DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

BIOHYDROGEN GAS PRODUCTION FROM SUGAR BEET

by
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October, 2015
İZMİR

BIOHYDROGEN GAS PRODUCTION FROM SUGAR BEET

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M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"BIOHYDROGEN GAS PRODUCTION FROM SUGAR BEET"** completed by **BURCU ERKUL** under supervision of **ASST. PROF. SERKAN EKER** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Burcu ERKUL

BIOHYDROGEN GAS PRODUCTION FROM SUGAR BEET

ABSTRACT

Environmental destruction is an important problem that affecting human life. Excessive usage of fossil fuel causes decrease of fossil fuel resources. This consumption causes to cleaner energy sources demand. Hydrogen is clean energy source and has high energy.

Hydrogen production is performed via chemical, physical, biological processes. Hydrogen can be produced by biophotolysis, photo-fermentation and dark fermentation or using combination of these biologically.

The aim of thesis, is to determine factors that affecting biohydrogen production from sugar beet via dark fermentation. For this, effects on hydrogen production yield was investigated by changing sugar beet concentration, microorganism concentration and sugar beet surface area. Experiments were conducted as batch dark fermentation experiments. Anaerobic sludge was used as microorganism source. Acid and heat pretreatments were applied, to eliminate methanogens and select hydrogen producing bacteria. Sugar beet was used as substrate. EX-FERM technique was used. Experiments were performed as three sets. Six bottles were prepared including control bottle for any set. Microorganism concentrations (1 gram per liter) and sugar beet surface areas (0.54 square centimeter) were kept constant and various sugar beet concentrations were used at first set. Maximum cumulative hydrogen production result (73.68 mililiter) was obtained from 60 gram per liter concentration. 60 gram per liter substrate was used at second set. Substrate concentrations and surface areas (0.54 square centimeter) were kept constant. Various microorganism concentrations were used. The highest cumulative hydrogen production result (116.05 mililiter) was obtained at 1 gram per liter biomass concentration. 60 gram per liter sugar beet, 1 gram per liter microorganism and various sugar beet surface areas were used at third set. The highest cumulative hydrogen result (146.25 mililiter) was obtained at 0.06 square centimeter surface area. Total gas volume, hydrogen percentage

measurements, residual sugar analysis and pH adjustments were performed everyday. Gompertz constants were calculated via Statistica program.

Keywords: Biohydrogen, batch dark fermentation, sugar beet, hydrogen production, ex-ferm

ŞEKER PANCARINDAN BİYOHİDROJEN GAZI ÜRETİMİ

ÖZ

Günümüzde, çevresel tahribat, insan yaşamını etkileyen önemli problemlerden biridir. Fosil yakıtların aşırı kullanımı, fosil yakıt kaynaklarının hızla tükenmesine sebep olmaktadır. Bu durum, yeni temiz enerji kaynakları arayışına yol açmıştır. Hidrojen, temiz enerji kaynaklarından biridir ve yüksek enerji değerine sahiptir.

Hidrojen gazı, kimyasal, fiziksel ve biyolojik yöntemler kullanılarak elde edilmektedir. Biyolojik olarak; biyofotoliz, ışıklı fermantasyon ve karanlık fermantasyon işlemleri aracılığıyla ve bu işlemlerin kombinasyonları kullanılarak elde edilmektedir.

