Bioresource Technology 161 (2014) 10–19

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Hydrogen production from cheese whey with ethanol-type fermentation: Effect of hydraulic retention time on the microbial community composition

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HIGHLIGHTS

- H_2 and EtOH production in anaerobic fluidized bed reactors from cheese whey.
- A maximum H_2 yield of 1.33 mol H_2 mol^{-1} lactose was produced for an HRT of 4 h.
- A maximum EtOH yield of 1.22 mol EtOH mol^{-1} lactose was obtained for an HRT of 4 h.
- The bacterial community was affiliated with *Selenomonas* and *Clostridium*.
- The archaeal community was affiliated with the genera *Methanobacterium*.

ARTICLE INFO

Article history:
Received 20 December 2013
Received in revised form 3 March 2014
Accepted 5 March 2014
Available online 13 March 2014

Keywords:
Cheese whey
Lactococcus lactis
Selenomonas sp.
Methanobacterium sp.
Continuous anaerobic fluidized bed reactors

ABSTRACT

The effects of different hydraulic retention times (HRTs) of 4, 2, and 1 h and varying sources of inoculum (sludge from swine and sludge from poultry) on the hydrogen production in two anaerobic fluidized bed reactors (AFBRs) were evaluated. Cheese whey was used as a substrate, and 5000 mg COD L^{-1} was applied. The highest hydrogen yield (HY) of 1.33 mol mol^{-1} lactose and highest ethanol yield (EtOHY) of 1.22 mol EtOH mol^{-1} lactose were obtained at the highest HRT (4 h). When the reactors were operated at an HRT of 1 h, methane (0.68 L CH_4 h^{-1} L^{-1}) was produced concurrently with hydrogen (0.51 L H_2 h^{-1} L^{-1}). The major metabolites observed were soluble ethanol, methanol, acetic acid, and butyric acid. Cloning of the 16S rRNA gene sequences indicated that the microbial community were affiliated with the genera *Selenomonas* sp. (69% of the sequences), and *Methanobacterium* sp. (98% of the sequences).

1. Introduction

The final disposal of dairy wastewater presents various issues. These effluents have different compositions according to the dairy product made, such as cheese, yogurt, milk, and butter (Prazeres et al., 2012). Cheese is a major agricultural product. Nine kilograms of cheese whey is produced for every kilogram of manufactured cheese. Cheese whey is considered a highly polluting effluent from dairy products due to its high organic load and the volume generated, representing a significant environmental impact for the dairy industry (Carvalho et al., 2013;. Prazeres et al., 2012).

Cheese whey is a highly biodegradable wastewater composed of lactose (70–72% dry extract), protein (8–10%), and minerals (12–15% dry extract) (Perna et al., 2013). The anaerobic digestion process is a particularly attractive solution for treating this type of wastewater due to the operational savings and bioenergy generated from a renewable source of inexpensive and abundant raw materials, promoting a reduction in wastewater pollution (Dareioti et al., 2009).

The fermentation of cheese whey, whose main component is lactose (a disaccharide consisting of one molecule of glucose and one molecule of galactose), may produce a maximum yield of 8 mol of hydrogen and 4 mol of acetate, or 4 mol of hydrogen and 2 mol of butyrate per mole of lactose (Davila-Vazquez et al., 2011).

Some researchers have demonstrated the potential of using cheese whey as a fermentation substrate for the production of

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http://dx.doi.org/10.1016/j.biortech.2014.03.020
0960-8524/

hydrogen (Carrillo-Reyes et al., 2012; Perna et al., 2013). However, several parameters are not yet well established and must be further investigated. The effect of using inocula of different origins has not been reported in the literature. The choice of inoculum is important for the selection of microorganisms and thus favorable hydrogen production (Tang et al., 2008). Using different inoculum sources at a controlled pH may result in different percentages of metabolites produced, such as acetate and ethanol, as well as differing amounts of organic substrate consumed. The acclimatization of the biomass chosen as the inoculum source is important to achieving greater consumption of the substrate and greater hydrogen production (Abreu et al., 2010).

The hydraulic retention time (HRT) is also an important parameter in fermentation processes. Higher rates of volumetric hydrogen production and increased percentages of hydrogen in biogas can be obtained by decreasing the HRT and thus increasing the organic loading rate (OLR) (Carrillo-Reyes et al., 2012; Davila-Vazquez et al., 2009). Changes in OLR have a considerable influence on the diversity of the microbial population and on the metabolism pathways of bacteria that may favor hydrogen production (Hafez et al., 2010). Some authors who used cheese whey as a substrate have verified an increase in the hydrogen yield with increasing OLR (Castello et al., 2009; Perna et al., 2013). However, other authors have observed a decrease in yield with increasing OLR (Azbar et al., 2009; Davila-Vazquez et al., 2009).

Cheese whey as a substrate has been studied in continuous stirred tank reactors (CSTRs) (Azbar et al., 2009; Davila-Vazquez et al., 2009; Yang et al., 2007). Although the stirring conditions of these systems can efficiently improve mass transfer, operating CSTRs at low HRTs may result in the washout of cells (Carrillo-Reyes et al., 2012).

