DARK FERMENTATIVE BIO-HYDROGEN PRODUCTION FROM SUGAR-BEET PROCESSING WASTES

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ABSTRACT

DARK FERMENTATIVE BIO-HYDROGEN PRODUCTION FROM SUGAR-BEET PROCESSING WASTES

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In this study, bio-hydrogen generation potential of sugar-beet processing wastes (sugar-beet processing wastewater and beet-pulp) through dark fermentation was investigated. For this purpose, four different experimental set-ups were used.

In the first set-up, sugar-beet processing wastewater was used along with four different cultures to investigate the effect of culture type on bio-hydrogen production. In addition, unseeded reactor was prepared to investigate bio-hydrogen production potential of indigenous microorganisms. The highest bio-hydrogen production yield (87.7 mL H_2/g COD) was observed in the unseeded reactor. In the second set-up, beet-pulp was compared with sugar-beet processing wastewater in terms of bio-hydrogen generation potentials at an initial COD level of 4.5 g/L. In the third set-up, bio-hydrogen productivities of only beet-pulp and co-digestion of beet-pulp and sugar-beet processing wastewater at high COD values were investigated. The results of third set-up revealed that the reactor fed by 20 g/L COD beet-pulp provided the highest bio-hydrogen production yield (95.6 mL H_2 / g COD). Finally, in the fourth set-up, the effects of five different pretreatment methods on solubilization of beet-pulp were investigated. Then, three out of five

pretreatment methods were chosen to compare the corresponding bio-hydrogen productivities. Maximum bio-hydrogen production yield (115.6 mL H_2/g COD) was observed in reactor which contained alkaline pretreated beet-pulp.

Based on the results obtained in this study, it is postulated that, bio-hydrogen production from sugar-beet processing wastes by dark fermentation can not only enable waste minimization but also contribute to sustainability via valuable bio-based product formation from wastes, namely bio-hydrogen.

Keywords: Bio-Hydrogen, Anaerobic Digestion, Sugar-Beet Processing Wastes, Pretreatment.

ÖZ

ŞEKER ENDÜSTRİSİ ATIKLARINDAN KARANLIK FERMENTASYONLA BİYO-HİDROJEN ÜRETİMİ

Özkan, Leyla

Yüksek Lisans, Çevre Mühendisligi Bölümü
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Bu çalışmada, şeker endüstrisi atıklarının (şeker endüstrisi atıksuyu ve pancar küspesi), karanlık fermentasyonla biyo-hidrojen üretim potansiyeli araştırılmıştır. Bu amaçla, dört farklı deneysel düzenek kullanılmıştır.

İlk deneysel düzenekte, şeker endüstrisi atıksuyu ile birlikte kullanılan dört farklı kültürün biyo-hidrojen üretimine etkileri araştırılmıştır. Bunlara ek olarak, şeker endüstrisi atıksuyu içerisinde halihazırda bulunan mikroorganizmaların biyo-hidrojen üretim potansiyellerini belirlemek için kültür eklenmemiş reaktör hazırlanmıştır. En yüksek biyo-hidrojen üretim verimi (87,7 mL H_2/gr KOİ), kültür eklenmemiş reaktörde gözlenmiştir. İkinci deneysel düzenekte, 4,5 gr/L KOİ değerinde, pancar küspesinin ve şeker endüstrisi atıksuyunun biyo-hidrojen üretim potansiyelleri karşılaştırılmıştır. Üçüncü deneysel düzenekte, yüksek KOİ değerlerinde yalnızca pancar küspesinin ve şeker endüstrisi atıksuyu ile birlikte kullanılan pancar küspesinin, biyo-hidrojen üretimleri araştırılmıştır. Üçüncü

deneysel düzenekte, en yüksek biyo-hidrojen üretim verimi (95,6 mL H_2 /gr KOİ), 20 gr/L KOİ değerinde pancar küspesi içeren reaktörde gözlenmiştir. Son olarak dördüncü deneysel düzenekte, beş farklı ön arıtım metodunun, pancar küspesinin çözünebilirliğine etkisi araştırılmıştır. Daha sonra, biyo-hidrojen üretimlerini karşılaştırmak için beş önarıtım metodundan üçü seçilmiştir. Maksimum biyo-hidrojen üretim verimi (115,6 mL H_2/gr KOİ), alkali önarıtımı yapılmış pancar küspesini içeren reaktörde gözlenmiştir.

Bu çalışmada elde edilen sonuçlar temel alındığında, şeker endüstrisi atıklarından karanlık fermentasyon yöntemiyle biyo-hidrojen eldesi, atık stabilizasyonu sağlamasının yanısıra atıklardan değerli biyoürün eldesi ile de sürdürülebilir üretime katkıda bulunmaktadır.

Anahtar Kelimeler: Biyo-hidrojen, Anaerobik Bozundurma, Şeker Endüstrisi Atıkları, Önarıtım.

To My Family…

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ABBREVIATIONS

AD	: Anaerobic Digestion
BES	: 2-Bromoethanesulfonate
BM	: Basal Medium
COD	: Chemical Oxygen Demand
GC	: Gas Chromatograph
H-Ac	: Acetic Acid
H-Bu	: n-Butyric Acid
H-Pr	: Propionic Acid
HRT	: Hydraulic Retention Time
MLSS	: Mixed Liquor Suspended Solids
MLVSS	: Mixed Liquor Volatile Suspended Solids
OLR	: Organic Loading Rate
sCOD	: Soluble Chemical Oxygen Demand
cCOD	: Consumed Chemical Oxygen Demand
SRT	: Solids Retention Time
tCOD	: Total Chemical Oxygen Demand
TKN	: Total Kjeldahl Nitrogen
TS	: Total Solids
TSS	: Total Suspended Solids
tVFA	: Total Volatile Fatty Acid
TVS	: Total Volatile Solids
VFA	: Volatile Fatty Acid
VFA_f	: Final Volatile Fatty Acid
VFA_i	: Initial Volatile Fatty Acid
$P_{T\,total}$	: Total Phosphorus

CHAPTER 1

INTRODUCTION

1.1. Background Information

The worldwide energy need has been increasing and the reserves of fossil fuels have been decreasing. Moreover, the utilization of fossil fuels cause environmental pollution problems due to the emission of pollutants like COx, NOx, SOx, CxHx, soot, ash, droplets of tars and other organic compounds. These reasons drawing into researchers to investigate new sustainable energy sources. Hydrogen is considered as a viable alternative fuel and "energy carrier" of future because of its clean, efficient, renewable, and non-polluting characteristics.

Some of the conventional hydrogen gas production methods are steam reforming of natural gas, gasification of coal and electrolysis of water (Nath and Das, 2004). However, these methods use non-renewable energy sources to produce hydrogen and are not sustainable. In addition, these non-environmentally friendly processes do not accomplish the dual goals of waste reduction and energy production concurrently. Bio-hydrogen production from renewable biomass by bacteria assumes paramount importance as an alternative energy resource because it has significant potential to meet the increasing energy needs in the future (Chowdhury et al., 2007).

Bio-hydrogen production can be realized by anaerobic (dark fermentation) and photosynthetic microorganisms (photofermentation) using carbohydrate rich and non-toxic raw materials and wastes. However, due to the low utilization efficiency

of light and difficulties in designing light reactor, the latter method is hard to be applied (Liu et al., 2008). The former possesses the ability to generate hydrogen without photoenergy. Moreover, bio-hydrogen production by dark fermentation, compared to alternatives such as bio-photolysis of water or photofermentation, is advantageous because of its ability to produce H_2 at higher rates (Magnusson et al., 2008). Dark fermentation has the ability to convert wide ranges of reduced carbon sources, which are found in many industrial effluents and agriculture residues, into bio-hydrogen (Ray et al., 2008). This is advantageous because not only a partial waste stabilization is achieved, but also valuable metabolites such as acetic, butyric and lactic acids are produced (Kim et al., 2006) from cheap and even free carbon sources which makes it more attractive from the economical point of view. So, dark fermentation is a viable alternative to the aforementioned methods for hydrogen gas production.

Recently, studies on bio-product (bio-fuel, bio-energy etc.) production are conducted with the use of various kinds of biomass sources, including wastes, as substrate instead of petroleum. Renewable bio-energy and industrial raw material production from various kinds of biomass sources including organic wastes is a considerably new concept. The well-known term of this concept is biorefining and with this process biofuels like biodiesel, ethanol, methane and hydrogen and a variety of chemicals such as alcohols, ketones, and organic acids are produced from wastes. This biorefining process will bring an innovative approach to the removal and treatment of industrial organic wastes. In this approach, organic wastes are not only considered as wastes to be treated or removed but also as resources or raw materials that can be utilized to produce energy or products. At the end of the biorefining process, the major objective is to get maximum gain with the utilization of substrate and minimum residue to be treated. In this context, the practice of biorefining that considers wastes as biomass resources to produce bio-energy, bio-chemical, bio-compost etc. will provide conservation of ecological and economical assets of Turkey.

Sugar production has considerable potential in Turkey which is one of the major sugar producing countries in the world (Zuhal and Kemal, 2004). Sugar-beet processing industry consumes large amount of energy and produces considerable amount of wastes. Therefore, several measures have been investigated to reduce energy consumption and enable waste minimization (Krajnc et al., 2007). Sugar-beet processing wastes can be considered a suitable source of renewable energy and bioproducts via anaerobic biological digestion because of their high content of biodegradable organics.

1.2. Aim and Scope of the Study

The main objective of this study was to investigate bio-hydrogen generation potential of sugar-beet processing wastes (sugar-beet processing wastewater and beet-pulp) through dark fermentation. According to past studies, expensive pure carbohydrate substrates (such as glucose, sucrose) has been widely used for H_2 production (Fang and Liu, 2002; Wooshin et al., 2005; Kanai et al., 2005; Chowdhury et al., 2007; Ray et al., 2008). On the other hand, bio-hydrogen production from organic wastes not only provide a partial waste stabilization but also it is an economically feasible process because organic wastes are free or very cheap carbon sources. Therefore, bio-hydrogen production from low cost substrates is the most promising hydrogen production method to meet the current renewable energy demand (Chowdhury et al., 2007; Liu et al., 2008). Sugar-beet processing wastes have significant potential as a renewable source of fuel (Zuhal and Kemal, 2004; Farhadian et al., 2007). Bio-hydrogen production from sugar-beet processing wastes by dark fermentation can not only enable waste minimization but also contribute to sustainability via valuable bio-based product, bio-hydrogen. In the literature, there are some studies on bio-hydrogen production from sugar factory wastewater, sugar-beet wastewater, molasses (Ueno et al., 1996; Tanisho et al., 1998; Logan et al., 2002; Wu and Lin, 2004; Hussy et al. 2005; Vatsala et al., 2008). However, there is not any study investigating bio-hydrogen generation

potential of sugar-beet processing wastes (sugar-beet processing wastewater and beet-pulp) together. In order to fill a gap in the literature, in this study, bio-hydrogen generation potential of sugar-beet processing wastes (sugar-beet processing wastewater and beet-pulp) through dark fermentation was investigated. Effects of different types of cultures, waste types, COD and pretreatment methods to bio-hydrogen generation potential of sugar-beet processing wastes were investigated.

For this purpose, four different experimental set-ups were used in a stepwise fashion (Figure 1.1). In the first set-up, sugar-beet processing wastewater (4.5 g/L COD) was used along with glucose acclimated acidogenic culture, mixed anaerobic culture, 2-bromoethanesulfonate added mixed anaerobic culture and heat treated mixed anaerobic culture to investigate the effect of culture type on bio-hydrogen production. In addition, unseeded reactor (US) was prepared to investigate bio-hydrogen production activities of indigenous microorganisms. As a result of Set-up 1, high bio-hydrogen production yields were observed in the unseeded reactor (US) which contained only sugar-beet processing wastewater and in the reactor which contained sugar-beet processing wastewater and mixed anaerobic culture (MAC). Thus, in the subsequent experimental set-up (Set-up 2), two types of reactors were used; reactor which contained only beet-pulp and reactor with mixed anaerobic cultures and beet-pulp.

In the second set-up, beet-pulp was compared with sugar-beet processing wastewater in terms of bio-hydrogen generation potentials at an initial COD level of 4.5 g/L. In the third set-up, bio-hydrogen productivities of only beet-pulp and co-digestion of beet-pulp and sugar-beet processing wastewater at high COD values (20, 25, 30 g/L COD) were investigated. The results of third set-up revealed that the reactor fed by 20 g/L COD beet-pulp yielded highest bio-hydrogen production yield. Based on the results obtained in the Set-up 3, in the fourth set-up, reactors were fed by 20 g/L COD of beet-pulp. In the first part of Set-up 4, the effects of

five different pretreatment method (alkaline pretreatment, thermal pretreatment, microwave pretreatment, thermal-alkaline pretreatment, microwave-alkaline pretreatment) on solubilization of beet-pulp were investigated. Then, three (alkaline, microwave-alkaline, thermal-alkaline pretreatments) out of five pretreatment methods which resulted in higher solubilization ratios (CODs/CODt) was chosen to compare bio-hydrogen productivities.

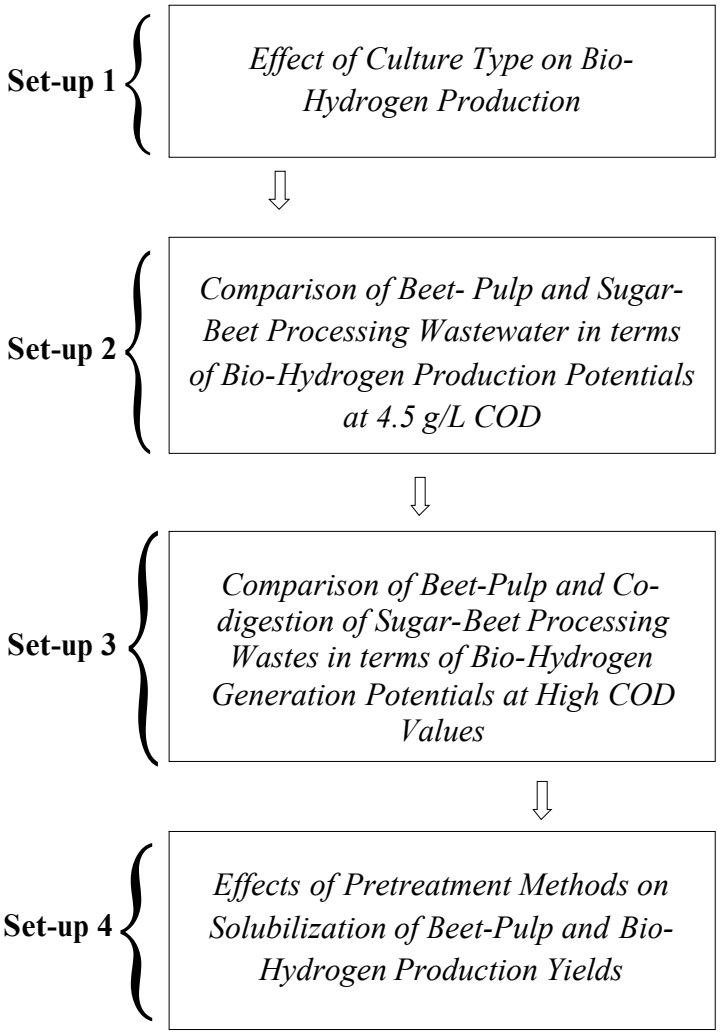


Figure 1.1 Schematic representation of the experimental set-ups

CHAPTER 2

LITERATURE REVIEW

2.1. Anaerobic Digestion

2.1.1. Process Description

Anaerobic digestion, a naturally occurring biological process, involves the decomposition of organic matter in the absence of free molecular oxygen. It is carried out by a group of microorganisms which involves different classes of microorganisms. These microorganisms convert organic matter into a mixture of methane and carbon dioxide by a series of interdependent metabolic reactions (Metcalf and Eddy, 2003).

Anaerobic digestion has been regarded as a viable option for the management of different kind of wastes for decades. Today, anaerobic digestion has significant role in the treatment of wide variety of organic wastes originated from domestic, industrial and agricultural activities. Some of the wastes which suitable for anaerobic biological treatment are waste activated sludge (Demirer and Othman, 2008; Romano and Zhang, 2008), organic fraction of municipal solid waste (Hartmann and Ahring, 2005), crude cheese whey (Ergüder et al., 2001; Ferchichi et al., 2005), domestic wastewater (Haandel et al., 2006), fruit and vegetable wastes (Ergüder et al., 2000; Bouallagui et al., 2005), food waste (Han and Shin, 2004; Lay et al., 2005), animal manure (Gungor-Demirci and Demirer, 2004; Demirer and Chen, 2004; Hartmann and Ahring, 2005; Karim et al., 2005; Maranon et al., 2008), sugar industry wastes (Hutnan et al., 2001; Farhadian et al., 2007), rice winery

wastewater (Yu et al., 2002), pharmaceutical wastewater (Oktem et al., 2006), brewery effluents (Connaughton et al., 2006), molasses wastewater (Wu and Lin, 2004). In addition, anaerobic treatment has several significant advantages over aerobic treatment, such as bio-product formation, comparatively less excess sludge production, no or little nutrient requirement, high-strength waste treatment ability, seasonal operation flexibility and lower operational costs (Gavrilescu, 2002).

2.1.2. Phases of Anaerobic Digestion

The microbial conversion processes in anaerobic treatment are typically described in four main stages. These stages are hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2.1). Each of these stages contains different groups of bacteria. These groups work in sequence, with the products of one group serving as the substrates of another group. Therefore, each group is linked to other groups. The efficient digestion process is performed by these well-organized groups of bacteria and established balance between consumption and production of intermediate metabolites.

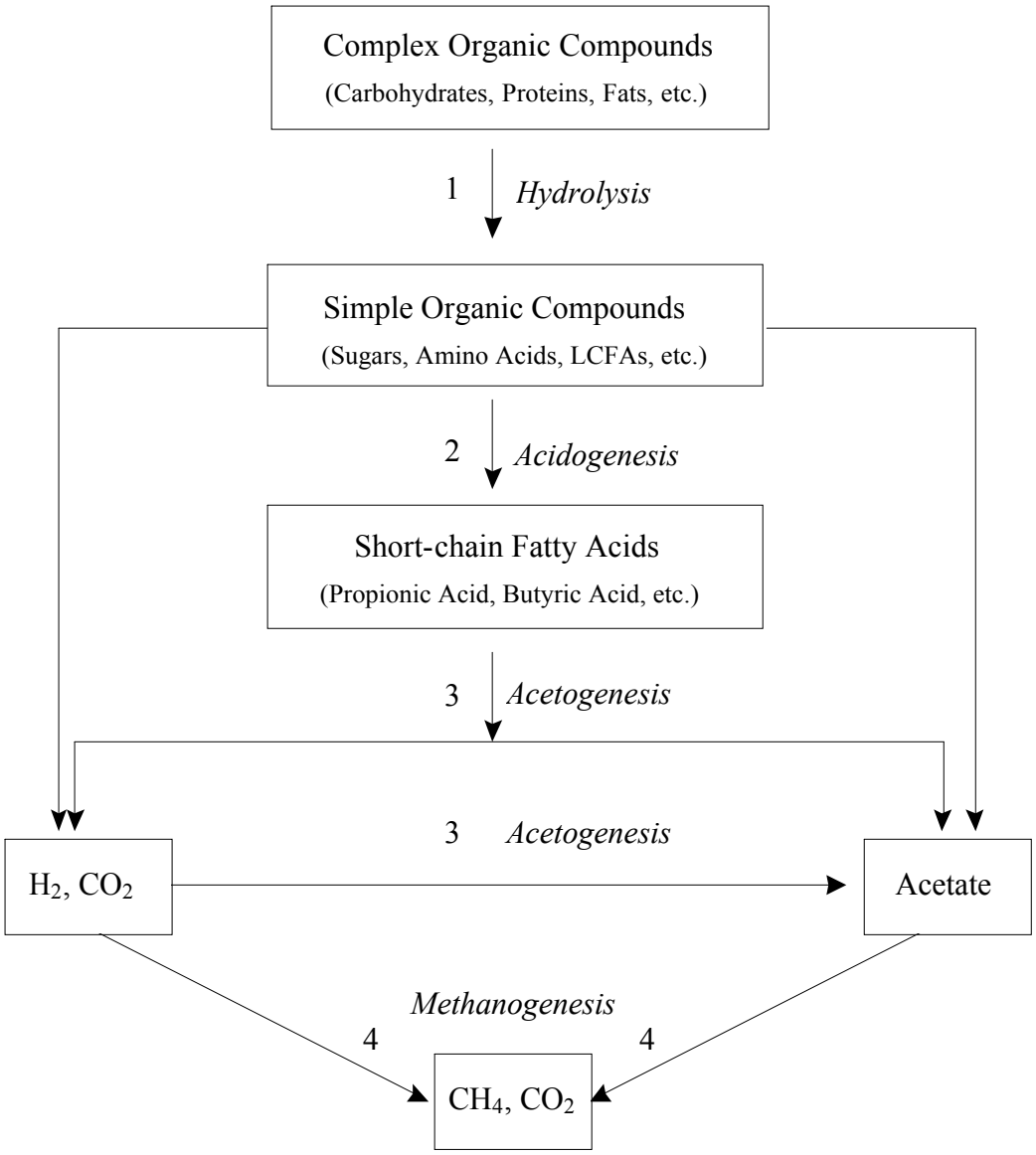


Figure 2.1 Phases of anaerobic digestion (Gerardi, 2003)

The first stage of anaerobic digestion involves the hydrolysis and solubilization of complex organic compounds (carbohydrates, proteins, fats, etc.) into simpler organics such as sugars, amino acids and long chain fatty acids (LCFAs). Hydrolysis is catalyzed by enzymes excreted from the hydrolytic bacteria (obligate

and facultative anaerobes). These extracellular enzymes cause the disintegration of complex organic compounds which can not penetrate through bacterial cells due to their polymeric structures (Gerardi, 2003). If the feedstock is complex, the hydrolytic phase is relatively slow. This is especially true for raw cellulolytic waste, which contains lignin (Ostrem, 2004).

Second stage is the acidogenesis, at this stage, soluble organic compounds produced through hydrolysis stage are degraded by a large diversity of obligate and facultative anaerobes through different fermentative pathways. The degradation of these compounds results in the production of short-chain fatty acids like, acetic, propionic and butyric acids as well as alcohols, hydrogen and carbon dioxide (Gerardi, 2003).

Third step is the acetogenesis in which higher organic acids (the volatile acids longer than two carbons) and alcohols produced during the acidogenesis stage are degraded to acetate, carbon dioxide and hydrogen. The production of acetate is accomplished through the activity of acetogenic or acetate-forming bacteria. This stage of anaerobic digestion process is crucial since the pronounced products of acetogenesis are used as a substrate by methane-forming bacteria in methanogenesis stage.

Finally, in the methanogenic stage, methanogenic microorganisms convert the acetate, hydrogen and carbon dioxide into methane and carbon dioxide. Methane is produced during methanogenesis in two ways: by means of cleavage of two acetic acid molecules (Equation 2.1) to generate carbon dioxide and methane, or by reduction of carbon dioxide [as bicarbonate (HCO_3^-) or carbonate (CO_3^{2-})] with hydrogen (Equation 2.2 and 2.3). The acetate reaction is the primary producer of methane (Gerardi, 2003).

$CH_3COOH \rightarrow CH_4 + CO_2$ ..... (2.1)

$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$ .....(2.2)

$4H_2 + CO_3^{2-} + 2H^+ \rightarrow CH_4 + 3H_2O$ ..... (2.3)

2.1.3. Operational Conditions Influencing Anaerobic Digestion Process

Several operational and environmental conditions must be satisfied in order to achieve proper digester operation, enhance the microbial activity and thus increase the anaerobic digestion efficiency. Some of these operational and environmental conditions are retention time, organic loading rate (OLR), nutrients, alkalinity, pH, and temperature, mixing.

Retention Time

There are two significant retention times in an anaerobic digester. These are solids retention time (SRT) and hydraulic retention time (HRT). The SRT is the average time that bacteria (solids) are retained in the anaerobic digester. The HRT represents the time that the wastewater is retained in the anaerobic digester. Because of the slow-growing time of methane-forming bacteria, typical SRTs for anaerobic digesters are higher than 12 days. Low SRT values (lower than 10 days) are not recommended for convention reactor configurations because of the risk of washout of methane-forming bacteria (Gerardi, 2003). SRT values vary with operational conditions such as temperature, waste characteristics, mixing, etc. The SRT and the HRT are the same for a suspended-growth anaerobic digester that has no recycle. However, SRT values of anaerobic digesters that utilize fixed-film media for the growth of bacteria are high. High SRT values maximize removal capacity, reduce required digester volume, and provide buffering capacity for protection against the effects of shock loadings and toxic compounds in wastewaters and sludges (Gerardi, 2003). Thus, high SRT values are advantageous for anaerobic digesters.

