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Sunflower-based biorefinery: Poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) production from crude glycerol, sunflower meal and levulinic acid

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HIGHLIGHTS

- A sunflower-based biorefinery concept was developed.
- Biodiesel industry by-products were valorized for PHA production.
- Commercial carbon sources and nutrient supplements were replaced.
- High P(3HB-*co*-3HV) concentrations were achieved using renewable resources.

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ABSTRACT

Polyhydroxybutyrate (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] production was developed in bioreactor cultures using the strain *Cupriavidus necator* DSM 7237 cultivated on crude glycerol, sunflower meal (SFM) hydrolysates and levulinic acid as the sole fermentation feedstocks. Bacterial growth and PHB production was influenced significantly by the free amino nitrogen and inorganic phosphorus content of the SFM hydrolysate. Fed-batch bioreactor fermentations led to the production of 27 g L^{-1} PHB with an intracellular content of 72.9% (w/w). Continuous feeding of levulinic acid led to the production of up to 23.4 g L^{-1} P(3HB-*co*-3HV) with an intracellular content of 66.4% (w/w) and a 3HV content of 22.5 mol%. A maximum 3HV content of 31 mol% was achieved at earlier fermentation time (53 h). Thus, levulinic acid could be combined with biodiesel industry by-products for the production of high P(3HB-*co*-3HV) concentration, intracellular content and industrially useful 3HV content.

1. Introduction

The development of sustainable industrial processes necessitates the exploitation of renewable sources of carbon. Koutinas et al. (2014) presented the potential to restructure various conventional industrial sectors (e.g., food industry, pulp and paper industry, 1st generation biofuel production processes) into integrated biorefineries through valorization of waste and by-product streams. Oilseed-based biodiesel production plants generate oilseed meals and crude glycerol as by-product streams. It is projected that by 2022, the annual worldwide oilseed production will increase by 28%, while the respective oilseed meal production is projected to increase by 23% reaching around 315×10^6 t. It is also expected that the annual worldwide biodiesel production from edible vegetable oils will increase by approximately 11×10^6 t to 30×10^6 t from nowadays to 2022 (Koutinas et al., 2014), corresponding to the generation of approximately 3×10^6 t of crude glycerol, assuming that 10% (w/w) is the approximate stoichiometric glycerol yield during transesterification of triglycerides with methanol. Crude glycerol will also be generated via transesterification of other sources of triglycerides including cooking oil, animal tallow and jatropha (Chatzifragkou and Papanikolaou, 2012). The development of advanced generation biorefineries for the production of microbial oil by oleaginous microorganisms will generate additional sources of triglycerides

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for biodiesel and oleochemical production leading to proportional quantities of crude glycerol (Koutinas and Papanikolaou, 2011). Sunflower is the third most important oilseed regarding annual oil extraction (around 14×10^6 t) for both food and biodiesel production and the fourth most important oilseed regarding annual oilseed meal production (around 15×10^6 t) (Koutinas et al., 2014).

Integrated oilseed biorefineries could be developed through microbial bioconversion of crude glycerol and extraction of value-added ingredients contained in oilseed meals. Crude glycerol has been evaluated as the carbon source, supplemented with commercial or crude nutrient supplements, for the production of 1,3-propanediol, citric acid, polyhydroxybutyrate (PHB) and 2,3-butanediol among several other metabolic products (Chatzifragkou and Papanikolaou, 2012; Chatzifragkou et al., 2014; Kachrimanidou et al., 2013). Besides conventional uses as animal feed supplements, oilseed meals could be fractionated into various value-added products (Coats et al., 2001; Das Purkayastha et al., 2013; Kannan et al., 2012; Long and Gibbons, 2013) such as:

- protein concentrates, isolates or respective hydrolysates with a wide spectrum of end-uses (e.g., feed and food additives, adhesives, bioactive peptides preventing chronic diseases),
- antioxidant-rich formulations for non-food applications, carbohydrate extraction (e.g., pectin from rapeseed meal) or hydrolysis,
- glucosinolate-derived biopesticides,
- fermentation products derived from molasses (e.g., soy-based) generated during protein extraction processes.

Ren et al. (2010) proposed "reactive extraction", where biodiesel is produced via direct contact of macerated seeds with methanol and catalyst, as an intensified process for both biodiesel production and extraction of value-added by-product streams (e.g., antioxidants).

