Research Article

Effects of Thermophilic Heat Pretreatment of Mixed Inoculum on Biohydrogen Production from Synthetic and Sugarcane Mill Wastewaters

Sandhya Babel*
School of Biochemical Engineering and Technology, Thammasat University
Rangsit Campus, Khlong Nueng, Khlong Luang, Pathum Thani 12120, Thailand

Emmanuel Pacheco Leaño
Department of Biology, College of Arts & Sciences, Central Mindanao University,
University Town, Musuan, Bukidnon 8710, Philippines

Abstract

Various pretreatment methods have been conducted on the mixed inoculums to enrich hydrogen producing bacteria (HPB). This study investigates the use of heat as a pretreatment method for the anaerobic sludge at 105°C, ranging from 45, 60, 90, and 120 minutes to determine the optimum time to suppress methanogenic activity and enhance hydrogen production using artificial wastewater in batch experiments. Experiments are also conducted with sugarcane mill wastewater with and without dilution and the results compared with synthetic wastewater. Optimum time of heating the seed inoculum obtained from the experiment with sucrose solution was applied to the sugar mill wastewater as substrate. Results of batch experiments showed that heat treatment at 105°C of the seed sludge, regardless of time duration of heating, showed no activity for methanogenic bacteria when using sucrose solution as substrate. There is an increasing trend of hydrogen production with an increase in heating time at 105°C regardless of sucrose loading (10 g/L and 20 g/L) with the highest value recorded at 120 minutes (3.24 mol H_2/g COD on 10g/L and 4.26 mol H_2/g COD on 20g/L). Results for sugarcane mill wastewater revealed an increasing trend of hydrogen production with the highest value of 2.38 mol H_2/gCOD observed for 100% substrate loading (without dilution) and 1.75 mol H_2/gCOD on 50% dilution, indicating a suitable source for clean energy production.

Keywords: Anaerobic Sludge; Heat Pretreatment; Hydrogen Production; Wastewater; Sucrose.

1. Introduction

Dark fermentation is widely reported in the literature for hydrogen production. The advantage is that it can utilize cheap organic substances. Although hydrogen can be efficiently produced from simple sugars in small scale laboratory experiments, application of this technology in organic waste or wastewater is a major challenge (Mu et al., 2007). Studies have investigated hydrogen production from wastewater (Bhaskar et al., 2008; Jung et al., 2010). In

***Correspondence :** sandhya@siit.tu.ac.th

general, it is hard to convert directly real wastewater and wastewater containing complex substances such as insoluble organic substances, oils, and fats into bio hydrogen using anaerobic fermentation. This might be due to low substrate conversion efficiency for hydrogen, and most of the organics remain as soluble fermentation products. Even under optimal conditions, about 40-60% of the original organic matter remains in solution in the form of Volatile Fatty Acids (VFAs) and solvents (Gadhamshetty et al., 2009, Mohan et al, 2007a). Thus, it is important to use several strategies, such as improving the digestibility of the substrate and pretreatment of seed inoculum for enhancing hydrogen production while achieving stabilization of the treated organic waste prior to disposal.

