in the presence of an abundant source of carbon. PHAs can be used as substitutes

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for petroleum-derived polymers in various commercial applications such as food packaging, biocomposite production, adhesives, medical purposes, agriculture and flushable hygiene products (Wolf et al., 2005). Polyhydroxybutyrate (PHB), the most well-known member of the PHA family, is a homopolymer of 3-hydroxybutyric acid units. Apart from PHB, research has also focused on the production of different copolymers such as P(3HB-*co*-3HV), a copolymer of 3-hydroxybutyric acid (3HB) and 3-hydroxyvaleric acid (3HV) units at various proportions (Lee et al., 2008; Salim et al., 2011).

PHAs can be produced by various commercial carbon sources such as carbohydrates (e.g. glucose, fructose, sucrose, lactose), methanol, alkanes (i.e. hexane to dodecane), vegetable oils and short and long chain fatty acids (e.g. butyrate upwards) (Wolf et al., 2005; Castilho et al., 2009; Koller et al., 2010). One of the major problems that hinder industrial PHA production is the cost of the carbon source and fermentation media formulation in general. For this reason, research aimed at the utilization of agricultural or industrial waste and byproduct streams for PHA production (Koller et al., 2010). Crude glycerol has been tested as fermentation feedstock for the production of PHB by *C. necator* DSM 545 (Cavalheiro et al., 2009; Mothes et al., 2007). Cavalheiro et al. (2012) reported on the production of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate-*co*-3-hydroxyvalerate) by *C. necator* DSM 545 cultivated on crude glycerol and 3HV/4HB precursors (i.e. propionic acid and γ-butyrolactone).

Oilseed meal could be converted into a nutrient-rich hydrolysate that could be used as fermentation media supplement. Wang et al. (2010) reported the production of hydrolysates from rapeseed meal that contained up to 2061.2 mg/L free amino nitrogen (FAN), 304 mg/L inorganic phosphorus (IP) and 1.8 g/L glucose by employing crude enzyme-rich extracts from solid state fermentations of *Aspergillus oryzae*. Crude hydrolysates or commercial formulations rich in amino acids and peptides have been used as nutrient supplements in microbial bioconversions for PHB production (Bormann et al., 1998; Lee et al., 1995; Xu et al., 2010). The utilization of wheat-derived hydrolysates enriched in glucose and various sources of nutrients (e.g. amino acids, peptides, phosphorus) led to the production of 162.8 g/L PHB (Xu et al., 2010). By using specific amino acids or their mixtures, PHB production by various microorganisms was enhanced (Fujita et al., 1993; Lee and Chang, 1994; Lee et al., 1995), and utilization of specific amino acids may lead to the production of P(3HB-*co*-3HV) (Kimura et al., 2003).

The present study explored the production of PHAs, including P(3HB-*co*-3HV), by *C. necator* DSM 545 using rapeseed meal hydrolysates and crude glycerol as nutrient supplements and main carbon source, respectively.

2. Methods

2.1. Microorganisms

An industrial strain of *A. oryzae* isolated from a soy sauce starter at the company Amoy Food, Ltd. (Hong Kong), kindly provided by Professor Colin Webb (Satake Centre for Grain Process Engineering, University of Manchester, UK), was utilized in solid state fermentations to produce crude enzymes essential for rapeseed meal hydrolysis. The isolation and purification of this fungal strain was reported by Wang et al. (2005). Fungal spores were maintained at 4 °C on slants containing 30 g/L rapeseed meal, 15 g/L wheat bran and 20 g/L agar.

Submerged fermentations for PHA production were carried out with *C. necator* DSM 545, a glucose consuming mutant of *C. necator* DSM 529. Since preliminary shake flask fermentations showed that the original bacterial strain was consuming glycerol at a very low rate (approximately 0.2 g/L per h of shake flask fermentation) when the initial glycerol concentration was 10 g/L, successive cultivations in shake flasks at gradually increasing glycerol concentrations (5–25 g/L) were carried out. After successive cultivations, the glycerol consumption rate by *C. necator* was improved significantly (more than 0.3 g/L per h of shake flask fermentation). In addition, glycerol was entirely consumed even at initial glycerol concentrations higher than 20 g/L. Bacterial stock cultures were stored at 4 °C in petri dishes containing 10 g/L glycerol, 10 g/L yeast extract, 5 g/L peptone and 20 g/L agar. Inoculums for shake flask and bioreactor fermentations were prepared by transferring bacteria cells into liquid medium containing 10 g/L glycerol, 10 g/L yeast extract and 5 g/L peptone.