Bu çalışmanın amacı, şeker pancarından karanlık fermantasyonla biyohidrojen gazı üretimini etkileyen bazı faktörleri belirlemektir. Farklı şeker pancarı konsantrasyonları, farklı mikroorganizma konsantrasyonları ve farklı yüzey alanlarına sahip substrat kullanarak, hidrojen gazı üretim verimi üzerindeki etkileri araştırılmıştır. Deneyler, karanlık kesikli fermantasyon deneyleri şeklinde yürütülmüştür. Deneylerde, mikroorganizma kaynağı olarak anaerobik arıtma çamuru kullanılmıştır. Anaerobik çamura, metanojenleri elimine edip hidrojen üreten bakterileri seçmek amacıyla ısı ve asit ön işlemleri uygulanmıştır. Substrat olarak oda sıcaklığında şeker pancarı kullanılmıştır. Deneylerde, EX-FERM tekniği kullanılmıştır. Deneyler 3 set halinde tasarlanmıştır. Her set için 6 adet (bir adet kontrol şişesi) deney şişesi hazırlanmıştır. İlk sette mikroorganizma konsantrasyonu litrede 1 gram, şeker pancarı yüzey alanı ise 0.54 santimetrekare olarak sabit tutulmuş, çeşitli şeker pancarı konsantrasyonları kullanılmıştır. Maksimum kümülatif hidrojen üretim sonucu (73.68 mililitre) litrede 60 gram şeker pancarı kullanılarak sağlanmıştır. İkinci sette şeker pancarı miktarı litrede 60 gramda substrat yüzey alanı ise 0.54 santimetrekarede sabit tutulup farklı konsantrasyonlarda mikroorganizma kullanılmıştır. Maksimum kümülatif hidrojen üretim sonucu (116.05 mililitre) litrede 1 gram mikroorganizma kullanılarak elde edilmiştir. İlk iki setin sonuçları doğrultusunda en yüksek sonuçların alındığı substrat konsantrasyonu (litrede 60

gram) ve en yüksek sonuçların alındığı mikroorganizma konsantrasyonu (litrede 1 gram) sabit tutularak farklı yüzey alanlarına sahip şeker pancarları kullanılmıştır. En yüksek sonuç (146.25 mililitre) 0.06 santimetrekare şeker pancarı kullanılarak sağlanmıştır. Toplam gaz ve hidrojen yüzdesi ölçümleri, kalan şeker analizleri ve pH ayarlamaları günlük olarak yapılmıştır. Gompertz sabitleri, "Statistica" programı ile hesaplanmıştır.

Anahtar kelimeler: Biyohidrojen, kesikli karanlık fermentasyon, şeker pancarı, hidrojen üretimi, ex-ferm

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CHAPTER ONE
INTRODUCTION

1.1 Biohydrogen Gas Production

Energy is one of the most important thing worldwide. Energy demand is increasing day by day because of industrial, technologic developments and population growth. Coal, fuel oil, petrol, natural gas are the most commonly used fuels worldwide. All of these are fossil fuels. Fossil fuels contain hydrocarbon. As result of combustion of fossil fuels, COx, NOx, SOx compounds and CH_4 gas are released. In addition, ash and soot occur. So fossil fuels are not environmentally friendly. They cause environmental pollution, global warming and harm to ozone layer. All of these reasons prompted to find a new energy source that non-pollutant and high energy carrier.

Hydrogen is the first element in the periodic table which has the smallest atomic number and mass. Its weight is light but the most abundant element all over the world (Winter & Winter, 2009). It combines with oxygen and creates water. Energy value is three times higher than gasoline.

Das & Veziroğlu (2008) reported that hydrogen (H_2) is the most reassuring alternative among the fuels. Because hydrogen is environmentally being. Also its energy content is substantially higher than other fuels (142 kJ/g or 61.000 Btu/lb), and it's convenient to transport for domestic and industrial usage. In addition low cost is an extra advantage.

There is only one disadvantage of hydrogen. It doesn't exist in nature as a fuel, like the other fuels. It must be produced from water or some renewable substances containing carbohydrates (Argun & Kargı, 2011).

Many technologies can be used for production of hydrogen today. International Energy Agency [IEA], (2006) reported that history of hydrogen production started by using electrolysis technology. Electrolysis was the most widely using way to pure hydrogen production from water in 1920's. Over time this technology give place to different technologies and materials. Hydrogen production has been based on fossil materials containing hydrocarbon since 1960's.

Biological production of hydrogen has gained importance since being more renewable and sustainable than the other technologies. There are considerable advantages way using for production of biohydrogen; water photolysis (direct - indirect), dark fermentation and photo fermentation. In addition these hybrid systems can be used (Das & Veziroğlu, 2001; Kapdan & Kargı, 2006).

