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Microbial selection strategies for polyhydroxyalkanoates production from crude glycerol: Effect of OLR and cycle length

André Freches, Paulo C. Lemos*

LAQV-REQUIMTE, Department of Chemistry, Faculty of Science and Technology, Universidade NOVA de Lisboa, Caparica, Portugal

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ABSTRACT

Crude glycerol from biodiesel manufacture can be used as carbon source for microbial fermentations. The production of polyhydroxyalkanoates by manipulating the Sequencing Batch Reactor (SBR) selection stage of microbial mixed cultures (MMC) using high organic loading rates (OLR, 50C mM/day) and different cycles lengths (6, 12 and 24 h) were optimized. Batch-production of polyhydroxybutyrate (PHB) presented an accumulation capacity in the high range (0.44 g/g) after 3 pulses of 50C mM, with a final content of 59% PHB/wt., for the culture selected with 50C mM/day and a 24 h cycle length. These values were in the range to those obtained with pure cultures and higher than the ones for MMC. With this strategy three main advantages in terms of the PHA production can be considered: utilization of a real waste without the resort to pure microbial cultures and a pre-fermentation step, consolidating the role of MMC in the valorisation of complex wastes/by-products.

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1. Introduction

Biofuels are a promising and sustainable source of energy, an alternative renewable fuel developed in order to overcome the world energy crisis caused by the exhaustive use of fossil resources. Their production in the last decade has grown exponentially in the European Union, particularly biodiesel. In fact, the biodiesel market was expected to reach 1400 million litres by 2016 with crude glycerol as the main by-product (10% (v/v) [1]. Both the conversion of glycerol into valuable products and the purification of crude glycerol have attained a growing interest in recent years due to the existing surplus of this by-product. In order to meet the requirements of the cosmetic and pharmaceutical industries crude glycerol needs to be refined to high levels of purity, a very costly process. Several new applications for crude glycerol, such as its utilization as feedstock for industrial fermentations, have been studied [2]. Crude glycerol has been demonstrated as a suitable substrate for production of 1,3-propanediol [3], H_2 [4] and bacterial cellulose [5]. The conversion of crude glycerol into polyhydroxyalkanoates (PHA) could be just as interesting. PHA have been reported to be present in more than 300 different organisms (both Gram-positive and Gram-negative), either from Eubacteria or Archaea domains, functioning as storage of energy and carbon. The thermoplastic and elastomeric characteristics and its non-toxic biodegradable nature classify PHA as a potential market replacement for conventional polymers [6]. Notwithstanding, PHA industrial production still depends on the use of pure bacterial cultures fermentation and synthetic substrates, both with high operation costs associated [7]. In order to oppose this tendency, different methods for PHA production, which include the use of agro-industrial wastes as feedstock and mixed microbial cultures (MMC) have been proposed. In fact, Gurieff and Lant [8] demonstrated that the use of MMC could work both as an economically interesting polymer production process and as an effective industrial wastewater treatment technology. Culture selection is a key step to set up a PHA accumulating system and activated sludge from wastewater treatment plants has been proved to be an excellent source of microorganisms capable of PHA accumulation [9,10]. Aerobic dynamic feeding (ADF, or feast/famine) strategies can be applied in order to establish a stable MMC with high PHA storage capacity. This method alternates between stages of carbon availability (feast) and carbon absence (famine). Sequencing Batch Reactor (SBR) processes are the most common for culture selection phase. Albuquerque et al. [11] compared the performance of a SBR and a continuous system for culture selection using fermented molasses as carbon source and concluded that both reactor configurations had similar stoichiometric and kinetic performances, in terms of PHA content, productivity and polymer yield. Generally, microbial cultures are sensitive to variations in growth conditions and several important

* Corresponding author.
E-mail address: paulo.lemos@fct.unl.pt (P.C. Lemos).

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process parameters, such as sludge retention time (SRT), organic loading rate (OLR) and pH must be optimized. Chua et al. [12] assessed the effect of SRT in the production of PHA by activated sludge and observed that a short SRT supplied a sufficient amount of sludge for polymer production with higher sludge yields for shorter SRTs. Other studies [12,13] reported the effect of pH in culture selection and it became clear that the culture response to pH variations may depend on the type of feed and the mixed microbial consortia selected. Albuquerque et al. [14] assessed the substrate concentration variation during the culture selection phase, ranging between 30 and 60C mM VFA. Kinetic (limiting concentration of carbon source) and physiological (internal growth limitation due to shorter length of the famine phase) effects were demonstrated. The MMC of this study was able to achieve a PHA content of 75% of cell dry weight with fermented molasses as substrate proving that this approach can be used as an alternative for the pure culture/refined substrate approach. Depending on the type and complexity of the substrate used as feedstock, PHA production processes via MMC can be operated in two or three steps. The two-step approach has been mainly applied when organic acids are used as feedstock and includes a selection step for PHA-accumulating organisms, followed by a batch step for maximizing PHA production [15,16]. Volatile fatty acids (VFA) are considered the main precursors for PHA production and for most real wastes used as feedstock, a pre-fermentation step is required in order to increase the VFA content. This initial step involves additional costs since it requires an additional reactor for pre-fermentation and possibly a filtration system for recovering the produced VFA, depending on the type of initial substrate. Hence, the utilization of feedstocks with readily available carbon sources, such as glycerol, could help to reduce the costs of the process, since a pre-fermentation should not be required. Indeed, the glycerol-based PHA production by an MMC was proven by Moralejo-Gárate et al. [17] when synthetic glycerol was used as feedstock and a PHA content of 67% in cdw was obtained (0.35 g PHB/g glycerol). Regarding the utilization of crude glycerol as feedstock for PHA production several researchers have already documented this possibility. Dobroth et al. [18] established a stable mixed culture with crude glycerol, but the culture produced polyhydroxybutyrate (PHB) exclusively using the methanol fraction with a low polymer yield on substrate (0.10 g PHB/g methanol). However, it was demonstrated that the resulting PHB material properties and characteristics are suitable for many commercial uses. More recently, Moita et al. [19] also used crude glycerol for PHA production and concluded that the selected culture preferred the glycerol fraction over the methanol fraction. This study reported the highest PHB content (47% cdw) achieved with a real VFA-free waste as substrate.

