Effects of the operational conditions on the production of 1,3-propanediol derived from glycerol in anaerobic granular sludge reactors

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ABSTRACT

The aim of this study has been to produce 1,3-propanediol (1,3-PDO) from glycerol (gly) fermentation by means of a microbial mixed culture (granular sludge), as well as to establish the operational conditions of two up-flow anaerobic sludge blanket (UASB) reactors in order to achieve a maximum 1,3-PDO yield. The UASB reactors with initial pH values set at 6.8 and 5.5 were operated at 30 °C during 165 days. Thirteen variables were previously screened by a Plackett-Burman (PB) design; results showed that yeast extract, MgSO₄ and methanogenesis inhibition (by heat shock) showed a positive effect, whereas high glycerol concentration, tryptone and CaCl₂ showed a negative impact on the 1,3-PDO produced by glycerol degradation. Following four experimental periods, the highest average yield of 0.43 mol 1,3-PDO mol⁻¹ gly was achieved when sodium bicarbonate was added to the reactors. Propionate and acetate were also produced and a high microorganism diversity was detected; however, the restrictive operational conditions of the reactors led to the death of the methanogenic archaea. Nevertheless, the continuous production of 1,3-PDO from glycerol within UASB reactors inoculated with granular sludge can be considered highly feasible.

Key words | 1,3-propanediol, glycerol, granular sludge, Plackett-Burman design, UASB reactor

INTRODUCTION

The decrease of petroleum reserves coupled to the environmental problems caused by the burning of fossil fuels has compelled the development of alternative fuel resources. Biofuels, such as biodiesel, bioethanol, biohydrogen or methane, have all become alternative energy sources to petroleum as they are renewable, biodegradable, less toxic and far less polluting; i.e. they generate less sulfur and aromatic hydrocarbon releases together with reduced greenhouse gas emissions (Demirbas 2008). Biodiesel is mainly obtained by the transesterification reaction of animal fats or vegetable oils in the presence of a primary alcohol catalyst (usually methanol) leading to a fatty acid methyl ester which is used as biofuel (Almeida et al. 2012).

As a consequence, glycerol, a byproduct of biodiesel production has massively been accumulated. Thus, glycerol conversion into other useful products comprises an attractive solution to the biodiesel industry by reducing disposal associated costs and increasing the value-added chemical production (Almeida et al. 2012). Glycerol can be converted into several compounds such as acrolein, n-butanol, 2,3-butanediol, citric acid, lactic acid, poly-hydroxyalkanoates (PHA), 1,3-propanediol (1,3-PDO), ethanol and hydrogen (Selmbo et al. 2009; Chookaew et al. 2014; Luo et al. 2016). Among these products, 1,3-PDO stands out as a monomer used for the production of polyesters, polylethers and polyurethanes. Moreover, 1,3-PDO is currently included in the formulation of cosmetics, lubricants, solvents, inks, adhesives, medicines and more.

1,3-PDO can be produced by chemical or biological processes; however, chemical synthesis requires high temperatures, high pressures and requires high cost catalysts, generating toxic pollutants as production relies on fossil fuels (Silva et al. 2014). Consequently, microbial production has received a considerable amount of attention owing to its simple process lacking toxic intermediates and producing less impurities compared to the 1,3-PDO...
chemically synthesized (Silva et al. 2014). Furthermore, 1,3-PDO production is highly specific for the glycerol fermentation and cannot be obtained from any other anaerobic conversion (Deckwer 1995).

So far, most of the research carried out on microbial conversion of glycerol to produce 1,3-PDO has been undertaken with pure cultures (Barbriato et al. 1995; Rossi et al. 2013; Jolly et al. 2014). However, use of pure microbial strains presents a number of challenges which can render the process economically unfeasible, such as the need to supplement the culture medium with additional organic material sources, the potential pathogenicity of some strains (enterobacteria and clostridia), together with the high maintenance costs due to the strict operational conditions.

Only few studies exist using mixed cultures for the production of 1,3-PDO (Gallardo et al. 2014) and some have examined production together with hydrogen (Selembro et al. 2009; Kivistö et al. 2013; Liu et al. 2013). Therefore, the aim of this study has been to produce 1,3-PDO derived from glycerol fermentation by means of a microbial mixed culture (granular sludge), also to establish the operational up-flow anaerobic sludge blanket (UASB) reactor conditions in order to achieve a maximum 1,3-PDO yield.