Hydrogen-producing bacteria (HPB) can produce hydrogen gas from organic wastewater. This not only helps in treating organic wastewater, but also generates very clean fuel (Wang and Wan, 2008; Mu et al., 2006; Levin et al., 2006; Cheong and Hansen, 2006; Fan et al., 2004). Processes using mixed cultures are more practical than those using pure culture, because the former are simpler to operate and may have a variety of microorganisms that serve as a broader source of inoculum; thus mixed cultures are preferred for wastewater treatment (Li and Fang, 2007). However, in a mixed culture system, under anaerobic conditions the hydrogen produced by hydrogen-producing bacteria, such as *Clostridium* and *Enterobacte*r, is often readily consumed by hydrogen-consuming bacteria, such as methanogens and homoacetogens (Li and Fang, 2007; Oh et al., 2003; Lay, 2001; Cai et al., 2004). Therefore, in order to harness hydrogen from a mixed culture system, the seed sludge needs a pretreatment to suppress as much hydrogen-consuming bacterial activity as possible while still preserving the activity of the hydrogen-producing bacteria (Wang and Wan, 2008). At present, the pretreatment methods reported for enriching hydrogen-producing bacteria from seed sludge mainly include heat-shock, acid, base, aeration, freezing and thawing, chloroform, sodium 2-bromoethanesulfonate or 2-bromoethanesulfonic acid and iodopropane. Some researchers reported that heat pretreatment on anaerobic sludge is the most suitable method to prepare HPB from mixed inoculums (Mu et al., 2007; Wang and Wan, 2008). Other pretreatment methods such as loading-shock (O-Thong et al., 2009), alkaline (Zhu and Beland, 2006), chloroform (Hu and Chen, 2007) and BESA (Mohan et al., 2008), were also reported as the most effective method to enrich HPB in different studies.

Studies have shown that the application of the heat-shock pretreatment method resulted in the highest H_2 production rate (HPR) (Mu et al., 2007; Wang and Wan, 2008). So far, there have been several studies comparing various pretreatment methods for enriching hydrogen-producing bacteria from seed sludge; however, some conclusions were conflicting (Wang and Wan, 2008). Thermophilic hydrogen production has been proven to be effective in some studies (Akutsua et al., 2009; Ueno et al., 2007). Heat pretreatment of sludge sample as seed will inhibit the bioactivity of hydrogen consumers and enhance biohydrogen-producing spore-forming bacteria.

Thus, in this study, experiments were carried out to investigate the use of heat as a pretreatment method for the sludge from an Upflow Anaerobic Sludge Blanket (UASB) reactor from a local brewery company. This, anaerobic sludge was heat-treated at 105^{o}C ranging from 45, 60, 90, and 120 minutes, to determine the optimum time to suppress methanogenic activity and enhance hydrogen production using synthetic wastewater (sucrose). Optimum time of heating the inoculum obtained from the experiment was applied to the sugar mill wastewater as substrate. The wastewater was also diluted to 50% to see the effect on hydrogen production.

2. Methodology

2.1 Seed Sludge

Anaerobic sludge collected from an Upflow Sludge Anaerobic Blanket (UASB) reactor of Pathum Thani Brewery, Thailand, was used as seed sludge. The sludge was stored at 4^{o}C prior to analysis of its parameters such as pH, Total Solids (TS), Total Volatile Solids (TVS) and Chemical Oxygen Demand (COD).

2.2 Sugarcane Mill Wastewater

Sugarcane mill wastewater was collected from Saraburi Sugar Co., Ltd., Saraburi, Thailand. The sample was immediately analyzed in the laboratory as to its pH, TS, TVS, and COD content and was then kept at 4^{o}C for further experiments.

2.3 Synthetic Wastewater

Two concentrations of artificial wastewater were used: 20 g/L and 10 g/L sucrose. These were prepared by dissolving 20 g and 10 g of sucrose in distilled water in a 1L volumetric flask, respectively. No additional nutrients or trace elements were supplemented and thus the solution can be referred to as sucrose solution. COD of the prepared synthetic water was analyzed.

2.4 Pretreatment of Seed Sludge

Heat treatment was done to inactivate methanogens in the sludge. Seed sludge was heat-treated at 105^{o}C for 45, 60, 90, and 120 minutes to deactivate methanogenic activity. This thermophilic temperature was selected based on the optimum conditions obtained from preliminary experiments. Results of the experiments conducted at 75^{o}C, 85^{o}C, 95^{o}C, and 105^{o}C for the pretreatment of seed sludge at different time periods showed that methanogenic activity was suppressed at 105^{o}C when using sucrose as substrate. Thus, 105^{o}C was used for the pretreatment of the seed sludge. This was then allowed to cool down at room temperature. Heat treatment was conducted to evaluate the effectiveness of biohydrogen producing bacteria.