2.2. Raw materials used as fermentation media

Crude glycerol, kindly provided by ADM Industries (Germany), contained 81% glycerol, 10–12% water, 5–6% potassium salts, 1% free fatty acids, and <1% methanol. Rapeseed (*Brassica napus*) meal a by-product of biodiesel production, contained 9.4% moisture, 57.9 mg/g (on a dry basis, db) total Kjeldahl nitrogen (TKN), 36.2% protein (db, 6.25 × TKN) and 7.9% ash.

2.3. Solid state fermentation

Solid state fermentations (SSF) of rapeseed meal were carried out in 250-mL Erlenmeyer flasks at 30 °C. Each flask, containing 5 g of rapeseed meal, was sterilised at 121 °C for 20 min prior to SSF. The moisture content of all flasks, after inoculation, was adjusted to 65% (w/w, db).

A suspension of *A. oryzae* spores was used as fermentation inocula. To increase the concentration of fungal spores, *A. oryzae* was cultivated on solid medium (containing 30 g/L rapeseed meal, 15 g/L wheat bran and 20 g/L agar) in 250-mL Erlenmeyer flasks. Sterile tap water (10 mL) was aseptically transferred into the agar slant tube containing the fungal spores and a spore suspension was formed by scrapping the surface of the slant with a wire loop. Subsequently, 1 mL of this spore suspension was transferred to solid medium (30 g/L rapeseed meal, 15 g/L wheat bran and 20 g/L agar) in a 250-mL Erlenmeyer flask. These flasks were incubated at 30 °C for 5 days or until complete sporulation on the surface of the flask. The inoculum for SSF was formulated by adding 50 mL of sterilised tap water and a few glass beads (4 mm diameter) into each flask containing fungal spores, followed by vigorous shaking until a fungal suspension was formed. The spore concentration employed was approximately 2×10^6 spores/mL.

To produce a nutrient-rich supplement for subsequent PHA fermentations, solids were collected after 44 h of SSF and used as source of crude enzymes (e.g. proteolytic enzymes) for the hydrolysis of rapeseed meal macromolecules.

2.4. Production of rapeseed meal hydrolysate

Rapeseed meal hydrolysates were produced in 1-L Duran bottles by mixing the remaining solids from SSF with 50 g/L (db) of fresh rapeseed meal suspension in tap water. Duran bottles were placed in a 55 °C water bath and agitated using magnetic stirrers. At the end of the reaction (approximately 24 h), the remaining solids were removed by vacuum filtration (Whatman No. 2). Rapeseed meal hydrolysates were filter sterilised using a 0.2-μm filter unit (Polycap™ AS, Whatman Ltd.). The pH of rapeseed meal hydrolysates was adjusted to the optimum pH range (6.7–6.9) for *C. necator* growth with 5 M NaOH.

2.5. Bacterial bioconversions in shake flasks

Bacterial fermentations in 250-mL Erlenmeyer shake flasks (50 mL broth volume) were carried out in an orbital shaker (ZHWY-211C Series Floor Model Incubator, PR China) at 180 rpm, 30 °C and initial pH in the range of 6.7–6.9. The pH during shake flask fermentations was re-adjusted manually with 5 M NaOH when each sample was taken. Inoculums of 1 mL were used for each flask. Each shake flask fermentation was repeated three times.

Five shake flasks were prepared with fermentation media that contained rapeseed meal hydrolysates with initial FAN concentrations in the range of 170 and 460 mg/L and crude glycerol with initial concentration of 25 g/L. Two shake flask fermentations were supplemented with mineral medium and a sterile stock solution of trace elements (1 mL), respectively. The composition of both mineral medium and stock solution of trace elements were taken from Kim et al. (1994). Another two shake flask fermentations were carried out at the best FAN concentration (460 mg/L) with initial glycerol concentrations of 9 and 16 g/L.

In a second set of batch shake flask fermentations, media were prepared with initial crude glycerol concentration of 25 g/L, an initial FAN concentration of 400 mg/L and initial concentrations of NaCl or K_2SO_4 of 2, 6, 10, 15, 20, and 25 g/L.

Shake flask fermentations were also carried out in fed-batch mode to identify the effect of rapeseed meal hydrolysate and crude glycerol addition. Two fed-batch fermentations were conducted in shake flasks with the same initial glycerol (21 g/L) and FAN (400 mg/L) concentrations. A third shake flask fermentation was carried out in batch mode under the same medium. A trace element solution of 1 mL was employed in all shake flasks. The initial volume in all shake flasks was 40 mL. The first two shake flasks were fed with the same crude glycerol solution and a concentrated rapeseed meal hydrolysate solution. The initial rapeseed meal hydrolysate was concentrated six times with a rotary evaporator (Buchi rotavapor R-114) in order to increase the FAN concentration. Rapeseed meal hydrolysate was added at 48 h and crude glycerol was added at 58 and 72.5 h.