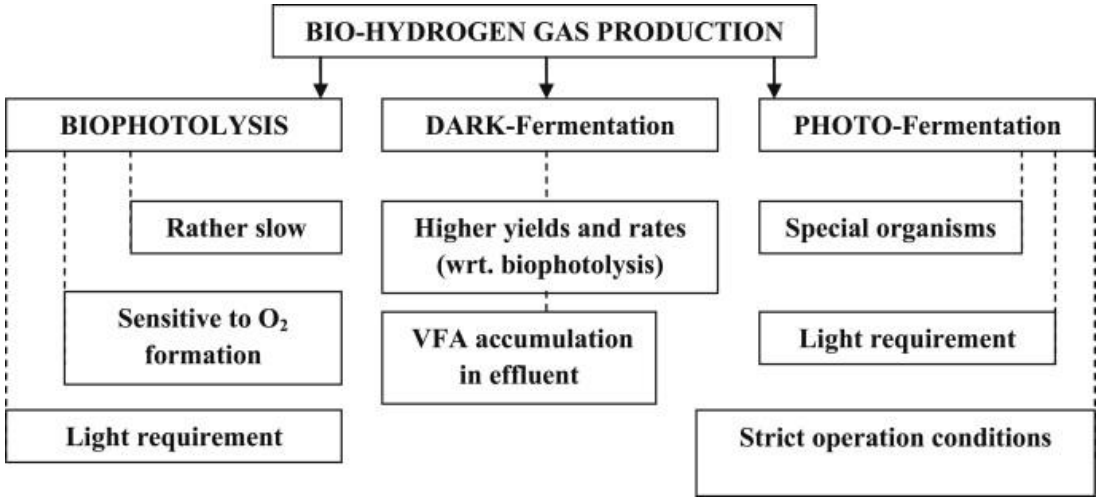


Figure 1.1 Comparison of bio-hydrogen production by algal and bacterial bioprocesses (Argun & Kargı, 2011)

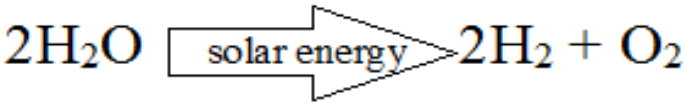
1.1.1 Bio-Hydrogen Production via Biophotolysis

Biophotolysis can be classified into two groups; direct biophotolysis and indirect biophotolysis.

1.1.1.1 Direct Biophotolysis

Photosynthesis is a process that converts water and carbondioxide to nutrient (carbohydrate) and oxygen. The same processes that used in this method found in plants and algae photosynthesis. The only difference is final product that hydrogen gas is obtained instead of carbohydrate (Das & Veziroğlu 2001).

Biohydrogen production by direct biophotolysis occurs via photosynthetic capability of algae (Holladay, Hu, King & Wang, 2009). Algaes photosynthetic system converts solar energy into chemical energy as hydrogen gas (Ni, Leung & Sumathy 2006).



$$\Delta G_0 = +749 \text{ kj/mol}^4 \qquad (1.1)$$

There are two photosynthetic systems that carry out biophotolysis. Photosystem I (PSI) responsible for CO_2 reduction and produces reductant. Photosystem II (PSII) decomposes water to obtain oxygen (Ni, Leung & Sumathy, 2006).

In the biophotolysis process, each electron from water uses a photon per photosystem (totally two photons). And photons are used in CO_2 or H_2 generation with hydrogenase. Hydrogenases are the catalyzer for hydrogen formation. Depending on lack of hydrogenases, H_2 generation is not the case for green plant. Only CO_2 reduction takes place.

Microalgaes (green and blue-green algaes) able to produce hydrogen under certain conditions because of containing hydrogenase enzymes (Hallenbeck & Bennemann, 2002).

During the process, PSII absorbs solar energy and electrons are obtained. Then electrons flow to ferrodoxin (Fd) via solar energy. Hydrogenase takes the electrons from ferrodoxin and generates hydrogen (Ni, Leung & Sumathy, 2006).

