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Microbial diversity of hydrogen-producing bacteria in batch reactors fed with cellulose using leachate as inoculum

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ABSTRACT

Hydrogen production using cellulosic residues offers the possibility of waste minimization with renewable energy recovery. In the present study, heat-treated biomass purified from leachate was used as inoculum in batch reactors for hydrogen production fed with different concentrations of cellulose (2.5, 5.0 and 10 g/L), in the presence and absence of exogenous cellulase. The heat-treated biomass did not degrade cellulose and hydrogen production was not detected in the absence of cellulase. In reactors with cellulase, the hydrogen yields were 1.2, 0.6 and 2.3 mol H_2/mol of hydrolyzed cellulose with substrate degradation of 41.4, 28.4 and 44.7% for 2.5, 5.0 and 10 g/L cellulose, respectively. Hydrogen production potentials (P) varied from 19.9 to 125.9 mmol H_2 and maximum hydrogen production rates (R_m) were among 0.8–2.3 mmol H_2/h. The reactor containing 10 g/L of cellulose presented the highest P and R_m among the conditions tested. The main acid produced in reactors were butyric acid, followed by acetic, isobutyric and propionic acids. Bacteria similar to *Clostridium* sp. (98–99%) were identified in the reactors with cellulase. The heat-treated leachate can be used as an inoculum source for hydrogen production from hydrolyzed cellulose.

1. Introduction

The common current energy sources (fossil fuels) have caused serious environmental problems such as global warming. Therefore, alternative energy sources should be developed to replace fossil fuels.

Hydrogen (H_2) is a promising candidate as an ideal fuel in the future due to its clean nature and high energy yield (122 KJ g^{-1}) [1,2].

Compared with conventional H_2 generation processes that require a high energy demand, biological H_2 production is more environmentally friendly and less energy intensive [3]. Furthermore, fermentative H_2 production can utilize different organic substrates and produce high hydrogen yields [4,5].

Hydrogen production from cellulose is a promising alternative to substitute fossil fuels because cellulose is the most abundant renewable resource in nature, is inexpensive and constitutes a major portion of agricultural and forest waste

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and industrial effluents such as those from the pulp/paper and food industries [6–8]. Therefore, the use of cellulosic residues offers the possibility of waste minimization with renewable energy recovery [4].

Leachate is generated in places of municipal solid waste disposal, such as landfills, as a consequence of the contact of water with the solid waste, and so it is a wastewater characterized by its high chemical oxygen demand (COD) [9,10]. It contains a complex mixture of organic and inorganic constituents and microorganisms performing anaerobic waste biodegradation [9]. Therefore, leachate is a source of hydrolytic and fermentative microorganisms and has already been used as a source of anaerobic biomass for hydrogen production from glucose [11]. According to Liu et al. [12], one of the main organic compounds of leachate is cellulose, since municipal solid wastes are composed mostly by lignocellulosic residues [13].

The conversion of cellulose to hydrogen involves hydrolysis into soluble sugars followed by their fermentation by anaerobic microorganisms [8,14]. The biological hydrolysis of cellulose is carried out by a cellulase enzyme complex [8,15] synthesized by a variety of microorganisms. The set of enzymes (endoglucanases, exoglucanases and β-glucosidases) act in synergy to release readily fermentable monomers [16].

Cellulolytic microorganisms are present in different anaerobic environments such as sediments, rice paddies, rumen, bioreactor sludge and landfill leachate [11,17–19]. Some bacteria of the genera *Clostridium*, *Bacillus*, *Ruminococcus*, *Bacterioides*, *Enterobacter*, *Erwinia*, *Acetovibrio* are known as cellulose degraders [4,20].

Huang et al. [9] did an overall description of the bacterial populations of a leachate from a full-scale recirculating landfill and reported that the clones were related to the genus *Clostridium*, which are described as the main group of cellulose degraders in landfills [21].

In addition to hydrolytic and fermentative bacteria, the leachate microbial community is composed of hydrogenotrophic methanogenic archaea [22], which is undesirable in anaerobic hydrogen production systems. Heat pretreatment is commonly used to inactivate hydrogen-consuming microorganisms [23–25].

Recent studies have used leachate as a substrate or as a source of nutrients for anaerobic hydrogen production [10,12]. However, there are few reports of hydrogen production that have used leachate as a source of anaerobic microorganisms. For this reason, the aim of this research was to investigate the biological hydrogen production from cellulose using microbial consortia purified from a leachate under mesophilic conditions, in the presence and absence of cellulase. The phylogenetic diversity of the hydrogen-producing community was analyzed using 16S rRNA-based techniques.

2. Materials and methods

2.1. Source of inoculum

Leachate samples were collected from a landfill in São Carlos, Brazil and used as a source of inoculum. The samples were transported to the laboratory and were stored at 4 °C prior to use for experimental purposes to minimize the effects of biological and chemical reactions.

The leachate was heat pretreated at 90 °C for 10 min to inactivate hydrogenotrophic methanogens archaea [26]. The pretreated biomass was transferred to 50 mL vials and submitted to centrifugation (8500 rpm/4 °C for 8 min). The pellet containing the microorganisms was transferred to a Duran flask containing fresh culture medium for biomass enrichment, described in Section 2.3.

2.2. Cellulose and cellulase

The cellulose used as the sole carbon source was a microcrystalline powder with a 20-μm particle size (Sigma–Aldrich).

The cellulase enzyme used (1,4-(1,3:1,4)-β-D-glucan 4-glucanohydrolase, Celluclast® 1.5 L from *Trichoderma reesei* ATCC 26921) is sold commercially by Sigma Aldrich (C2730). The amount of enzyme added to each reactor took into account the theoretical number of enzyme units required to catalyze the total breakdown of cellulose into glucose. Each milliliter of cellulase contained 700 units, and each unit is capable of liberating 1 μmol of glucose/hour at pH 5.0 and 37 °C [27]. The concentrations of cellulase added to the reactors are presented in Table 1.

2.3. Batch enrichment of mesophilic H_2-producing microorganisms

The enrichment of the pretreated biomass (microorganisms) was done in Del Nery medium prepared as previously described by Maintinguer et al. [25] and modified by the substitution of peptone with yeast extract (1000 mg/L). The culture medium was sterilized by filtration and the pH was adjusted to 7 with 1.0 M NaOH.

The enrichment was performed in 1 L anaerobic bottles with a 0.5 L working volume made up of Del Nery medium, 10% (v/v) inoculum, cellulose (0.5 g/L) and cellulase (4 ml/L or 2800 units of cellulase). The headspace in the anaerobic bottles was filled with N_2 (100%) and incubated at 37 °C. This biomass was maintained in the exponential growth phase and used for the hydrogen production assays.

2.4. Hydrogen production in batch fermentation

All batch fermentation tests were performed in anaerobic batch reactors with a total volume of 5 L and a working volume of 1 L, consisting of Del Nery medium modified as described above under the three conditions outlined in Table 1. Reactors without cellulase for each substrate concentration were also included in the experiment. The biomass in exponential growth phase was submitted to successive washes (8500 rpm/

Table 1 – Concentration of cellulase added to the reactors fed with cellulose.

Cellulose concentration (g/L)	Cellulase (mL)	Units of cellulase (U)
2.5	7.5	5250
5.0	15	10,500
10	30	21,000