2.6. PHA extraction

PHA extraction was carried out by the dispersion-extraction method reported by Hahn et al. (1994). Cells were harvested by centrifugation at 3000*g* for 10 min. Subsequently, 30 mL of chloroform and 30 mL of sodium hypochlorite (30%, v/v in water) were used per gram of dried cell mass. This mixture was treated at 30 °C for 90 min in an orbital shaker at 150 rpm. The mixture was centrifuged at 3000*g* for 15 min and three distinct phases were obtained. The upper phase was the hypochlorite solution, the middle phase contained non-PHA cell materials and the bottom phase was chloroform containing PHA. The upper phase was carefully removed with a pipette and the chloroform phase was obtained by filtration (Whatman No. 1 filter paper, VWR Scientific, USA) of the two remaining phases. PHA was recovered by precipitation using 10 volumes of ice-cold methanol.

2.7. Nuclear magnetic resonance (NMR)

NMR spectra were recorded with a BRUCKER 400 NMR spectrometer at 125 MHz (^{13}C NMR) using deuterated chloroform ($CDCl_3$) as solvent. For each analysis, 10 mg of biopolymer sample and 1 mL of solvent were employed.

2.8. Differential scanning calorimetry (DSC)

Thermal properties of PHAs were determined by DSC using a PERKIN-ELMER PYRIS 1 calorimeter calibrated with indium and *n*-heptane standards. Samples (around 10 mg) were heated from 25 °C to 190 °C, cooled to 25 °C and heated again to 190 °C at a rate of 10 °C/min. Runs for determining glass transition temperatures (T_g) were carried out between −50 °C and 50 °C by using liquid nitrogen as cooling agent and helium as purging gas.

2.9. Thermogravimetric analysis (TGA)

Thermal stability of samples was determined with a thermogravimetric analyzer (PERKIN ELMER II). Samples were heated from 30 to 500 °C at a constant heating rate of 10 °C/min under a nitrogen atmosphere.

2.10. Analytical methods

Samples from bacterial fermentations were centrifuged at 3000*g* for 10 min (Hettich Universal 320-R, Germany). The supernatant was used to determine glycerol and FAN concentrations. The solid sediment was washed with distilled water and centrifuged at 3000*g* for 10 min. The solids were suspended in acetone and transferred into 14-mL McCartney universal bottles. Total dry weight (TDW) was measured after drying the solids at 50 °C and cooling in a desiccator until a constant weight was obtained. Residual microbial biomass (RMB) concentration was determined by substracting PHA concentration measured by GC analysis from TDW.

Glycerol concentration was determined with a high performance liquid chromatograph (HPLC, Waters 600E) equipped with an Aminex HPX–87H (300 mm × 7.8 mm, Bio Rad, CA) column coupled to a differential refractometer (RI waters 410). Operating conditions were as follows: sample volume 40 μL; mobile phase 0.005 M H_2SO_4; flow rate 0.6 mL/min; column temperature 65 °C. Before injection, samples were diluted to appropriate concentration with deionized water and filtered through a 0.45-μm membrane filter.

Free amino nitrogen (FAN) concentration in liquid samples was determined by the ninhydrin colorimetric method promulgated in the European Brewery Convention (Lie, 1973). Total Kjeldahl nitrogen (TKN) concentration was measured using a KjeltekTM 8100 distillation Unit (Foss, Denmark). Ash content was determined following the AACC Approved Method 08-01.

PHA concentration was determined by the chromatographic method described by Riis and Mai (1988) using benzoic acid as internal standard. A gas chromatographic analyzer (Fisons 8060) equipped with a Flame Ionization Detector (FID) and a Chrompack column (60 m × 0.25 mm, film thickness 0.25 μm, J&W Scientific) was used for PHA concentration measurements. Helium was used as carrier gas at a flow rate of 2 mL/min. The oven temperature was set initially at 120 °C for 1 min, ramped at 10 °C/min to 200 °C, held constant for 5 min, and increased to 220 °C at 10 °C/min with a final isothermal period of 3 min. The injector and detector temperatures were 230 °C. The appearance of each PHA monomer (propyl-esters of 3HB and 3HV) in the chromatogram was confirmed based on the retention time of respective monomers from commercial PHB and P(3HB-*co*-3HV) standards (Sigma).