2.5 Hydrogen Gas Production

Heat-treated sludge (10 mL) at different heating time duration and artificial wastewater (50 mL, sucrose) are added into 120 mL serum bottle at a working volume of 60 mL. For control, seed sludge without heat pretreatment was used. The solutions are adjusted to an initial pH of 7 with 1M HCl or 1 M NaOH. The bottles are sparged with nitrogen gas for 3 minutes to generate anaerobic condition. These are then sealed tightly with a rubber septum and aluminum cap before being put on a shaker operated at a constant rate of 90 rpm. The batch experiments done in duplicates are conducted in the dark for 168 hours at 37^{o}C. Experiments are carried out following the same procedure with sugarcane mill wastewater with and without dilution (50% and 100%) only for sludge heat-treated at 120 minutes.

During the test, gas samples are collected routinely and analyzed for hydrogen and methane contents. At the end of the test, mixed samples from each serum bottle are collected to determine the final pH and COD.

2.6 Determination of Gas Composition

The gas accumulating the head space of the serum bottles was measured and sampled initially after 12 hours of contact time and 24 hours thereafter up to 168 hours. The gas produced was collected using a syringe. Before each sampling, a 60-mL syringe was used to equilibrate the pressure inside the bottle to the ambient pressure, and the volume in the syringe was recorded as the total gas volume. A Hamilton Syringe 1710N with a capacity of 100μL was used to draw the gas from each serum bottle. This volume was reduced to 50μL by releasing the rest of the gas to ensure that no amount of liquid was drawn from the serum bottle. This was then injected into the gas chromatograph. The components of the gas were analyzed using a gas chromatograph (GC) (PerkinElmer, USA) equipped with a thermal conductivity detector (TCD). The GC operating conditions used in the determination of gas composition were similar to that employed by Khanal et al. (2004). Column, detector, and injection temperature were 45°C, 100°C, and 100°C, respectively. Carrier gas flow rate (Helium) flow rate was 25 mL/min.

2.7 Determination of Wastewater and Sludge Characteristics

The pH value was measured by a pH meter (WTW, Model pH 330). COD, TS, and TVS are determined in accordance with the procedures described in the Standard Methods for the Examination of Water and Wastewater (1998). COD was measured using the Closed Reflux Colorimetric Method.

3. Results and Discussions

3.1 Characterization of wastewater and sludge

Characteristics of wastewater and sludge are presented in Table 1. Synthetic wastewater both at 20 g/L and 10 g/L have higher COD values (21,900 mg/L and 13,000 mg/L, respectively), compared with sugarcane mill wastewater which has only 11,550 mg/L (no dilution) and 5,480 mg/L at 50% dilution. This COD value for sugarcane mill wastewater is slightly above the reported values of between 2,300–8,000 mg/L (World Bank Group, 2007). There are more volatile solids in sugarcane mill wastewater (287 mg/L), compared to synthetic wastewater (8.93 mg/L).

The sugar industry has high water consumption and generates huge amounts of wastewater with large quantities of suspended solids and organic matter; COD ranges from 2,300–8,000 mg/L from sugar cane processing and up to 10,000 mg/L in beet processing (World Bank Group, 2007). The wastewater basically consists of sugars and organic material from cane or beets. The wastewater quantity and composition depends on the final products, production processes, and equipment (Macarie and Le Mer, 2006).

The conversion efficiency of organics to hydrogen is one of the significant problems encountered in the fermentation process as most of the organics remain as soluble fermentation products. Also, it depends on the fermentation pathways. The highest theoretical yields of hydrogen are associated with acetate as fermentation end product. The yields are normally low since the end products of mixed-acid type fermentation include lactic, acetic, propionic, and formic acids and alcohols.