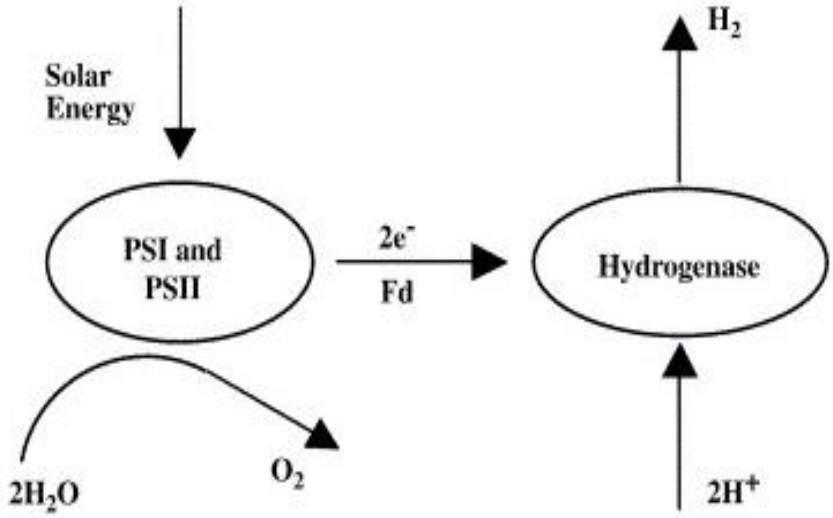


Figure 1.2 Schematics of direct biophotolysis (Ni, Leung & Sumathy, 2006)

Hydrogenase enzyme is sensitive to oxygen. So oxygen presence inhibits the hydrogen production. Oxygen level must be less than 0.1%. Green algae *Chlamydomonas reinhardtii* can provide this condition, because this is oxygen breathing micoorganism and consumes oxygen. Despite this, efficiency is low. Various kinds mutants derived from microalgae can be used to increase the efficiency.

Studies show that mutants' O_2 tolerance is high, therefore higher efficiency and so higher hydrogen production can be obtained (Ni, Leung & Sumathy, 2006).

Nath and Das (2004) reported that biophotolysis process is theoretically advantageous but low efficiency and low hydrogen production show that this method must be developed.

1.1.1.2 Indirect Biophotolysis

Indirect biophotolysis is an alternative to direct biophotolysis to escape from inhibitive effect of O_2. Indirect biophotolysis can be observed in three steps.

i) Biomass production in photosystem

ii) Production of 2 mol acetate and 4 mol hydrogen per mol glucose in the algae cells via aerobic dark fermentation.

iii) The conversation of two mol acetate into hydrogen (Genç, 2009).

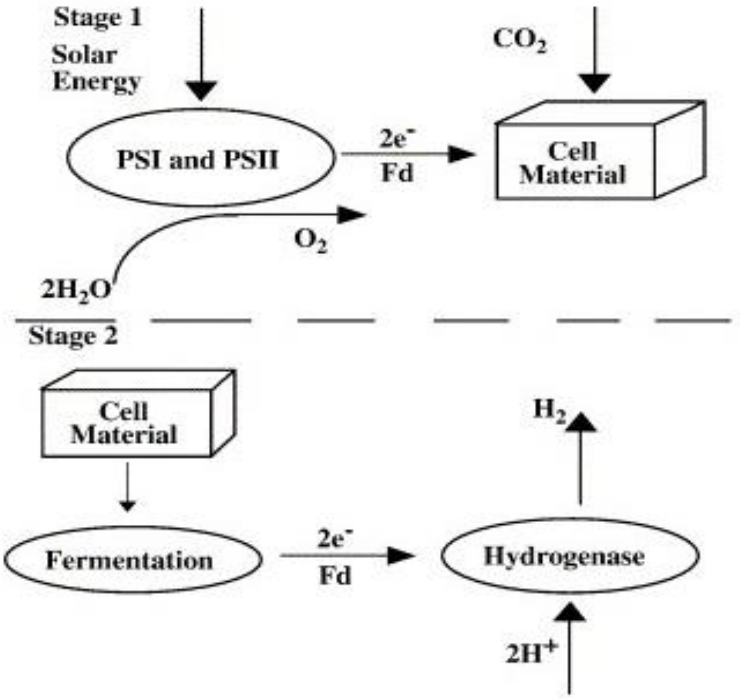


Figure 1.3 Indirect biophotolysis for hydrogen production (Ni, Leung & Sumathy, 2006)

Usually, cyanobacteria that can produce hydrogen using nitrogenase with absence of nitrogen are used in an indirect biophotolysis (Hallenbeck & Bennemann, 2002). Cyanobacteria use solar energy as energy source and use CO_2 as carbon source. Cells take CO_2 and use for H_2 production (Manish & Banerjee, 2008).