3. Results and discussion

3.1. Shake flask fermentations

Five shake flask fermentations (Table 1) were carried out using crude glycerol and rapeseed meal hydrolysates to obtain varying initial FAN concentrations (170, 240, 320, 400 and 460 mg/L) and 25 g/L initial glycerol concentration. The total dry weight (TDW), residual microbial biomass (RMB), PHA, final glycerol

Table 1
Shake flask fermentations of *C. necator* using media derived from by-products of biodiesel production (all values represent the mean of three replicates).

Initial glycerol (g/L)	Initial FAN[a] (mg/L)	T_F[b] (h)	Total dry weight (g/L)	Residual microbial biomass (g/L)	PHA[c] (g/L)	3HB[d] (mol%)	3HV[e] (mol%)	PHA[c] content (%)	Final glycerol (g/L)	PHA[c] yield (g/g)[f]
Rapeseed meal hydrolysate										
25	170	64	5.1	3.7	1.4	92	8	27.4	12.4	0.11
25	240	62	5.3	3.9	1.4	92.5	7.5	27.4	10.4	0.09
25	320	60	7.4	5.2	2.2	95.9	4.1	29.7	1.3	0.09
25	400	74	10.1	5.9	4.2	96.9	3.1	41.5	0.4	0.17
25	460	74	15.1	8.1	7	97.2	2.8	46.3	0.2	0.28
16	460	48	12.5	7.3	5.2	96.8	3.2	41.6	0.2	0.32
9	460	28	8.2	5.1	3.1	96.5	3.5	37.8	0.2	0.34
Rapeseed meal hydrolysate supplemented with trace elements[g]										
25	400	74	11.4	5.5	5.9	96.4	3.6	51.8	0.2	0.24
Rapeseed meal hydrolysate supplemented with mineral medium[g]										
25	400	57	13.7	7.9	5.8	98.5	1.5	42.6	0.2	0.23

[a] Free Amino Nitrogen.
[b] Final fermentation time.
[c] Polyhydroxyalkanoate.
[d] 3-Hydroxybutyrate content.
[e] 3-Hydroxyvalerate content.
[f] g PHA produced per g glycerol consumed.
[g] Kim et al. (1994).

concentration and glycerol to PHA conversion yield presented in Table 1 were determined when glycerol was completely consumed or when glycerol consumption was prematurely stopped due to a likely depletion of other nutrients (e.g. FAN). Glycerol consumption stopped only at initial FAN concentrations of 170 and 240 mg/L (Table 1). It is obvious that rapeseed meal hydrolysates can be used as nutrient supplements to achieve microbial growth and P(3HB-*co*-3HV) intracellular accumulation. Total dry weight, RMB and PHA concentration and content increased when initial FAN concentrations up to 460 mg/L were used. Since the initial glycerol concentration was constant, these results show that rapeseed hydrolysate provide carbon sources (*e.g.* amino acids) for bacterial growth and PHA accumulation. The highest total dry weight (15.1 g/L), PHA concentration (7 g/L) and PHA content (46.3%) were achieved at an initial FAN concentration of 460 mg/L. FAN concentrations lower than approximately 250 mg/L resulted in incomplete glycerol consumption, whereas concentrations higher than 500 mg/L resulted in sufficient microbial growth but significantly reduced or even negligible PHA accumulation (data not shown).

Table 1 shows that rapeseed cake hydrolysates and crude glycerol led to the production of P(3HB-*co*-3HV) without addition of precursors for the production of 3HV monomer. The final 3HV content produced in each fermentation carried out at a constant glycerol concentration (25 g/L) declined with increasing FAN concentrations. In addition, the molar percentage of 3HV in the biopolymer decreased during fermentation (Fig. 1). For instance, at an initial FAN concentration of 170 mg/L, the 3HV content at 10 and 64 h of fermentation were 12.3 mol% and 8 mol%, respectively. When the initial FAN concentration was 400 mg/L, the 3HV content at 10 and 74 h of fermentation were 9.2 mol% and 3.1 mol%, respectively. Glycerol and most amino acids lead to 3HB production, while only certain amino acids lead to 3HV production. Thus, the gradual consumption of amino acids that lead to 3HV production result in the reduction of 3HV content during fermentation.

