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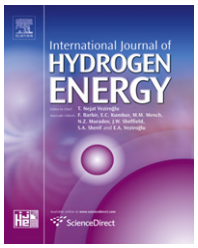

Effects of substrate loading and co-substrates on thermophilic anaerobic conversion of microcrystalline cellulose and microbial communities revealed using high-throughput sequencing

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ARTICLE INFO

Article history:
Received 9 December 2011
Received in revised form
13 February 2012
Accepted 15 February 2012
Available online 17 March 2012

Keywords:
Co-substrate
Microcrystalline cellulose
Thermophilic
Anaerobic
Illumina
High-throughput

ABSTRACT

Batch tests were conducted to investigate the effect of co-substrates, including glucose, xylose and starch, on thermophilic anaerobic conversion of microcrystalline cellulose using mixed culture enriched from anaerobic digestion sludge (ADS). Up to 30.9% of cellulose was utilized with xylose as co-substrate. When using glucose as co-substrate, cellulose conversion rate reached the maximum of 0.048 g/l/h at cellulose loading of 5.0 g/l. Illumina high-throughput sequencing of the 16S rRNA gene revealed that the thermophilic consortium exclusively consisted of *Clostridium* (more than 70% of all sequences). Growth of *Thermoanaerobacterium* over *Clostridium* would inhibit cellulose conversion capacity of the consortium. But the growth of *Thermoanaerobacterium* could be repressed by pH higher than pH 6.0. Co-substrates caused noticeable variation of bacterial community structure. Predominance of *Thermoanaerobacterium* over *Clostridium* was observed when monosugars (glucose and xylose) were used as co-substrate without pH control. Starch was ineffective as co-substrate because it competed with cellulose for *Clostridium*.

1. Introduction

The pressing need for clean renewable energy sources has aroused worldwide research interest on the exploration of biofuels. Over the last decades, research revolved around this topic mainly focused on bioethanol and biodiesel generation from food crops. This first generation of biofuels has been seen as possible alternatives to release the global dependence on gasoline or diesel, however, the edible feedstock used for this approach competed with food supply and thus aggravate the recent food crisis. Hence, the next generation biofuels produced from lignocellulosic feedstocks (e.g. forestry or agricultural residues and municipal wastes) are now essential for the development of renewable biofuels.

Another particularly attractive advantage for biofuel generation from cellulosic materials is their relatively low cost and plentiful supply. It is estimated that the United States has the potential to produce in excess of 1.3 billion tons of biomass feedstock each year for the production of lignocellulosic biofuels [1]. The general absence of low-cost technology to

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0360-3199/$ – see front matter
doi:10.1016/j.ijhydene.2012.02.079

overcome the recalcitrance of cellulosic biomass is the central impediment for more widespread utilization of this important resource [2].

In recent years, cellulose fermentation at thermophilic condition by mixed microbial consortium had been extensively studied as a promising strategy to overcome the above impediment [3–5]. A number studies have been carried out on the process parameters and corresponding microbial communities. However, much remains to be elucidated at the time. Normally co-substrate was added in order to improve the treatability and bioavailability of the more refractory matter for microbial degradation. Its effect has been widely studied in bioremediation of contaminated soil and wastewater containing inhibitory organic compounds or highly chlorinated environmental pollutants [6–8]. However, the idea of dosing co-substrate was seldom applied in biodegradation of recalcitrant lignocellulosic materials. Thereby, the objective of this study was to investigate the effects of co-substrate and cellulose loading on thermophilic anaerobic conversion of microcrystalline cellulose. In addition, hydrogen is the most attractive fermentation product because as energy carrier it releases more heat and produces only water during combustion. However, hydrogen generation process is very delicate to microbial environment that the activity of methanogens must be effectively repressed in order to get good yield of hydrogen. In this study, the effect on cellulose conversion by repressing the activity of methanogen in order to enhance hydrogen generation will be investigated by comparing the cellulose conversion capacity of hydrogen and methane producing consortium. Furthermore, Illumina high-throughput sequencing was applied for the first time to reveal the microbial community of anaerobic digestion seed sludge and the enriched thermophilic cellulolytic microflora.