3.2 Effect of heat pretreatment of seed sludge

Results of batch experiments showed that anaerobic sludge used as seed sludge without heat pretreatment with the synthetic wastewater responded after 12 hours and has a detectable amount of methane (Figure 1). There is no observed marked difference between the sucrose load (20 g/L and 10 g/L) in terms of the hydrogen production. This might be due to the formation of volatile fatty acids (VFAs) which might cause the drop of pH, hence, a lesser amount of H_2 production. The decrease of gas production at 48 h and increase at 72 h could be due to the availability, concentration, and influence of fermentation products as pointed out by Venkata Mohan et al. (2007). It is known that bacteria which undergoes mixed acid fermentation or butane 2,3 diol fermentation evolves hydrogen through formate decomposition. The pathway of pyruvate decomposition through acetyl-CoA produces formate and the acetyl-CoA gives rise to either acetate or ethanol (Tanisho et al., 1998).

The gas produced from sugar by untreated seed sludge in the studies of Hu and Chen (2007) and Chen et al. (2002) both contained methane gas. This could be due to the presence of a certain amount of hydrogen-consuming bacteria, specifically the methanogens. This means that the anaerobic sludge contains both types of microbes, the hydrogen-producing and hydrogen-consuming bacteria. The physiological differences between hydrogen-producing bacteria and hydrogen-consuming bacteria could be a gauge for alternative methods for preparation of hydrogen producing seeds. Spore forming hydrogen-producing bacteria can form protective spores when subjected to an extreme environment such as high temperature or extreme acidity and alkalinity while methanogens are devoid of such capability (Li and Fang, 2007; Oh et al., 2003). Heat pretreatment of anaerobic sludge offers one strategy to enrich hydrogen-producing bacteria for the conversion of substrate into hydrogen.

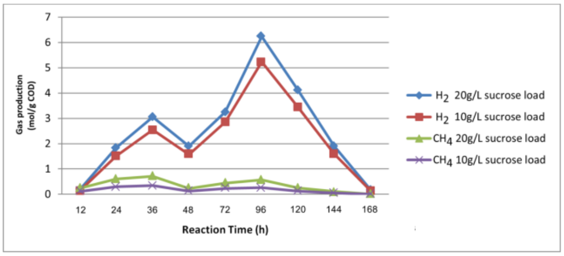
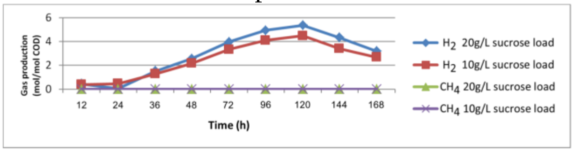
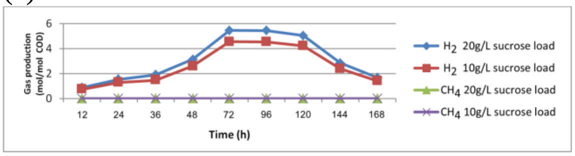


Fig.1. Effect of using seed sludge with no pretreatment on gas production using synthetic wastewater as substrate at 37°C for 168 hours.

Heat pretreatment at 105°C, regardless of time duration of heating (45, 60, 90, and 120 min), showed no activity for methanogenic bacteria (Figure 2). Methanogenic activity is eradicated from anaerobic sludge. This suggests that methanogens are not able to survive at 105°C. This result agrees with the work of Hasyim et al. (2011) where heat shock pretreatment was performed in an autoclave at 105°C for 20 minutes in order to inhibit the methanogenic activity. Batch experiments were conducted for adaptation of inoculums with glucose as a carbon source at 35°C. The results showed that the heat shock pretreatment completely repressed methanogenic activity during the cultivation when compared with the control. Furthermore, this study agrees with the results obtained by Mu et al. (2007). They concluded that the heat-shock pretreatment method was the best among the three pretreatment methods (acid, base, and heat-shock) studied for enriching hydrogen-producing bacteria from anaerobic sludge. Figure 2 further shows the pattern and changes of hydrogen production at different pretreatment time durations. It can be seen that regardless of pretreatment duration, synthetic wastewater responded with hydrogen yield immediately after 12 hours, though the comparatively least yield was observed with 45 minutes heat treatment. Initial response for hydrogen yield varies with the pretreatment time. The longer the pretreatment time, the higher is the response for hydrogen yield. Longer time duration of heat pretreatment resulted in an earlier peak of hydrogen gas evolution. Heat pretreatment time duration therefore had a great influence on the initial and peak responses of hydrogen gas evolution. Pretreatment at 120 minutes showed the best initial response after 12 h, though it dropped after 24 h, but continuously rose after 72 h and sustained this response even after 168 h.