As shown in Table 1, two shake flask fermentations were carried out at the best FAN concentration (460 mg/L) and lower initial glycerol concentrations (9 and 16 g/L). Decreasing glycerol concentrations resulted in a reduction in total dry weight, residual microbial biomass and P(3HB-*co*-3HV) concentration and content; however, the 3HV content was slightly increased probably due to the increased ratio of 3HV (i.e. specific amino acids) to 3HB (mainly glycerol) precursors. Higher PHA production was achieved when

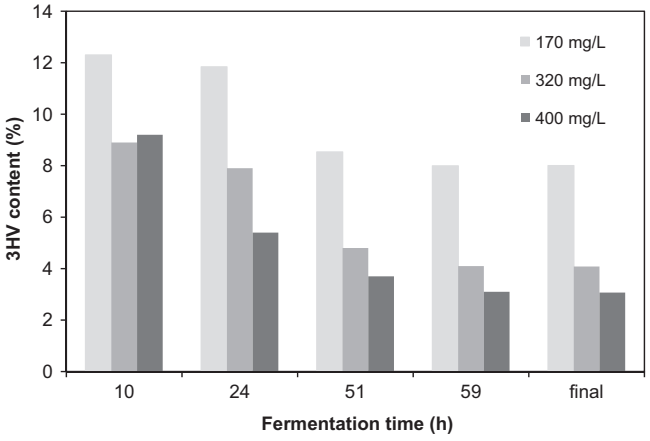

Fig. 1. 3-Hydroxyvalerate (3HV) content during shake flask fermentations carried out with 170, 320 and 400 mg/L initial FAN concentration.

pure glycerol was used in shake flask fermentations (results not presented). At initial pure glycerol and FAN concentrations of 9–25 g/L and 300–470 mg/L, the highest P(3HB-*co*-3HV) concentration and content achieved were 9.1 g/L and 65%, respectively. Cavalheiro et al. (2009) also reported improved PHB production in bioreactor experiments when pure glycerol was used.

Table 1 also presents two shake flask fermentations that were supplemented with a trace element solution and a mineral medium, respectively (Kim et al., 1994). Initial glycerol and FAN concentration in those fermentations were 25 g/L and 400 mg/L, respectively. The addition of trace elements or mineral medium led to an increase in total dry weight and P(3HB-*co*-3HV) concentration as compared to shake flask fermentations carried out with only rapeseed meal hydrolysates at an initial FAN concentration of 400 mg/L. Ongoing research is focused at increasing the release of nutrients from oilseed cakes aiming at the production of nutrient-rich supplements for fermentation processes.

Fig. 2 presents profiles of glycerol and FAN consumption as well as TDW, RMB and P(3HB-*co*-3HV) production during fermentation with initial glycerol and FAN concentrations of 25 g/L and 460 mg/L, respectively. FAN consumption coincided with PHA production; however, during shake flask fermentations, the dissolved oxygen concentration is most likely below the critical point for exponential

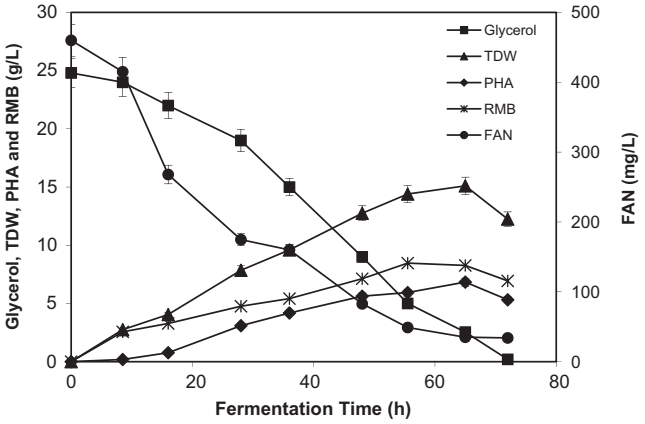

Fig. 2. Glycerol, total dry weight (TDW), polyhydroxyalkanoate (PHA), residual microbial biomass (RMB) and free amino nitrogen (FAN) concentrations during shake flask fermentation carried out with initial glycerol and FAN concentrations of 25 g/L and 460 mg/L (data presented are the mean values of three replicates and error bars represent their respective st. dev.).

microbial growth. Thus, oxygen could be one of the limiting factors leading to PHA accumulation. When wheat-based media were used for PHB production using *C. necator* NCIMB 11599, PHB accumulation mainly occurred after the exhaustion of FAN (Koutinas et al., 2007; Xu et al., 2010). In all shake flask fermentations with rapeseed hydrolysates, PHA production stopped when FAN was completely consumed, regardless the fact that glycerol was still present in the broth. *C. necator* DSM 545 produced copolymers containing 3HV monomers when it was cultivated in rapeseed hydrolysates. These observations indicate variations in the mechanism of PHA production when crude hydrolysates generated from different renewable resources are employed. It has been reported that amino acids (maybe even short peptides) and other micronutrients (e.g. phosphorus) may influence PHA accumulation and copolymer production (Steinbüchel and Lütke-Eversloh, 2003; Lee et al., 2008; Venkateswar and Venkata, 2012).

