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Anaerobic granular sludge as a biocatalyst for 1,3-propanediol production from glycerol in continuous bioreactors

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HIGHLIGHTS

- The production of 1,3-propanediol in EGSB reactors is described for the first time.
- Relatively high 1,3-propanediol yields were obtained without methane production.
- The pre-treatment applied to the biomass has no significant effect at low HRT.
- Anaerobic granular sludge is suitable for the continuous production of 1,3 PDO.

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ABSTRACT

1,3-Propanediol (1,3-PDO) was produced from glycerol in three parallel Expanded Granular Sludge Blanket (EGSB) reactors inoculated with granular sludge (control reactor-R1), heat-treated granular sludge (R2) and disrupted granular sludge (R3) at hydraulic retention times (HRT) between 3 and 24 h. Maximum 1,3-PDO yield (0.52 mol mol^{-1}) and productivity (57 g L^{-1} d^{-1}) were achieved in R1 at HRTs of 12 and 3 h, respectively. DGGE profiling of PCR-amplified 16S rRNA gene fragments showed that variations in the HRT had a critical impact in the dominant community of microorganisms. However, no appreciable differences in the bacterial population were observed between R2 and R3 at low HRTs. Production of H_2 was observed at the beginning of the operation, but no methane production was observed. This study proves the feasibility of 1,3-PDO production in EGSB reactors and represents a novel strategy to valorise glycerol generated in the biodiesel industry.

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1. Introduction

The increasing demand for energy, environmental concerns related to fossil fuels use and depletion, together with incentives for biofuels production have led to a rapid growth of worldwide biodiesel production in the last years. As a consequence, a surplus of crude glycerol, a by-product of biodiesel production that represents 10% w/w of the product, has been generated thus creating a glut in the market (Johnson and Taconi, 2007). Furthermore, the disposal of these massive amounts of glycerol became a complex and expensive process, imposing a great pressure in this industry. Since the supply of oils to be converted to biodiesel is becoming increasingly competitive, the profitability of the biodiesel industry will depend on the ability to confer value to its by-products and therefore, the conversion of crude glycerol into other useful products is required (Choi, 2008; Johnson and Taconi, 2007).

The anaerobic biological conversion of glycerol is of interest due to the highly reduced nature of this compound (Choi, 2008). Its fermentation results in the generation of more reducing equivalents when it is converted to glycolytic intermediates as compared with glucose fermentation (Yazdani and Gonzalez, 2007). This excess of reducing equivalents must be oxidized which can be accomplished by the production of H_2 and/or via various NAD(P)H consuming pathways towards reduced or neutral end products (Heyndrickx et al., 1991). In fact, glycerol can be converted into several compounds such as citric acid, lactic acid, formic acid, acetic acid, butyric acid, propionic acid, succinic acid, dihydroxyacetone (DHA), 1,3-PDO, dichloro-2-propanol (DCP), acrolein, hydrogen, butanol, ethanol, among others (Choi, 2008; Dharmadi et al., 2006; Yang et al., 2012).

Particularly interesting is 1,3-PDO, a versatile organic chemical used for the production of polyesters, polyethers and polyurethanes. This product is highly specific for glycerol fermentation and cannot be obtained from any other anaerobic conversion (Homann et al., 1990). The metabolic pathways involved in glycerol degradation have been reviewed (da Silva et al., 2009; Saxena et al., 2009). In *Klebsiella*, *Citrobacter*, *Clostridium* and *Enterobacter*, in the absence of an external oxidant, glycerol is fermented by a dismutation process involving two parallel pathways. The

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production of 1,3-PDO occurs in the reductive pathway that is carried out in two enzymatic steps. The first enzyme coenzyme B12-dependent glycerol dehydratase (EC 4.2.1.30) removes a water molecule from glycerol to form 3-hydroxypropionaldehyde, which is then reduced to 1,3-propanediol by the $NADH_2$-dependent enzyme 1,3-propanediol dehydrogenase (1,3-propanediol-oxydoreductase, EC 1.1.1.202). An exception is *C. butyricum*, which possesses a vitamin-B12 independent glycerol dehydratase (Saint-Amans et al., 2001). In the oxidative pathway glycerol is transformed in pyruvate which is subsequently converted to various fermentation products depending on the microorganism and culture conditions. Such products include lactate, acetate, ethanol, butyrate, succinate (Choi, 2008), 2,3-butanediol (Saxena et al., 2009), and butanol (Biebl, 2001). Glycerol can also be assimilated by *K. pneumoniae* via glycerol 3-phosphate (G3P) to pyruvate under aerobic conditions (Choi, 2008; da Silva et al., 2009).

As alternative to pure cultures, 1,3-PDO could be produced using open mixed cultures operated under non-sterile conditions. Expanded granular sludge bed (EGSB) reactors, a system where microorganisms are naturally immobilized, allowing maintaining a high biomass concentration inside the reactors under low hydraulic retention times (HRT), represents an interesting alternative to use open mixed cultures. Even though this kind of system has been widely used to treat wastewater, it has not been fully exploited for the production of added-value compounds from industrial by-products such as glycerol.

The aim of this work was to investigate the production of 1,3-PDO from glycerol in EGSB reactors inoculated with granular sludge. Three parallel continuous high-rate EGSB reactors were operated at hydraulic retention times between 3 and 24 h and glycerol concentration of 25 g L^{-1}. A control reactor was inoculated with granular sludge without any treatment (control reactor-R1). In order to test strategies to promote the elimination of methanogens, heat-treated and disrupted granules were used as inoculum in reactors R2 and R3, respectively. The microbial community is investigated for the first time in 1,3-PDO producing EGSB reactors.