Nitrogenase activity inhibits oxygen. So production of hydrogen comes over under anaerobic conditions. CO_2 may inhibit the photo production of H_2. But CO_2 is necessary for some cultures at the step of hydrogen improvement. Studies show that low CO_2 concentration (%4-18 w/v) increases the cell density at growing phase and gives rise to higher hydrogen production at following phases (Das & Veziroğlu, 2008).

1.1.2 Photofermentation

Photosynthetic microorganism able to produce hydrogen via nitrogenase by using solar energy and organic acids or biomass. This activity is called as photofermentation (Ni, Leung & Sumathy, 2006).

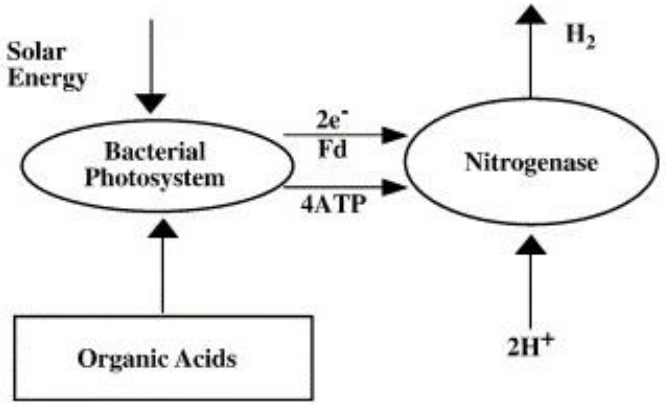


Figure 1.4 Various types of biomass can be used for hydrogen production by photofermentation (Ni, Leung & Sumathy, 2006)

Light level affects the rate of hydrogen production efficiency for photosynthetic bacteria species (Nath & Das, 2004).

There are some disadvantages of biohydrogen production by photofermention;

- Nitrogenase enzyme usage with high energy need,
- Low conversion yield of solar energy,
- Anaerobic photobioreactor need with large area demand (Ni, Leung & Sumathy, 2006).

Ni, Leung & Sumathy (2006) reported that photofermentation method is not good option because of disadvantages above.

1.1.3 Dark Fermentation

Hallenbeck & Bennemann (2002) reported that the dark fermentation of biomass is the most promising way to biohydrogen production in comparison to others. It's regarded as favorable method because of simplicity of process, high hydrogen production rates, variable feed material utilization especially wastes. It doesn't require oxygen. All of these advantages make dark fermentation more favorable among other biological methods.

Anaerobic or facultative anaerobic bacteria achieve biohydrogen production by dark fermentation under anaerobic conditions (Antonopoulou, Ntaikou, Stamaelatou & Lyberatos, 2011). Various kinds of microorganism are available for biological production of hydrogen. However, the most commonly used microorganisms are Clostridium (sporeforming), Enterobacter (facultative), Bacillus, thermophilic bacteria species and treatment sludge taken from asidogenic phase of treatment plant (Argun & Kargı, 2011). Pure microorganism or mixed microorganism culture can be used for biohydrogen production. But mixed microorganism culture is more preferable. Since pure microorganism culture can be contaminated by some hydrogen consuming bacteria species (Liu, Min & Angelidaki, 2008).

The major requirement is carbon for fermentative hydrogen gas production. Sugar containing biomass (sugar beet, sugar cane), starch containing biomass (potato, wheat etc.) and lignocellulosic matter containing biomass can be used to provide carbon requirement. Except all these, wastewater treatment plant sludge can be used as carbon source also (Argun & Kargı, 2011).