Fig. 2 also shows that the RMB concentration increased with PHA accumulation. Bacterial growth occurred simultaneously with PHA accumulation in all fermentations carried out in shake flasks. Although *C. necator* usually produces PHAs when bacterial growth has stopped, in some bacteria PHA accumulation may coincide with bacterial growth. For instance, simultaneous bacterial growth and PHB accumulation has been reported for mutant strain *Alcalinenes eutrophus* GE1 (DSM 7237) when it was cultivated in glycerol as carbon source and a mineral medium (Eggink et al., 1994).

Fig. 3 presents the glycerol, FAN, TDW, RMB and PHA concentrations measured during shake flask fermentations carried out in fed-batch (Fig. 3a) and batch modes (Fig. 3b) using the same initial glycerol (21 g/L) and FAN (400 mg/L) concentrations. Rapeseed meal hydrolysate was added at 48 h, while crude glycerol was added at 58 and 72.5 h. The profiles of TDW and PHA are similar until 58 h, but from this point on, PHA production continued only in the case of fed-batch fermentation until glycerol was completely consumed. The highest PHA concentration (10.9 g/L) and content (55.6%) were achieved in fed-batch fermentation. These values are 49.5% and 18.7% higher than the corresponding values of 5.5 g/L and 45.2% achieved in batch fermentations. The addition of rapeseed meal hydrolysates was necessary because PHA production ceased when FAN was depleted even though glycerol was still present in the broth. In both cases presented in Fig. 3, the 3HV content was 9.8 mol% and 3.3 mol% at 10 and 58 h, respectively. The 3HV content remained constant after 58 h in fed-batch fermentation. Microbial growth occurred simultaneously with PHA accumulation in fed-batch (Fig. 3a) and batch fermentations (Fig. 3b).

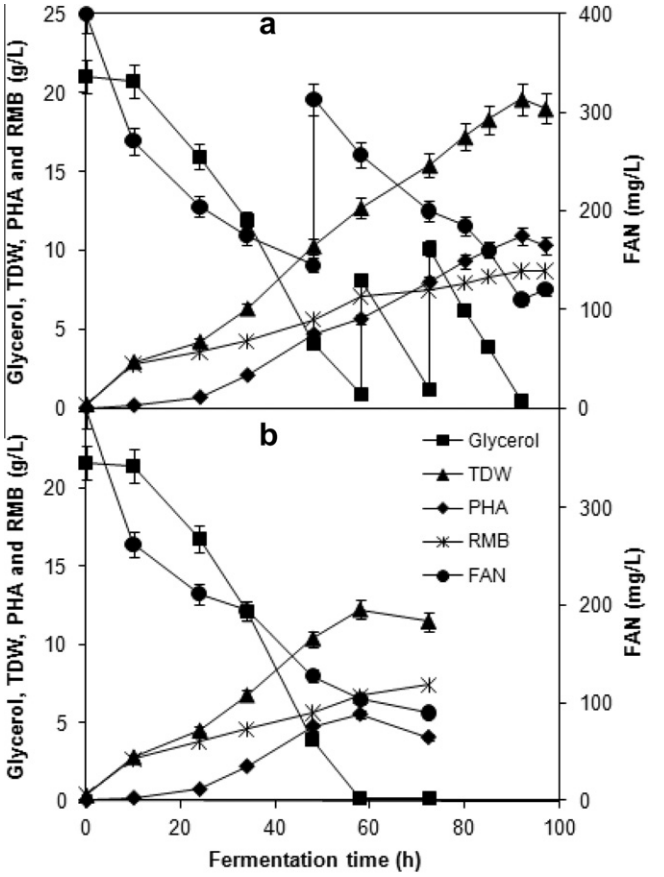

Fig. 3. Glycerol, total dry weight (TDW), polyhydroxyalkanoate (PHA), residual microbial biomass (RMB) and free amino nitrogen (FAN) concentrations during shake flask fermentations carried out on fed-batch (a) or batch (b) mode (data presented are the mean values of three replicates and error bars represent their respective st. dev.).