2. Methods

2.1. Inoculum source

Granular sludge was obtained from an up-flow anaerobic sludge blanket reactor used to treat brewery wastewater. The water treatment facility is located in Lisbon, Portugal.

2.2. Experimental procedure

Two pre-treatments were applied to the granular sludge: heat, which consisted in (i) autoclaving the granular sludge at 100 °C for 15 min; and (ii) disruption of the granules using a 0.6 × 25 mm syringe. Granular sludge without treatment was used as control.

Continuous fermentations were conducted at 37 °C in three EGSB reactors (working volume of 375 ml) operated in parallel. Each reactor was inoculated with 100 ml of sludge and fed with a semi-defined culture medium containing per liter; 25 g glycerol, 1 g yeast extract, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.02 g $CaCl_2{\cdot}2H2O$, 0.2 g $MgSO_4{\cdot}7H_2O$, 3 g $NaHCO_3$, 0.5 g cysteine–$HCl{\cdot}H_2O$, 3 g NH_4Cl, 1 ml acid micronutrients solution (1.8 g L^{-1} HCl, 61.8 mg L^{-1} H_3BO_3, 61.3 mg L^{-1} $MnCl_2$, 943.5 mg L^{-1} $FeCl_2$, 64.5 mg L^{-1} $CoCl_2$, 12.9 mg L^{-1} $NiCl_2$, 67.7 mg L^{-1} $ZnCl_2$) and 1 ml alkaline micronutrients solution (0.4 g l^{-1} NaOH, 17.3 mg l^{-1} Na_2SeO_3, 29.4 mg L^{-1} Na_2WO_4, 20.5 mg L^{-1} Na_2MoO_4). Internal liquid recirculation was used in order to keep a suitable expansion of the granules inside the reactor. The initial pH of the medium was adjusted to 6.8. Different HRTs were applied starting with 24 h and afterwards changing it to 20, 12, 6 and 3 h. Steady states were assessed by measuring the concentration of 1,3-PDO.

2.3. Analytical methods

Acids, glycerol and 1,3-PDO were measured through high performance liquid chromatography (Jasco, Japan) equipped with UV and RI detectors. The column (Aminex cation-exchange HPX-*87H)* was eluted isocratically with H_2SO_4 0.01 N at 60 °C using a flow rate of 0.7 ml min^{-1}.

Biogas composition was analyzed by gas chromatography (Chrompack 9001) equipped with a thermal conductivity detector and two columns: Porapack Q (100–180 mesh) 2 m × 1/8″ × 2.0 mm SS column, and a MolSieve 5A (80–100 mesh) 1.0 m × 1/8″ × 2.0 mm SS. Argon was the carrier gas at a flow rate of 16 ml min^{-1}. The oven, injector and detector temperatures were 35, 110 and 110 °C, respectively.

2.4. Statistical analysis

The values of yields and productivities were compared among the three reactors for each operating condition. An *F*-Test Two-Sample for Variances was applied and subsequently a *t*-test was used assuming equal or unequal variances.

2.5. Microbial community analysis

2.5.1. DNA Extraction and PCR–DGGE

For the inoculum and all HRTs studied, representative samples of biomass were collected and stored at −20 °C until further treatment. Total genomic DNA was extracted using a FastDNA SPIN kit for soil (Qbiogene, Carlsbad,CA, USA) according to the manufacturer's instructions. The V6–V8 region of bacterial 16S rRNA genes was amplified by PCR using the primers U968GC-f and L1401-r (Nübel et al., 1996). PCR products were separated by DGGE in a polyacrylamide gel (8%) containing a linear denaturing gradient ranging from 30% to 60% (100%-denaturing solution containing 7 M urea and 40% formamide) using the DCode System (Bio-Rad Laboratories Inc., CA, USA). Electrophoresis was performed for 16 h at 85 V in 0.5× TAE buffer at 60 °C. Gels were then stained with silver nitrate and scanned in an Epson Perfection V750 PRO (Epson, USA). Similarity indices (Si) were calculated from the densitometric curves of the scanned DGGE profiles using the Pearson product-moment correlation coefficient (Häne et al., 1993).

2.5.2. Cloning and sequencing

Bacterial 16S rRNA genes were amplified by PCR using the primers Bact27-f and 1492-r (Lane, 1991). The PCR products were purified with Nucleo Spin Extract II kit (Clontech Laboratories), ligated into pGEM-T vector (Promega, WI, USA) and introduced into competent *Escherichia coli* cells *E. cloni*®10G (Lucigen, WI, USA), according to the manufacturer's instructions. Cells of positive transformants were lysed and the 16S rRNA genes were amplified by PCR using the primers U968GC-f and L1401-r and screened in DGGE by comparison with the band-patterns of the sludge sample (template for cloning). Clones matching different bands in the total community profile were selected for further analysis. Selected clones were amplified using pGEM-T vector-targeted primers SP6/T7, purified with the Nucleo Spin Extract II kit (Clontech Laboratories, USA) and subjected to DNA sequence analysis. Sequencing reactions were performed at Eurofins MWG Operon (Germany). Similarity searches for the 16S rRNA gene sequences were performed using the BLAST search program within the GenBank database (http://www.ncbi.nlm.nih.gov/blast/) (Altschul et al., 1990).

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