Organic Loading Rate

Organic loading rate (OLR) is a measure of the biological conversion capacity of the AD system. It is the mass of substrate added per unit volume of digester per unit period of time. It can be calculated according to equation 2.4 where C_{in} stands for influent volatile solids (VS) concentration, V_{in} stands for influent feeding volume per day and V stands for reactor volume.

$$OLR = (C_{in} * V_{in}) / V \quad \text{..... (2.4)}$$

OLR is closely linked to removal of organics and SRT of the digester. High SRT ensures efficient removal of organics (high COD removal) and permits high OLR values. Optimal OLRs are dependent on various operational parameters such as the substrate, type of reactor, HRT, nutrients and alkalinity (Romano and Zhang, 2008). Feeding the system above sustainable OLR results in low biogas yield due to accumulation of inhibiting substances such as fatty acids. According to Rajeshwari et al. (2000), typical OLR values in suspended and attached growth reactors are reported as 0.25–3 and 10–100 g COD/L-day, respectively.

Nutrients

Sufficient amount of nutrients are essential for anaerobic microbial growth. For an efficient anaerobic digestion, macronutrients like nitrogen, phosphorus and micronutrients like cobalt, iron, nickel, and sulfur must be present in the digestion medium. Compared with the aerobic processes, in anaerobic processes nutrient requirements are less due to lower cell (sludge) yield (Gerardi, 2003).

pH and Alkalinity

The optimal pH values for the each of the microbial groups in anaerobic digestion are different. However, most anaerobic bacteria perform well within a pH range of 6.8 to 7.2 (Gerardi, 2003). According to Speece (1996), methanogens operate optimum at a range of 6.5 to 8.2 while acidogens prefer between 4 and 6.5.

Sufficient alkalinity is essential for proper pH control. Alkalinity serves as a buffer that prevents rapid change in pH. Calcium, magnesium and ammonium bicarbonates are examples of buffering substances found in a digester. A well-established digester has alkalinity concentration of 3000 to 5000 mg/L as $CaCO_3$ (Metcalf and Eddy, 2003).

Temperature

In anaerobic digestion, temperature is important in determining the rate of digestion, particularly rates of hydrolysis and methane formation. Anaerobic digestion has two optimal operating temperature ranges, the mesophilic range from 30 to 35°C and the thermophilic range from 50 to 60°C. Thermophilic operation has numerous advantages reported over mesophilic operation such as the higher destruction level of pathogens, the faster rate of anaerobic digestion, and the higher loading rates (Gerardi, 2003). However, higher sensitivity to toxicants and higher operation cost (higher energy demand) make the thermophilic operation more problematic than mesophilic operation. In addition, methanogenesis is also possible in psychrophilic conditions (temperatures below 20 ^{0}C), but occurs at lower rates (Connaughton et al., 2006).

Mixing

Adequate mixing is a critical issue for anaerobic digestion. Mixing improves the contact between substrate and microorganisms. In addition, mixing enables rapid dispersion of any toxic materials entering the reactor (minimizing toxicity), prevents the formation of scum and the deposition of grit, distributes nutrients throughout the digester as well as equalizing temperature of digester (eliminating thermal stratification) (Gerardi, 2003).

2.2. Hydrogen Production

2.2.1. The Importance of Hydrogen

Increasing worldwide energy need, diminishing fossil fuel supplies and increase levels of greenhouse emissions from the combustion of fossil fuels are the major reasons drawing into researchers to investigate new sustainable energy sources that could substitute fossil fuels.

Hydrogen is a clean and environmentally friendly fuel since the combustion of hydrogen produces only water vapor instead of greenhouse gases like CO_2. Furthermore, hydrogen has a high energy yield of 122 kJ/g, which is about 2.75 times greater than that of hydrocarbon fuels (Kapdan and Kargi, 2006). On these grounds, hydrogen has appeared as a promising green alternative to fossil fuels for the future to meet the growing energy demands. However, several challenges need to be overcome before a full hydrogen economy can be realized such as the development of hydrogen storage systems and infrastructure (Reith et al., 2003).

Hydrogen is currently produced industrially in large quantities from fossil fuels, most commonly by steam reforming of natural gas to form hydrogen and carbon

dioxide. Emissions of the latter contribute to climate change. Thus, sustainable methods of production are required if hydrogen is to replace fossil fuels in a move towards a low-carbon or hydrogen economy. From the perspective of global environmental impacts, such as greenhouse effect and resource recovery, microbial hydrogen production (biological hydrogen production) from renewable biomass reduces dependence on fossil fuel, decrease carbon dioxide emission and recovers bio-energy. Moreover, in order to establish a sustainable and cost-effective hydrogen production process, hydrogen should be produced from wastes. Hydrogen production from organic wastes not only enables a partial stabilization of waste, but also it is an attractive process because organic wastes are free or low cost carbon sources (Valdez-Vazquez et al., 2005). However, the yield and rate of bio-hydrogen production are still low at present. Yield improvement is necessary for improving the economic feasibility of bio-hydrogen production (Bartacek et al., 2007).

Numerous studies have been conducted to produce hydrogen by using organic wastes as substrate such as crude cheese whey (Ferchichi et al., 2005), wastewater sludge (Wang et al., 2003), rice winery wastewater (Yu et al., 2002), food waste (Han and Shin, 2004), molasses (Logan et al., 2002), sugar factory wastewater (Ueno et al., 1996), bean curd manufacturing waste (Noike and Mizuno, 2000; Noike, 2002), cattle wastewater (Tang et al., 2008), cassava starch manufacturing wastewater (Reungsang et al., 2004), sweet potato starch residue (Yokoi et al., 2001; Yokoi et al., 2002), domestic wastewater (Van Ginkel et al., 2005), patato industry wastewater (Van Ginkel et al., 2005), organic fraction of municipal solid waste (Lay et al., 1999). According to these studies, hydrogen production from organic wastes was feasible. However, in order to improve overall energy efficiency and enable economic feasibility of bio-hydrogen production from wastes, additional treatment processes need to be done (Bartacek et al., 2007). For further energy recovery, the energy residues remain in the by-products in the forms of acids need to be converted to bio-hydrogen and carbon dioxide by two stage

photobiological hydrogen production process or to be converted to methane and carbon dioxide by the dark fermentation of these readily degradable organic compounds. If the dark hydrogen fermentation is not followed by further conversion, the hydrogen yield will not warrant economic feasibility (Reith et al., 2003).

In Turkey, numerous studies have been conducted on bio-hydrogen production through photofermentation (Arık et al., 1996; Eroğlu et al., 1997; Eroğlu et al., 1999; Koku et al., 2002; Koku et al., 2003; Öztürk et al., 2006; Uyar et al., 2006; Kars et al., 2008; Kapdan et al., 2009). In addition, several studies have been conducted to produce bio-hydrogen through photofermentation by using organic wastes as substrate such as milk industry wastewater (Türkarslan et al.,1998), sugar refinery wastewater (Yiğit et al., 1999; Yetiş et al., 2000), olive mill wastewater (Eroğlu et al., 2004). However, studies on the use of dark fermentation in bio-hydrogen production are still new and limited (Eroğlu et al. 2006; Öztekin et al., 2008; Argun et al., 2008).

2.2.2. Hydrogen Production Methods

Hydrogen is produced mainly from fossil fuels (Steam reforming of natural gas, thermal cracking of natural gas, coal gassification, partial oxidation of heavier than naphtha hydrocarbons), biomass (Dark fermentation, photofermentation) and water (Electrolysis, biophotolysis, thermolysis, thermochemical process) (Das and Veziroğlu, 2001).

Hydrogen can be obtained via biological, thermochemical and electrochemical processes. However, to be sustainable, hydrogen production technologies need to utilize renewable sources instead of non-renewable energy sources. Thermochemical and electrochemical processes use fossil fuels as a source for hydrogen production. Thus, biological hydrogen production processes are

considered to be more environment friendly and less energy intensive as compared to thermochemical and electrochemical processes (Das and Veziroğlu, 2001; Hussy et al., 2005).

Biological hydrogen production processes can be divided into two groups; light-dependant processes (direct biophotolysis, indirect biophotolysis, photofermentation) and dark fermentation. Among these biological processes, photofermentation and dark fermentation may be two of the most promising processes because of lower cost and higher hydrogen yields (Lee et al., 2002; Yang et al., 2006). Between these, dark fermentation is gradually more attractive due to the high hydrogen production rates, the absence of light sources and the capability to convert large range of organic wastes to more valuable energy sources (Das and Veziroğlu, 2001).

Biological hydrogen production processes (direct biophotolysis, indirect biophotolysis, photofermentation, dark fermentation) were described briefly below.

Light-dependant processes

- *Direct biophotolysis*

In direct biophotolysis solar energy is used to convert water to oxygen and hydrogen which are produced together. In this process, solar energy is directly converted to hydrogen via photosynthetic reactions (Equation 2.5) (Manish and Banerjee, 2008).

$$2H_2O + \text{‘light energy’} \quad 2H_2 + O_2 \qquad (2.5)$$

The generated hydrogen ions are converted into hydrogen gas by hydrogenase enzyme that is extremely sensitive to oxygen (Manish and Banerjee, 2008). Some

green algae such as *Chlamydomonas reinhardtii* naturally possess an hydrogenase enzymes and could be used in a direct biophotolysis scheme (Bartacek et al., 2007). The main drawback of the process is that hydrogen and oxygen are produced together, which causes oxygen inhibition of the hydrogen producing enzymes.

- *Indirect biophotolysis*

In indirect biophotolysis oxygen evolution and hydrogen evolution are temporally and spatially separated. In this process, cyanobacteria produce hydrogen by using light as the energy source and carbon dioxide as sole carbon source (Equation 2.6). The cells take up CO_2 first to produce cellular substances, which are subsequently used for hydrogen production (Equation 2.7). Hydrogenase enzyme is used in the indirect biophotolysis processes (Manish and Banerjee, 2008).

$$12H_2O + 6CO_2 + \text{‘light energy’} \quad C_6H_{12}O_6 + 6O_2 \quad \text{.....(2.6)}$$

$$C_6H_{12}O_6 + 12H_2O + \text{‘light energy’} \quad 12H_2 + 6CO_2 \quad \text{.....(2.7)}$$

- *Photofermentation*

The photosynthetic bacteria produce hydrogen through the action of their nitrogenase system under nitrogen-deficient conditions. They use light as the energy source and organic compounds as the carbon source. The photosynthetic bacteria convert organic compounds to hydrogen and carbon dioxide under anaerobic conditions in the presence of light (Equation 2.7) (Manish and Banerjee, 2008).

$$C_6H_{12}O_6 + 6H_2O + \text{‘light energy’} \quad 12H_2 + 6CO_2 \quad \text{.....(2.7)}$$

Dark fermentation

Hydrogen production via dark fermentation is a special type of anaerobic digestion comprising only hydrolysis, acidogenesis and acetogenesis. In dark fermentative hydrogen production, anaerobic bacteria (mainly clostridium and enterobacter) use organic compounds to produce hydrogen and carbonaceous products like CO_2 and acetic acid and ethanol in the absence of a light source (Bartacek et al., 2007).

The majority of microbial hydrogen production is driven by the anaerobic metabolism of pyruvate. The breakdown of pyruvate is catalyzed by one of two enzyme systems; pyruvate- formate lyase (PFL) (Equation 2.8) and pyruvate-ferredoxin oxido reductase (PFOR) (Equation 2.9) (Manish and Banerjee, 2008).

$$\text{Pyruvate} + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{formate} \quad \text{.....(2.8)}$$

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd(ox)} \rightarrow \text{acetyl-CoA} + \text{CO2} + 2\text{Fd(red)} \quad \text{.....(2.9)}$$

Figure 2.2 shows the pathway of dark fermentation using glucose as the model substrate. According to the Figure 2.2, glucose is first converted to pyruvate, producing adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and the reduced form of nicotinamide adenine dinucleotide (NADH) via the glycolytic pathway. Pyruvate is then further converted to acetylcoenzyme A (acetyl-CoA), carbon dioxide, and reduced ferredoxin (Fd (red)) from which hydrogen can be derived hydrogen by PFOR (Equation 2.9). Strict anaerobes derive hydrogen from Fd (red) by hydrogenase. Pyruvate may also be converted to acetyl-CoA and formate (Equation 2.8), which may be readily converted to hydrogen and carbon dioxide by bacteria such as *Escherichia coli*. Acetyl-CoA is finally converted into acetate, butyrate, and ethanol, depending on the microorganisms and the environmental conditions. NADH is used in the formation of butyrate and ethanol and the residual NADH may be oxidized, producing hydrogen and NAD+. ATP is

generated in the formation of butyrate and acetate from acetyl-CoA (Li and Fang, 2007).

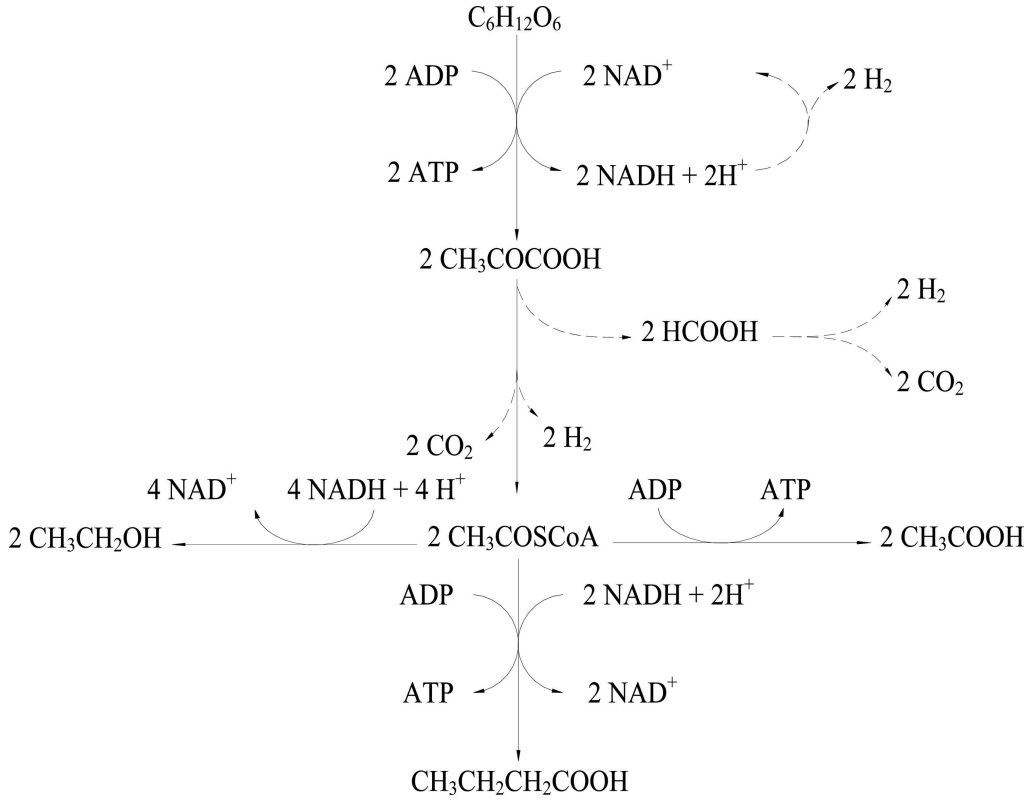


Figure 2.2 Pathway of hydrogen production from fermentation of glucose (Tanisho, 2001)

2.2.3. Operational Conditions Influencing Dark Fermentative Bio-Hydrogen Production

Hydrogen production by dark fermentation is highly dependent on the operational conditions such as pH, temperature, hydraulic retention time (HRT), mixing.

pH

pH is the important parameter for the suppression of the hydrogen-consuming methanogenic activities. In addition, it may directly affect the hydrogenase activity (Dabrock et al., 1992) as well as the metabolism pathway (Lay, 2000). Hydrogen production efficiencies and the composition of soluble metabolites are sensitive to the change in pH. The optimal pH was in the range of 5.2–7.0 with an average of pH 6.0 for hydrogen conversion from carbohydrates (Li and Fang, 2007). According to literaure, pH 6.0 provided the highest hydrogen production performance (Logan et al., 2002; Zhang et al., 2003; Ferchichi et al., 2005; Kotay and Das, 2006; Lee et al., 2008).

Temperature

Temperature is the one of the most important operational parameter that affects the growth rate and metabolic activity of microorganism. Dark fermentative hydrogen production reactions can be operated in three temperature ranges, mesophilic (30-35 ^{0}C), thermophilic (50-60 ^{0}C), or psychrophilic (5-25 ^{0}C) (Gerardi, 2003). Due to the drastic differences in reactor, substrate, seed sludge, and other process conditions, it is difficult to compare hydrogen yield at the three temperature ranges. Most of the work in the area of fermentative hydrogen production has focused on mesophilic temperatures (about 37°C), because most of the hydrogen-producing *Clostridium sp.* prefer these conditions (Bartacek et al., 2007). According to the literature, dark fermentative hydrogen production is the most commonly applied at mesophilic temperatures (Wang et al., 2003; Han and Shin, 2004; Lay, 2004; Khanal et al., 2004; Hussy et al., 2005; Li et al., 2008; Davila-Vazquez et al., 2008).

Hydraulic Retention Time

The hydraulic retention time (HRT) corresponds to the average time the added substrate resides in the reactor (the total liquid volume of the reactor divided by the amount of liquid added and withdrawn per day). In order to establish hydrogen fermentation, a fermenter has to be operated at low hydraulic retention times to suppress methanogenic activity in the pre-fermentation stage as the methanogens are known to grow more slowly than acidogenic bacteria. The optimal HRT values are changing depending on substrate such as optimal HRT value was 2 h for a rice winery wastewater (Yu et al., 2002), 12 h for a sugar factory wastewater (Ueno et al., 1996), 6–9 h for bean curd waste (Noike et al., 2003), 3–8 h for glucose and sucrose (Li and Fang, 2007), 17 h for starch (Lay, 2000).

Mixing

Mixing enhances the dark fermentative hydrogen production process by distributing bacteria, substrate, and nutrients throughout the digester as well as equalizing temperature. According to Lamed et al. (1988), mixing enhanced the hydrogen yield from cellulose and cellobiose.

2.2.4. Pretreatment methods for Hydrogen Production

Several studies have been carried out using pure cultures of anaerobic bacteria, such as *Clostridium sp.* to study conversation of carbohydrates to hydrogen (Zeikus, 1980; Taguchi et al., 1992). Among the hydrogen-producing bacteria, *Clostridium sp.* and *Enterobacter* are the most widely studied (Tanisho et al., 1983; Heyndrickx et al., 1986; Taguchi et al., 1992; Tanisho and Ishiwata, 1994; Rachman et al., 1998; Kumar and Das, 2000). However, processes using mixed cultures are more practical, because they are simpler to operate and easier to control, and may have a

broader choice of feedstock (Valdez-Vazquez et al., 2005). On these grounds, nowadays researchers are more interested in using mixed cultures, instead of pure cultures, for wastewater and waste treatment (Noike, 2002; Oh et al., 2003; Han and Shin, 2004; Shin et al., 2004; Hussy et al., 2005; Valdez-vaquez et al., 2005, Fang et al., 2006).

In mixed cultures, hydrogen produced by hydrogen producing bacteria (such as *Clostridium sp*. and *Enterobacter*) are consumed immediately by hydrogenotrophic methanogens (Ray et al., 2008). Thus, in order to produce hydrogen from mixed anaerobic cultures, hydrogen consuming bacterial activity (methanogens) should be inhibited or eliminated while preserving the activity of the hydrogen-producing bacteria.

Several methods have been reported to inhibit methanogenic activity and selectively enrich hydrogen producing acidogenic bacteria from mixed anaerobic cultures are heat treatment (Han and Shin, 2004; Zhu and Béland, 2006; Mu et al., 2007; Ren et al., 2008), aeration (Zhu and Béland, 2006; Ren et al., 2008; Wang and Wan, 2008), acid and base treatment (Chen et al., 2002; Lin et al., 2006; Mu et al., 2007; Wang and Wan, 2008; Ren et al., 2008), inhibiting chemical addition such as 2-bromoethanesulfonate (BES), iodopropane, chloroform (Chidthaisong and Conrad, 2000; Zhu and Béland, 2006; Mohan et al., 2008; Wang and Wan, 2008), acidogenic culture preparation (Yu et al., 2002).

The physiological differences between hydrogen producing bacteria (acidogenic bacteria) and hydrogen uptake bacteria (methanogenic bacteria) form the fundamental basis behind the development of the various pretreatment methods proposed for preparation of hydrogen producing seeds. Effective pretreatment processes included heat treatment, acidic or basic treatment, aeration treatment, chemical treatment and so on.

Heat Treatment

The heat treatment is achieved mostly by relying on the spore-forming characteristics of the hydrogen-producing clostridium. *Clostridium sp*. can form protective spores under harsh conditions such as high temperature, but methanogens have no such capability (Zhu and Béland, 2006). Methanogens are sensitive to temperature changes. Heat-treatment temperatures ranging from 75 ^{0}C to 121 ^{0}C and exposure times ranging between 15 min to 2 hr (Li and Fang, 2007). The most common condition in heat treatment is 100 ^{0}C for 15 min.

Mohan et al. (2008) evaluated the influence of the heat treatment of anaerobic mixed inoculum at 100 ^{0}C for 1 hr on hydrogen production. Hydrogen production yield increased from 0.002 mmol H_2/g COD to 0.122 mmol H_2/g COD with heat treatment procedure. Mu et al. (2007) used heat, acid and base treatment methods to suppress methanogenesis in mixed cultures and to enrich H_2-producing inoculum. Thus, highest H_2 yield of 2.0 mol-H_2/mol-glucose was achieved with the heat treated sludge. Ren et al. (2008) studied four treatment methods including heat-shock treatment, acid treatment, alkaline treatment and repeated-aeration treatment to enrich hydrogen producing bacteria. Thus, hydrogen production increased from 180.4 mL (control) to 189.5 mL by heat treatment of sludge. On the other hand, the study by Zhu and Béland indicated that the cumulative hydrogen production of the seed sludge treated by heat was lower than that of the control test (Zhu and Béland, 2006). Heat treatment of digested wastewater sludge reduced hydrogen production yield to 2.59 mol H_2/ mol sucrose from 5.17 mol H_2/ mol sucrose (untreated sludge). Kawagoshi et al. (2005) observed no difference between non-heat-treated and heat-treated digested sludge in hydrogen production.

Acidic or Basic Treatment

In the conventional methanogenic process, the pH is controlled at near pH 7. At pH below 6.3 or above 7.8, methane production rate would drop sharply (van Haandel and Lettinga, 1994; Chen et al., 2002). Thus, the bioactivity of methanogens can be inhibited by adjusting the pH of anaerobic sludge away from pH 7. On the other hand, the hydrogen-producing clostridium can resist to extreme acidity and alkalinity by forming protective spores.

Wang and Wan (2008) indicated that the hydrogen production potentials of the sludge treated by acid (96.8 mL), base (125.9 mL) were higher than hydrogen production potential of the control test (65.7 mL) during the fermentative hydrogen production using glucose as the substrate. Similarly, Mohan et al. (2008) investigated the effect of acid treatment of anaerobic mixed inoculum on hydrogen production. Thus, hydrogen production yield increased from 0.002 mmol H_2/g COD (control) to 0.008 mL by acid treatment of sludge. On the other hand, Ren et al. (2008) indicated that hydrogen production potential of control (180.4 mL) decreased to 134.1 mL in reactor which contained base treated sludge and 51.9 mL in reactor which contained acid treated sludge. Mu et al. (2007) indicated that lowest hydrogen yield of 0.5 mol-H_2/mol-glucose was obtained with the base treated sludge when compared with yield of 2.0 mol-H_2/mol-glucose was achieved with the heat treated sludge and yield of 1.3 mol-H_2/mol-glucose was achieved with the acid treated sludge.

Aeration Treatment

Methanogens are obligative anaerobic archaeobacteria (strict anaerobic bacteria). Thus, activity of hydrogen consuming methanogens inhibited when they are exposed to an aerobic environment. The bacteria capable of producing hydrogen are mainly *Clostridium sp.* (strict anaerobic bacteria), *Escherichia coli* (facultative

anaerobic bacteria) and *Enterobacter* (facultative anaerobic bacteria). Facultative anaerobes are resistant to oxygen (Zhu and Béland, 2006).

Ren et al. (2008) indicated that higher hydrogen production (224.5 mL) was achieved by aeration treatment of sludge when compared to hydrogen production (180.4 mL) was obtained from non-treated sludge. Similarly, Wang and Wan et al. (2008) indicated that hydrogen production of seed sludge treated by aeration (80.2 mL) was higher than that of non- treated seed sludge (65.7 mL). Zhu and Béland (2006) investigated the effect of aeration treatment of sludge on hydrogen production. Results of the study showed that lower hydrogen production yield (4.84 mol H_2/ mol sucrose) was obtained from the bottle with aeration treated sludge when compared to hydrogen production yield of the bottle with untreated sludge (5.17 mol H_2/ mol sucrose).

