Production of 1,3-propanediol in EGSB reactors by open mixed cultures using glycerol as the carbon source

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Abstract
Two pre-treatments (granules disruption and heat) were applied to granular sludge to favour the production of 1,3-propanediol (1,3-PDO) from glycerol by non-methanogenic bacteria in expanded granular sludge blanket (EGSB) reactors. Different hydraulic retention times (HRT) were tested to achieve a maximum yield of 0.59 mol of 1,3-PDO/mol of glycerol in the control (no treatment) reactor at an HRT of 12 h. Molecular biology tools were used to evaluate the microbial community present in the inoculum and biomass for each HRT studied. Variations in the HRT had a critical impact in the dominant community of microorganisms. No appreciable differences in microbial population were observed between the reactors operated with heat–treated and disrupted granules at low HRTs. Additionally, the production of H_2 was observed at the beginning of the operation, however this was associated with low production of 1,3-PDO. In turn, no methane production was observed. This study proves the feasibility of 1,3-PDO production in EGSB reactors, which have the advantage of being operated under non-sterile conditions and represent a novel strategy to valorise glycerol being generated as by-product in the biodiesel industry.

Keywords
Glycerol, 1,3-propanediol, EGSB reactors, DGGE

INTRODUCTION

Glycerol represents 10% of the by-products generated in the biodiesel production (da Silva et al., 2009). Due to the fast growth of the biodiesel industry in the last decades, disposal of these massive amounts of glycerol became a complex and expensive process, imposing a great pressure in this industry. Therefore, finding a way to valorise this by-product is imperative. Nowadays, pure glycerol is mainly used in the food, pharmaceutical, and cosmetic industries as solvent, sweetener, and thickener (Wang et al., 2001). In addition, glycerol can be used as a feedstock for the production of several compounds such as lactic acid, ethanol, propionic acid, succinic acid and 1,3-propanediol, among others (Dharmadi et al., 2006). Due to its highly reduced state, the microbial fermentation of glycerol is of special interest (Choi 2008).

Particularly, 1,3-PDO has gained great attention by being a versatile organic chemical used for the production of polyesters, polyethers and polyurethanes (Chuah et al., 1995, Kaur et al., 2012). Glycerol can be fermented into 1,3-PDO by *Klebsiella*, *Clostridium*, *Enterobacter* and *Citrobacter* (Kaur et al., 2012). Alternatively, 1,3-PDO can be produced using open mixed cultures to be operated under non-sterile conditions, which is the goal of the current work. These cultures are composed by a great microbial diversity and are able to degrade a variety of substrate mixtures. Furthermore, in continuous culture this microbial diversity decreases leading to a community constituted only by the best-adapted microorganisms.

MATERIALS AND METHODS

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.

Experimental procedure

Two pre-treatments were applied to the granular sludge: heat, which consisted in autoclaving the granular sludge at 100ºC for 15 minutes; and disruption of the granules using a 0.6x25 mm syringe. Granular sludge without treatment was used as control.

Continuous fermentations were carried out at 37 ºC in three EGSB reactors (working volume of 375 ml) operated in parallel. . Each reactor was inoculated with one hundred ml of sludge and fed with a semi-defined culture medium containing 25 g l^{-1} glycerol, salts and 1 g l^{-1} yeast extract. The initial pH of the medium was adjusted to 6.8. Different HRTs were applied starting with 24 h and afterwards changing to 12 h, 6 h and 3 h. Steady states were assessed by measuring the 1,3-PDO concentration. Biogas production was measured with a Ritter MilliGascounter®.

Analytical methods

Gas samples were analyzed by gas chromatography (Chrompack 9001) equipped with a thermal conductivity detector and two columns: Porapack Q (100-180 mesh) 2 m x 1/8'' x 2.0 mm SS column, and a MolSieve 5A (80-100 mesh) 1.0 m x 1/8'' x 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.

Acids, glycerol and 1,3-propanediol 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 0.01 N H_2SO_4 at 60 °C at a flow rate of 0.7 ml min^{-1}.

Molecular biology analysis

PCR-DGGE: For the inoculum and all HRTs studied, representative samples were collected and stored at -20 ºC until further treatment. Total genomic DNA was extracted using a FastDNA SPIN kit for soil (Qbiogene, Carlsbad,CA, USA), the V6 to V8 region of bacterial 16S rRNA genes was amplified by PCR using the primers U968GC-f and L1401-r and the 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 7M urea and 40% formamide), using the DCode System (Bio-Rad Laboratories Inc, CA, USA).

Cloning and sequencing: bacterial 16S rRNA genes amplified with primers Bact27-f and 1492-r were ligated into pGEM-T vector (Promega, WI, USA) and introduced into competent *Escherichia coli* ®10G & 10GF (Lucigen, WI, USA), according to the manufacturer's instructions. Cells of positive transformants were lysed and the 16S rRNA genes were 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 and subjected to DNA sequence analysis (Eurofins MWG Operon, Germany). Similarity searches for the 16S rRNA gene sequences were performed using the BLAST search program within the GenBank database.

RESULTS & DISCUSSION

Soluble fermentation products

Samples collected from the effluent showed that glycerol was mainly converted in 1,3-PDO.