MATERIAL AND METHODS

Inoculum and media composition

Anaerobic granular sludge was collected from a brewery full-scale UASB reactor (Mahou – San Miguel SA, Alovera, Spain). The medium composition for glycerol degradation and 1,3-PDO production was prepared according to a Plackett-Burman (PB) screening design, based on the first-order polynomial model (Equation (1)):

\[ Y = \beta_0 + \sum \beta_i X_i \]  

(1)

where \( Y \) is the response (1,3-PDO yield), \( \beta_0 \) is the model intercept, \( \beta_i \) is the linear coefficient and \( X_i \) is the level of the independent variable. A total of 13 variables (glycerol, pH, yeast extract, tryptone, peptone, K₂HPO₄, KH₂PO₄, (NH₄)₂SO₄, NH₄Cl, MgSO₄, FeSO₄, CaCl₂ and methanogenesis inhibition) were screened in 20 experimental runs (Table A, supplementary material, available with the online version of this paper) carried out in duplicate allowing the averaging of results. The experiment was carried out in 35-mL serum bottles containing 20 mL useful volumes. The variables and their levels (−1 for low level and +1 for high level) were selected based on literature reports concerning the production of 1,3-PDO (Selembro et al. 2009; Chatzifragkou et al. 2011; Gallardo et al. 2014). An analysis of variance (ANOVA) was performed to find the variables that had a significant effect (\( p < 0.05 \)) on the responses (1,3-PDO yields measured as mol·mol⁻¹ of consumed glycerol) following 24 h of the experiment. Based on the PB results, the next experiment was adjusted. Glycerol 85% was purchased from Merck KGaA (Germany). The pH adjustment was carried out with 1M NaOH or HCl.

Batch reactors

The experiment corresponding to the PB design was carried out in batch reactors (35-mL serum bottles). Each reactor contained a useful volume of 20 mL. Glycerol concentration, medium composition, initial pH and pre-treatment of the inoculum varied according to the PB design matrix (Table A, supplementary material). The serum bottles were sealed with butyl rubber stoppers and aluminum stoppers prior to autoclaving for 15 min at 1 atm. After cooling down, 5 g of sludge were inoculated into each reactor. To inhibit the methanogenesis process, granular sludge was heat pre-treated in a boiling water bath during 15 min; next, the sludge was cooled down and filtered through a sterilized gauze (to remove the excess water) previous to its use as an inoculum. Subsequently, the bottles were purged with 80:20 N₂:CO₂ during 3 min. The incubation was static, in the absence of light, in a thermostatic chamber at 30 °C.

UASB reactors

Two laboratory-scale UASB reactors (R1, R2) were operated under mesophilic conditions (30 ± 2 °C). The reactors made out of acrylic comprised a total volume of 5.4 L. Inflow was controlled by a peristaltic pump and the average hydraulic retention time was 20 h. Reactor inoculations were accomplished by completing a third of their total volume with granular sludge (approximately 18 g-VSS L⁻¹-reactor). Four phases were executed during the operation of the reactors. Throughout Phase 1, the two reactors were fed with a simple mineral medium (medium 1, Table 1). During Phase 2, medium 1 was supplemented with a micronutrient solution (Table 1) (1 mL-micronutrient solution · L⁻¹ medium). In Phase 3, medium 2 was used (Table 1). In the course of Phase 4, both reactors made use of medium 2 with 0.3 g of sodium bicarbonate (NaHCO₃) per g of chemical oxygen demand (COD) added. All the way through Phases 1 to 3
the reactor’s initial pH values were 6.8 (R1) and 5.5 (R2). With respect to Phase 4, the initial pH value of both reactors was 6.8. The glycerol loading rate (GLR) applied varied between 5 to 15 g-gly·L⁻¹·d⁻¹.