3.2. Effect of salt concentration during shake flask fermentations

Two series of shake flask fermentations were carried out to study the inhibitory effect of salts (K_2SO_4 and NaCl) present in crude glycerol that will gradually accumulate in the broth during fed-batch fermentation. All fermentations were conducted with initial FAN and crude glycerol concentrations of 400 mg/L and 25 g/L, respectively. In the case of K_2SO_4 addition (Fig. 4a) at the beginning of the fermentation, bacterial growth and PHA production was not significantly affected up to a concentration of 20 g/L. When initial K_2SO_4 concentrations of 2–20 g/L were used, glycerol was totally utilized. TDW varied between 11.8 and 10.2 g/L and PHA production between 4 and 3.1 g/L (PHA contents of 34.7–30.4%). However, in shake flask fermentations supplemented with a K_2SO_4 concentration of 25 g/L, TDW and PHA content were reduced to 6.6 g/L and 0.7 g/L, respectively (PHA content of 10.6%).

In the case of NaCl addition, the inhibitory effect was observed at lower concentrations (Fig. 4b). An NaCl concentration of 2 g/L resulted in 10.4 g/L TDW and 3.7 g/L PHA concentration (PHA content of 35.6%). At an NaCl concentration of 6 g/L, TDW and PHA concentration were reduced to 6.5 g/L and 1.4 g/L, respectively. The reduction in cell growth and PHA synthesis continued as the initial concentration of NaCl was increased. The inhibitory effect of NaCl concentrations higher than 6 g/L was also observed regarding glycerol and FAN utilization. These results are similar to those reported by Mothes et al. (2007) regarding the effect of different salts on PHB fermentation using *C. necator* DSM 4058.

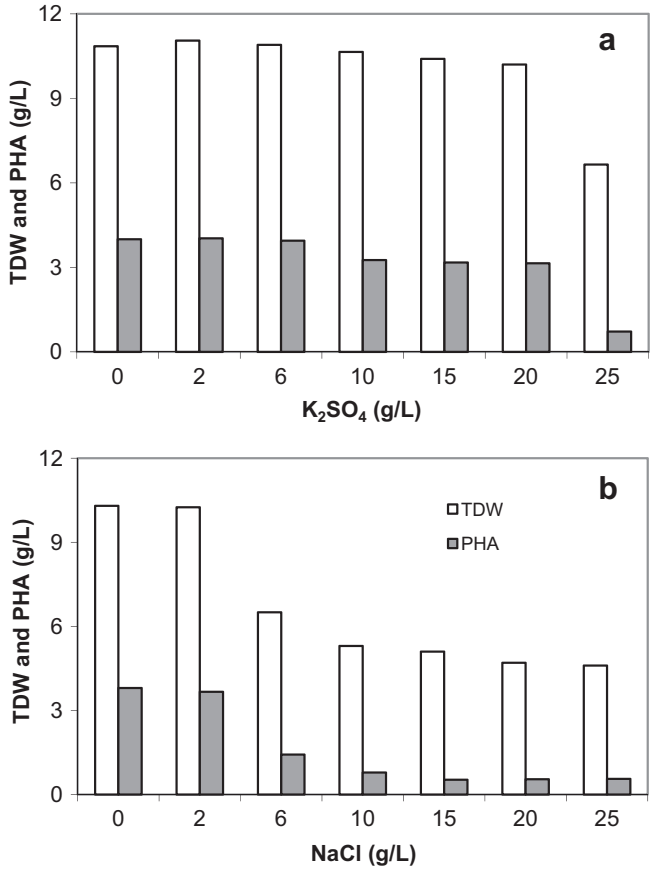

Fig. 4. Effect of K_2SO_4 (a) and NaCl (b) concentration on total dry weight (TDW) and polyhydroxyalkanoate (PHA) production during shake flask fermentations.

3.3. Chemical structure and thermo-physical properties of P(3HB-co-3HV)

Fermentations in a bioreactor were carried out to isolate P(3HB-*co*-3HV) samples for physicochemical analysis by ^{13}C NMR, DSC and TGA. Fig. 5 presents the chemical displacements of P(3HB-*co*-3HV) samples into the ^{13}C NMR spectrum corresponding to the carbons of the PHA chain. The major peaks at 19.67, 40.99 and 67.54 ppm are due to chemical signals of methyl (4), methylene (2), and methine (3) groups, respectively in the 3HB unit. Additional peaks located at 9.31, 26.59, 38.74, and 71.73 ppm are assigned to chemical displacements of the C–H stretching vibrations of groups CH_3 (5), CH_2 (2 and 4), and CH (3), respectively in the 3HV unit. These signals are in agreement with chemical displacements reported by other authors for P(3HB-*co*-3HV) copolymers (Bluhm et al., 1986; Kamiya et al., 1989). The ^{13}C NMR spectrum confirmed that *C. necator* was able to produce P(3HB-*co*-3HV) copolymers by employing a bioprocessing strategy based on the utilization of rapeseed meal hydrolysates and crude glycerol generated from industrial biodiesel production.