2. Material and methods

2.1. Inoculum

The sludge seed was anaerobic digestion sludge collected from Shek Wu Hui wastewater treatment plant (Hong Kong SAR, China), containing volatile suspended solid (VSS) of 13.6 g/L. The sludge was enriched at 55 °C using microcrystalline cellulose (50 μm, Sigma, USA) as substrate and glucose as co-substrate at chemical oxygen demand (COD) ratio 10:1 for 120 days before inoculation. The VSS of the enriched ADS reduced to1.37 g/L after enrichment.

2.2. Growing medium

The chemical composition of the growing medium was as follows (gram per liter): K_2HPO_4 0.5, KH_2PO_4 0.5, $CaCl_2$ 0.05, $MgSO_4 \cdot 7H_2O$ 0.05, NH_4Cl 0.5, and 1 ml trace element solution which was prepared according to a previous report [9]. The concentration of total phosphorus (TP) and total nitrogen (TN) in the medium was 200 mg/L and 130 mg/L respectively.

2.3. Experiments

2.3.1. Effect of co-substrate

Sequential batch experiments were conducted in 50 ml serum bottles with working volume of 30 ml. Each reactor was inoculated with enriched ADS at 60% (v/v) to achieve an initial biomass concentration around 820 mg/l. 4.0 g/l microcrystalline cellulose was used as the substrate. Glucose, xylose and soluble starch of 0.4 g/l were dosed individually as the co-substrate. The initial pH was adjusted to around pH 6.6 with 1N HCl and 1N NaOH. The serum bottle was then sealed with the butyl rubber stopper and the aluminum seal, and then purged with argon for 5 min to ensure the anaerobic environment inside. The bottles were then incubated in water bath at 55 °C with constant stirring at 120 rpm. The first batch ceased after 48 h, and then the sludge was centrifuged at 4000 rpm for 10 min and the obtained sludge pellet was applied to inoculate the second batch with fresh supplement of substrate and co-substrate the same as the previous batch. All the batch tests were conducted in duplicates, plus control without dosing co-substrate and blank dosing neither cellulose substrate nor co-substrate. The results presented were the average value of two replicates subtracted by the blank value.

2.3.2. Effect of substrate loading

In order to provide better pH control and mixing, 1-L five-neck flask was used as the reactor for the loading tests. 800 ml enriched ADS was fed with increasing amount (2.0, 3.0, 4.0, and 5.0 g/l) of microcrystalline cellulose with glucose as the co-substrate. Cellulose and glucose concentrations were controlled at COD ratio of 10:1. The reactors were then purged with argon for 15 min. pH was controlled above pH 6.0 by adding 2N NaOH via the automatic pH controller (pH-201, MSITECH, Singapore). Each batch was stopped when pH value started to rise above pH 6.2 because this time point usually indicated the highest cellulose conversion. Two replicate tests were conducted for each loading and the data presented were the average value.

2.4. Analytical methods

The volume of gas produced was monitored by a glass syringe. Gas contents, including hydrogen, methane and carbon dioxide, were determined by a gas chromatograph (GC) (Hewlett–Packard 5890II, USA) equipped with a thermal conductivity detector (TCD). Argon was used as the carrier gas at a flow rate of 30 ml/min. Injector, detector and column temperatures were kept at 57, 180 and 50 °C, respectively. The COD value of the liquid was determined based on the practical assumption that COD equals to three times the amount of total organic carbon (TOC) which was measured using TOC-VCPH (SHIMADZU, Japan). The composition of products in liquid including volatile fatty acids (VFAs) and alcohols, was measured using a second GC (6890N, Agilent Technologies, USA) with a flame ionization detector (FID) [10]. TSS (total suspended solid), VSS, TN, and ammonia–nitrogen (NH_4^+–N) were determined according to the Standard Methods [11]. The conversion of cellulose substrate was calculated based on the mass balance as described before in Ref. [12]. The accumulative amount of converted cellulose (in terms of COD) was calculated as the sum of the cumulative CH_4 and H_2 in gas