Analysis

Gas productions were measured by gas-meters (Schlumberger). The gas phase was analyzed by gas chromatography (Bruker 450-GC), concentrations of CO₂ and H₂ were determined with a thermal conductivity detector and CH₄ with a flame ionization detector. Glycerol concentration and related byproducts were measured using a high performance liquid chromatography (HPLC) system (Agilent Technologies 1260 Infinity) equipped with a refractive index detector. The volatile suspended solids (VSS) and COD determinations followed Standard Methods for the Examination of Water and Wastewater (APHA/AWWA/WEF 2012), methods 2540E and 5220C, respectively. The PB design and all the statistical analysis were performed with Design-Expert® software version 6 (DX6).

Scanning electron microscopy

The structural integrity and the morphology of the microorganisms present in the granular sludge samples derived from the inoculum and the UASB reactors (at the final operation stages) were analyzed by means of scanning electron microscopy (SEM) (Philips XL30 microscope) as described by Alphenaar et al. (1994). To preserve the granular structure, samples were fixed in glutaraldehyde; next, samples were washed with sodium cacodylate and dehydrated with ethanol solutions. Prior to undertaking the analysis with the microscope, samples were critical-point dried with CO₂ and then metallized in a gold bath.

RESULTS AND DISCUSSION

Media composition to produce 1,3-PDO

The influence of the 13 variables on the 1,3-PDO yields (mol·mol⁻¹ of consumed glycerol) are reported in Table 2. The statistical model was significant (p = 0.0127) regarding 1,3-PDO yield and the coefficient of determination (R²) amounted to 0.94. Glycerol concentration, yeast extract, tryptone, MgSO₄, CaCl₂ and methanogenesis inhibition presented significant effects on the 1,3-PDO yield response (p-value < 0.05). Yeast extract, MgSO₄, and methanogenesis inhibition evidenced a positive effect on 1,3-PDO yield. This means that the influence of these variables on the 1,3-PDO yield is considered to be greatest at their higher level; in contrast, glycerol concentration, tryptone and CaCl₂ caused a negative effect: in this case, the influence of these variables would be greatest at their lower level. The ANOVA disclosed that the fitting model for H₂ and ethanol yields were statistically non-significant (p = 0.78 and p = 0.55, respectively) (Table B, supplementary material, available with the online version of this paper).

In basis of the results obtained in the PB experiments and the media reported by other authors, it was decided to operate two UASB reactors (R1 and R2) using a simple mineral
medium (medium 1, Table 1) based on the medium reported by Varrone et al. (2015) used for the bioconversion of glycerol to hydrogen and ethanol by using a complex inoculum. The fact that the high glycerol concentration triggered a negative effect on the 1,3-PDO yield suggests that the inoculum should be adapted to use glycerol as the only substrate; thus, the reactors were initially operated with low glycerol concentrations (5 g-gly·L⁻¹). Because methanogenesis inhibition evidenced a positive effect on 1,3-PDO yield, the reactor R2 was operated at a pH of 5.5 in order to inhibit the methanogenic activity. The method of methanogenesis inhibition by heat shock was replaced by acid inhibition in the UASB reactors because, after five days, methane production was observed in the batch reactors of the PB design test. The variables peptone, K₂HPO₄, KH₂PO₄, (NH₄)₂SO₄, NH₄Cl and FeSO₄ showed little or no influence within the considered range (p > 0.05), and thus could be excluded or kept under other concentrations in the medium. Due to the positive effect of yeast extract on 1,3-PDO yield, yeast extract was added to Varrone’s mineral medium.

### UASB performances

The results obtained during the four-phase operational periods of the two UASB reactors are displayed in Figure 1. During Phase 1, after both reactors had experienced a short lag-phase, glycerol consumption was equal to almost 100% in the course of the first 15 days, thus the GLR was gradually increased up to 15 g-gly·L⁻¹·d⁻¹. However, this led to a drastic reduction of the removal efficiency (glycerol consumption), so that after operating for a period of 50 days, in Phase 2, the GLR was adjusted to 8 g-gly·L⁻¹·d⁻¹ and a micronutrient solution (Table 1) was added to medium 1. A yield decrease of 1,3-PDO (R1: 0.27 ± 0.15 mol-1,3-PDO mol-gly / C₀₁ and R2: 0.29 ± 0.10 mol-1,3PDO mol-gly / C₀₁) was observed in a parallel manner with an increase of propionate production. Past 83 days, in Phase 3, the mineral medium 1 was substituted by medium 2 (Table 1), seeking to increase the yield of 1,3-PDO. This led to decrease the yield of propionate allowing the attainment of higher 1,3-PDO yields equal to 0.40 ± 0.07 and 0.33 ± 0.07 mol-1,3-PDO mol-gly / C₀₁ with respect to R1 and R2.