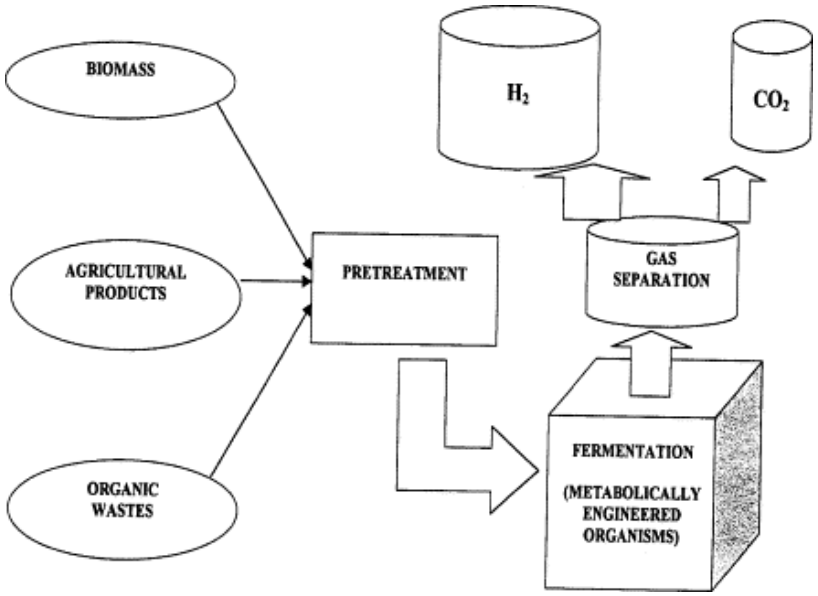


Figure 1.5 Hydrogen production through dark fermentation (Hallenbeck & Bennemann, 2002)

The reaction of glucose conversion to acetate is widely considered as reference although various kinds of substrates can be used, while estimating the theoretical yields (Ntaikou, Antonopoulou, Stamaelatou & Lyberatos, 2011).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (1.2)$$

According to the reaction above, maximum theoretical fermentation yield is 4 mol H_2 per mol glucose when acetic acid is end product (Ni et al., 2006).

If there is butyrate instead of acetic acid, the maximum theoretical yield is 2 mol H_2 per mol glucose as seen on the equation below (Ni et al., 2006).

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_2CH_2CH_2OOH + 2H_2 + 2CO_2 \quad (1.3)$$

Despite the equations above, 4 mol H_2 production per mol glucose is not possible in practice. Because both acetate and butyrate are exist as end product together (Ni et al., 2006).

There are some important environmental parameters for this process. Such as organic loading rate, hydraulic retention time, oxidation reduction potential, temperature, pH, nitrogen to carbon ratio.

Microorganism uses the organic matter while hydrogen production process and it is evaluated by organic loading rate (OLR) that expresses immensity of chemical oxygen demand. The unit of OLR is g/(Lday). OLR is an important parameter for hydrogen production process and to acquire optimal microbial growth. But higher organic loading rate doesn't provide higher hydrogen production yield. There is not certain optimum range of OLR for hydrogen production via fermentation. There are various values in different studies that obtained maximum hydrogen production yield (Mohammadi et al., 2012).

Hydraulic retention time (HRT) can be defined as time period that a volume of liquid which is detained in active volume of reactor (Mohammadi et al., 2012). Khanna & Das (2012) reported that HRT is also used for selection of microbial population where growth rates match the mechanical dilution rates that caused by volume of flow. If HRT is too short or too long, microorganism will be affected adversely. Because HRT is inversely proportional with F/M. According to some studies HRT affects bacterial competition. Lower HRT levels increase hydrogen yield. Studies show that there is a range 0.25 to 60 hours for HRT which is experienced with different organic matters and there is not certain optimum HRT. Because HRT depends on different factors such as microorganism, substrate, reactor design etc. (Mohammadi et al., 2012).

Oxidation-reduction potential (ORP) is another parameter that must be held under control. ORP value shows aerobic or anaerobic conditions. Negative (-) values show that fermentation medium has anaerobic conditions. Cai, Liu & Wei, (2004) reported that the optimum ORP range is between -200 mV and -250 mV for Clostridium species.