(a)



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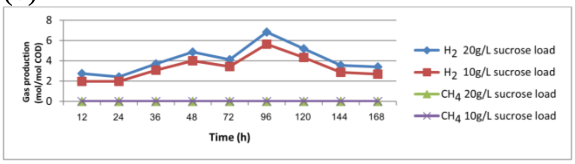

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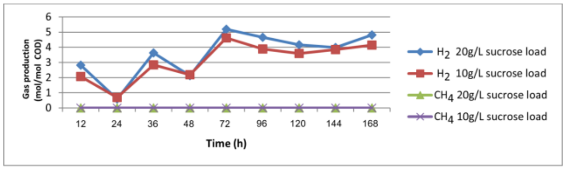

(d)

Fig.2. Gas production from sludge pretreated at 105°C at different heating times of (a) 45 min (b) 60 min (c) 90 min (d) 120 min using synthetic wastewater as substrate.

3.3 Hydrogen production from synthetic wastewater

Results of batch experiments showed that heat pretreatment of anaerobic sludge at 105°C adjusted to pH 7 and run at 37°C at 90 rpm showed an increasing trend of cumulative hydrogen production with an increase in heating time from 45 to 120 minutes at 105°C regardless of substrate loading (10g/L and 20g/L). The highest cumulative value was recorded at 120 minutes (3.54 mol H_2/g COD with 10g/L and 4.26 mol H_2/g COD with 20g/L) (Figure 3). It has been reported by Gadhamshetty et al. (2009), that not all sucrose is degraded when used as substrate for bio-hydrogen production. Degradation was found to be only 43% when 10 g/L sucrose was used as substrate. However, higher degradation of 81.8% was observed when glucose at 10 g/L was used as substrate (Alalayh et al., 2009). Thus, bio-hydrogen production depends on the type of substrate, biodegradability of organics present, and the fermentation end products. In the control, a small amount of methane could be detected while heat treated sludge at 105°C could suppress methanogens completely. Methanogens, unlike hydrogen-producing bacteria, when exposed to high temperature cannot resist this condition because they are devoid of forming protective spores (Li and Fang, 2007; Oh et al., 2003). Thus, heat treatment of the mixed inoculum culture sludge can enhance hydrogen production and is effective.

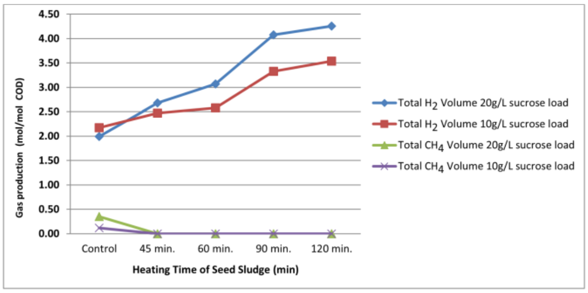

Fig.3. Effect of heating time of pretreated seed sludge on cumulative gas production from synthetic wastewater at 168 hours reaction time.

3.4 Hydrogen production from sugar mill wastewater

Analysis for sugarcane mill wastewater as substrate and heat pretreatment of anaerobic sludge at 105°C adjusted to pH 7 and run at 37°C at 90 rpm showed that the gas produced from the anaerobic fermentation contained no detectable methane. Figure 4 illustrates the effect of gas production with time on the cumulative hydrogen production in batch tests. Both concentrations responded positively for hydrogen yield at 12 hours. There seems to be very little increase of H_2 production at 24 to 48 hours for 100% sugarcane mill wastewater and it abruptly increases at 72 hours. An increasing trend was observed until 168 hours. On the other hand, less production of hydrogen gas was observed for 50% sugarcane mill wastewater at 24 and 36 hours, but it started to increase at 48 hours until 168 hours.