Anaerobic fluidized bed reactors (AFBRs) are high-rate systems that favor mass transfer between the medium and biofilm. Moreover, AFBRs have been used successfully for biological hydrogen production (Barros and Silva, 2012; Shida et al., 2012). Thus, the present study aims to evaluate the effect of HRT (4, 2, and 1 h) and different inoculum sources on hydrogen production using cheese whey as a substrate in AFBRs.

2. Methods

2.1. Analytical methods

The Carbohydrate analyses were performed according Dubois et al. (1956). The biogas produced in the reactors was measured by a Ritter MilliGas counter (TG1; Ritter Inc., Germany). The composition of the biogas was analyzed by gas chromatography (Shimadzu GC-2010) using a Supelco Carboxen Plot 1010 column (length: 30 m, internal diameter: 0.53 mm) with argon as the carrier gas. Acids and alcohols were determined with a liquid chromatograph (HPLC Shimadzu) equipped with a pump (LC-10ADVP), auto sampler (SIL-20A HT), column oven (CTO- 20A) at 43 °C, refractive index detector (RID-10A), controller (SCL-10AVP) system, and Aminex column HPX-87H (300 mm, 7.8 mm, BioRad). The mobile phase consisted of H_2SO_4 (0.01 N) at 0.5 mL min^{-1}.

2.2. PCR/DGGE and phylogenetic analysis of 16S rRNA gene

At the end of each fermentation, biomass samples were taken for each HRT applied (4, 2, and 1 h) in both reactors to analyze the effect of HRT on the microbial community using denaturing gradient gel electrophoresis (DGGE). The organisms were sequenced and identified using samples from AFBR2 with an HRT of 1 h.

Genomic DNA was extracted by cell lysis with glass beads (Sigma), phenol, chloroform, and phosphate buffer. The 16S rRNA fragments were amplified by polymerase chain reaction (PCR) using primers 968FGC-1401R (Nübel et al., 1996) for the bacteria community and primers Parch519fGC-Arch915r (Vissers et al., 2009) for the archaea community. The 16S rRNA fragments were then subjected to DGGE.

Using denaturing gradient concentrations of 45% and 65%, DGGE was prepared for community of bacteria and archaea. The electrophoresis conditions were 75 V and 60 °C for 16 h. The DGGE band profiles were obtained using an Eagle Eye III TM (Stratagene) at 254 nm and Eagle Sight software. Pearson's correlations for the band profiles were calculated by BioNumerics software version 2.5.

Cloning and 16S rRNA sequence determination used primers 27F-1100R (Lane, 1991) and 21F-Arch958R (Vissers et al., 2009) to identify the bacteria and archaea populations, respectively. The purified PCR products were cloned into plasmid pGEM Easy Vector System I according to the manufacturer's specifications. Transformation was achieved in competent cells of *Escherichia coli*. The cloned DNA fragments were recovered by PCR using the primers M13F and M13R. The PCR products were sequenced by Macrogen (Macrogen; http://www.macrogen.com). These sequences were compared with the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/) and Ribosomal Database Project (http://rdp.cme.msu.edu/seqmatch). The phylogenetic tree was developed using the software MEGA version 5.2 using the neighbor joining method. The confidence of tree was evaluated by the bootstrap method using 1000 replications. The sequences from this study were deposited in GenBank, with accession numbers ranging from KF848958 to KF848979.

2.3. Sludge and substrate

Two inocula from different origin sources were used in this study. AFBR1 was inoculated with sludge from a UASB reactor used in the treatment of swine wastewater (In_1). AFBR2 was inoculated with sludge from a UASB reactor that treated poultry slaughterhouse wastewater (In_2). These inocula were subjected to heat treatment according to Kim et al. (2006).

The cheese whey powder used as substrate in this study was supplied by Cargill Agrícola S/A. Micronutrients were added with the following concentrations (in mg.L^{-1}): $NiSO_4.6H_2O$ (0.48), $FeSO_4.7H_2O$ (2.40), $FeCl_3.6H_2O$ (0.24), $CoCl_2.2H_2O$ (0.04), $CaCl_2.6H_2O$ (23.40), SeO_2 (0.04), urea (624.00), KH_2PO_4 (42.40), K_2HPO_4 (10.83), and $Na_2HPO.4H_2O$ (16.67). The cheese whey was diluted to 5000 mg COD.L^{-1} and had an initial pH of 6 and a total carbohydrate content of 4 g.L^{-1}. The pH was controlled within the range of 4.0–4.5 with $NaHCO_3$.

2.4. Set-up and operating conditions of the reactors

Two reactors were constructed of transparent acrylic, with a volume of 770 mL each (Fig. 1). Polystyrene was used as a support material for biomass immobilization, with a mean diameter of 2.2 cm. The reactor was fed with cheese whey powder, diluted to 5000 mg.L^{-1}, and heat-treated sludge (10% *v/v*). The total liquid flow (Q) was maintained at 76 L.h^{-1}, and the superficial velocity was maintained at 1.3 times the minimum fluidization velocity.

Nitrogen gas was used to sparge the fermentation medium to create an anaerobic environment. The reactor temperature was maintained at 30 °C. The system was initially operated in batch mode for 72 h to promote the adhesion of microorganisms to the support material and then operated continuously at an HRT of 4 h. When steady-state conditions were reached (i.e., hydrogen