Chemical Treatment

Methanogens are very sensitive to many chemicals. 2-Bromoethanesulfonate (BES), iodopropane and chloroform can be used to prevent methanogenic activity in the reactors. BES is a structural analog of the coenzyme-M which is specifically found in methanogens but not in other microorganisms (Zhu and Béland, 2006). Moreover, iodopropane is a corrinoid antagonist that prevents the functioning of the B12 enzymes as a methyl group carrier (Kenealy and Zeikus, 1981). Adding chloroform to the seed sludge can also inhibit activity of methanogens (Li and Fang, 2007).

Mohan et al. (2008) indicated that BES treatment of inoculum resulted in higher H_2 yield (0.032 mmol/g COD) than the control (0.002 mmol/g COD). Zhu and Béland (2006) examined the effect of iodopropane and BES treatment of sludge on hydrogen production. The maximum hydrogen yield (5.64 mol H_2/ mol sucrose) was obtained from the bottle with iodopropane treated sludge, followed by the

bottle with BES treated sludge (5.28 mol H_2/ mol sucrose) while hydrogen yield of the bottle with non-treated sludge was 5.17 mol H_2/ mol sucrose.

2.3. Beet-sugar Industry

2.3.1. Sugar-beet Processing

The objective of sugar-beet processing is to extract the sucrose stored in the beet and to transform it into sugar crystals. The production of sugar from sugar beet is based on the five fundamental processing steps: Beet preparation, sugar extraction, juice purification, juice concentration/evaporation, and crystallization (Barjol and Chavanes, 2003).

Figure 2.3 illustrates the process of sugar production. First, sugar-beets are transported via conveyor belts or flume water to the washing installation in which beets are separated from soils, leaves and stones. Then, cleaned sugar beets are cut into slices which is called cossettes. In the sugar extraction step, the sugar in the cossettes is extracted. As a result of this process, diffusion juice and exhausted beet pulp is generated. Produced diffusion juice contains not only sugar but also other components. In a purification process, these are removed by the addition of lime and clear solution of sugar called thin juice is obtained. In the fourth step, water is removed by successive evaporating vessels from the thin juice until a syrup with a solid content around 70% is obtained. Resulting thin juice is further evaporated in specially designed pans until sugar crystals form. Finally, crystals are separated from liquid phase by centrifugation and the centrifuged sugar dried and stored in silos (Barjol and Chavanes, 2003).

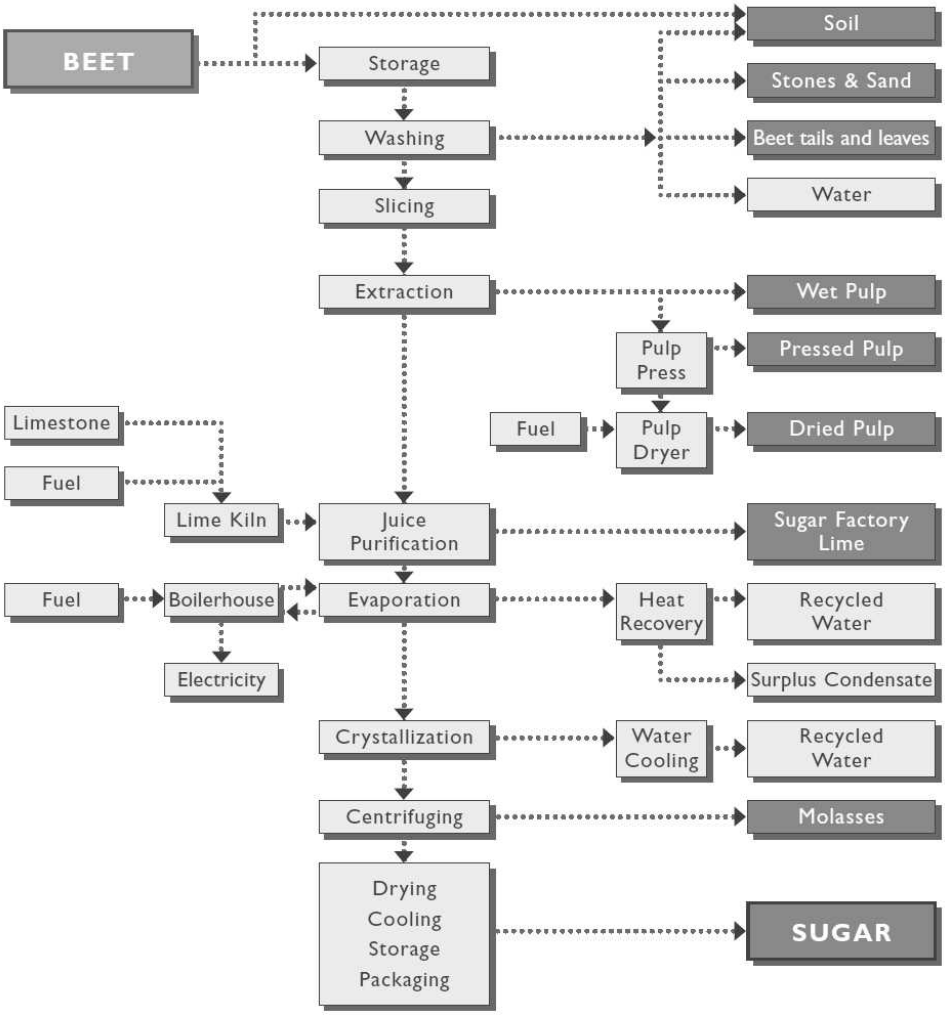


Figure 2.3 The process of sugar production (Barjol and Chavanes, 2003)

2.3.2. Characteristics and Management of Sugar Production Wastes

Environmental problems connected with traditional sugar-beet processing practices are mainly related to the production of large amounts of wastes and by-products and the high consumption of energy, water, lime (Vaccari et al., 2005). By-products of

the sugar-beet processing operation are wastewater, exhausted beet-pulp, molasses, lime sludge, adhering soil, stones and beet leaves (Krajnc et al., 2007).

The manufacture of sugar from sugar beet uses large volumes of water. In beet-sugar industry numerous sources of wastewaters exist such as cooling waters, cleaning waters, pulp-press waters. However, volume of these wastewater streams are insignificant as compared to flume waters which represents about 70% of the total waste volume (Dilek et al., 2003). Beet constituents are leached from cut and damaged surfaces into the flume water so flume water contains high concentrations of hydrocarbons and soluble organic matter. The most common treatment strategy for beet-sugar industry wastewaters is biological treatment after sedimentation (Shore et al., 1984). Anaerobic biological digestion is preferable for effective treatment of the wastewater since it contains high concentrations of hydrocarbons (Wang et al., 1986). Thus, there are numerous studies in the literature on anaerobic digestion of sugar-beet processing wastewater (Shore et al., 1984; Wang et al., 1986; Iza et al., 1990; Farhadian et al., 2007).

Beet-pulp remaining after the extraction of sugar is one of the by-products of sugar beet industry. The processing of 1 ton of beet produces approximately 250 kg of exhausted pressed pulp with 75-80 % water content (Spagnuolo et al., 1997). Beet-pulp composed mainly of cellulose (22–30%), hemicelluloses (24–32%) (essentially arabinan) and pectin (24–32%), with a small amount of lignin (3–4%) (Spagnuolo et al., 1997). Beet-pulp is mainly utilized for animal feed, either used as part of compound feed products or fed directly, in countries with an intensive cattle-raising industry (Hutnan et al., 2000). On the other hand, in other countries, it is dumped in landfills (Voragen et al., 1997). However, composition of beet-pulp is suitable for biological degradation. Therefore, a possible alternative for the utilization of beet-pulp can be anaerobic digestion (Weiland, 1993; Hutnan et al., 2000, 2001; Koppar and Pullammanappallil, 2007).

The final syrup from the crystallization step is called molasses which contains 50% sugar and it is used in a variety of market applications. Molasses is used as a feedstock by fermentation and alcohol distillation industries to produce high value pharmaceuticals, alcohol, citric acid, yeast and other specialty products (Barjol and Chavanes, 2003). In addition, molasses is also used as supplement for animal feed because of its sugar and protein content (Krajnc et al., 2007).

In a purification process, impurities in sugar juice are removed by the addition of large quantities of lime because of that high amounts of lime sludge is formed. Lime sludge is used as soil conditioning product for agricultural land and as a raw material in the cement production (Vaccari et al., 2005).

During beet preparation step, transport and the washing process, adhering soil, stones and leaves are separated from the beet. The soil can be directly returned to the fields by land spreading or dewatered to form high quality arable soil which is used in a wide range of productive applications such as agricultural land improvement and civil engineering and housing construction. Recovered stones can be used in road building and in the construction industry and leaves can be used for animal feed (Barjol and Chavanes, 2003).

Sugar-beet processing wastes have significant potential as a renewable source of fuel (such as bio-ethanol, bio-hydrogen) (Zuhal and Kemal, 2004). Thus, numerous studies have been conducted on bio-hydrogen production using sugar industry wastes as a carbon source such as Ueno et al. (1996) acquired a yield of 2.52 mol-H_2/ mol-glucose from sugar factory wastewater. Wu and Lin (2004) obtained hydrogen production yield 47.1 mmol-H_2/g COD by using molasses wastewater as a substrate. Tanisho et al. (1998) and Logan et al. (2002) molasses obtained hydrogen production yield 0.52 mol H_2/mol substrate and 109 mL H_2/g hexose by using molasses. Hussy et al. (2005) used co-digestion of sucrose, pulped sugarbeet and a water extract of sugarbeet as substrate and hydrogen yields for refined

sucrose and pulped sugarbeet were, 1.7–1.9 and 1.7 mol/mol hexose, respectively, with nitrogen sparging.

2.3.3. Pretreatment of Lignocellulosic Biomass

Lignocellulosic material consists of mainly three different types of polymers (lignocelluloses), namely cellulose, hemicellulose and lignin. Lignocelluloses comprise a large fraction of municipal solid waste, crop residues, animal manures, woodlot arisings, forest residues and dedicated energy crops (Sims, 2003). The digestibility of the hemicellulose and cellulose present in the lignocellulosic biomass (the enzymatic hydrolysis of lignocellulose) is limited by several factors such as crystallinity of cellulose, lignin content, accessible surface area (Chang and Holtzapple, 2000). In addition, solubilization of lignocellulose components also depends on some factors like temperature, moisture content and pH (Fengel and Wegener, 1984). Thus, in order to improve the rate of enzyme hydrolysis, increase yields of fermentable sugars from cellulose or hemicellulose and increase solubilization of lignocellulose components, numerous studies have been conducted on pretreatment of lignocellulosic biomass (Playne, 1984; Vaccarino et al., 1987; Ramos et al., 1992; Delgenés et al., 2002; Laser et al., 2002; Park et al., 2004; Zhu et al., 2005 and 2006; Eskicioğlu et al., 2007; Zhao et al., 2007; Silverstein et al., 2007; Ruiz et al., 2008).

The commonly used pretreatment methods for lignocellulosic biomass are mechanical (Sidias and Koukios, 1989; Delgenés et al., 2002), thermal (Ramos et al., 1992; Lawther et al., 1996; Ruiz et al., 2008), alkaline (Playne, 1984, Vaccarino et al., 1987; Zhu et al., 2005 and 2006; Saha and Cotta, 2007; Silverstein et al., 2007; Zhao et al., 2007), acid (Torget et al., 1991; Sanchez et al., 2004; Karimi et al., 2006; Silverstein et al., 2007), ozone (Silverstein et al., 2007), microwave (Park et al., 2004; Eskicioğlu et al., 2007) and combination of these such as microwave-

alkali (Zhu et al., 2005 and 2006), thermal- alkali (Playne, 1984), thermal-acid (Emmel et al. 2003), microwave-acid-alkali (Zhu et al., 2006).

Several methods have been studied for pretreatment of lignocellulosic wastes for conversion to ethanol or methane (Hendriks and Zeeman, 2009). On the other hand, since it is still in the research and development phase (Reith et al, 2003); limited number of studies have been conducted on pretreatment of lignocellulosic wastes for hydrogen production.

Li and Chen et al. (2007) produced hydrogen from thermal pretreated corn straw using *Clostridium butyricum* AS1.209. Corn straw was pretreated into the stream exploded vessel at 1.5MPa for 10 min. The hydrogen yield produced from steam-exploded corn straw was 68 ml H_2/g corn straw which is higher than 9 ml H_2/g corn straw which was obtained from raw corn straw.

In the Fan et al. (2006), the performance of hydrogen production using the raw wheat straw and HCl pretreated wheat straw was compared in batch fermentation tests. It was reported that before the substrate were degraded by microorganisms, wheat straw and dilute HCl was boiled in a teflon digestor by microwave heating or in a beakers. The results showed that the pretreatment of the substrate plays a key role in the conversion of the wheat straw wastes into hydrogen. Under the condition of microwave heating, the accumulative hydrogen yield increased remarkably with the increase of HCl concentration in the range of 0.5–2.0%. Maximum hydrogen yield of 22.9 ml H_2/g TVS was observed in the test using the pretreated substrate (2.0% HCl). Then, the hydrogen yield gradually declined from 22.9 ml H_2/g TVS at HCl concentration of 2.0% to 6.0 ml H_2/g TVS at HCl concentration of 5.0%. Under the condition of boiling, the change curve of hydrogen yield was similar to that by microwave heating, except that the maximum hydrogen production yield was only 17.9 ml H_2/g TVS.

Datar et al. (2007) pretreated a corn stover using a steam-explosion process and studied its fermentability for hydrogen production. Corn stover were treated with high-pressure steam with and without acid during pretreatment. Hydrogen molar yields of 2.84 and 3.0 were measured with the hydrolyzates derived from neutral and acidic pretreatment, respectively.

Vrije et al. (2002) used miscanthus pretreated by a combination of a mechanical and chemical method as a substrate to produce hydrogen. Mechanical treatment existed of either milling or extrusion. Miscanthus was chemically pretreated with NaOH at 70 C for 4 hour. It was observed that alkaline treatment of miscanthus increased hydrogen yields by 15.6%.

Claassen et al. (2004) produced hydrogen from sweet sorghum bagasse by thermophilic bacteria. Sweet sorghum bagasse was pretreated at 110°C in a pressure cooker for 2 hours together with aqueous solution of either NaOH or sulfuric acid.

Zhang et al. (2007) investigated the effect of acidification pretreatment of substrate on hydrogen yield. The cornstalk wastes used as substrate were pretreated by dilute HCl. The cornstalk wastes and dilute HCl were boiled in a beaker for 30 min. It was showed that the acidification pretreatment of the substrate plays a crucial role in conversion of the cornstalk wastes into hydrogen. It was found that the hydrogen yield increased with the increase of HCl concentration in the range of 0.04–0.2%, and then decreased with increasing HCl concentration. Maximum hydrogen yield of 149.7 ml H_2/ g TVS was observed at 0.2% HCl concentration for the substrate of acidification pretreatment.

Wang et al. (2003) produced hydrogen from wastewater sludge pretreated by five different method (ultrasonication, acidification, sterilization, BES addition, freezing-thawing) by *Clostridium biofermentans*. It was observed that freezing-

thawing and sterilization of wastewater sludge increased the specific hydrogen yield from /0.6 mmol-H_2/g-COD for the original sludge to 1.5-2.1 mmol-H_2/g-COD. Acidifying sludge did not significantly promote the production of hydrogen. Furthermore, adding BES and ultrasonication reduced the hydrogen yield.

Guo et al. (2008) produced hydrogen from sterilization, microwave and ultrasonication pretreated waste sludge. *Pseudomonas sp. GZ1* was inoculated in pretreated waste sludge to produce hydrogen. It was stated that sterilized sludge had the largest yield of hydrogen production (15.02 mL/g tCOD). Hydrogen yields of microwave and ultrasonication pretreated sludges were 11.04 mL/g tCOD and 4.68 mL/g tCOD, respectively.

CHAPTER 3

MATERIALS AND METHODS

3.1. Waste Characteristics

3.1.1. Sugar-Beet Processing Wastewater

Sugar-beet processing wastewaters used for Set-up 1 and 3 were obtained from a private beet-sugar factory located near Amasya and Ankara Beet-Sugar Factory, respectively. Characterizations of the sugar-beet processing wastewaters were carried out and the results were provided in Table 3.1 and Table 3.2. After the characterization, wastewaters were kept frozen at –20 ^{o}C in order to inhibit biological activity prior to the use in the experimental studies.

Prior to the characterization and the use in the study, sugar-beet processing wastewaters were settled for 2-hour to remove the suspended materials which are very common for sugar-beet processing wastewater. The time period of 2-hour was chosen to represent the typical hydraulic retention time of primary sedimentation before the secondary treatment systems (Metcalf and Eddy, 2003).

Table 3.1 Wastewater characteristics for Set-up 1 (2 hr settled)

Parameter	Value (mg/L)
tCOD	6621 ± 113.2
sCOD	6165 ± 517.1
TS	6062 ± 53.0
VS	2832 ± 25
TSS	665 ± 21.2
VSS	335 ± 7.1
pH	6.82
Alkalinity (as $CaCO_3$)	1760
TKN	10
P_{Total}	2.7
tVFA (as H-Ac)	1115 ± 20
H-Ac	394 ± 5
H-Pr	610 ± 12
H-Bu	46 ± 1

Table 3.2 Wastewater characteristics for Set-up 3 (2 hr settled)

Parameter	Value (mg/L)
tCOD	3418 ± 94.2
sCOD	3168 ± 237.3
TS	4516 ± 25.1
VS	2110 ± 12.2
TSS	483 ± 14
VSS	160 ± 5.4
pH	7.08
Alkalinity (as $CaCO_3$)	1120
TKN	10
P_{Total}	3.4
tVFA (as H-Ac)	578 ± 6
H-Ac	329 ± 5
H-Pr	149 ± 4
H-Bu	28 ± 1

3.1.2. Pressed Beet-pulp

Pressed beet-pulp used for Set-up 2, 3 and 4 was obtained from a private beet-sugar factory located near Amasya. Characterization of the beet-pulp was carried out and the results were tabulated (Table 3.3). After the characterization, beet-pulp was kept frozen at –20 ^{o}C in order to inhibit biological activity prior to the use in the experimental studies.

In order to achieve physical homogeneity, first the frozen beet-pulp was thawed at room temperature and further dried at 105 ^{o}C for 24 hours. Then, the dried pulp

particles were grinded by the help of a pestle and the homogenized powdered pulp was used for reactor feeding.

Table 3.3 Pressed beet-pulp characteristics

Parameter	Value
Moisture (%)	85 ± 0.1
TS (%)	15 ± 0.1
VS (%TS)	94 ± 0.01
COD (g/g dry weight)	1.22 ± 0.15
TKN (%TS)	7.28
P_{Total} (%TS)	1.0 ± 0.28

3.2. Inoculum

Four different types of cultures were used as seed in the experiments; mixed anaerobic culture, heat treated mixed anaerobic culture, 2-bromoethanesulfonate added mixed anaerobic culture and acidogenic culture.

Mixed anaerobic culture

The mixed anaerobic cultures were obtained from the anaerobic sludge digesters at the Ankara Wastewater Treatment Plant. Before being used as inoculum, the mixed anaerobic culture was concentrated by settling 24 hour period of time. By this way, seed VSS concentration was increased. Then, concentrated sludge was filtered through a screen with a pore size of 1 mm before use. The volatile suspended solids concentration of the concentrated seed cultures was 18730± 189 mg/L.

Heat treated mixed anaerobic culture

To inhibit the bioactivity of the hydrogen consumers and to enrich spore-forming, hydrogen-producing acidogens, mixed anaerobic culture was heated at 100 ^{o}C for 15 min (Han and Shin, 2004).

2-bromoethanesulfonate added mixed anaerobic culture

To prevent methanogenic activity in the reactors, 2-bromoethanesulfonate added mixed anaerobic culture was prepared by adding 10 mM 2-bromoethanesulfonate (BES) in mixed anaerobic culture (Chidthaisong and Conrad, 2000).

Acidogenic culture

Acidogenic anaerobic culture was enriched from mixed anaerobic culture through acidification of glucose in fed-batch continuously mixed acidogenic reactor. Experiment was performed in a continuously stirred 2000 mL reactor with effective volume of 1500 mL. Reactor was operated by daily fed-batch feeding strategy and kept in a temperature controlled room at 35±1 ^{o}C for 81 days. To enable all the necessary micro and macro nutrients required for optimum anaerobic microbial growth, BM was added. For the start-up 300 mL BM and 1200 mL mixed anaerobic culture taken from Ankara Wastewater Treatment Plant were added. Glucose stock solution was prepared and each day reactor was fed by 250 mL BM and 250 mL glucose solution.

A fermenter has to be operated at low hydraulic retention times to suppress methanogenic activity in the pre-fermentation stage as the methanogens are known to grow more slowly than acidogenic bacteria (Hawkes et al., 2002). Because of the slow-growing time of methane-forming bacteria, typical HRTs for anaerobic digesters are higher than 12 days. Low HRT values (lower than 10 days) are not recommended for convention reactor configurations because of the risk of washout

of methane-forming bacteria (Gerardi, 2003). Hence, low HRT (3 days) was chosen to enrich spore-forming, hydrogen-producing acidogens and to wash out methanogens. Lower HRT values could not choose as culture enrichment reactor was run with a fed-batch strategy.

Reactor's hydraulic retention time, initial organic loading rate and initial pH were 3 days, 2.5 g/L-day glucose and 8.03 respectively. The value and stability of pH in the reactor are important to suppress hydrogen consumption and obtain enriched cultures of hydrogen producing acidogenic bacteria. The pH range of 5.0–6.0 is accepted as optimum for the growth of anaerobic acidogenic microorganisms (Speece, 1996). Since, it was aimed to stabilize pH value in this range; OLR adjustments were done (2.5 g glucose/L to 4 g glucose/L). Final VSS concentration of acidogenic culture (540 mg/L) was concentrated to 6630 mg/L by gravity settling prior to the use in the experimental studies.

As the control parameters of reactor, pH, MLSS, MLVSS and VFA were measured. Among these parameters, pH was measured daily; MLSS and MLVSS were measured every 2 days. VFA concentrations were determined daily for 5 days and then measured every 2 days.

3.3. Basal Medium

Basal Medium (BM) contains all the necessary micro- and macro-nutrients for an optimum anaerobic microbial growth. So in order to supply adequate nutrients for an optimum microbial growth, reactors were fed by basal medium (BM). In this study, the medium composition was as follows (concentrations are given in parentheses as mg/L): NH_4Cl (1200), $MgSO_4 \cdot 7H_2O$ (400), KCl (400), $Na_2S \cdot 9H_2O$ (300), $CaCl_2 \cdot 2H_2O$ (50), $(NH_4)_2 \cdot HPO_4$ (80), $FeCl_2 \cdot 4H_2O$ (40), $CoCl_2 \cdot 6H_2O$ (10), KI (10), $MnCl_2 \cdot 4H_2O$ (0.5), $CuCl_2 \cdot 2H_2O$ (0.5), $ZnCl_2$ (0.5), $AlCl_3 \cdot 6H_2O$ (0.5),

$NaMoO_4 \cdot 2H_2O$ (0.5), H_3BO_3 (0.5), $NiCl_2 \cdot 6H_2O$ (0.5), $NaWO_4 \cdot 2H_2O$ (0.5), Cysteine (10), (Güngor-Demirci and Demirer, 2004).

3.4 Analytical Methods

Total Solids and Volatile Solids

TS and VS determinations were carried out as described in standard methods (2540 B. Total Solids Dried at 103–105 ^{o}C, 2540 E. Fixed and Volatile Solids Ignited at 550 ^{o}C) (APHA, 2005).

Suspended Solids and Volatile Suspended Solids

SS and VSS determinations were carried out as described in standard methods (2540 D. Total Suspended Solids Dried at 103–105 ^{o}C) (APHA, 2005).

pH

pH values were measured with a pH meter (HI 8314, Hanna Instruments) and a pH probe (HI 1230, Hanna Instruments).

Total Chemical Oxygen Demand

tCOD determination for beet-pulp characterization were accompanied in accordance with Standard methods (5220 B. Open Reflux Method) (APHA, 2005). tCOD determinations for sugar-beet processing wastewaters were carried out by EPA approved reactor digestion method (for COD range of 0-1500 mg/L) and spectrophotometric determinations were performed by using a spectrophotometer (SN 05827, PC Multidirect).

Soluble Chemical Oxygen Demand

First, samples were filtered through 0,45 μm pore sized filters (Millipore). Then, sCOD determinations were carried out by EPA approved reactor digestion method (for COD range of 0-1500 mg/L) and spectrophotometric determinations were performed by using a spectrophotometer (SN 05827, PC Multidirect).