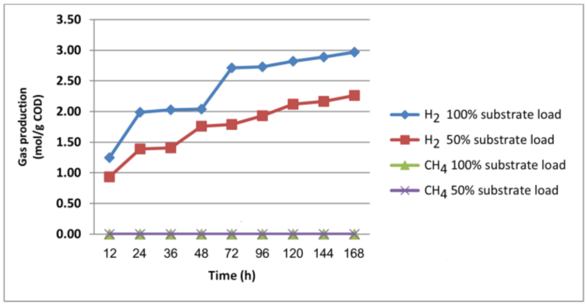

Fig.4. Gas production from sugar mill wastewater (with and without dilution) and seed sludge heated for 120 min at various reaction times.

Results for cumulative gas production with time for sugarcane mill wastewater revealed an increasing trend of hydrogen production as to the substrate loading with 2.97 mol H_2/g COD of hydrogen yield observed for 100% substrate loading (without dilution), compared with 2.26 mol H_2/g COD hydrogen production for 50% dilution after 168 hours of incubation (Figure 4).

Sugarcane mill wastewater had a total production of 2.97 mol H_2/g COD compared to 4.26 mol H_2/g COD (Figure 4) in synthetic wastewater. For organic materials to be potentially useful as substrates for sustainable biohydrogen production, they must be not only abundant and readily available, but also cheap and highly biodegradable. This shows that sugarcane mill wastewater can be a good source for biohydrogen production as it is rich in organics as reflected from the COD values showed in Table 1 and is also readily available. Heat pretreatment of the seed sludge enhanced the activity of hydrogen producing bacteria.

3.5 Comparison of heat pretreatment methods with other studies

The hydrogen conversion rate is calculated based on the assumption that the maximal hydrogen yield is 4 mol/mol glucose using glucose as the substrate and 8 mol/mol sucrose using sucrose as the substrate (Logan et al., 2002; Zhang et al., 2005). In this study, the hydrogen conversion rates of the seed sludge pretreated by heat at 105^{o}C using sucrose 10 g/L and 20g/L are 44.3% and 53.3%, respectively. This is in line with the study of Gadhamshetty et al. (2009). They reported a conversion rate of 43% when using sucrose 10 g/L.

Table 2 summarizes the key information in 18 studies, including this study, related to seed sludge heat pretreatment for the enrichment of hydrogen-producing bacteria. Generally, the most commonly used substrate is sucrose. As presented in Table 2, the hydrogen conversion rate from heat pretreatment of anaerobic seed sludge in this study is higher than most other studies using heat pretreated seed sludge as inoculum and was only lower than that of the study of Lin and Lay (2004a) using activated sludge as seed inoculum. Heat pretreatment of anaerobic sludge using sucrose as a substrate (100%) in this study yielded better results, compared with the result obtained by Mu et al. (2007), Fan et al. (2004), Zu and Be'land (2006), Logan et al. (2002), Zhang et al. (2005), Lin and Lay (2005; 2004b), and Chang and Lin (2004). Such differences may result from the kind of seed inoculum used, the heat pretreatment conditions employed, substrates types and concentrations, cultivation temperatures, fermentation pH, and so on. In addition, most of the studies were conducted on pure cultures using substrate such as glucose or sucrose, and also most studies were conducted under mesophilic conditions. The UASB sludge used as inoculum was collected from a thermophilic reactor. The heat pretreatment condition used in this study is higher compared to other authors.