The main objective of this study was to assess the effect of cycle duration in microbial selection in order to optimize PHA production. For this purpose a two-stage system was used which comprehended the selection of acceptable PHA-production oriented cultures and batch PHA accumulation tests with each selected culture under pulse-feeding regime.

2. Materials and methods

2.1. Crude glycerol composition

The crude glycerol used in this study was provided by Sovena – Portugal, an industrial biodiesel manufacturing company. This industry uses several sources of vegetable oils as raw material in the production of biodiesel. The crude glycerol used on this study was collected immediately after the biodiesel production and before any purification step. This fraction was mainly composed by glycerol and methanol (≈70:30%, g C/g TOC). A small fraction (2.58% w/w) of free fatty acids and fatty acids methyl esters (FFA/FAME) was also present in crude glycerol.

2.2. Selection of PHA-accumulating microbial culture

The PHA-accumulating culture enrichment using crude glycerol was performed in a cylindrical reactor operated as a sequencing batch reactor (SBR) with a working volume of 1500 ml. The SBRs were operated under ADF conditions. Each SBR had different working cycles (24 h, 12 h, 6 h). The initial reactor (24 h) was inoculated with a PHA-accumulating MMC already acclimatized to crude glycerol as feedstock [19] and later operated under 12 h cycle. The 6 h cycle reactor was seeded from the 24 h one. The aerobiosis, fill and withdraw periods were adapted to keep HRT at 2 days and SRT at 5 days. The settling time was constant for each SBR (10 min). In order to maintain the crude glycerol feed at 50 CmM/day the feeding duration at the beginning of every cycle was also adapted. The aerobic period always comprised more than 96% of the total duration of the cycle. In order to keep the C:N:P ratio (on a molar basis) at 100:6:1, a nitrogen (NH_4Cl) and phosphorus source (KH_2PO_4/K_2HPO_4) were mixed with tap water and added separately to the reactor. Thiourea (10 mg/l) was added to this mineral solution to prevent nitrification. Magnetic stirring was kept at 400 rpm and a ceramic diffuser was used for aeration of the reactor (1 l/min). NaOH 1 M and HCl 0.5 M solutions were used to keep pH between 8.0 and 8.4. Both pH and dissolved oxygen (DO) were measured on-line and all pumps were controlled by a software developed in-house based on Lab-View. All SBRs stood in a temperature-controlled room (20–22 °C).

2.3. Analytical methods

The volatile suspended solid (VSS) procedure described in Standard Methods (APHA, 1995) was used to determine biomass concentration. PHA determination protocol was already described by Moita et al. [19]. A digestion (3.5 h at 100 °C) of lyophilized biomass using 1:1 solutions of chloroform with heptadecane, as internal standard, and a 20% acidic methanol solution was performed. The resulting organic phase was extracted and injected into a gas chromatograph coupled to a Flame Ionization Detector (GC-FID, Bruker 400-GC). A Bruker BR-SWAX column (30 m x 0.25 mm x 0.25 μm) with splitless injection at 240 °C was used. The oven temperature program was as follows: 40 °C; then 20 °C/min until 100 °C; then 3 °C/min until 135 °C; and finally 20 °C/min until 220 °C. The detector temperature was set at 230 °C. Hydroxybutyrate (HB) and hydroxyvalerate (HV) concentrations were determined using standards of a commercial P(HB-HV) polymer (88%/12%, Aldrich). Glycerol and methanol samples (supernatant after centrifugation, no pre-treatment required) were also analysed by gas chromatography using a different Bruker BR-SWAX column (30 m x 0.53 mm x 1 μm). In this case the splitless injection was performed at 290 °C. The oven temperature program used was: 80 °C; 10 °C/min until 200 °C. The detector temperature was set at 280 °C. Commercial glycerol (87%, Pancreac) and methanol (99%, VWR Chemicals) were used as standards and 1,4-butanodiol was used as internal standard. An acidic digestion (2 h at 100 °C) of lyophilized cells using 1 ml HCl for each sample was used to extract the glucose biopolymer (GB). The digested samples were analysed by high performance liquid chromatography (HPLC). For this purpose, a Refractive Index detector (Merck, Germany) and Aminex HPX-87H column (Bio-Rad Laboratories, CA, USA) were used with sulphuric acid (H_2SO_4) 0.01 M as eluent at a flow rate of 0.6 ml/min and 50 °C operating temperature. An ammonia gas sensing combination electrode (ThermoOrion 9512) was used for ammonia concentration determination. A calibration curve was obtained using NH_4Cl standards (0.01–10 N mM).

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