Gas Composition

Gas samples were taken through rubber stoppers from gas phase with a syringe for gas composition analyses. Gas compositions were determined with a gas chromatograph (Thermo Electron Co.) equipped with a thermal conductivity detector (TCD). Produced gases were separated as hydrogen (H_2), carbon dioxide (CO_2), oxygen (O_2), methane (CH_4) and nitrogen (N_2) by using parallel connected columns (CP-Moliseve 5A and CP-Porabond Q) at a fixed oven temperature of 45 ^oC. Helium was used as carrier gas at 100 kPa constant pressure. The inlet and detector temperatures were set to 50 ^oC and 80 ^oC respectively.

Gas Production

The gas accumulated in the head space of each reactor was taken through rubber stoppers from gas phase with a 10 mL gas-tight syringe (Sanitex, Italy). Gas-tight syringe was used to equilibrate the pressure inside the reactor to the ambient pressure and the total gas volume measured with gas-tight syringe was recorded. Hydrogen, methane, carbon dioxide gases percentages in total gas was determined with a gas chromatograph. Finally, with the help of the total gas volume and gas percentages; hydrogen, methane, carbon dioxide gas volumes were calculated.

Volatile Fatty Acids and Ethanol

For volatile fatty acid measurements samples were filtered by 0.22 μm pore sized filters. Then, the samples were diluted with deionized water to assure the VFA concentration of the sample to be in the range of pure VFA calibration of gas chromatograph. After filtering and dilution, samples were acidified with 98% formic acid to a pH less than 2.5, in order to convert the fatty acids to their undissociated forms (i.e. acid forms).

Acidified samples were measured in a gas chromatograph (Thermo Electron Co., Thailand) for their VFA contents. Nukol column (Model 25326, 15 m × 0.53 mm) was used to separate VFAs (acetic, propionic, n-butyric, iso-butyric, n-valeric, iso-valeric, n-caproic, iso-caproic and n-heptanoic acids). Flame ionization detector (FID) was used for this purpose which was adjusted to 280 ^{o}C as operating temperature. Helium was used as carrier gas with a constant flow rate of 6 mL/min and the inlet temperature was kept at 250 ^{o}C. Oven temperature was initially set to 100 ^{o}C with 2 min holding time and then increased to 200 ^{o}C with 8 ^{o}C/min ramping. The concentrations of separate VFAs were expressed in terms of acetic acid equivalents by dividing the concentration value by its molecular weight and multiplying with the molecular weight of acetic acid. Total volatile fatty acid (tVFA) was determined by the sum of concentrations of all VFA species expressed as acetic acid equivalents. For ethanol measurement, samples were filtered by 0.22 μm pore sized filters. Then filtered samples were measured in a gas chromatograph (Thermo Electron Co., Thailand) equipped with a flame ionization detector for their ethanol contents.

Total Kjeldahl Nitrogen

TKN was measured according to the procedure described in standard methods (4500-N_{org} B. Macro-Kjeldahl Method) (APHA, 2005).

Total Phosphorus

P_{Total} determinations were carried out as described in standard methods (4500-P) (APHA, 2005).

Alkalinity

Alkalinity was measured according to standard methods (2320-B Titration Method) (APHA, 2005).

3.5 Experimental Set-ups and Procedures

3.5.1. Set-up 1: Effect of Culture Type on Bio-Hydrogen Production

This experiment was run to investigate the effects of four different types of cultures (mixed anaerobic culture, heat treated mixed anaerobic culture, 2-bromoethanesulfonate added mixed anaerobic culture and acidogenic culture) on the bio-hydrogen production from sugar-beet processing wastewater. Heat treated mixed anaerobic culture, 2-bromoethanesulfonate added mixed anaerobic culture and acidogenic culture were prepared to use in Set-up 1 (Section 3.2). In Set-up 1, sugar-beet processing wastewater (4.5 g/L COD) was used along with glucose acclimated acidogenic culture (at AC), mixed anaerobic culture (at MAC), 2-bromoethanesulfonate added mixed anaerobic culture (at BMAC) and heat treated mixed anaerobic culture (at HMAC) (Table 3.4). In addition, unseeded reactor (US) was also prepared to investigate bio-hydrogen production activities of indigenous microorganisms (Table 3.4).

Experiment was performed in 110-mL glass reactors with effective volume of 60 mL. Each reactor contained 4.5 g/L COD of sugar-beet processing wastewater and

VSS concentration in the reactors were adjusted to 1800 mg/L. BM was added into the reactors to supply adequate macro- and micro-nutrients.

After seeding and adding substrate, the initial anaerobic environment in the reactors was established by purging with nitrogen gas for 3 min at the start of cultivation. Then, the reactors were sealed with natural rubber stoppers and plastic screw-caps. Prepared reactors were incubated in a constant temperature room (35±2 ^{o}C). Continual mixing was applied at 175 rpm by using a mechanical shaker. To avoid sunlight, reactors were covered with aluminum foil. By doing this, bio-hydrogen which may be produced by photosynthetic bacteria was prevented (Fang and Liu, 2002).

Gas productions and compositions of each reactor were measured daily and initial-final pH measurements were also carried out.

Table 3.4 Reactors used in Set-up 1

Reactor	Including
US	- 4.5 g/L COD of sugar-beet processing wastewater - Basal Medium
MAC	- 4.5 g/L COD of sugar-beet processing wastewater - Mixed anaerobic culture - Basal Medium
BMAC	- 4.5 g/L COD of sugar-beet processing wastewater -2-bromoethanesulfonate added mixed anaerobic culture - Basal Medium
AC	- 4.5 g/L COD of sugar-beet processing wastewater - Acidogenic culture - Basal Medium
HMAC	- 4.5 g/L COD of sugar-beet processing wastewater - Heat treated mixed anaerobic culture - Basal Medium

3.5.2. Set-up 2: Comparison of Beet-Pulp and Sugar-Beet Processing Wastewater in terms of Bio-Hydrogen Generation Potentials at 4.5 g/L COD

This set-up was used to compare bio-hydrogen generation potentials of beet-pulp and sugar-beet processing wastewater at an initial COD level of 4.5 g/L. In Set-up 1, more bio-hydrogen production was observed at MAC and US compared with the BMAC, HMAC and AC. Thus, in Set-up 2, two types of reactors were used; reactor which contained only beet-pulp without any external seed addition (P1) and reactor with mixed anaerobic culture and beet-pulp (P2). Reactors were run as duplicates,

totally 4 reactor was run (two P1 reactor and two P2 reactor). The mean values and standard deviations of duplicate reactors were used in all tables.

In Set-up 2, 250 mL glass reactors with effective volume of 180 mL were used. Each reactor contained 4.5 g/L COD beet-pulp and VSS concentration in the reactors were adjusted to 1800 mg/L. BM was added into the reactors in order to supply adequate nutrients for an optimum microbial growth. Initial pHs of the reactors were adjusted to 6 (Lee et al., 2008; Pakarinen et al., 2008) by 2M NaOH and 2M HCI solutions.

Prior to incubation, reactors were flushed with nitrogen gas for 3 min in order to maintain anaerobic conditions and then sealed with natural rubber stoppers and plastic screw-caps. The reactors were kept continuously mixing at 175 rpm by using a mechanical shaker in a temperature controlled room (35 ± 1 ^{o}C). To avoid sunlight, reactors were covered with aluminum foil. By doing this, bio-hydrogen which may be produced by photosynthetic bacteria was prevented (Fang and Liu, 2002).

For all of the reactors, gas productions and compositions were measured daily during digestion period. After the digestion period was ended, VFA concentrations of the reactors were measured. In addition, initial and final pH measurements were carried out.

3.5.3. Set-up 3: Comparison of Beet-Pulp and Co-Digestion of Sugar-Beet Processing Wastes in terms of Bio-Hydrogen Generation Potentials at High COD Values

Set-up 3 was run to investigate bio-hydrogen productivities of only beet-pulp and co-digestion of beet-pulp and sugar-beet processing wastewater at high COD values (20, 25, 30 g/L COD) when compared to COD values of Set-up 1 and 2 (4.5 g/L COD). 250 mL glass reactors with effective volume of 180 mL were used. Set-up 3 contained six different types of reactors (Table 3.5). R3, R5, R6 were run as duplicates. The mean values and standard deviations of duplicate reactors were used in all tables.

Based on the results of Set-up 2, indigenous microorganisms in wastewater are not found in beet-pulp or not effective as much as in sugar-beet processing wastewater. Thus, all reactors were inoculated with mixed anaerobic sludge, establishing a VSS concentration of 1800 mg/L. BM was added into the reactors to supply adequate macro- and micro-nutrients and initial pHs of the reactors were adjusted to 6 (Mohan et al., 2007; Lee et al., 2008; Pakarinen et al., 2008) by 2M NaOH and 2M HCI solutions.

Reactors were purged with nitrogen gas for 3 min at the start of cultivation in order to maintain anaerobic conditions and then capped tightly with natural rubber stoppers and plastic screw-caps. Prepared reactors were incubated in a mechanical shaker at 175 rpm in a constant temperature room (35±2 ^{o}C). To avoid sunlight, reactors were covered with aluminum foil.

Gas productions and compositions of each reactor were measured daily during digestion period. Initial and final pH, VFA, tCOD, sCOD, ethanol measurements were carried out.

Table 3.5 Reactors used in Set-up 3

Reactor	Including
R1	- 20 g/L COD of sugar-beet processing wastewater and beet-pulp - Mixed anaerobic culture - Basal Medium
R2	- 25 g/L COD of sugar-beet processing wastewater and beet-pulp - Mixed anaerobic culture - Basal Medium
R3	- 30 g/L COD of sugar-beet processing wastewater and beet-pulp - Mixed anaerobic culture - Basal Medium
R4	- 20 g/L COD of beet-pulp - Mixed anaerobic culture - Basal Medium
R5	- 25 g/L COD of beet-pulp - Mixed anaerobic culture - Basal Medium
R6	- 30 g/L COD of beet-pulp - Mixed anaerobic culture - Basal Medium

3.5.4. Set-up 4: Effects of Pretreatment Methods on Solubilization of Beet-Pulp and Bio-Hydrogen Production Yields

This set-up mainly consists of two parts: Effects of pretreatment methods on solubilization of beet-pulp and effects of pretreatment methods on bio-hydrogen production yields. In the first part, the effects of five different pretreatment method (alkaline pretreatment, thermal pretreatment, microwave pretreatment, thermal-alkaline pretreatment, microwave-alkaline pretreatment) on solubilization of beet-pulp were investigated. In the second part, three out of five pretreatment methods (Alkaline, microwave-alkaline, thermal-alkaline pretreatment) were used to compare effects of pretreatments on bio-hydrogen productivity.

Effects of Pretreatment Methods on Solubilization of Beet-Pulp

In this part of set-up 4, beet-pulp was pretreated by five different pretreatment methods (alkaline pretreatment, thermal pretreatment, microwave pretreatment, thermal-alkaline pretreatment, microwave-alkaline pretreatment). These methods were used to observe whether the solubilization of beet-pulp could be improved or not. Control reactor was also run to determine initial sCOD, VFA of beet-pulp. Reactors were run as duplicates (total of 12 reactors) and the mean values of duplicate reactors were used in all tables. For this part of the Set-up 4, each reactor contained 36 g/L COD of beet-pulp. After pretreatments, sCOD and VFAs of the reactors were measured.

For alkaline pretreatments, the pH was set at 12 by the addition of 2M NaOH then mixed with magnetic stirrer for 30 min. (Kim et al., 2003). Alkaline pretreatment duration was extended to 30 minutes in order to achieve a more homogenous distribution of alkaline agent and application of NaOH was continued for the whole 30 minutes to keep the pH at the desired value. Thermal pretreatments were done with autoclave at 121 ^{0}C, 1.5 atm for 30 min. Similarly, Wang et al. (2003)

pretreated wastewater sludge at 121 ^{o}C for 30 minute and Kim et al. (2003) pretreated waste activated sludge at 121°C and 1.5 atm pressure for 30 min to increase soluble COD. Microwave pretreatments were achieved with Berghof, MWS-2 Microwave System, having a maximum temperature of 220°C, maximum power of 1000 W and maximum pressure of 40 bars and the frequency of 2450 MHz. For microwave pretreatment 700 W; 170 ^{o}C; 30 min were chosen. Similarly, Zhu et al. (2005) and Park et al. (2004), also choose 700 W for 7 and 30 min for microwave pretreatment, respectively. For thermal-alkaline pretreatments, alkaline pretreated beet-pulp (at pH 12 for 30 min) was exposed to thermal pretreatment (autoclave at 121 ^{0}C, 1.5 atm for 30 min). In addition, for microwave-alkaline pretreatments, alkaline pretreated beet-pulp (at pH 12 for 30 min) was exposed to microwave pretreatment (700 W; 170 ^{o}C; 30 min).

Effects of Pretreatment Methods on Bio-Hydrogen Production Yields

In the second part of the set-up 4, three out of five pretreatment methods were chosen to compare effects of pretreatments on bio-hydrogen productivity. Alkaline, microwave-alkaline, thermal-alkaline pretreatments were chosen to compare effects of pretreatments on bio-hydrogen productivity since they increased initial sCOD of beet-pulp significantly when compared to thermal and microwave pretreatments. 250 mL glass reactors with effective volume of 180 mL were used. Four different types of reactors were set-up (Table 3.6) and run as duplicates (total of 8 reactors). The mean values and standard deviations of duplicate reactors were used in all tables.

In Set-up 3, a substrate concentration of 20 g COD/L resulted in the highest bio-hydrogen yield at reactors which contained only beet-pulp. Thus, in this part of the Set-up 4, each reactor contained 20 g/L COD of beet-pulp. All reactors were inoculated with mixed anaerobic sludge, VSS concentration in the reactors were adjusted to 1800 mg/L. BM was added into the reactors in order to supply adequate

nutrients for an optimum microbial growth. Initial pHs of the reactors were adjusted to 6 (Lee et al., 2008; Pakarinen et al., 2008) by 2M NaOH and 2M HCI solutions.

Table 3.6 Reactors used in second part of the Set-up 4

Reactor	Including
S1	- Reactor which contained beet-pulp (20 g/L COD) with no pretreatment - Mixed anaerobic culture - Basal Medium
S2	- Reactor which contained beet-pulp (20 g/L COD) with thermal-alkaline pretreatment - Mixed anaerobic culture - Basal Medium
S3	- Reactor which contained beet-pulp (20 g/L COD) with alkaline pretreatment - Mixed anaerobic culture - Basal Medium
S4	- Reactor which contained beet-pulp (20 g/L COD) with microwave-alkaline pretreatment - Mixed anaerobic culture - Basal Medium

Reactors were flushed with nitrogen gas for 3 min at the start of cultivation in order to maintain anaerobic conditions and then sealed with natural rubber stoppers and plastic screw-caps. Prepared reactors were placed in a mechanical shaker and

stirred at 175 rpm in a constant temperature room (35^0C±2). To avoid sunlight, reactors were covered with aluminum foil.

For all of the reactors, gas productions and compositions were measured daily during digestion period. In addition, initial and final pH, VFA, tCOD, sCOD measurements were carried out.

CHAPTER 4

RESULTS AND DISCUSSIONS

In this chapter, the experimental results obtained are presented. The effects of different culture types on bio-hydrogen production were explained. Bio-hydrogen generation potentials of beet-pulp and sugar-beet processing wastewater were compared at an initial COD level of 4.5 g/L. Then, bio-hydrogen generation potentials of only beet-pulp and co-digestion of beet-pulp and sugar-beet processing wastewater at high COD values (20, 25, 30 g/L COD) were discussed. After that, the effects of five different pretreatment methods (alkaline, thermal, microwave, thermal-alkaline, microwave-alkaline pretreatments) on solubilization of beet-pulp were compared. Three (alkaline, microwave-alkaline, thermal-alkaline pretreatments) out of five pretreatment methods used which resulted in higher solubilization ratios (CODs/CODt) were chosen to compare the corresponding bio-hydrogen productivities. Figure 4.1 illustrates the four different experimental set-ups used in this study.

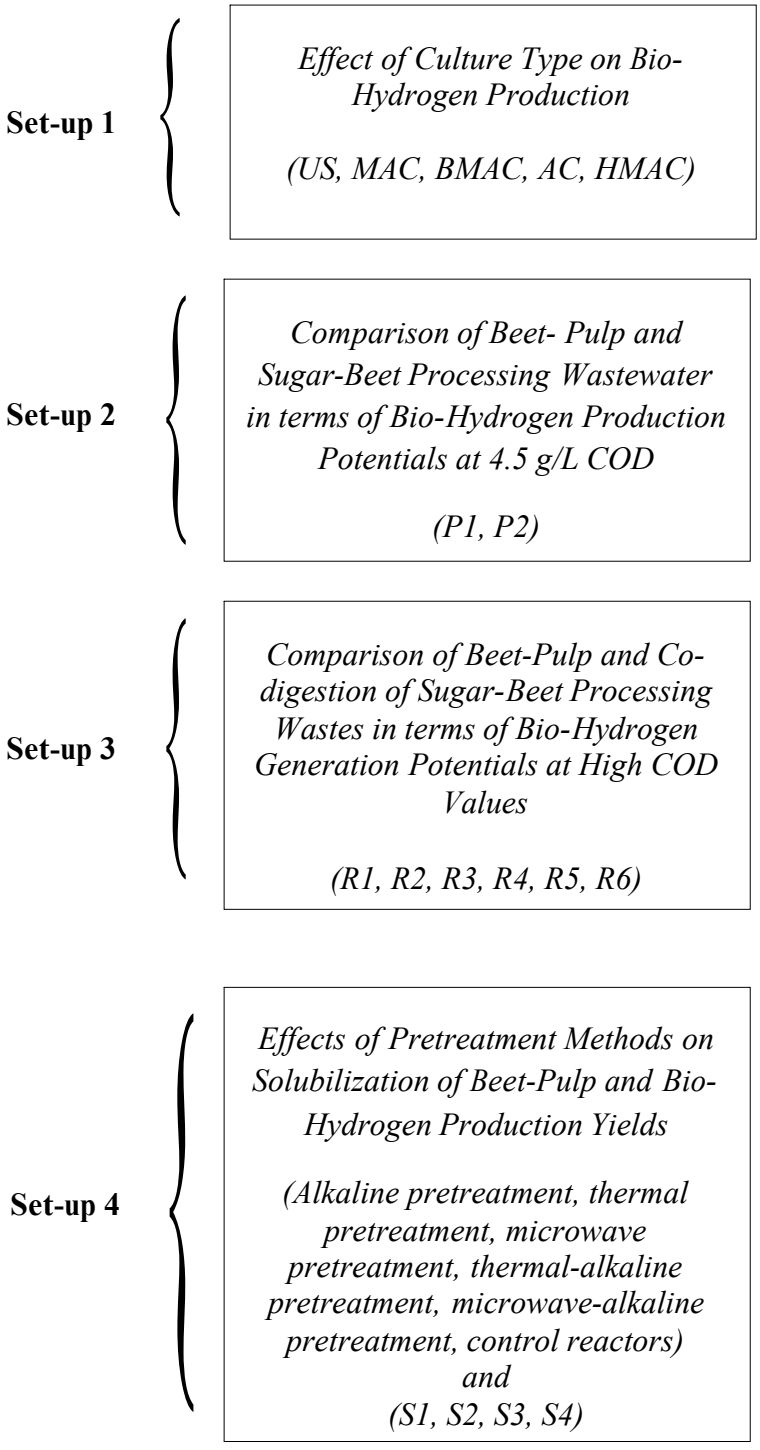


Figure 4.1 Four different experimental set-ups used in this study

4.1. Set-up 1: Effect of Culture Type on Bio-Hydrogen Production

Heat treated mixed anaerobic culture, 2-bromoethanesulfonate added mixed anaerobic culture and acidogenic culture were prepared to use in Set-up 1 (Section 3.2). In Set-up 1, sugar-beet processing wastewater (4.5 g/L COD) was used along with glucose acclimated acidogenic culture (at AC), mixed anaerobic culture (at MAC), 2-bromoethanesulfonate added mixed anaerobic culture (at BMAC) and heat treated mixed anaerobic culture (at HMAC) to investigate the effect of culture type on bio-hydrogen production. In addition, unseeded reactor (US) was prepared to investigate bio-hydrogen production activities of indigenous microorganisms.

4.1.1. Culture Enrichment

Acidogenic anaerobic culture was enriched from mixed anaerobic culture through acidification of glucose in fed-batch continuously mixed acidogenic reactor for 81 days of operation (Section 3.2). Glucose acclimated acidogenic culture was prepared to use in AC reactor in Set-up 1.

The results of VFA analyses indicated that acetate (H-Ac), propionate (H-Pr), and butyrate (H-Bu) were the major VFAs observed during the pre-acidification of glucose [Figure 4.2 (a)]. H-Ac, H-Pr and H-Bu productions comprising 51.3–66.4; 11.2–37.4; 2.4–21.2 % (w/w) of tVFA. According to literature, these short-chain fatty acids were found to be dominant in acidogenic reactors (Yu and Fang, 2002). In addition, acetate is not normally present at very high levels in methanogenic anaerobic systems because acetate serves as a substrate for methanogens. In methanogenic anaerobic systems, total VFA concentration must be between 50-500 mg/L as H-Ac for acceptable activity of methanogens (Gerardi, 2003). For this reason, high total VFA concentrations (2439–15252 mg/L as H-Ac) and high acetate levels (1620–9241 mg/L) in acidogenic reactor were associated with higher acidogenic activity and/or lower methanogenic activity.

As shown in Figure 4.2 (c), the initial VSS of 12050 mg/L was reduced to 540 mg/L after the experiment because hydrogen-consuming methanogens were washed out from the reactor. In addition, initial pH of 8.03 was decreased to 4.5- 6.0 probably as a result of VFA formation. OLR was increased from 2.5 to 4 g glucose/L-d to stabilize pH around 5.0– 6 .0 since pH range of 5.0-6.0 is optimum for acidogens (Ginkel et al., 2001) [Figure 4.2 (b), (d)].