Table 2 presents 3HV molar fraction, glass transition temperature (T_g), melting temperature (T_m), enthalpy of fusion (ΔH_m), percentage of crystallinity (% X_c) and degradation temperature ($T_{d(50\%)}$) for P(3HB-*co*-3HV). The 3HV molar fraction was calculated based on the proportionality between the 3HV fraction and the melting temperature as reported by Bluhm et al. (1986) for copolymer containing between 3 and 29% of 3HV on a molar basis. This value was confirmed by calculating the area ratio of peaks for methyl groups (4 and 5) corresponding to the 3HB and 3HV units in the ^{13}C NMR spectrum (Fig. 5).

Fig. 6 presents the TGA curves for P(3HB-*co*-3HV). The degradation profile of P(3HB-*co*-3HV) shows two degradation stages. A first stage until approximately 250 °C with a mass loss of 10% was probably due to plasticizers (e.g. rest of solvents from the extraction procedure) or low molar mass molecules present in the sample and a second stage between 250 °C and 280 °C with a mass loss of 90% due to the polymer itself. Results reported in the literature indicate that P(3HB-*co*-3HV) degradation began at 250 °C (Verhoogt et al., 1996; Wang et al., 2008), which agrees with the value found for the copolymer obtained in this work. The degradation is caused by chain scission and hydrolysis, resulting in a lower molar mass polymer and the formation of crotonic acid (Modi et al., 2011).

Previously, Cavalheiro et al. (2012) employed *C. necator* DSM545 for the production of P(3HB-*co*-4HB-*co*-3HV) terpolymers using crude glycerol and 4HB/3HV precursors (i.e. γ-butyrolactone and propionic acid). In the present study, P(3HB-*co*-3HV) was produced entirely from industrial by-product streams. The production of this type of polymer could have been due to the presence of different amino acids in rapeseed hydrolysates. The protein of rapeseed meals contains predominantly glutamic acid, while other amino acids (e.g. threonine, methionine, isoleucine and valine) are present in significantly lower and more or less comparable quantities (Shahidi et al., 1992). Steinbüchel and Lütke-Eversloh (2003) reported that specific amino acids such as valine, isoleucine, threonine and methionine are precursors for 3HV synthesis because they are catabolized via propionyl-CoA. In addition, Yoon et al. (1995) have reported that small amounts of threonine, isoleucine and valine increased production of 3HV during fermentation with *Alcaligenes* sp. SH-69, which was identified as a microorganism capable of producing the copolymer P(3HB-*co*-3HV) from single carbon sources. Lee et al. (2008) reported that 3HV monomer composition in P(3HB-*co*-3HV) produced by *C. necator* H16 can be regulated in the range of 0–23 mol% by changing culture parameters such as initial pH, nitrogen source and nitrogen concentration.

Table 2
Thermo-physical properties of P(3HB-*co*-3HV) produced during bioreactor fermentations and comparison with values reported in the literature.

Polymer	3HV[a] (mol%)	T_g[b] (°C)	T_m[c] (°C)	ΔH_m[d] (J/g)	%X_c[e]	$T_{d(50\%)}$[f] (°C)	Reference
P(3HB-*co*-3HV)	12	−1.9	155	72.61	49	271	This work
P(3HB-*co*-3HV)	4.1	2.3	163	–	–	252	Han et al. (2010)
P(3HB-*co*-3HV)	8	−1.2	169	49.00	33	–	Lee et al. (2008)
P(3HB-*co*-3HV)	12	–	153	66.40	45	–	Gunaratne and Shanks (2005)
P(3HB-*co*-3HV)	30	2.1	111	–	–	352	He et al. (2001)

[a] 3-Hydroxyvalerate content.
[b] Glass transition temperature.
[c] Melting temperature.
[d] Enthalpy of fusion.
[e] Percentage of crystallinity.
[f] Degradation temperature.

4. Conclusions

This study shows the potential of rapeseed hydrolysates as nutrient supplements for the production of P(3HB-*co*-3HV). The utilization of crude by-products as the sole raw materials for P(3HB-*co*-3HV) production could lead to the replacement of expensive commercial carbon sources, nutrient supplements and monomer precursors. This advantage was accompanied by the production of PHA copolymers with better thermal and mechanical properties than PHB.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.11.088.

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