Acetate and butyrate were present but always in low concentrations (<1 g/l).

The 1,3-PDO yield for the reactors containing the disrupted, heat-treated and non-treated granules at an HRT of 24 h were 0.12, 0.29 and 0.22 g g^{-1}, respectively. Interestingly, these values increased with the decrease of the HRT applied. The maximum 1,3-PDO yield was reached at an HRT of 12 h for the reactor working with non-treated biomass (0.49 g g^{-1}) and for the other reactors at an HRT of 6 h (0.47 g g^{-1}).

The highest yields of 1,3-PDO from glycerol have been reported for pure cultures of *Clostridium butyricum* by Saint-Amans et al. (1994) and Gonzalez-Pajuelo et al. (2005) corresponding to 0.56 g g^{-1} and 0.65 mol mol^{-1} respectively; and for mixed cultures from wastewater (Selembo et al., 2009) corresponding to 0.69 mol mol^{-1}. The maximum theoretical yield for 1,3-PDO in co-production with acetate is 0.70 mol 1,3-PDO/mol glycerol (Beauprez et al., 2010; Biebl et al., 1999).

In this work, we obtained a yield of 0.59 mol 1,3-PDO/mol glycerol. Our results show the same metabolic profile as the one described by Gonzalez-Pajuelo et al. (2005), in which 1, 3-PDO production is significantly lower when H_2 is being produced. On the other hand, the highest volumetric productivities of 1,3-PDO obtained in the current work did not correspond to the highest yields but with the lower retention time (3 h). These values were 1.79, 2.25 and 3.42 g $l^{-1}h^{-1}$ for the reactors operated with disrupted granular sludge, heat-treated granular sludge and the control, respectively.

Gas composition

Methane production was not observed in any HRT meaning that the used conditions were unfavorable to methanogenic bacteria. Biogas production varied over time, being intense immediately after inoculation and gradually decreasing with the decrease of the HRT. CO_2 was the most abundant gas in the process and H_2 production was only significant at an HRT of 24 h for the reactors containing non-treated and heat-treated granular sludge. These results point to a shift in the overall metabolism that can be explained by the selection process that occurs through the different HRTs.

Microbial community profiles

The results obtained by DGGE clearly show that the microbial community changed significantly through the time of operation of the reactors (Figure 1).

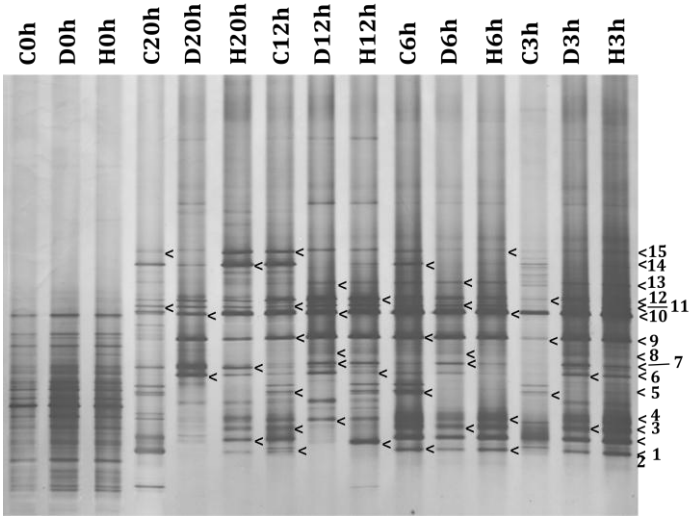

Figure 1: DGGE profile of the granular sludge at time 0 h, 20 h, 12 h, 6 h and 3 h for the control (no treatment) (C), disrupted granules (D) and heat-treated granules (H) reactors. Numbers mark the band position of the sequenced 16S RNA genes.

When the samples from different reactors are compared it is perceptible that the microbial community evolved differently according to the initial treatment. Nevertheless, no significant differences were found at low HRT, at least between the reactors operated with heat-treated and disrupted granular sludge. Bacterial 16S rRNA gene sequences retrieved from de sludge samples showed high levels of similarity (>97%) to those of members of the genus *Lactobacillus* (bands 1,2,3,4, and 10), *Vagococcus* (11,12 and 13), *Clostridium* (bands 14 and 15), *Enterococcus* (bands 6 and 7), *Klebsiella* (band 8), *Enterobacter* (band 9) and to uncultured bacteria from the *Clostridiaceae* family (band 5).

CONCLUSIONS

Continuous production of 1,3-PDO from glycerol using granular sludge in EGSB reactors is described for the first time. The production yield (0,59 mol 1,3-PDO/ mol glycerol) is not as high as the values reported for pure cultures, however the advantage herein is that fermentations are conducted in non-sterile conditions which has the potential of decreasing significantly the operational costs. Also, the conditions used suppressed the metabolism of the methanogenic bacteria resulting in the absence of methane production. Furthermore, the results suggest that pre-treatment of the biomass is not required.

ACKNOWLEDGEMENTS

R. Gallardo acknowledges the *Fundação para a Ciência e a Tecnologia* for the PhD Grant ref. SFRH/BD/42900/2008.

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