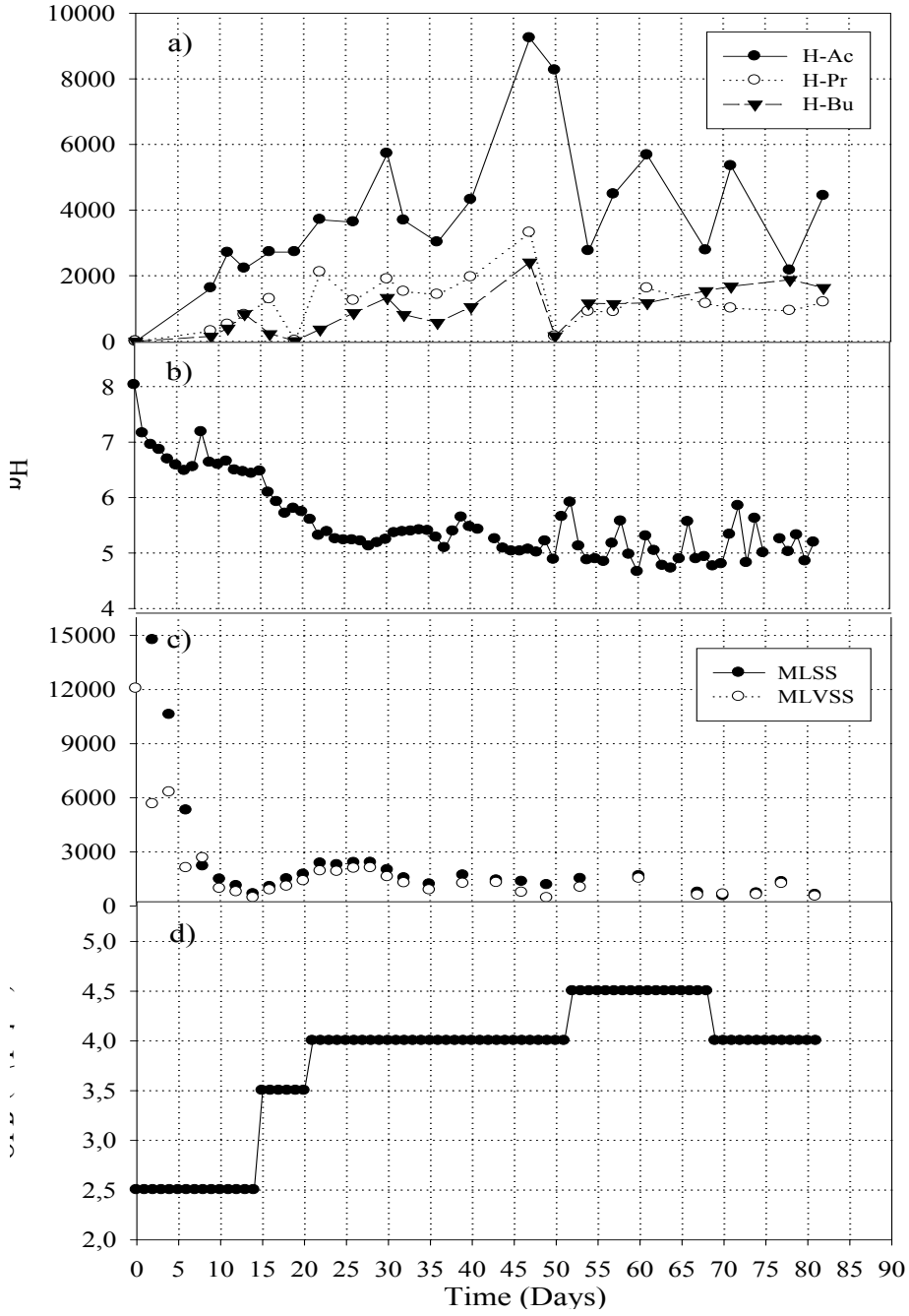


Figure 4.2 Temporal variations of control parameters of the acidification reactor: a) VFA; b) pH; c) MLSS and MLVSS; d) OLR

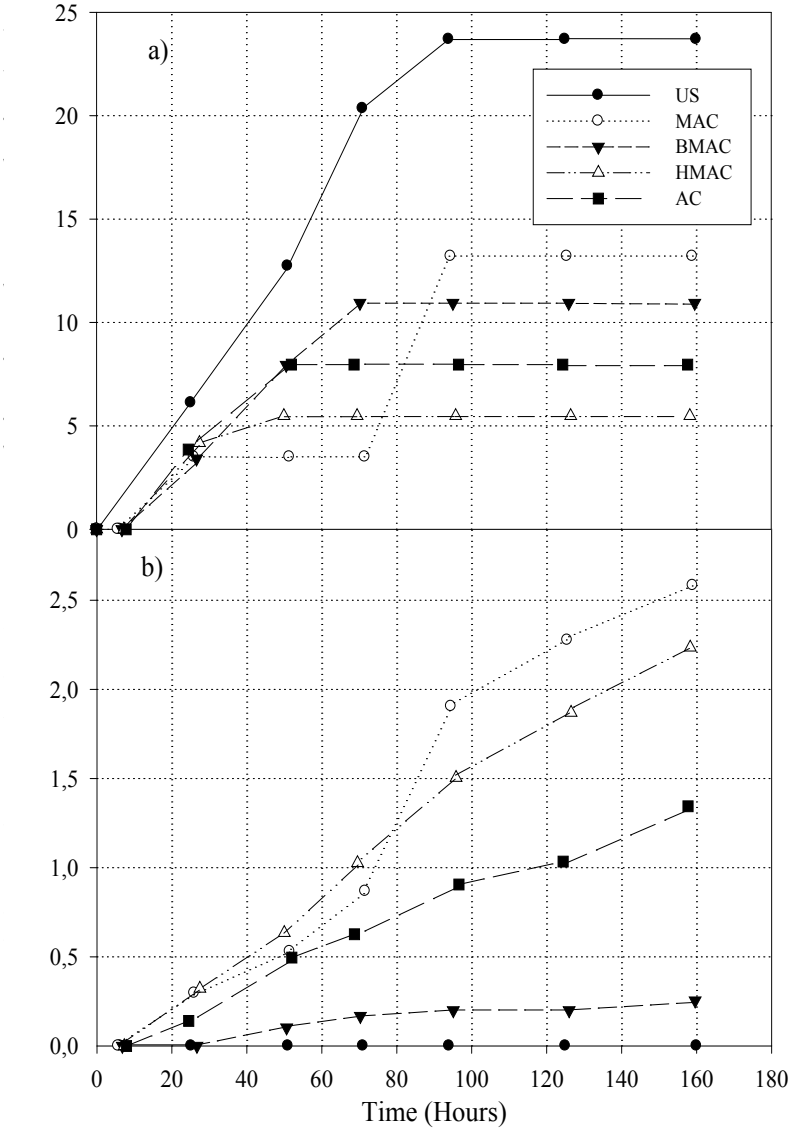
4.1.2. Effect of Culture Type on Bio-Hydrogen Production

In Set-up 1, the effects of four different culture types on bio-hydrogen production were investigated. To this purpose, four different types of reactors (MAC, HMAC, BMAC and AC) were run which contained four different types of cultures (Table 3.4). In addition, unseeded reactor (US) was prepared to investigate bio-hydrogen production activities of indigenous microorganisms.

Total gas productions of reactors in Set-up 1 were illustrated in Table 4.1. Total gas compositions in reactors consisted of bio-hydrogen, carbon dioxide and no or insignificant amounts of methane (0–2.6 mL). The cumulative bio-hydrogen and methane productions of US, MAC, BMAC, HMAC, AC reactors were illustrated in Figure 4.3 (a) and (b), respectively. High initial bio-hydrogen production rates were observed in first 2-3 day period by consumption of easily degradable substrate.

Table 4.1 Total gas productions observed in Set-up 1

Reactors	Total gas production (mL)
US	44.6
MAC	49.3
BMAC	48.6
AC	34.5
HMAC	49.6



US → 4.5 g/L COD wastewater

MAC → 4.5 g/L COD wastewater+mixed anaerobic culture

HMAC → 4.5 g/L COD wastewater+heat treated mixed anaerobic culture

BMAC → 4.5 g/L COD wastewater+BES added mixed anaerobic culture

AC → 4.5 g/L COD wastewater+acidogenic culture

Figure 4.3 Cumulative bio-hydrogen (a) and methane (b) productions of US, MAC, BMAC, HMAC, AC reactors

Indigenous microorganisms

The maximum bio-hydrogen production yield in Set-up 1 (87.7 mL H_2 /g COD) was observed in US relative to other reactors (MAC, BMAC, HMAC, AC) (Table 4.2). In US, bio-hydrogen was produced directly by using sugar-beet processing wastewater without any external seed addition. This means that, bio-hydrogen production from sugar-beet processing wastewater do not need the presence of any seed because wastewater serves as a raw material and also the seed sludge for bio-hydrogen production.

Table 4.2 Bio-hydrogen production yields observed in Set-up 1

	Bio-hydrogen production yields (mL H_2/g COD)
US	87.7
MAC	48.9
BMAC	40.5
AC	29.5
HMAC	20.3

It is important to explain why the maximum bio-hydrogen was observed in the US, despite the fact that the other reactors contained external seed addition. Anaerobic microflora naturally present within the sugar-beet processing wastewater (indigenous bacteria) have already been acclimated to substrate that could be the reason for maximum bio-hydrogen production. Shore et al. (1984) stated that sugar wastes mainly included sucrose, and the sucrose may rapidly be catabolized to acetic, propionic and butyric acid by indigenous microorganisms in anaerobic conditions. In Table 3.1, it can be seen that sugar-beet processing wastewater has already contained acetic acid, propionic acid and butyric acid. This means that,

investigated wastewater has already been acidified by indigenous microorganisms. As seen from Fig. 4.3 (b), methane production was not detected in US, demonstrating inhibition of methanogenic activity.

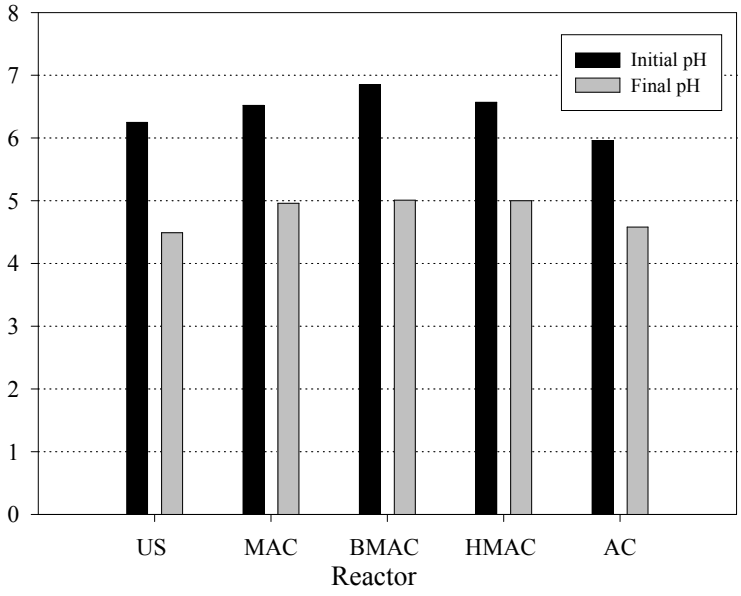
Maximum bio-hydrogen production yield (87.7 mL H_2 /g COD or 616 ml H_2 per liter of wastewater) was comparable with the related literature. Batch studies using dairy cow waste slurry in a temperature range from 37 to 85°C without addition of any external seed, have shown yields reaching 392 ml H_2 per liter of slurry (Yokoyama et al., 2007).

Mixed Anaerobic Culture

In MAC, relatively higher amount of bio-hydrogen per g COD was produced than in BMAC, AC, HMAC (Table 4.2). However, lower bio-hydrogen production yield was observed in MAC (48.9 mL H_2 /g COD) when compared to US (87.7 mL H_2 /g COD). Thus, it can be seen from this result, at 4.5 g/L COD, indigenous microorganisms in sugar-beet processing wastewater more efficient than microorganisms in mixed anaerobic cultures.

In MAC, which contained mixed anaerobic culture and sugar-beet processing wastewater, no method was applied to enrich hydrogen-producing microorganisms. On this account, higher diversity of microorganisms could be associated with the higher bio-hydrogen production compared to other culture types (Zhu and Beland, 2006). Furthermore, in MAC, low methane production (2.6 mL) was observed [Figure 4.3 (b)]. This means that, there were some factors apart from pretreatment that repressed the activity of methanogens and enable suitable environment for hydrogen-producing microorganisms. Fig. 4.4 illustrates the initial and final pH value of MAC. A considerable decrease in the pH value was observed at the end of the experiment, probably as a result of carboxylic acids formation (Ray et al., 2008). In addition, in order to enable acidic conditions external alkalinity was not

added to the medium composition. Therefore, it is speculated that the inherent and uncontrolled pH drop are the main factors, responsible for repression of methanogenic activity in MAC. This result is expected because optimum pH range for methanogens is 6.8-7.2 (Gerardi, 2003). On the contrary, inherent pH drop enable suitable environment for hydrogen-producing microorganisms since pH range of 5.0-6.0 is optimum for acidogens (Ginkel et al., 2001).



US → 4.5 g/L COD wastewater
MAC → 4.5 g/L COD wastewater+mixed anaerobic culture
HMAC → 4.5 g/L COD wastewater+heat treated mixed anaerobic culture
BMAC → 4.5 g/L COD wastewater+BES added mixed anaerobic culture
AC → 4.5 g/L COD wastewater+acidogenic culture

Figure 4.4 Initial and final pH values of reactors

2-Bromoethanesulfonate Added Mixed Anaerobic Culture

Co-enzyme-M is required for methyl-transfer reactions in the metabolism of methanogens (Balch and Wolfe, 1979). BES, which is a structural analog of the co-enzyme-M, was used to prevent methanogenic activity in the reactor because only methanogens included this enzyme; other microorganisms do not include this co-enzyme (Zhu and Béland, 2006). BES has been widely used to prevent methanogenic activity in the reactor (Wang et al., 2003; Zhu and Béland, 2006).

As depicted in Fig. 4.3 (b), the addition of 10 mM of BES did not lead to a complete cessation of the methane production. However, minimum methanogenic activity (0.3 mL) was observed in BMAC when compared to MAC, HMAC and AC [Fig. 4.3 (b)]. Low methanogenic activity suggested that 10 mM BES addition was sufficient to repress methanogenic activity significantly. BES addition (at BMAC) reduced the methane production 90% by volume compared to MAC [Figure 4.3 (b)]. Although BES addition would successfully suppress the methanogenic activity, the activity of hydrogen producers was also inhibited. Bio-hydrogen production yield in BMAC (40.5 mL H_2 /g COD) was not enhanced, it was less when compared to in MAC (48.9 mL H_2 /g COD) (Table 4.2). Apart from methanogens other hydrogen consuming microorganisms such as sulfate reducing bacteria and homoacetogens could be the reason of low bio-hydrogen production yield. BES might not be able to prevent bio-hydrogen consuming pathways taken by these bacteria.

The results of Set-up 1 were consistent with the results from Wang et al. (2003). It indicated that adding 1 M of BES on wastewater sludge did not promote the production of bio-hydrogen on the contrary reduced the bio-hydrogen yield and reduced the methane production 50% compared to the original sludge. In Set-up 1, methanogenic activity was inhibited (90%) and bio-hydrogen production was suppressed with the addition of BES.

Heat Treated Mixed Anaerobic Culture

Heat treatment of sludge inactivates or eliminates non-spore-forming bacteria such as methanogens and prevents conversion of hydrogen into methane (Sung et al., 2002). In HMAC, heat treated mixed anaerobic culture was used to repress the non-spore forming methanogenic bacteria and to dominate spore forming hydrogen producing bacteria since hydrogen producing bacteria, mostly *Clostridium*, can form protective spores under extremely strict living environment such as high temperature.

The results indicated that the activity of methanogens was decreased with heat treatment. The lower methanogenic activity was observed in HMAC as compared to in MAC by taking into account the results indicated in Fig. 4.3 (b). In addition, lower bio-hydrogen production yield was recorded (20.3 mL H_2 /g COD) when compared with the other methods (BMAC and AC) which was applied to enrich hydrogen-producing microorganisms (Table 4.2). In addition, bio-hydrogen production yield in HMAC (20.3 mL H_2 /g COD) was also lower than in MAC (48.9 mL H_2 /g COD). This result is consistent with Zhu and Béland (2006). The study which was conducted by Zhu and Béland (2006) indicated that cumulative bio-hydrogen production of the seed sludge pretreated by heat-shock was lower than that of the control test with untreated sludge. In summary, heat treated sludge did not significantly curtailed methanogenic activity but considerably suppressed the bio-hydrogen production [Fig. 4.3 (a), (b)].

Factors have to be considered to explain significant decrease in bio-hydrogen production yield in HMAC (20.3 mL H_2 /g COD) when compered to bio-hydrogen production yield in MAC (48.9 mL H_2 /g COD). Kim et al. (2006) indicated that activities of spore-forming acetogens resulted in low hydrogen production yield. Activities of the spore-forming acetogens could be the reason of low hydrogen productivity in HMAC because spore-forming acetogens like *Clostridium aceticum*

produce acetic acid from hydrogen and carbon dioxide (Brock and Madigan 1988). *Clostridium* and *Entrobacter* are most known bio-hydrogen producers. *Clostridium* species are spore-forming bacteria and they can form protective spores when they are in a restrictive environment such as high temperature, extreme acidity and alkalinity (Zhu and Béland, 2006). However, *Entrobacter* species are non-spore forming facultative anaerobes (Hussy et al., 2005). Possible inhibition of non-spore forming facultative *Enterobacters* by heat treatment could be another reason of low bio-hydrogen production yield (Zhu and Béland, 2006).

Acidogenic Culture

Bio-hydrogen production activity of previously prepared acidifying culture was investigated by AC. Fig. 4.3 (b) indicates that, culture enrichment did not result in notable inhibition on methanogenic activity when compared with the MAC. Furthermore, acidogenic activity was repressed and low bio-hydrogen production yield (29.5 mL H_2 /g COD) was observed (Table 4.2).

As seen in Fig. 4.4, pHs of reactors were around 6.0–7.0 and these pH values reduced to 4.5- 5.0 with the production of VFAs at the end of the experiment. The final pHs of reactors in Set-up 1 were consistent with Zhang et al. (2005) who reported that the final pH in the batch tests were about 4.6. Initial pH of AC (5.96) was slightly lower when compared with the other reactors. On these grounds, it can be stated that pH is not a factor in observing low bio-hydrogen yield. On the other hand, bio-hydrogen yield could be decreased by acetogens. Activities of acetogenic bacteria could be the reason for low bio-hydrogen production yield in AC as acetogens produce acetic acid from hydrogen and carbon dioxide (Brock and Madigan, 1988).

4.2. Set-up 2: Comparison of Beet-Pulp and Sugar-Beet Processing Wastewater in terms of Bio-Hydrogen Generation Potentials at 4.5 g/L COD

This set-up was used to compare bio-hydrogen production generation potentials of beet-pulp and sugar-beet processing wastewater at an initial COD level of 4.5 g/L. In set-up 1, it was observed that methanogenic activity inhibition methods (BES addition, heat treatment, acidogenic culture enrichment) did not enhance bio-hydrogen production; in MAC, which contained mixed anaerobic culture and wastewater, more bio-hydrogen production was observed than in BMAC, HMAC and AC. For this reason, in Set-up 2, two types of reactors were used; reactor which contained only beet-pulp without any external seed addition (P1) and reactor which contained mixed anaerobic culture and beet-pulp (P2).

Although sugar-beet processing wastewater produced bio-hydrogen at a value of 4.5 g/L COD (Set-up 1), beet-pulp did not produce bio-hydrogen at this COD value (Table 4.3). The reason of this was a low sCOD concentration in reactors because of the low solubility of beet-pulp. Thus, it can be stated that sugar-beet processing wastewater is more effective than beet-pulp in terms of bio-hydrogen production at a value of 4.5 g/L COD.

Table 4.3 Total gas productions, final pHs, final tVFAs in Set-up 2

	P1*	P2*
Total Gas (mL)		
H_2	0	0
CO_2	0.1±0.06	15.8±0.43
CH_4	0	8.6±0.28
Final pH	5.4±0.00	5.9±0.01
Final tVFA (mg/L as Hac)	519±259	734±243

* Mean ± standard deviation

In Set-up 1, maximum bio-hydrogen production yield (87.7 mL H_2 /g COD) was observed in US which contained sugar-beet processing wastewater without adding any external seed. In this set-up, in P1 which contained only beet-pulp without adding any external seed, small amount of CO_2 gas was observed; bio-hydrogen production was not observed (Table 4.3). Similarly, bio-hydrogen production was not observed; CO_2 and CH_4 gases were observed in P2 (Table 4.3). In addition, P2 produced more VFA and CO_2 gas compared to P1. Therefore, it can be said that more acidification was observed in P2 compared the P1. This data reveals that indigenous microorganism in sugar-beet processing wastewater not found in beet-pulp or not effective as much as in wastewater.

Substrate addition naturally increased the amount of acidification products (VFAs) which lead to natural reduction of the pH. Generally, initial pHs in bio-hydrogen producing acidification reactors drop around 4.6 due to effective acidification (Zhang et al., 2005; Li et al., 2008). However, in this set-up 4.5 g/L COD was not high enough to produce sufficient VFA to decrease the pH this level (Table 4.3). In addition, biogical activities of methanogens in P2 resulted in higher pH value in this

reactor when compared to P1 since methanogens consume the volatile acids and increase the pH of the reactor.

Total VFA productions of reactors are given in Table 4.3. In this set-up, two P1 reactor and two P2 reactor, totally 4 reactor was run. Although two of the P1 reactors contained same amount of beet-pulp and mixed anaerobic culture, different total VFA concentrations were observed (Table 4.3). The same situation was also observed in P2 reactors. Since it is a biological process, 33-50% variations in total VFA concentration are thought to be acceptable.

Results of the VFAs analyses indicated that H-Ac, H-Pr and H-Bu were the main VFA constituents found in all reactors and these comprising 57.2–68.9; 18.8–20.2; 3.4–7.5% (w/w) of tVFA, respectively. According to Yu and Fang (2002), substrate characteristics play a major role on product distribution. These VFAs are generally found in carbohydrate fermentation (such as beet-pulp).

4.3. Set-up 3: Comparison of Beet-Pulp and Co-Digestion of Sugar-Beet Processing Wastes in terms of Bio-Hydrogen Generation Potentials at High COD Values

Set-up 3 was designed to investigate the bio-hydrogen productivities of only beet-pulp and beet-pulp and sugar-beet processing wastewater together at high COD values (20, 25, 30 g/L COD) when compared to Set-up 1 and 2. To this purpose, six different types of reactors were run (Table 3.5).

In Set-up 1, maximum bio-hydrogen production yield was observed in reactor which contained 4.5 g/L COD sugar-beet processing wastewater without any external seed addition. However, in Set-up 2, bio-hydrogen production was not observed in reactor which contained 4.5 g/L COD beet-pulp without any external seed addition (P1). In addition, more acidification was observed in reactor which

contained mixed anaerobic culture and beet-pulp (P2) when compared to P1. Based on these results, it can be said that indigenous microorganism in sugar-beet processing wastewater not found in beet-pulp or not effective as much as in wastewater. Thus, the mixed anaerobic culture was used as seed in Set-up 3.

Effects of COD Values on Bio-Hydrogen Production Yields

The results of Set-up 3 indicate inverse relationship between substrate concentration and bio-hydrogen production yields (Table 4.4). Increasing COD concentrations from 20 to 30 g COD/L resulted in decrease in bio-hydrogen yields. This might attribute to substrate concentration inhibition. Substrate concentration has a significant effect on bio-hydrogen production. When a certain substrate threshold is exceeded, high substrate concentration would result in the accumulation of VFA and a fall in pH, which would inhibit bio-hydrogen producers (Pakarinen et al., 2008). According to results of set-up 3, higher substrate concentration (25 and 30 g COD/L) curtailed bio-hydrogen production yield (Table 4.4). These results were consistent with Ginkel and Logan (2005) which reported that biological hydrogen production increased with reduced organic loading. Increased substrate concentration, naturally increased the amount of acidification products (VFAs) (Table 4.4) which lead to a decrease in pH. Thus, the reduction in pH curtailed bio-hydrogen production in reactors which contained 25 and 30 g COD/L substrate (R2, R3, R5, R6) when compared the reactors which contained 20 g COD/L substrate (R1, R4) (Table 4.4). The optimum substrate concentration for bio-hydrogen production is dependent on several parameters such as the substrate used, type of reactor and HRT. In Set-up 3, the highest bio-hydrogen production yields (89.5 and 95.6 mL/g COD) were observed in reactors which contained 20 g/L COD substrate. Therefore, results indicated that, 20 g COD/L was the optimum substrate concentration for fermentative bio-hydrogen production from only beet-pulp and sugar-beet processing wastewater and beet-pulp in the range investigated. This is agreement with Lin et al. (2008) which tested different substrate

concentrations (5-60 g COD/L) to observe the effects on bio-hydrogen production from starch. Similary, when starch concentrations were higher than 20 g COD/L, bio-hydrogen production yields decreased.

Bio-hydrogen production efficiency of only beet-pulp were high when compared to co-digestion of beet-pulp and sugar-beet processing wastewater (Table 4.4). In Set-up 3, maximum bio-hydrogen production yield was calculated as 95.6 mL /g COD in R4 which contained 20 g/L COD of only beet-pulp. Maximum bio-hydrogen production yield in Set-up 3 (95.6 mL H_2/g COD) is higher than 89.2 mL H_2/g COD from sucrose (Sung et al., 2002) and 71.3 mL H_2/g COD from cassava starch manufacturing wastewater (Reungsang et al., 2004).

Table 4.4 Bio-hydrogen production yields in Set-up 3

		H_2 yield (mL/g COD)
R1	20 g COD /L (ww+pulp)	89.5
R2	25 g COD /L (ww+pulp)	76.3
R3	30g COD /L (ww+pulp)	57.5±8.1*
R4	20 g COD /L (pulp)	95.6
R5	25 g COD /L (pulp)	81.8±3.5*
R6	30 g COD /L (pulp)	54.2±3.9*

* Mean ± standard deviation

Volatile Fatty Acid Productions

Initial and final tVFAs of the reactors were illustrated in Table 4.5. The VFA production in the reactors increased with the increased COD concentrations (from 20 to 30 g COD/L). In addition, higher tVFA concentrations were observed in

reactors which contained beet-pulp and sugar-beet processing wastewater together (R1, R2, R3) when compared to reactors which contained only beet-pulp (R4, R5, R6). Sugar-beet processing wastewater is highly biodegradable with its soluble carbohydrates (Shore et al., 1984). However, ligno-cellulosic composition of beet-pulp causes a slight difficulty for degradation when compared to wastewater. Thus, high rate acidification became in reactors which contained sugar-beet processing wastewater and beet-pulp together (R1, R2, R3) (38.7–42.0 mL/day) when compared with the reactors which contained only beet-pulp (19.9–28.6 mL/day). High rate acidification in R1, R2 and R3 resulted in a sharp drop of reactor pH and subsequent inhibition of bacterial hydrogen production. Therefore, higher tVFA concentrations were observed in R1, R2 and R3 as the metabolic pathway might be shifted to VFA production instead of bio-hydrogen production.

Table 4.5 Initial and final tVFAs in Set-up 3

		mg/L as H-Ac	
		tVFA initial	tVFA final
R1	20 g COD /L (ww+pulp)	388	2175
R2	25 g COD /L (ww+pulp)	395	2329
R3	30g COD /L (ww+pulp)	409	3271±252*
R4	20 g COD /L (pulp)	76	1490
R5	25 g COD /L (pulp)	107	1770±142*
R6	30 g COD /L (pulp)	105	2177±307*

* Mean ± standard deviation

Acidification degree gives information about the production of VFAs due to anaerobic acidification of substrate. Acidification degrees of the reactors were calculated and depicted in Figure 4.5 to express the acidification efficiency.