Table1. Characteristics of artificial wastewater, sugarcane mill wastewater and sludge used in the experiment**.**

Parameters	**Unit**	**Sucrose**		**Sugarcane**		**Sludge**
		20g/L	**10g/L**	**100%**	**50%**	
1. pH	-	7.1	7.2	6.8	6.6	8.09
2. Total Solids	mg/L	0	0	583	235	17,706.67
3. Total Volatile Solids	mg/L	8.93	no data	287	no data	9,785.00
4. COD	mg/L	21,900	13,000	11,550	5,480	4,664.00

Table2. Comparison of hydrogen production by heat pretreatment methods with other studies**.**

Seed Inoculum	**Heat pretreatment of Seed Sludge**	**Substrate**	**Maximum H_2 yield**	**H_2 conversion rate (%)**	**Reference**
Anaerobic sludge	Heated at 105^{o}C for 120 min.	Sucrose 20g/L	4.26 mol/mol sucrose	53.3	This study
Anaerobic sludge	Heated at 105^{o}C for 120 min.	Sucrose 10g/L	3.54 mol/mol sucrose	44.3	This study
Anaerobic sludge	Heated at 105^{o}C for 120 min.	Sugarcane wastewater 100%	2.97 mol H_2/g COD		This study
Anaerobic sludge	Heated at 105^{o}C for 120 min.	Sugarcane wastewater 50% dilution	2.26 mol H_2/g COD		This study
Anaerobic sludge	Heated at 102^{o}C for 90 min.	Sucrose	4.00 mol/mol sucrose	50.0	Mu et al., 2007
Digested sludge	Boiled at 100^{o}C for 15 min.	Glucose	1.78 mol/mol glucose	44.5	Wang and Wan, 2008
Anaerobic sludge	Baked at 104^{o}C for 2 h	Glucose	0.968 mol/mol glucose	24.2	Oh et al., 2003
Anaerobic sludge	Heated in boiling water bath for 30 min.	Glucose	1.1 mol/mol glucose	27.5	Hu and Chen, 2007
Soil	Dried at 104^{o}C for 2 h	Glucose	0.92 mol/mol glucose	23.0	Logan et al., 2002
Soil	Dried at 105^{o}C for 2 h	Glucose	1.8 mol/mol glucose	45	Iyer et al., 2004
Cow dung compost	Baked at 100-105^{o}C for 2 h	Sucrose	2.24 mol/mol sucrose	28	Fan et al., 2004
Digested sludge	Boiled for 20 min.	Sucrose	3.18 mol/mol sucrose	38.8	Zhu and Be´land, 2006
Soil	Dried at 104^{o}C for 2	Sucrose	1.8 mol/mol	22.5	Logan et al., 2002

	h		sucrose		
Cracked cereals	Baked for 2 h and then boiled for 30 min.	Sucrose	2.73 mol/mol sucrose	34.1	Zhang et al., 2005
Activated sludge	Heated at 100^oC for 45 min	Sucrose	3.43 mol/mol sucrose	42.9	Lin and Lay, 2005
Activated sludge	Heated at 100^oC for 45 min	Sucrose	0.0015 mol/mol sucrose	0.02	Chang and Lin, 2004
Activated sludge	Heated at 100^oC for 45 min	Sucrose	4.8 mol/mol sucrose	60.0	Lin and Lay, 2004a
Activated sludge	Heated at 100^oC for 45 min	Sucrose	1.84 mol/mol sucrose	23.0	Lin and Lay, 2004b

4. Conclusions

Heat pretreatment of anaerobic sludge from UASB at 105^oC, regardless of time duration of heating, inhibited the activity of methanogenic bacteria. Results of batch experiments further showed that there is an increasing trend of hydrogen production with the increase in heating time from 45 to 120 minutes, regardless of sucrose substrate loading (10 g/L and 20 g/L). The hydrogen conversion rates of the seed sludge pretreated by heat at 105^oC using sucrose 10 g/L and 20 g/L are 44.3% and 53.3%, respectively. Sugarcane mill wastewater can be a potential substrate for biohydrogen production. The heat pretreatment is a practical pretreatment method for enriching hydrogen-producing bacteria from UASB. COD reduction is found to be about 49-52% in all cases. Thus, this study shows that it is possible to use the wastewater from the sugarcane industry to produce hydrogen, a clean source of energy.

5. Acknowledgment

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