Polyhydroxyalkanoates (PHA) are biodegradable poly-esters accumulated intracellularly as energy-reserve granules that could replace petroleum-derived plastics. In most bacterial strains, such as *Cupriavidus necator*, intracellular PHA production is triggered via nutrient limiting conditions (e.g., nitrogen or phosphorus) and a surplus availability of the carbon source. PHB can be produced from pure or crude glycerol supplemented with synthetic media or crude nutrient supplements (Ashby et al., 2011; Cavalheiro et al., 2009; Ibrahim and Steinbuchel, 2009; Mothes et al., 2007; Zhu et al., 2010). Ibrahim and Steinbuchel (2009) reported production of 54.3 g L^{-1} PHB at an intracellular content of 66.9% (w/w), a glycerol to PHB conversion yield of 0.25 g g^{-1} and a productivity of 1.09 g L^{-1} h^{-1} in bioreactor cultures using the strain *Zobellella denitrificans* MW1 cultivated on glycerol supplemented with 20 g L^{-1} NaCl. Cavalheiro et al. (2009) used the strain *C. necator* DSM 545 in bioreactor cultures for the production of 38.1 g L^{-1} PHB at an intracellular content of 50% (w/w), an overall crude glycerol to PHB production yield lower than 0.22 g g^{-1} and a productivity of 1.1 g L^{-1} h^{-1}. The vast majority of literature-cited studies used inorganic chemicals as nutrient supplements.

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] as well as other PHA co-polymers can be produced from crude glycerol via supplementation with various precursors (Ashby et al., 2004; Cavalheiro et al., 2012; Kenny et al., 2012). Cavalheiro et al. (2012) produced P(3HB-*co*-4HB) and P(3HB-*co*-4HB-*co*-3HV) with the strain *C. necator* DSM 545 cultivated on crude glycerol and inorganic chemicals supplemented with either γ-butyrolactone alone or a combination of γ-butyrolactone and propionic acid, respectively. Kachrimanidou et al. (2013) demonstrated the potential to produce a crude hydrolysate from sunflower meal (SFM) that was subsequently used as a nutrient supplement for the production of 9.9 g L^{-1} P(3HB-*co*-3HV) containing 3 mol% of 3HV in shake flask cultures using the bacterial strain *C. necator* DSM 545. SFM was hydrolyzed with crude enzymes produced by a fungal strain of *Aspergillus oryzae* cultivated on solid state fermentation (SSF) using SFM as substrate.

Levulinic acid (LA) is likely to become a cost-efficient precursor for P(3HB-*co*-3HV) production as it will eventually be produced in bulk in future biorefineries utilizing a variety of lignocellulosic resources or waste streams (Bozell et al., 2000). The significant production of by-products leading to a reduction of the maximum theoretical yield, the high cost of equipment and difficulties with catalyst recycling currently hinder the commercialization of LA production. The Biofine process is the most known procedure that has been developed for LA production (Hayes et al., 2006). The cost of LA production could be reduced from its current high price of 6 – 9 €/kg, at an annual production level of 450 t, to less than 0.2 $/kg depending on the production capacity (Bozell et al., 2000). This unitary cost is much lower than for other precursors (e.g., 0.95 $/kg to more than 2 $/kg for propionic acid in the period 2003–2009) employed for the production of 3HV co-monomer units in P(3HB-*co*-3HV) fermentations. The combination of crude glycerol with LA could provide low cost carbon sources for P(3HB-*co*-3HV) synthesis. Ashby et al. (2012) reported P(3HB-*co*-3HV) production with the strain *Pseudomonas oleovorans* NRRL B-14682 using LA and crude glycerol as carbon sources, but the reported concentrations and intracellular contents were rather low to support industrial implementation. Furthermore, although other carbon sources (e.g., glucose, fructose, xylose, hemicellulosic hydrolysates) have been used together with LA for the production of P(3HB-*co*-3HV), the reported concentrations and intracellular contents achieved in most studies are not satisfactory to warrant industrial implementation (Jang and Rogers, 1996; Chung et al., 2001; Keenan et al., 2006). A relatively high P(3HB-*co*-3HV) concentration of 12.6 g L^{-1} and a significantly high intracellular content (81.2%, w/w) were reported by Wang et al. (2013).

A biorefinery concept has been developed for the production of PHB from wheat (Koutinas et al., 2007; Xu et al., 2010). In a similar manner, the main aim of this work is to develop advanced sunflower-based biorefinery concepts using by-products from biodiesel production processes. The approach followed is to initially verify the utilization of crude glycerol and whole SFM hydrolysates as sole renewable resources for the production of either PHB or P(3HB-*co*-3HV) using LA as precursor (Fig. 1 – Process I) and subsequently optimize fractionation of SFM for the production of protein isolate, antioxidants and fermentation media for PHA production (Fig. 1 – Process II). The present study focuses on fermentation media optimization for PHB and P(3HB-*co*-3HV) production in a bench-scale bioreactor. The P(3HB-*co*-3HV) concentration and intracellular content produced in this study using LA as precursor were among the highest reported in the literature. The development of Process II (Fig. 1) and the techno-economic evaluation of both processes will be presented in forthcoming studies.

2. Methods

2.1. Microorganisms

Solid state fermentations of SFM were carried out with an *A. oryzae* strain that was kindly provided by Professor Colin Webb (University of Manchester, UK). Its origin as well as isolation, purification and maintenance protocols have been reported by Kachrimanidou et al. (2013).

The bacterial strain *C. necator* DSM 7237 was used in fermentative production of PHB and P(3HB-*co*-3HV). Inoculum preparation for bioreactor fermentations was carried out using bacterial stock cultures stored at 4 °C in petri dishes. Liquid media contained