Dinopoulou et al. (1988) calculated acidification degree as the proportion of the substrate which is converted to VFAs. So, in this set-up, acidification degrees were calculated as ratio of COD-equivalent of acidogenic products (VFAs) and consumed COD concentrations for each of the reactors (Equation 4.1). The COD equivalents of each VFA: Acetic acid, 1.066; Propionic acid, 1.512; Butyric acid, 1.816; Valeric, 2.036; Caproic acid, 2.204 (Yilmaz and Demirer, 2008).

$$\text{Degree of acidification (\%)} = \frac{S_f}{S_i} \times 100 \quad \text{..... (4.1)}$$

Where; S_i : Consumed substrate concentration, measured in COD (mg/L),

S_f : Produced VFAs, expressed as theoretical equivalents of COD concentrations (mg/L).

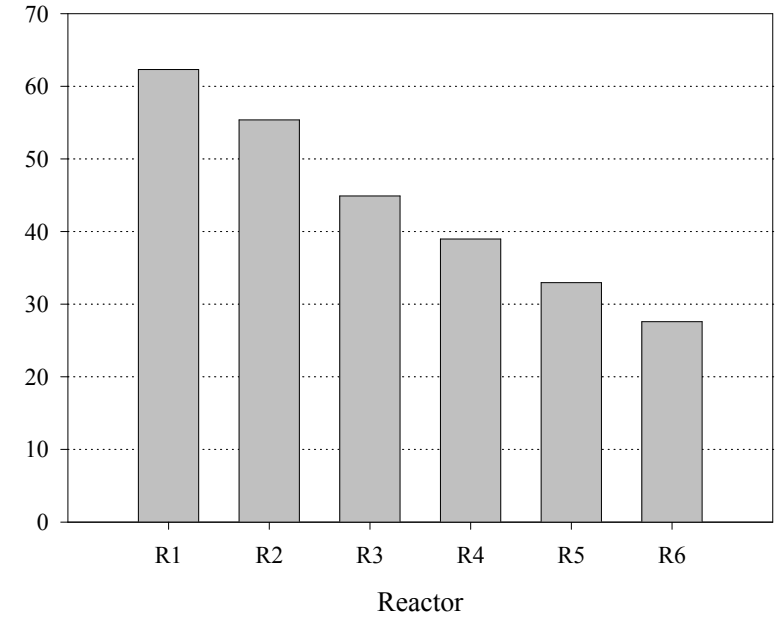


Figure 4.5 Acidification degrees in the reactors

Higher acidification degrees were observed in reactors which contained beet-pulp and sugar-beet processing wastewater together (R1, R2, R3) when compared to reactors which contained only beet-pulp (Figure 4.5). This is probably due to the shifting of the metabolic pathway to VFA. Moreover, increase in the substrate concentration decreased the acidification degree (Figure 4.5) which can be explained by stress on acidogenic bacteria with extra organic load (Oktem et al., 2006). Acidification degree of R1 (62.3%) was higher than that of R2 (55.4%) and R3 (44.9%). Similarly, R4 had higher acidification degree (39.0%) than R5 (33.0%) and R6 (27.6%). These results were consistent with Dinopoulou et al. (1988) which stated that the degree of acidification decrease with the increase in the influent

substrate concentration. Acidification degrees estimated in this set-up (27.6–62.3%) were comparable with values stated in the literature; 60% for complex wastewater (Dinopoulou et al., 1988), 29.7–44.5% for solid waste (Raynal et al., 1998), 61% for wastewater (Fang and Yu, 2001), 40.3% for fruit and vegetable wastes (Bouallagui et al., 2004).

During an anaerobic digestion process, the formation of bio-hydrogen is usually accompanied by formation of soluble metabolites (such as VFAs) which reflects the metabolism of hydrogen-producing cultures. The distribution of metabolites formed during bio-hydrogen production is often a crucial signal in assessing the efficiency of hydrogen-producing cultures (Dinopoulou et al., 1988). In all of the reactors, main acidification products were H-Ac, H-Pr, H-Bu and these comprising 51.4–69.0; 4.0–13.9; 16.4–30.8% (w/w) of tVFA, respectively. The higher molecular weight VFAs (valeric, caproic etc.) and ethanol (8.2–60.4 mg/L) were produced with insignificant amounts. According to the literature, substrate characteristics are important parameter on product distribution in an acidification reactor (Dinopoulou et al., 1988). The major products in bio-hydrogen production by anaerobic dark fermentation of carbohydrates are acetic, butyric and propionic acids (Kaptan and Kargı, 2006). In this set-up, the dominance of H-Ac, H-Pr and H-Bu can be associated with the carbohydrate fermentation as both sugar-beet processing wastewater and beet-pulp contain sugars (Shore et al., 1984; Hutnan et al., 2000). In addition, high bio-hydrogen yields are associated with a mixture of acetate and butyrate as fermentation products, and low bio-hydrogen yields are associated with reduced resulting products (such as alcohols) (Zhang et al., 2005). To maximize the yield of bio-hydrogen, the metabolism of the bacterium must be directed away from alcohols (such as ethanol) towards VFAs. Thus, the dominance of H-Ac, H-Pr and H-Bu can also be associated with high bio-hydrogen production yields (54.2–95.6 mL/g COD) in Set-up 3.

pH Values

Most of the studies indicated that initial pH value 6.0 are the best in anaerobic bio-hydrogen production (Mohan et al., 2007; Lee et al., 2008). Thus, initial pHs of all the reactors were adjusted to 6.0 in Set-up 3. Fan et al. (2006) stated that, acetate and butyrate producers are assumed to overcome propionate producers at the optimal pH, thereby increasing the bio-hydrogen yield. So, high bio-hydrogen production yields (54.2–95.6 mL/g COD) and distributions of acidic metabolites of the reactors in this set-up supported that the initial pH value 6.0 was suitable for fermentative bio-hydrogen production from sugar industry wastes.

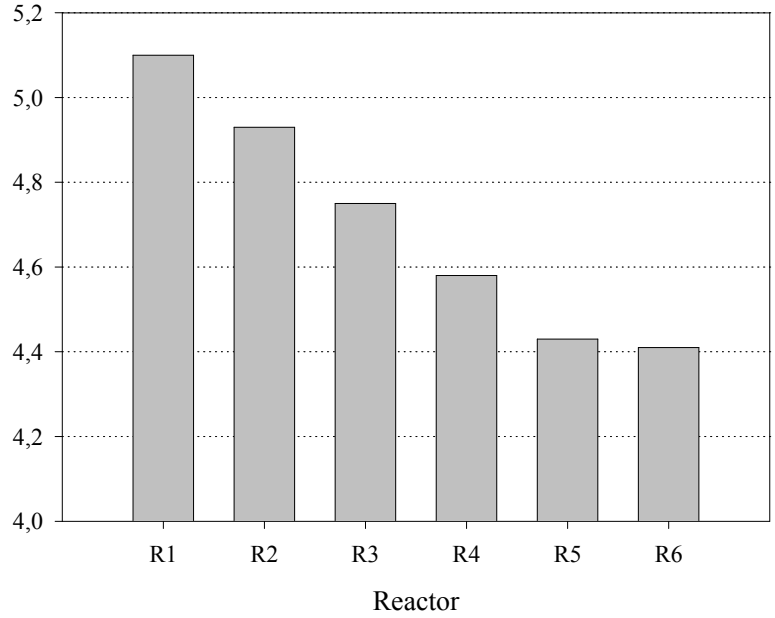


Figure 4.6 Final pHs of reactors in Set-up 3

The final pHs of the reactors were illustrated in Figure 4.6. It indicated that, pH decreased at all reactors due to fermentation. It indicated that, the final pH in all tests were lower than the initial pH 6.0 due to fermentation. The decrease in pH during incubation is due to production of organic acids which depletes the buffering capacity of the medium resulting in low final pH (Khanal et al., 2004). In all reactors, the final pH ranged from 4.41 to 5.10. This observation illustrates that the microbial activity in all the bottles were typically of acidogenic nature. It can be supported by the high bio-hydrogen yields in reactors (54.2–95.6 mg/L COD).

Based on the results of this set-up, increased substrate concentration (20 to 30 g/L COD) naturally enhanced the amount of acidification products (VFAs) (Table 4.5) which led to natural reduction of the pH. Final pH of R1 (5.10) was higher than R2 (4.93) and R3 (4.75). Similarly, final pH of R4 (4.58) was higher than R5 (4.43) and R6 (4.41).

Soluble and Consumed COD Concentrations

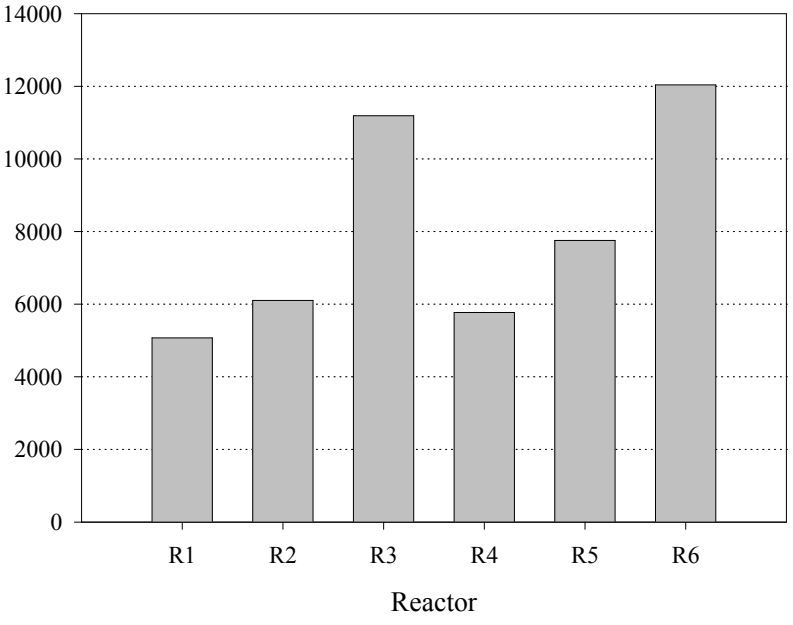
sCOD concentrations of the reactors were given in Table 4.6. It is clear from Table 4.6 that, substrate addition increased sCOD concentration. Higher sCOD concentrations were observed for the reactors with higher initial substrate concentrations. It can be seen from Table 4.5 and 4.6 that, the rise in VFA concentrations was compatible with the increase in sCOD concentrations. In other words, tVFA concentrations in the reactors rose as the substrate concentration increased and this situation resulted in increases in sCOD concentrations for all reactors. Since, the hydrolysis of particulate organic matter occurs simultaneously during the acidification of soluble organics. Moreover, sCOD concentrations were higher (6765–8165 mg/L) in the reactors which contained beet-pulp and sugar-beet processing wastewater together (R1, R2, R3) since solubility of wastewater was higher than beet-pulp. Thus, highest sCOD concentration (8165 mg/L) was observed in R3.

Table 4.6 Initial and final sCOD concentrations of the reactors in Set-up 3

		Initial (mg/L)	Final (mg/L)
		sCOD	sCOD
R1	20 g COD /L (ww+pulp)	3668	6765
R2	25 g COD /L (ww+pulp)	3861	7698
R3	30 g COD /L (ww+pulp)	3957	8165±0 *
R4	20 g COD /L (pulp)	1958	3966
R5	25 g COD /L (pulp)	2112	6065±990 *
R6	30 g COD /L (pulp)	2496	7349±1154 *

* Mean ± standard deviation

cCOD (Consumed COD) concentrations of the reactors were depicted in Figure 4.7. Bio-hydrogen production in R1, R2 and R3 ceased after 2–3 days of incubation while in R4, R5 and R6 ceased after 4–5 days, because of faster pH drop (Fig. 4.7). Thus, for the same COD value, cCOD concentrations of the reactors which contained only beet-pulp were higher than that of reactors which include sugar-beet processing wastewater and beet-pulp together. cCOD concentration of R4 was higher than R1 at an initial COD level of 20 g/L. Similarly, cCOD concentration of R5 was higher than R2 and R6 was higher than R2 at an initial COD level of 25 g/L and 30 g/L, respectively. In addition, there is a direct relationship between tCOD and cCOD concentrations. Increasing tCOD concentrations from 20 to 30 g COD/L resulted in increase in cCOD concentrations. cCOD concentration of R3 was higher than R1 and R2. Similarly, cCOD concentration of R6 (4.58) was higher than R4 and R5 (Figure 4.7).

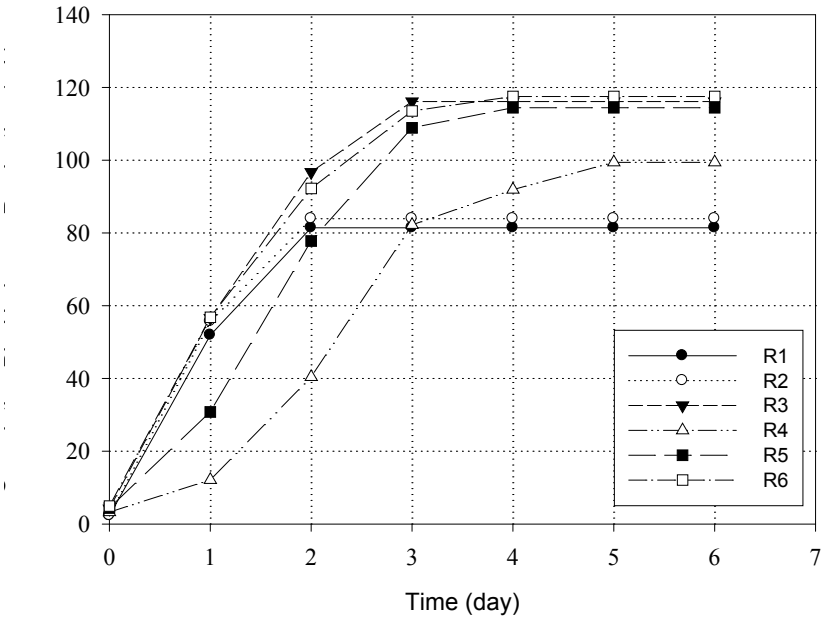


R1→ 20 g/L COD (ww+beet-pulp)
R2→ 25 g/L COD (ww+beet-pulp)
R3→ 30 g/L COD (ww+beet-pulp)
R4→ 20 g/L COD (beet-pulp)
R5→ 25 g/L COD (beet-pulp)
R6→ 30 g/L COD (beet-pulp)

Figure 4.7 cCOD concentrations of the reactors in Set-up 3

Bio-Hydrogen and Methane Productions

The operation of reactors was ceased when bio-hydrogen production stop. Cumulative bio-hydrogen productions of reactors were illustrated in Figure 4.8. Bio-hydrogen production in reactors which contained sugar-beet processing wastewater and beet-pulp together (R1, R2 and R3) ceased after 2–3 days of incubation. On the other hand, in reactors which contained only beet-pulp (R4, R5 and R6), bio-hydrogen production continued for 4–5 days (Fig. 4.8).



R1→ 20 g/L COD (ww+beet-pulp)
R2→ 25 g/L COD (ww+beet-pulp)
R3→ 30 g/L COD (ww+beet-pulp)
R4→ 20 g/L COD (beet-pulp)
R5→ 25 g/L COD (beet-pulp)
R6→ 30 g/L COD (beet-pulp)

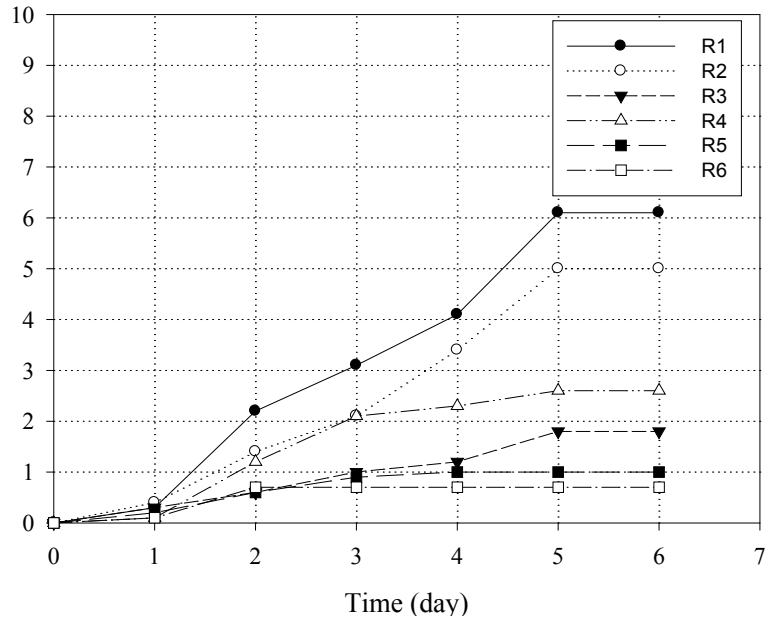
Figure 4.8 Cumulative bio-hydrogen productions of reactors in Set-up 3

The formation of organic acids as metabolic products causes a drop in pH (Figure 4.6). Gradual decreases in pH inhibit bacterial bio-hydrogen production since pH affects the activity of hydrogenase enzyme (Darock et al., 1992). Sugar-beet processing wastewater is more biodegradable than beet-pulp. For this reason, high rate acidification became in reactors which contained wastewater and beet-pulp together (38.7–42.0 mL/day) when compared with the reactors which contained only beet-pulp (19.9–28.6 mL/day). High rate acidification in R1, R2 and R3 caused an abrupt decrease of reactor pH because of this acid/pH inhibition; bio-hydrogen production was inhibited and stopped in 2–3 days. In R4, R5 and R6, pH

decreased slowly because of low biodegradability of waste so bio-hydrogen production continued for 4–5 days.

The direct relationship between substrate addition and bio-hydrogen production can be observed in Figure 4.8. Higher bio-hydrogen productions were observed in the reactors with higher substrate concentrations. In addition, for the same substrate concentration, in reactors containing only beet-pulp (R4, R5 and R6) more bio-hydrogen production was observed when compared to reactors containing beet-pulp and wastewater (R1, R2, R3) (Figure 4.8). High rate acidification in R1, R2 and R3 resulted in lower COD consumptions and lower bio-hydrogen productions, compared to R4, R5 and R6. Thus, highest bio-hydrogen production was calculated as 117.5 mL in R6.

Cumulative methane productions of reactors were depicted in Figure 4.9. Small amount of methane gas (0.7–6.1 mL) was observed at all reactors. In this set-up, pHs of the reactors were set to 6.0 to suppress methanogenic activitiy as methanogens operate optimum at a range of 6.5 to 8.2 while acidogens prefer between 4 and 6.5 (Speece, 1996). High bio-hydrogen productions (81.4–117.5 mL) and low methanogenic activity (0.7–6.1 mL) revealed that pH is the important parameter for bio-hydrogen production. In addition, Figure 4.9 indicates the inverse relationship between substrate concentrations and methane productions. It can be observed that, increased substrate concentrations caused decreases in methane productions because of acid/pH inhibition.



R1→ 20 g/L COD (ww+beet-pulp)
R2→ 25 g/L COD (ww+beet-pulp)
R3→ 30 g/L COD (ww+beet-pulp)
R4→ 20 g/L COD (beet-pulp)
R5→ 25 g/L COD (beet-pulp)
R6→ 30 g/L COD (beet-pulp)

Figure 4.9 Cumulative methane productions of reactors in Set-up 3

4.4. Set-up 4: Effects of Pretreatment Methods on Solubilization of Beet-Pulp and Bio-Hydrogen Production Yields

Set-up 4 mainly consists of two parts: Effects of pretreatment methods on solubilization of beet-pulp and effects of pretreatment methods on bio-hydrogen production yields. In the first part of set-up 4, beet-pulp was pretreated by five different pretreatment methods (alkaline pretreatment, thermal pretreatment, microwave pretreatment, thermal-alkaline pretreatment, microwave-alkaline pretreatment). These methods were used to observe whether the solubilization of beet-pulp could be improved or not. In addition, control reactor was also used to

determine initial sCOD, VFA of beet-pulp. In the second part of the set-up 4, three (alkaline, microwave-alkaline and thermal-alkaline pretreatments) out of five pretreatment methods were used to compare effects of pretreatments on bio-hydrogen productivity. In set-up 3, the highest bio-hydrogen yield was observed at reactor which contained 20 g COD/L of beet-pulp. Thus, in the second part of the set-up 4, each reactor contained 20 g/L COD of beet-pulp.

4.4.1. Effects of Pretreatment Methods on Solubilization of Beet-Pulp

Soluble COD Concentrations

sCOD values increased from 4266 mg/L (control) to 4920 mg/L, 5990 mg/L, 6739 mg/L, 19129 mg/L and 20884 mg/L at microwave, thermal, alkaline, microwave-alkaline, thermal-alkaline pretreatment reactors, respectively. In terms of the solubilisation ratio (or percentages of soluble to total COD), the ratio increased from 11.8% (control) to 13.7% in microwave, 16.6% in thermal, 18.7% in alkaline, 53.1% in microwave-alkaline, 58.0% in thermal-alkaline pretreatment reactors (Figure 4.10). Thus, highest sCOD concentration (20884 mg/L) and solubilization ratio (58.0%) were observed in thermal-alkaline pretreatment reactor (Table 4.7 and Figure 4.10).

Table 4.7 sCOD concentrations in pretreatment reactors

	sCOD* (mg/L)
Control	4266±125
Alkaline Pretreatment	6739±587
Thermal Pretreatment	5990±471
Microwave Pretreatment	4920±81
Thermal-alkaline Pretreatment	20884±818
Microwave-alkaline Pretreatment	19129±660

* Mean ± standard deviation

Kim et al. (2003) reported that initial soluble COD (8.1%) increased to 51.8% after thermal-alkaline pretreatment (at 121°C for 30 min, pH 12) and in the first part of Set-up 4, initial soluble COD (11.8%) increased to 58.0% after thermal-alkaline pretreatment (at 121°C for 30 min, pH 12). Therefore, the result of combine effect of thermal pretreatment and alkaline pretreatment was in line with the result of Kim et al. (2003) in terms of the amount of soluble COD release (solubilization ratio).

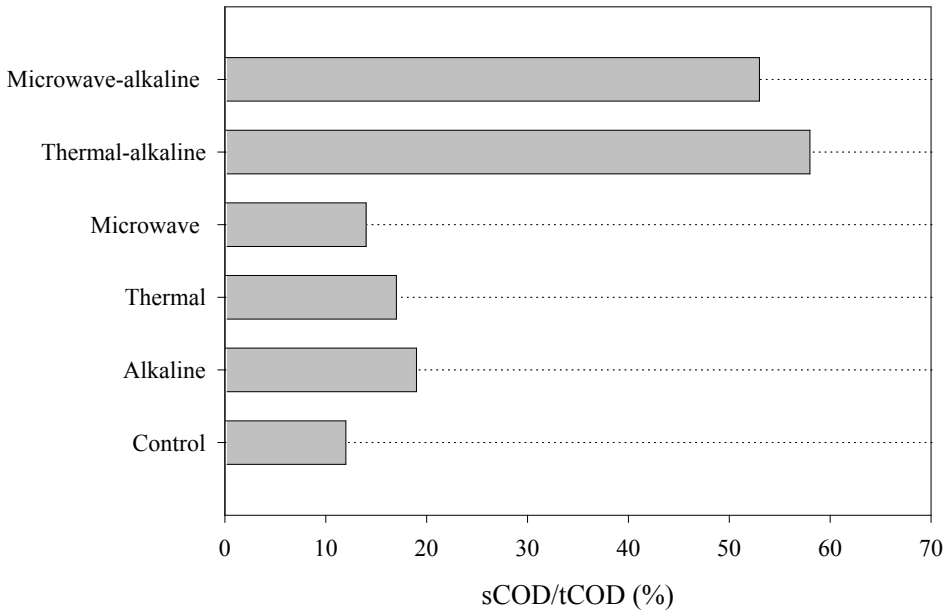


Figure 4.10 sCOD /tCOD ratios of pretreatment reactors

Synergistic effect was observed in microwave-alkaline and thermal-alkaline pretreatment reactors in terms of sCOD values. Thus, it was observed that the percent soluble COD release values of combined thermal and alkaline pretreatment were well above the releases achieved by each individual method. Similarly, the reactor at which microwave pretreatment combined with the alkaline pretreatment had higher solubilization ratio (53.1%) than individual microwave (13.7%) or

alkaline (18.7%) pretreatment. The results of this study were in agreement with literature findings such as Kim et al. (2003) reported that COD solubilization efficiencies of pretreatment methods as follow; thermal and alkaline pretreatment > alkaline pretreatment > thermal pretreatment. Similarly, Zhu et al. (2005) reported that rice straw pretreated by microwave/alkali had a higher hydrolysis rate and glucose content in the hydrolysate in comparison with the one by alkali alone.