The average pH values of the effluent during Phases 1 to 3 were 3.9 (R1) and 3.6 (R2). In a previous study making use of different microbial strains such as Clostridium, Klebsiella and Citrobacter, these microorganisms were reported to be producers of 1,3-PDO derived from glycerol as part of their NADH regeneration pathway via glycerol dehydratase activity, followed by 1,3-PDO oxidoreductase activity (Biebl et al. 1999). The optimum pH value for the production of 1,3-PDO by these microorganisms ranged between 5.5 to 7.0 (Saxena et al. 2009). Therefore, in the present study, during Phase 4 from day 105 of the operation onwards, 0.3 g of NaHCO₃·g⁻¹ of influent COD were added to both

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<th>Variable levels and statistical analysis of the Plackett-Burman design</th>
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reactors in order to increase their alkalinites. The addition of NaHCO$_3$ proved to be effective since the glycerol consumption remained higher than 90% concerning both reactors and glycerol was again mainly converted into 1,3-PDO (Figure 1). The pH of the effluent throughout Phase 4 settled at a value of about 5 in the case of both reactors. The 1,3-PDO yields amounted to 0.42 ± 0.16 mol mol-gly$^{-1}$ with regard to R1 and 0.43 ± 0.14 mol mol-gly$^{-1}$ with
reference to R2, both reactors evidencing a peak of 0.8 mol-1,3-PDO mol-gly⁻¹ (days 107 and 109). In addition, small amounts of ethanol were detected (less than 0.07 mol-ethanol mol-gly⁻¹). The diversity of the end products generated during the fermentation process of glycerol is directly related to the metabolic flexibility of the biomass. Glycerol fermentation by enterobacteria commonly results in the accumulation of two major products, i.e. 1,3-PDO and acetate, while the minor byproducts (lactate, formate, succinate and ethanol) are differentially produced according to the culture conditions. When fermentation of glycerol is carried out by C. butyricum, 1,3-PDO is produced as the main compound, with two acids (butyrate and acetate) as byproducts and CO₂ and H₂ as gaseous products (Barbirato et al. 1998). In the present study, 1,3-PDO was generated, resulting in the production of propionate and acetate as the main byproducts, followed by discrete amounts of butyrate, ethanol and butanol.

Although in our study the average yield considering the overall process was equal to 0.56 ± 0.17 mol-1,3-PDO mol-gly⁻¹ regarding R1 and 0.37 ± 0.13 mol-1,3-PDO mol-gly⁻¹ with respect to R2; however, when NaHCO₃ was added to the medium, the glycerol degradation rate increased and the 1,3-PDO yield rose up to 0.43 mol mol-gly⁻¹ (Phase 4). Durgapal et al. (2014) achieved a yield of 0.42 mol-1,3-PDO mol-gly⁻¹ when K. pneumoniae was grown in a successive feed-batch mode using a mineral medium supplemented with yeast extract (1 or 5 g·L⁻¹). Metsoviti et al. (2015) attained a yield of approximately 0.48 mol-1,3-PDO mol-¹ of consumed glycerol using C. freundii with feed-batch fermentation, in this case the culture medium was supplemented with peptone (5 g·L⁻¹), meat extract (5 g·L⁻¹) and yeast extract (2.5 g·L⁻¹). Joly et al. (2014) used Lactobacillus reuteri in a repeated feed-batch fermentation with a co-feeding of glucose and glycerol in a molar ratio of 1.5, achieving a yield of 0.97 mol-1,3-PDO mol⁻¹ of consumed glycerol, very close to accomplishing the theoretical value (1 mol·mol⁻¹). In the present research, glycerol encompassed the only carbon source. This was done intentionally in order to avoid the addition of other co-substrates which would result in a more expensive final product, making the whole process economically unfeasible.