Volatile Fatty Acid Productions

In all pretreatment reactors, tVFA concentrations (199-1153 mg/L) was higher than that of control reactor (190 mg/L). tVFA values increased from 190 mg/L (control) to 199 mg/L, 248 mg/L, 951 mg/L, 981 mg/L and 1153 mg/L at microwave, thermal, alkaline, microwave-alkaline, thermal-alkaline pretreatment reactors, respectively (Figure 4.11). In addition, the results revealed that the major acidification products were H-Ac, H-Pr and H-Bu in all of the reactors. Moreover, there is a direct relationship between tVFAs and sCOD concentrations (Table 4.7 and Figure 4.11). That means, after pretreatments organic particles in beet-pulp were liquidized to soluble carbohydrates, acids.

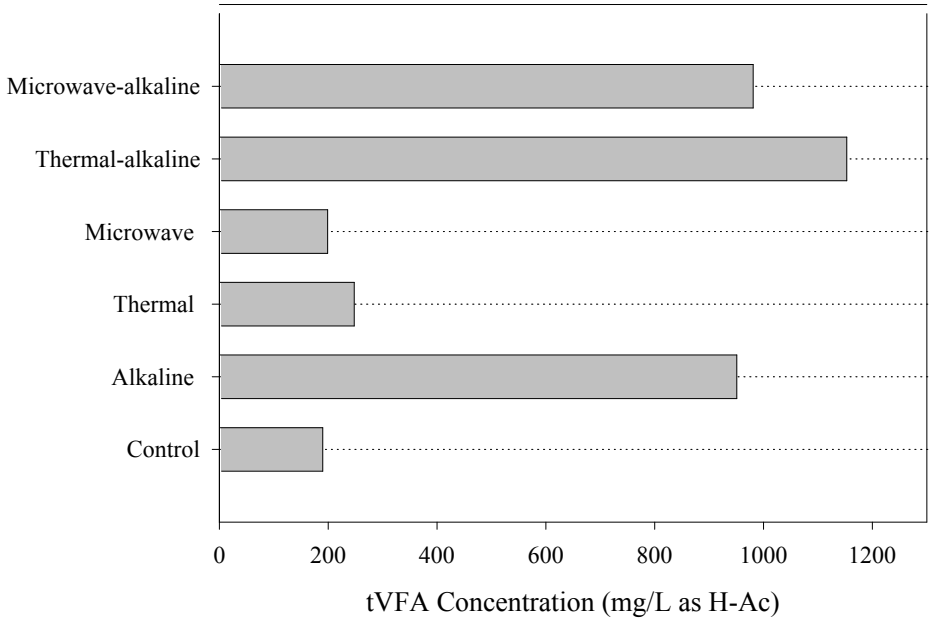


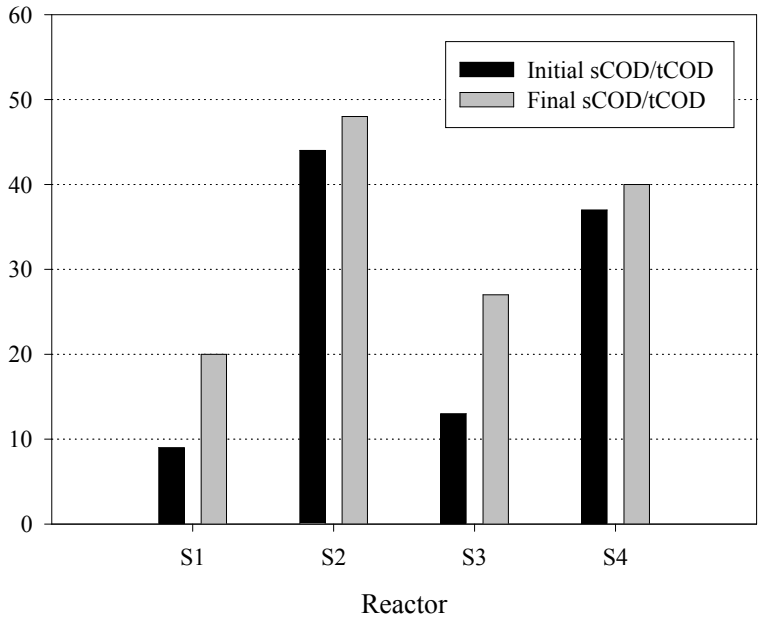
Figure 4.11 tVFA concentrations in pretreatment reactors

4.4.2. Effects of Pretreatment Methods on Bio-Hydrogen Production Yields

Solubilization Ratios

Initial and final sCOD/tCOD (solubilization ratio) values of the reactors were illustrated in Figure 4.12. In this part of Set-up 4, initial solubilization ratio was the percentages of initial soluble CODs of reactors before incubation for bio-hydrogen production to total COD and final solubilization ratio was the percentages of final soluble CODs of reactors after incubation for bio-hydrogen production to total COD.

It can be seen from Figure 4.12 that, highest initial solubilization ratios were observed in reactors which contained thermal-alkaline pretreated beet-pulp (S2: 43.6%) and in reactor which contained microwave-alkaline pretreated beet-pulp (S4: 36.9%). Lower solubilization ratio (12.6%) was achieved with alkaline pretreatment (S3) when compared to other pretreatments. Initial solubilization ratios in this part of Set-up 4 were consistent with the results which were observed in the first part of Set-up 4. In reactors which contained microwave-alkaline and thermal-alkaline pretreated beet-pulp (S2 and S4), synergistic effect was observed in terms of sCOD/tCOD values. Solubilisation ratios of these reactors is much higher than reactor which with alkaline pretreated beet-pulp (Figure 4.12). Initial sCOD/tCOD values estimated in this part of Set-up 4 (12.6–43.6%) comparable with values stated in the literature; 51.8% after thermal-alkaline pretreatment (Kim et al., 2003), 15% after microwave-thermal pretreatment (Eskicioglu et. al., 2006), 26.2% after alkaline-gamma ray irradiation (Kim et al., 2007), 40.7% after thermal-alkaline pretreatment (Carmona et al., 2007).



S1→ 20 g/L COD beet-pulp without pretreatment
S2→ 20 g/L COD thermal-alkaline pretreated beet-pulp
S3→ 20 g/L COD alkaline pretreated beet-pulp
S4→ 20 g/L COD microwave-alkaline pretreated beet-pulp

Figure 4.12 Initial and final sCOD/ tCOD ratios of the reactors in the second part of Set-up 4

During fermentation, the ratio of sCOD/tCOD increased further (Fig. 4.12). Final solubilization ratios were 19.5% in reactor which contained beet-pulp without pretreatment (S1), 47.7% in reactor which contained thermal-alkaline pretreated beet-pulp (S2), 27.0% in reactor which contained alkaline pretreated beet-pulp (S3), 40.1% in reactor which contained microwave-alkaline pretreated beet-pulp (S4). These results revealed that not only pretreatment but also fermentation was effective for solubilizing organic matters from beet-pulp.

Soluble and Consumed COD Concentrations

Initial sCOD values increased from 1711 mg/L (S1) to 8721 mg/L, 2528 mg/L, and 7382 mg/L at S2, S3 and S4, respectively (Table 4.8). Thus, COD solubilization efficiencies of pretreatment methods are as follows: thermal-alkaline pretreatment > microwave-alkaline pretreatment > alkaline pretreatment. COD solubilization efficiencies of pretreatment methods depicted that the pretreatments could destroy cellular wall, and release dissoluble matters efficiently. Maximum initial sCOD was observed in S2 (8721 mg/L) which contained thermal-alkaline pretreated beet-pulp. Initial soluble COD in S1 (8.5%) increased to 43.6% after thermal-alkaline pretreatment of beet-pulp. The result of thermal-alkaline pretreatment was in line with the result of Kim et al. (2003) which stated that initial soluble COD (8.1%) increased to 51.8% after thermal-alkaline pretreatment.

Table 4.8 Initial and final sCOD concentrations of the reactors in the second part of Set-up 4

		Initial (mg/L)	Final (mg/L)
		sCOD*	sCOD*
S1	No Pretreatment	1711±37	3900±141
S2	Thermal-alkaline Pretreatment	8721±115	9546±398
S3	Alkaline Pretreatment	2528±45	5400±28
S4	Microwave-alkaline Pretreatment	7382±86	8020±255

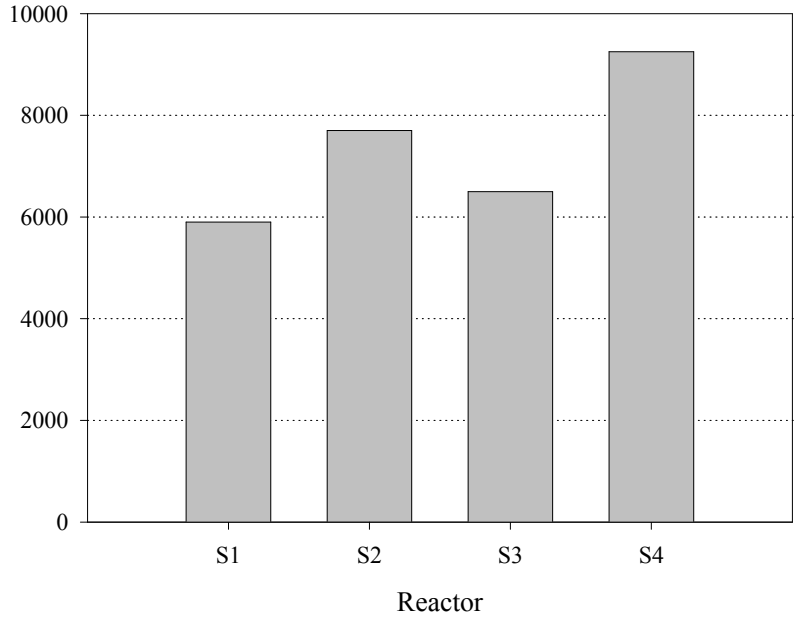
* Mean ± standard deviation

Final sCOD concentrations were 3900, 9546, 5400 and 8020 mg/L in S1, S2, S3 and S4, respectively. Thus, final sCOD concentrations of reactors were higher than the initial sCOD concentrations (Table 4.8). sCOD concentrations increased further during fermentation due to production of organic acids (VFA) (Table 4.9). These

acids are assumed to catalyze the further hydrolysis of the hemicellulose (Gregg and Saddler, 1996). These results revealed that both pretreatment and fermentation were effective for solubilizing organic matters from beet-pulp.

Highest sCODs were observed in reactors which contained thermal-alkaline and microwave-alkaline pretreated beet-pulp (S2 and S4) (Table 4.8). However, maximum bio-hydrogen production yield (115.6 mL H_2/g COD) was observed in S3. Hence, it can be said that not all organic matter released from beet-pulp is readily anaerobically fermented to bio-hydrogen. In addition, thermal-alkaline and microwave-alkaline pretreatment includes risk on solubilization of phenolic compounds besides the solubilization of hemicelluloses (Hendriks and Zeeman, 2009). Thus, toxic effects of these compounds might reduce bio-hydrogen production yields.

Consumed COD (cCOD) concentrations of the reactors were depicted in Figure 4.13. As can be seen from Figure 4.13, in reactors which contained pretreated beet-pulp (S2, S3 and S4) more COD consumptions were observed when compared to reactor which contained beet-pulp without pretreatment (S1). cCOD concentration of S1 (5900 mg/L) increased to 7700 mg/L in S2, 6500 mg/L in S3, 9250 mg/L in S4. In terms of the COD removal efficiency (percentages of consumed to total COD), the efficiency increased from 29.5% in S1 to 32.5% in reactor which contained alkaline pretreated beet-pulp, 38.5% in reactor which contained thermal-alkaline pretreated beet-pulp, 46.3% in reactor which contained microwave-alkaline pretreated beet-pulp. In other words, COD removal efficiencies of reactors varied between 29.5–46.3%.

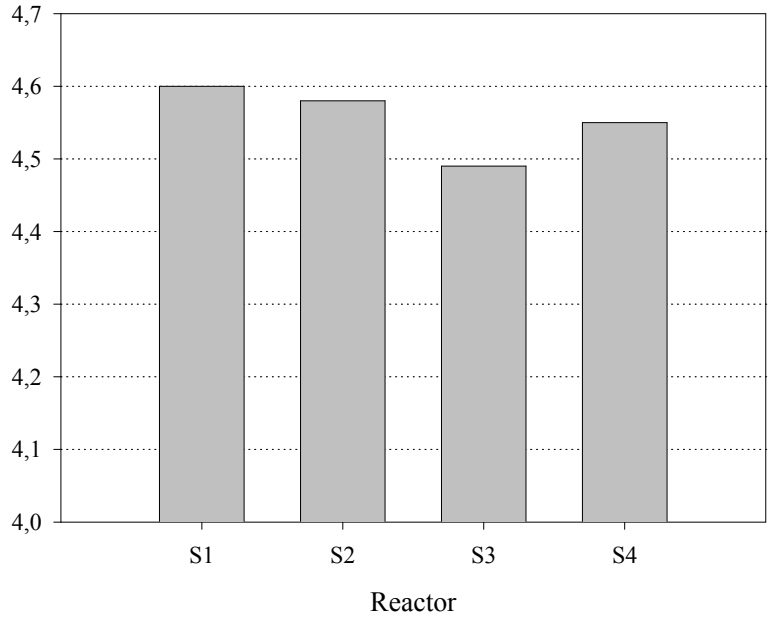


S1→ 20 g/L COD beet-pulp without pretreatment
S2→ 20 g/L COD thermal-alkaline pretreated beet-pulp
S3→ 20 g/L COD alkaline pretreated beet-pulp
S4→ 20 g/L COD microwave-alkaline pretreated beet-pulp

Figure 4.13 cCOD concentrations of the reactors in the second part of Set-up 4

pH Values

The effect of different pretreatment methods on the final pH was illustrated in Figure 4.14. It indicated that, pH decreased at all reactors due to fermentation. The final pHs in all reactors were lower than the initial pH 6.0. Organic acid productions due to fermentation which resulted in these expected pH drops. In all reactors, the final pH ranged from 4.49 to 4.60. Such low pH values may indicate successful acidification in the reactors.



S1→ 20 g/L COD beet-pulp without pretreatment
S2→ 20 g/L COD thermal-alkaline pretreated beet-pulp
S3→ 20 g/L COD alkaline pretreated beet-pulp
S4→ 20 g/L COD microwave-alkaline pretreated beet-pulp

Figure 4.14 Final pHs of reactors in the second part of Set-up 4

The final pHs in second part of Set-up 4, were consistent with Zhang et al. (2005) reported that the final pH in the batch tests were about 4.6 and Ren et al. (2006) observed that the final pHs in reactors were between 4.5–5. Lowest final pH value (4.49) was observed in reactor which contained alkaline pretreated pulp (S3) due to effective acidification. It can be supported by the highest bio-hydrogen yield (115.6 mg/L COD).

Volatile Fatty Acid Productions

Initial tVFAs of the reactors were illustrated in Table 4.9. Initial VFAs of the reactors which contained pretreated beet-pulp (S2, S3 and S4) were higher than that of reactor which include beet-pulp without pretreatment (S1) (Table 4.9). In addition, the initial VFA concentrations in the reactors increased parallel with the increased initial sCOD concentrations as organic particles in beet-pulp were liquidized to soluble carbohydrates, acids with pretreatment (Table 4.8 and 4.9).

Table 4.9 Initial and final tVFAs in the second part of Set-up 4

		mg/L as H-Ac	
		tVFA initial*	tVFA final*
S1	No Pretreatment	116±23	1393±272
S2	Thermal-alkaline Pretreatment	446±18	2455±465
S3	Alkaline Pretreatment	360±13	2721±176
S4	Microwave-alkaline Pretreatment	375±19	2398±267

* Mean ± standard deviation

The results indicated that, the soluble metabolites of initial VFAs included mainly H-Ac, H-Pr and H-Bu in all of the reactors (S1, S2, S3 and S4). H-Ac was the most abundant product, which accounted for 69.8–87.8% of tVFA for all of the reactors. In addition, the production of H-Bu and H-Pr was also significant, which accounted for 1.4–5.2% and 6.4–12.9% of tVFA, respectively. H-Ac percentage in S1 increased from 69.8% to 84.9 % in reactors which contained thermal-alkaline (S2), 87.8% in reactor which contained alkaline pretreated beet-pulp (S3) and 84.8 % in reactors which contained microwave-alkaline pretreated beet-pulp (S4). On the other hand, H-Pr percentage in S1 decreased from 12.9% to 9.2% in reactor

which contained thermal-alkaline beet-pulp (S2), 6.4% in reactor which contained alkaline pretreated beet-pulp (S3) and 8.3% in reactor which contained microwave-alkaline pretreated beet-pulp (S4). Similarly, H-Bu percentage in S1 decreased from 5.2% to 2.0% in reactors which contained thermal-alkaline (S2), 1.4% in reactor which contained alkaline pretreated beet-pulp (S3) and 1.9% in reactors which contained microwave-alkaline beet-pulp (S4). Wang and Wan (2008) stated that H-Bu and H-Pr might repress the process of fermentative bio-hydrogen production. In addition, Wang et al. (2006) stated that the rate of converting propionic acid to hydrogen is very slow, resulting in the accumulation of propionic acid that can repress the activity of hydrogen-producing bacteria and decrease the hydrogen production. Thus, the decrease in H-Pr and H-Bu percentages at all reactors which contained pretreated beet-pulp might lead to higher bio-hydrogen productions (Figure 4.15).

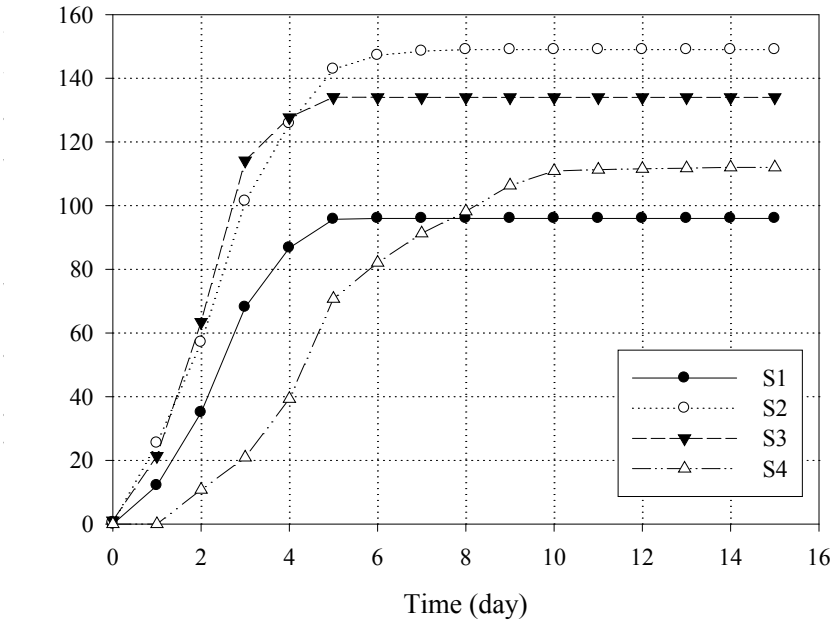
Final tVFAs of the reactors were shown in Table 4.9. Bio-hydrogen production was accompanied with the formation of VFAs throughout the fermentation of beet-pulp. These phenomena were expected because hydrogen production appears to be usually accompanied with the formation of VFAs is the main by-products in the metabolism of bio-hydrogen fermentation. Final VFAs of the reactors which contained pretreated beet-pulp (S2, S3 and S4) were higher than that of reactor which include beet-pulp without pretreatment (S1) (Table 4.8). Highest tVFA concentration (2721 mg/L) was observed in S3 which contained alkaline pretreated beet-pulp. In addition, lowest final pH value (4.49) was also observed in S3 as higher tVFA productions lead to lower pH values.

In all of the reactors, main acidification products were H-Ac (65.3–75.2% w/w of tVFA), H-Pr (4.9–11.6% w/w of tVFA) and H-Bu (9.8–19.9% w/w of tVFA) comprising 91.1–96.6% of tVFAs. The higher molecular weight VFAs (valeric, caproic etc.) were produced with insignificant amounts. Ethanol production (219.6 mg/L) was observed only in reactor which contained microwave-alkaline pretreated

beet-pulp (S4). Zhang et al. (2005) stated that high bio-hydrogen yields are associated with a mixture of acetate and butyrate as fermentation products, and low bio-hydrogen yields are associated with reduced resulting products (such as alcohols). For this part of Set-up 4, H-Ac and H-Bu accounted for 85.0–87.0% of (w/w) tVFA. Thus, the dominance of H-Ac, H-Bu can also be associated with high bio-hydrogen production yields (66.7–115.6 mL/g COD). The results of VFA production part were in line with the results of Fan et al. (2002) reported that in biohydrogen fermentation from glucose, VFAs mainly consists of acetate and butyrate and Fan et al. (2006) which stated that acetate and butyrate accounted for about 76–80% of VFAs in bio-hydrogen fermentation from wheat straw wastes.

Bio-Hydrogen and Methane Productions

The operation of reactors was ceased when bio-hydrogen production stopped. Fig. 4.15 depicted the effects of the different pretreatment methods on bio-hydrogen productions at the fixed initial pH 6.0 and substrate concentration 20 g/L COD. As can be seen from Fig. 4.15, cumulative bio-hydrogen productions in all pretreatment reactors (111.7–148.5 mL) were higher than that of reactor which contained beet-pulp without pretreatment (95.7 mL). Maximum bio-hydrogen production of 148.5 mL was observed in the reactor which contained the thermal-alkaline pretreated beet-pulp (S2). Reactors which contained alkaline pretreated beet-pulp (S3) and microwave-alkaline pretreated beet-pulp (S4) achieved cumulative bio-hydrogen production of 134.0 and 111.7 mL, respectively. Maximum bio-hydrogen production in Set-up 4 (148.5 mL or 50.3 mL/g beet-pulp) was comparable with that of Li and Chen et al. (2007). In that study, hydrogen produced from thermal pretreated corn straw was 68 ml H_2/g corn straw.



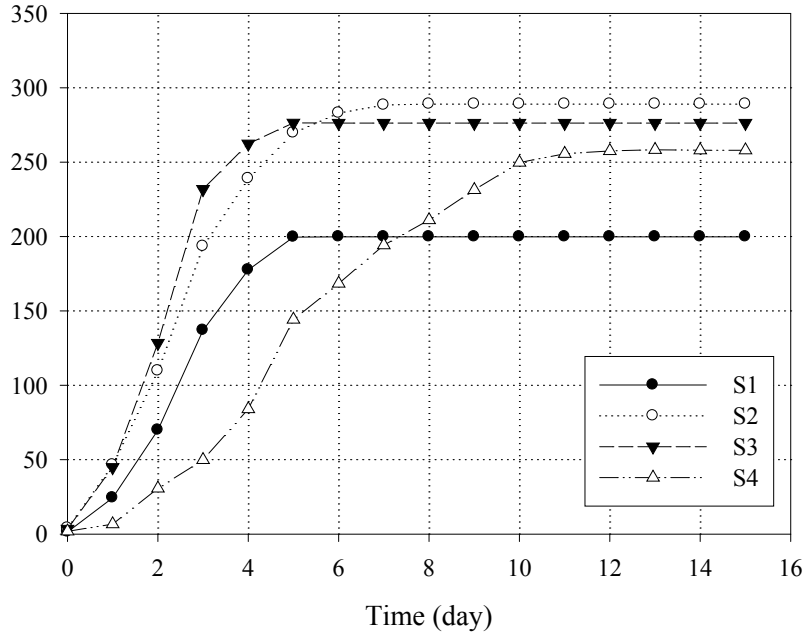
S1→ 20 g/L COD beet-pulp without pretreatment
S2→ 20 g/L COD thermal-alkaline pretreated beet-pulp
S3→ 20 g/L COD alkaline pretreated beet-pulp
S4→ 20 g/L COD microwave-alkaline pretreated beet-pulp

Figure 4.15 Cumulative bio-hydrogen productions of reactors in the second part of Set-up 4

The longest lag time (2 days) was observed in reactor which contained microwave-alkaline pretreated beet-pulp (S4) when compared the others (1 day). In addition, the longest bio-hydrogen production was also observed in S4 (Figure 4.15). Bio-hydrogen production in S4 ceased after 12 days of incubation while in reactors which contained beet-pulp without pretreatment (S1), alkaline pretreated beet-pulp (S3), thermal-alkaline pretreated beet-pulp (S4) ceased after 5–7 days of incubation. However, less bio-hydrogen production was observed in S4 which contained

microwave-alkaline pretreated beet-pulp than other reactors which contained pretreated beet-pulps (S2 and S3).

Gossett et al. (1982) stated that pretreatment with temperatures of 160 ^{0}C and higher, causes, besides the solubilization of hemicellulose, also the solubilization of lignin. The produced compounds are almost always phenolic compounds and have in many cases an inhibitory or toxic effect on bacteria, yeast and methanogens/archae. Microwave-alkaline pretreatment of beet-pulp was conducted at 170 ^{0}C for 30 min. Thus, this might be the one of the reason that less bio-hydrogen production in S4 when compared to S2 and S3. Not only microwave-alkaline pretreatment, but also thermal-alkaline pretreatment includes a risk on production of phenolic compouns. However, bacteria are cabaple of adapting to these compounds at least certain concentrations (Hendriks and Zeeman, 2009). Microwave-alkaline pretreatment was conducted at severe pretreatment conditions (at 170 ^{0}C) when compared to thermal-alkaline pretreatment (at 121 ^{0}C). Moreover, microwave-assisted extraction has been developed for the extraction of a variety of toxic organic contaminants, such as phenols and pesticides from soils, sediments and other solid matrices (Xiong et al. 1999). Nakazato et al. (2006) also indicated that heavy metals in soil like Cd, Cr, Cu, Ni, Pb, and Zn could be extracted by microwave. These toxic matters were deleterious for bio-hydrogen production bacteria. So, when the organic nutrients released, the toxic matters could be released simultaneously and they would restrain the bio-hydrogen production bacteria and this might be the other reason of less bio-hydrogen production observed in S4.

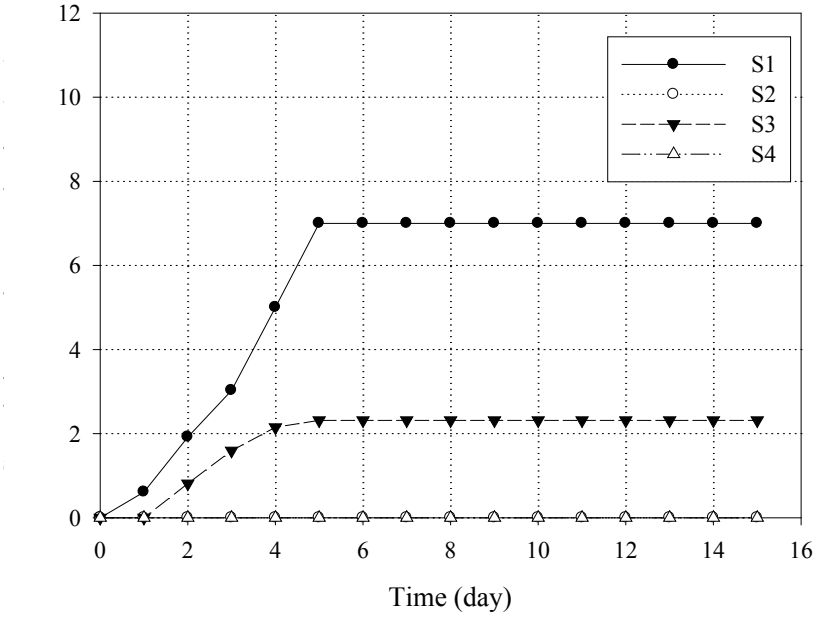


S1→ 20 g/L COD beet-pulp without pretreatment
S2→ 20 g/L COD thermal-alkaline pretreated beet-pulp
S3→ 20 g/L COD alkaline pretreated beet-pulp
S4→ 20 g/L COD microwave-alkaline pretreated beet-pulp

Figure 4.16 Cumulative total gas productions of reactors in the second part of Set-up 4

Figure 4.16 depicts the accumulated total gas productions inoculated with different pretreated beet-pulp and beet-pulp without pretreatment during fermentation period. Cumulative total gas productions in all pretreatment reactors (S2, S3 and S4) were higher (258.2–289.0 mL) when compared reactor which contained beet-pulp without pretreatment (199.8 mL). The increase in cumulative total gas production was due to the transfer of organic matter from the particulate fraction to the soluble fraction. Highest total gas production was calculated as 289.0 mL in S2 which incubated with thermal-alkaline beet-pulp. This represents an increase of 44.6% in

total gas compared with reactor which contained beet-pulp without pretreatment. Total gas composition consisted of bio-hydrogen, carbon dioxide and no or insignificant amounts of methane (0–3.5%). The bio-hydrogen and carbon dioxide percentage in the total gas was 43.4–51.6% and 48.4–56.6%, respectively. The bio-hydrogen and methane percentages were consistent with Zhang et al. (2007) who produced hydrogen from acid pretreated cornstalks and reported that the hydrogen percent in the total gas was 45–56% and there was no significant methane observed in the batch tests.



S1→ 20 g/L COD beet-pulp without pretreatment
S2→ 20 g/L COD thermal-alkaline pretreated beet-pulp
S3→ 20 g/L COD alkaline pretreated beet-pulp
S4→ 20 g/L COD microwave-alkaline pretreated beet-pulp

Figure 4.17 Cumulative methane productions of reactors in the second part of Set-up 4

Cumulative methane productions of reactors were depicted in Figure 4.17. As it can be seen from Figure 4.17, small amount of methane gas was observed in S1 and S3. Cumulative methane production was 6.8 mL in S1 which contained beet-pulp without pretreatment and 2.3 mL in S3 which contained alkaline pretreated beet-pulp. No methane was detected in reactors which contained thermal-alkaline pretreated beet-pulp (S2) and microwave- alkaline pretreated beet-pulp (S4). In this part of Set-up 4 , pHs of the reactors were also adjusted to 6.0 to suppress methanogenic activitiy. Methanogens prefer nearly neutral pH conditions with a generally accepted optimum range of 6.5 to 8.2 while acidogens prefer between 4 and 6.5 (Speece, 1996). In addition, toxic effects of phenolic compounds or heavy metals might also be the reason of the inhibition of methanogenic activity in S2 and S4. Most of the acidogenic bacteria (hydrogen producing bacteria) form protective spores when they are in a restrictive environment but methanogens have no such capability.

Bio-Hydrogen Production Yields

Bio-hydrogen production yields in the second part of Set-up 4 were illustrated in Table 4.10. The results showed that thermal-alkaline and alkaline pretreatment of the substrate improves the conversion of beet-pulp into bio-hydrogen by microorganisms. Bio-hydrogen production yields in thermal-alkaline and alkaline pretreatment reactors (S2 and S3) were higher than that of reactor which contained beet-pulp without pretreatment (Table 4.10). Bio-hydrogen production yield of S1 (contained beet-pulp without pretreatment) increased from 90.1 mL/g COD to 108.2 mL/g COD after thermal-alkaline pretreated beet-pulp and 115.6 mL/ gCOD after alkaline pretreatmented beet-pulp. On the other hand, microwave-alkaline pretreatment of beet-pulp (in S4) reduced bio-hydrogen production yield to 66.7 mL/g COD from 90.1 mL/g COD (Table 4.10) because of inhibitory or toxic effect of phenolic compounds or heavy metals. In S2 which contained thermal-alkaline pretreated beet-pulp higher bio-hydrogen production (148.5 mL) was observed than

S3 (134.0 mL) which contained alkaline pretreated beet-pulp. However, the maximum bio-hydrogen production yield (115.6 mL H_2/g COD) was observed in S3 because of the difference in COD consumptions (Figure 4.13).

Table 4.10 Bio-hydrogen production yields in the second part of Set-up 4

		H_2 yield* (mL/g COD)
S1	No Pretreatment	90.1±6.1
S2	Thermal-alkaline Pretreatment	108.2±15.8
S3	Alkaline Pretreatment	115.6±12.5
S4	Microwave-alkaline Pretreatment	66.7±10.1

* Mean ± standard deviation

The demands for higher efficiency processes, have prompted the need for pretreatment methods in order to improve substrate solubilization. The best method of pretreatment depend greatly on the type of lignocelluloses. Climent et. al. (2007) examined the effect of microwave pretreatment of activated sludge on anaerobic digestion with batch reactors; microwave pretreatment of activated sludge did not cause any improvement in biogas production. Guo et al. (2008) stated that sterilization, microwave and ultrasonication were all effective for solubilizing organic matters from waste sludge and the maximal yield of hydrogen was observed using sterilization pretreatment. Fox et al. (2003) reported an improvement in methane production with a factor of 3 to 4.5 after pretreating newsprint waste with alkaline heat pretreatment. Pavlostathis and Gossett (1985) mentioned a 100% increase in methane production after an alkaline pretreatment of wheat straw. Ghosh et al. (2000) stated that alkaline treatment of municipal solid waste improved the formation of biogas by 35%. Vrije et al. (2002) reported that

mechanical and alkaline treatment of miscanthus increased hydrogen yields by 15.6%. In this part of Set-up 4, beet-pulp was pretreated by alkaline, thermal-alkaline and microwave-alkaline pretreatments to increase substrate solubilization with the purpose of elevating bio-hydrogen production yields. Based on the results of second part of Set-up 4, it can be said that alkaline pretreatment was the best pretreatment method for the beet-pulp.

Bio-hydrogen production yield in S1 (90.1 mL /g COD) increased 20.1% by thermal-alkaline pretreatment of beet-pulp and 28.3% by alkaline pretreatment of beet-pulp. In Set-up 3, highest bio-hydrogen production yield was calculated as 95.6 mL /g COD in R4 which contained 20 g/L COD beet-pulp without pretreatment. Thus, thermal-alkaline pretreatment and alkaline pretreatment of beet-pulp increased bio-hydrogen production yields 13.2% and 20.9% when compared to highest bio-hydrogen production yield in Set-up 3 (95.6 mL /g COD). Percentages of increment in bio-hydrogen production yield (13.2- 28.3%) were comparable with the literature such as Ghosh et al. (2000) stated that alkaline treatment of municipal solid waste improved the formation of biogas by 35%. Vrije et al. (2002) reported that alkaline treatment of miscanthus increased hydrogen yields by 15.6%.

Numerous studies have been conducted to produce hydrogen by using wastes as substrate such as crude cheese whey (7.89 mmol H_2/g lactose; Ferchichi et al., 2005), wastewater sludge (1.5-2.1 mmol-H_2/g-dried solids; Wang et al., 2003), rice winery wastewater (1.37–2.14 mol H_2/mol-hexose; Yu et al., 2002), domestic wastewater (0.01 L H_2/L ww; Van Ginkel et al., 2005), patato industry wastewater (2.8 L H_2/L ww; Van Ginkel et al., 2005), organic fraction of municipal solid waste (150 mL H_2/g solid waste; Lay et al., 1999), sugar factory wastewater (2.52 mol H_2/mol glucose; Ueno et al., 1996), molasses wastewater (47.1 mmol-H_2/g COD; Wu and Lin, 2004), cassava starch manufacturing wastewater (71.3 mL H_2/g COD; Reungsang et al., 2004), cornstalk wastes (149.7 ml H_2/ g TVS; Zhang et al., 2007), wheat straw (22.9 ml H_2/g TVS; Fan et al., 2006), corn straw (68 ml H_2/g corn

straw; Li and Chen et al., 2007), sugar cane distillery effluent (2.76 mol/ mol glucose; Vatsala et al., 2008), co-digestion of municipal food waste and sewage sludges (112 mL/gVS added; Zhu et al., 2008), cattle wastewater (319 ml H_2/g cCOD; Tang et al., 2008), sewage sludge (18.14 L H_2/ kg dry solids; Massanet-Nicolau et al., 2008), sweet potato starch residue (7.0 mol H_2/mol glucose; Yokoi et al., 2001), molasses (0.52 mol H_2/mol substrate; Tanisho et al., 1998 and 109 mL H_2/g hexose; Logan et al., 2002). Maksimum bio-hydrogen production yield in this study (115.6 mL H_2/g COD) was comparable with 125 mL /g COD from starch (Khanal et al., 2004) and higher than 89.2 mL H_2/g COD from sucrose (Sung et al., 2002) and 71.3 mL H_2/g COD from cassava starch manufacturing wastewater (Reungsang et al., 2004).

Maksimum bio-hydrogen production yield in this study was 115.6 mL/g COD (from beet-pulp). In addition, the energy yield of hydrogen is 122.0 kJ/ g (Bartacek et al., 2007). Thus, 1.3 kJ energy per gram of COD can be generated from hydrogen gas under standard temperature and pressure (1 atm and 0^0C).

CHAPTER 5

CONCLUSIONS

The main objective of this study was to investigate bio-hydrogen generation potential of sugar-beet processing wastes (sugar-beet processing wastewater and beet-pulp) through dark fermentation. Effects of different types of cultures, waste types, COD and pretreatment methods to bio-hydrogen generation potential of sugar-beet processing wastes were investigated. From the obtained results of experimental set-ups (Set-up 1, 2, 3, 4) the following conclusions can be drawn:

- Bio-hydrogen production from sugar-beet processing wastewater does not need the presence of any seed because wastewater serves as a raw material and also the seed sludge for bio-hydrogen production.

- Methanogenic activity inhibition methods (BES addition, heat treatment, acidogenic culture enrichment) did not enhance bio-hydrogen production.

- In reactors which contained 4.5 g/L COD beet-pulp (Set-up 2), bio-hydrogen production was not observed while it was observed in reactors which contained 4.5 g/L COD sugar-beet processing wastewater (Set-up 1). Thus, it can be said that sugar-beet processing wastewater is more effective than beet-pulp in terms of bio-hydrogen production at 4.5 g/L COD value.

- The results of Set-up 3 indicated that, 20 g COD/L was the optimum substrate concentration for fermentative bio-hydrogen production from sugar-beet processing wastes in the range investigated.

- In the first part of set-up 4, in all pretreatment reactors sCOD and tVFA concentrations increased when compared to control reactor. Thermal-alkaline pretreatment, microwave-alkaline pretreatment and alkaline pretreatment methods increased initial sCOD and tVFA of beet-pulp significantly when compared to thermal and microwave pretreatments.

- In the second part of Set-up 4, COD solubilization efficiencies of pretreatment methods are as fallows: thermal-alkaline pretreatment > microwave-alkaline pretreatment > alkaline pretreatment. During fermentation, the ratio of sCOD/tCOD increased further. This result revealed that not only pretreatment but also fermentation was effective for solubilizing organic matters from beet-pulp.

- Thermal-alkaline and alkaline pretreatment of beet-pulp increased bio-hydrogen production yield while microwave-alkaline pretreatment of beet-pulp reduced bio-hydrogen production yield (The second part of Set-up 4).

Based on the results obtained in this study, it is postulated that, sugar-beet processing wastes especially beet-pulp have high bio-hydrogen generation potential. Thus, bio-hydrogen production from sugar-beet processing wastes via dark fermentation can not only enable waste minimization but also contribute to sustainability via valuable bio-based product formation from wastes, namely bio-hydrogen. Similarly, Alkaya (2008) worked on biorefining of sugar-beet processing wastes and produced biobased products (methane, organic acids) while treating sugar-beet processing wastes by anaerobic digestion. Therefore, it can be said that biorefining of sugar-beet processing wastes; enables not only waste management but also value-added product generation.

Biorefining concept will bring an innovative approach to the removal and treatment of industrial organic wastes. Biorefining process abides by the consecutive production of liquid (ethanol, organic acids) and gaseous (hydrogen, methane) energy carriers. At the end of the biorefining process, the major objective is to get maximum gain with the utilization of substrate and minimum residue to be treated. In this context, sugar-beet processing wastes can be utilized to produce value-added products such as methane, hydrogen, organic acids. By this way, minimum residues will remain for treatment.

Future Work

In order to improve overall energy efficiency and enable economic feasibility of dark fermentative bio-hydrogen production from sugar-beet processing wastes:

The energy residues remain in the by-products in the forms of acids should be converted to bio-hydrogen and carbon dioxide by two stage photobiological hydrogen production process or should be converted to methane and carbon dioxide by the dark fermentation of these readily degradable organic compounds.

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APPENDIX

CALIBRATION CURVES

Acetic acid calibration data was fit in a linear curve. R^2 and the equation of the fit curve are 0.965 and y = 12140x, respectively.

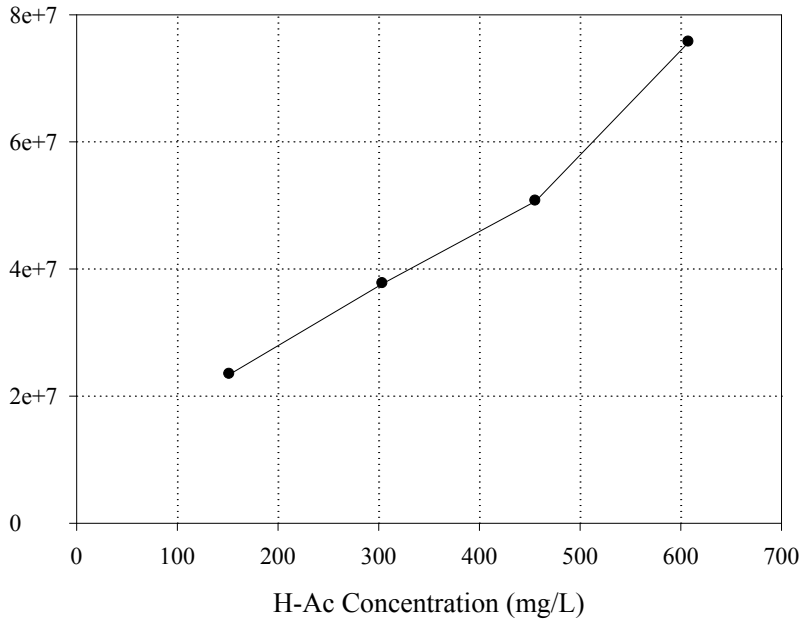

Figure A 1 Acetic acid calibration curve

Propionic acid calibration data was fit in a linear curve. R^2 and the equation of the fit curve are 0.995 and y = 22670x, respectively.

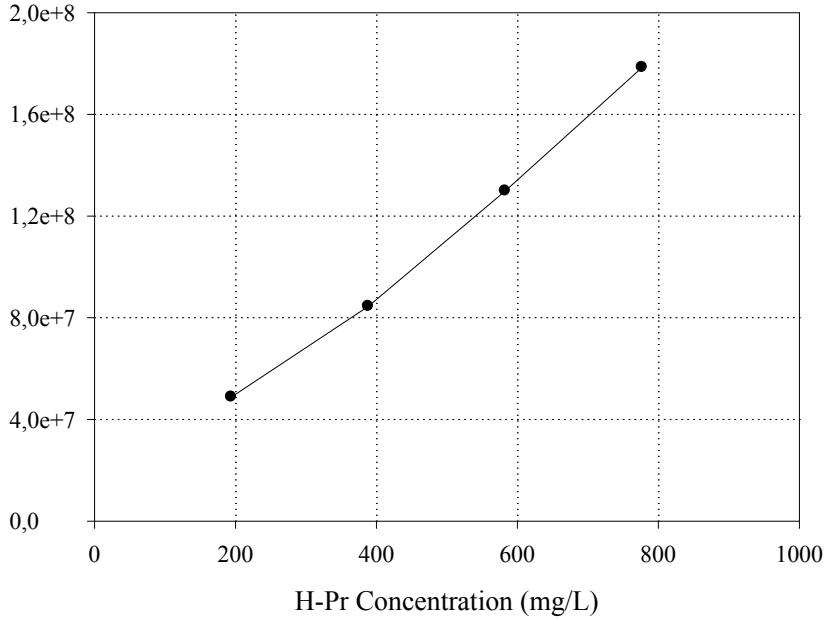

Figure A 2 Propionic acid calibration curve

Butyric acid calibration data was fit in a linear curve. R^2 and the equation of the fit curve are 0.995 and y = 27029x, respectively.

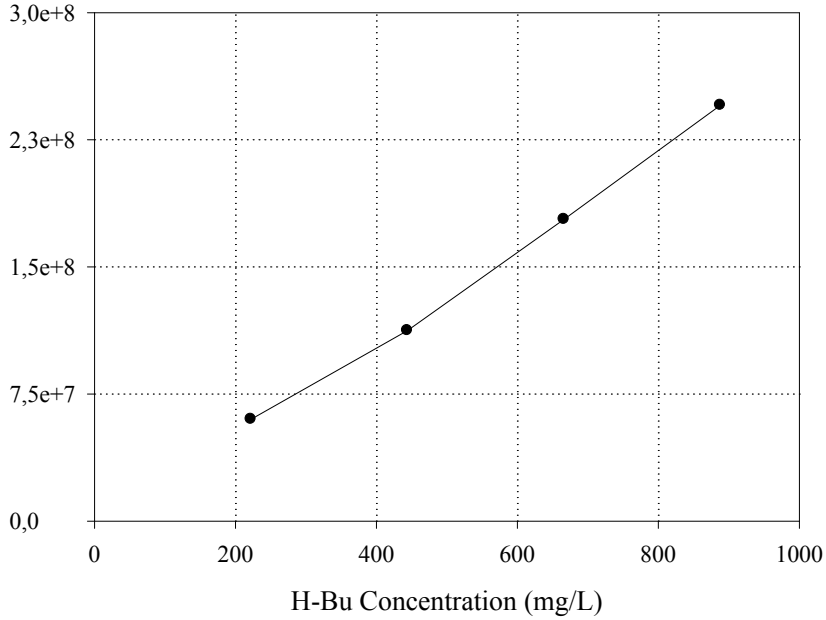

Figure A 3 Butyric acid calibration curve

Hydrogen gas calibration data was fit in a linear curve. R^2 and the equation of the fit curve are 0.998 and y = 1209x, respectively.

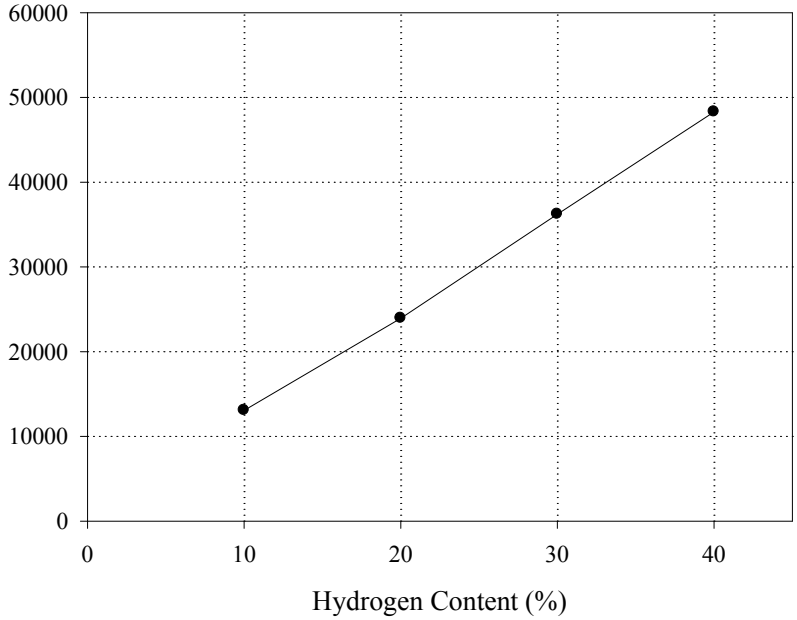

Figure A 4 Hydrogen gas calibration curve

Methane gas calibration data was fit in a linear curve. R^2 and the equation of the fit curve are 0.999 and y = 39868x, respectively.

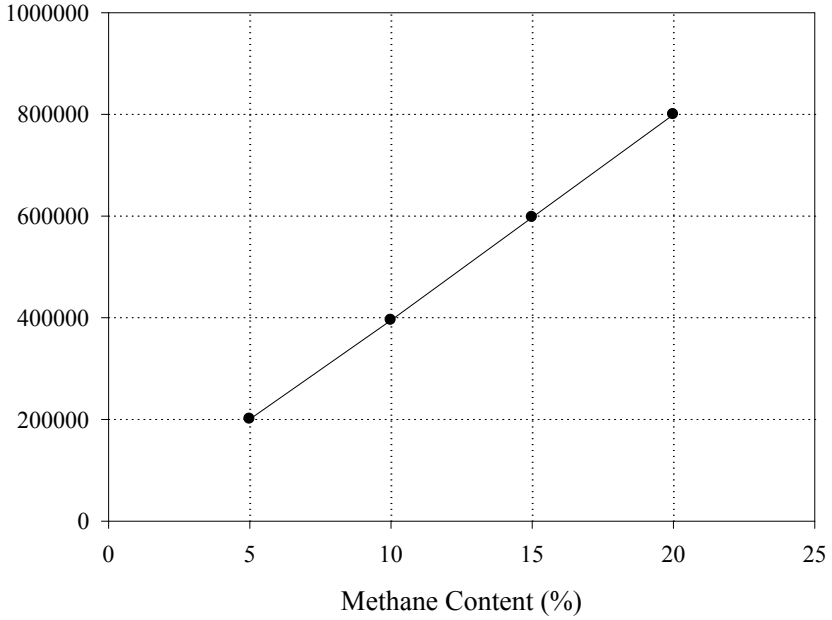

Figure A 5 Methane gas calibration curve