Remarkably, in all of the studies reported above, only pure bacterial strains were used. Furthermore, the use of genetically modified organisms has been reported. Przystałowska et al. (2015), for example, evaluated the use of a recombinant E. coli strain, reaching 0.4 mol-1,3-PDO mol-gly⁻¹ during a batch fermentation experiment. In this case, the limiting factor consisted of the growth medium removal to avoid the accumulation of secondary metabolites (acetate, etc.) which could inhibit the growth of the recombinant E. coli strain. Few studies have resorted to a mixed culture or to non-sterile operation conditions. Gallardo et al. (2014) obtained a maximum yield of 0.52 mol-1,3-PDO mol-gly⁻¹ during the operation of an expanded granular sludge blanket reactor type that was inoculated with granular sludge obtained from a UASB reactor used for the treatment of brewery wastewater. Selembo et al. (2009) reached 0.69 mol-1,3-PDO mol-gly⁻¹ and 0.28 mol-H₂ mol-gly⁻¹ by using mixed cultures (from soil, anaerobic sludge and dewatered sludge), however, they used preheated inoculants that favored the predominance of Clostridium species, known for their ability to produce 1,3-PDO (Biebl et al. 1999). Kivistö et al. (2013) reported a yield of 0.66 mol-1,3-PDO mol-gly⁻¹ using in their study non-sterile cultures of Halanaerobium saccharolyticum (halophilic bacteria) and Clostridium butyricum, nevertheless, these authors provided B₁₂ vitamin (500 mg·L⁻¹) throughout of the fermentation process, thus promoting the metabolic pathway of these microorganisms for the production of 1,3-PDO. Nonetheless, this practice would also lead to high production costs if adopted on a large scale.

The biogas analysis evidenced that the average methane yield of R1 was 0.01 mol-CH₄ mol-gly⁻¹ throughout of the experiment. With respect to R2, no methane was produced during the first 104 days of operation (Phases 1 to 3) corresponding to an initial acid pH (Figure 1(b)). After adding NaHCO₃ (Phase 4) a slight increase of CH₄ production (maximum yield of 0.01 mol-CH₄ mol-gly⁻¹) was noticed. The production of H₂ ranged between 0.01 to 0.08 mol·H₂ mol-gly⁻¹ with respect to both reactors. It is noteworthy to point out that the production of H₂ and 1,3-PDO involve competing routes in terms of reducing equivalents (Gallardo et al. 2014). The production of 1 mol of 1,3-PDO requires 1 mol of H₂, thus the production of the first compound inevitably affects the yield of the second (Selermo et al. 2009). Nevertheless, 1,3-PDO production by means of mixed cultures could result advantageous since fermentation in non-sterile conditions implicates a potential factor to reduce operation costs (Gallardo et al. 2014). Nevertheless, further studies are needed to improve the operating conditions that lead to higher yields of the desired products.

SEM images of the inoculum and sludge granules are presented in Figure 2. The observed granular structure of the inoculum (Figure 2(a)) was maintained throughout the operation process (Figure 2(b)), with both reactors being capable of degrading glycerol. Figure 2(c) demonstrate the high microorganism biodiversity detected at the end of the operation Phases 4, although at the end of this phase
filamentous cells with disrupted ends were observed inside of the granules (Figure 2(d)).

CONCLUSIONS

The continuous production of 1,3-PDO derived from glycerol within of two UASB reactors carried out by mixed cultures proved to be highly feasible, despite of yields in a range of 0.3 to 0.6 mol-1,3-PDO mol-gly−1 consumed not being as high as some returns previously reported for pure cultures. However, maintaining a non-sterile operational condition may represent a significant glycerol fermentation cost decrease and this would certainly benefit the final biodiesel industry cost. Mineral medium with the addition of sodium bicarbonate favored the production of 1,3-PDO as the main byproduct of the glycerol fermentation. The hydrogen and methane outputs remained low during the operation of the reactors, although the addition of sodium bicarbonate slightly increased the methane production of one of the reactors. There was a high microorganism diversity within of the reactors after an operation period of 100 days, keeping the structure of the granules. However, some restrictive conditions imposed during the operation of the reactors, such as the exclusive feeding with glycerol and the low initial pH values, seemingly led to the death of some kinds of microorganisms.

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