Operation temperature is one of important parameters influencing microorganism metabolic activity and growth rate in fermentative hydrogen production. Temperature depends on the type of microorganism. For psychrophilic bacteria 0-20°C, for mesophilic bacteria 20-42°C and for thermophilic bacteria 42-75°C is suitable temperature. 37 - 45°C is suitable temperature if it is utilized pure mesophilic bacteria cultures (*Clostridium* and *Enterobacter*strains etc.). But studies show that when mixed culture population is used there is no exact optimum temperature (Khanna & Das, 2012). Mohammadi et al., (2012) reported that temperature is also depends on carbon source used in hydrogen production process in addition to type of microorganism. There are various studies that show different suitable temperatures for hydrogen production via dark fermentation. Wu et al., (2013) utilized the pure cultures *Clostridium* or *Enterobacter* and reported optimum range as 37-45°C. But Sompong et al., (2008) used *Thermoanaerobicterium thermosaccharolyticum* PSU-2 and optimum temperature was reported as 60°C. According to the study reports there is no exact optimum temperature for biohydrogen production by dark fermentation (Mohammadi et al., 2012).

pH is one of the most important conditions. Medium pH plays role on production yield, characteristic of biogas and organic acids and specific hydrogen production rate. It's reported that the most appropriate range is between pH 5.0 - 6.0 for highest hydrogen yield or specific hydrogen production. Because of organic acid generation which decreases buffering capacity, pH level reduces. This reduction inhibits hydrogen production. Because hydrogenase enzyme is affected directly. So it's important to control pH level (Kapdan & Kargı, 2006).

Microorganism activity and microbial growth are affected by nitrogen to carbon ratio (C/N). Optimal C/N value is important for microbial activity and increase hydrogen production. But it's reported that optimum C/N value differs among different studies (Mohammadi et al., 2012).

1.1.4 Hybrid Systems

Hybrid systems comprise combination of dark and photo fermentation. Nonphotosynthetic and photosynthetic bacteria are necessary for hybrid systems. C.butyricum bacterium converts the carbohydrates to hydrogen without light demand. As a result, organic acids are generated and be source for photofermentation. Organic acids are converted to hydrogen by photosynthetic bacteria (Das & Veziroğlu, 2001).

1.2 Literature Review

In this thesis EX-FERM technique was used. EX-FERM technique is a process that extraction and fermentation of sucrose processes were carried out simultaneously in a substrate - water suspension (Rolz & Cabrera,1980).

Rolz and Cabrera (1980) studied on ethanol production from sugar cane by using the EX-FERM technique. They carried out the experiments in a cane - water suspension in flasks. They studied with 37 strains of Saccharomyces spp. Two types of treated sugar canes were used: chips and shredded pith, either fresh or dried.

They obtained the yeast strains from laboratories from different countries and their own collection. The mother culture of yeast was prepared in cane juice. Then it was added to the cane - water suspension. Static fermentation process was carried out for 40 hours at 30°C. This was first cycle. After static fermentation the sugar cane was removed and fresh sugar cane was added to yeast-alcohol broth. After this addition, it was waited for 24 hours and sugar cane was removed again. Then the

liquor was analyzed. According to results of analysis, sugar consumption was above 99% and ethanol concentrations were 1.29 to 4.00 g per 100 ml, depending on the yeast strain for the first cycle with fresh sugar cane as chips or pith for 10 of the 37 stains tested. Sugar consumption was nearly 98% and ethanol concentrations reached 4.27 to 5.37 g per 100 ml for 3 of 18 yeast strains tested with dried sugar cane in the second EX-FERM cycle. Product yields were acceptable values, and differences found among yeast strains for either cane chips or shredded pith were not at high levels and significant. Sugar cane treatment didn't affect the results at this level.

According to results of experiments Rolz and Cabrera, (1980) reported that the concept of EX-FERM ethanol fermentation is technically feasible.

Large amount of studies have been carried out hitherto about biohydrogen production via dark fermentation. Various environmental conditions, various microorganism species and different kinds of substrates as carbon source were investigated in earlier studies.

Kargı and Pamukoğlu, (2009) studied on bio-hydrogen production from ground wheat starch via dark fermentation. They used heat-treated anaerobic sludge. Anaerobic sludge was taken from asidogenic phase of anaerobic wastewater treatment plant of a yeast company. Sludge was boiled for 5 hours to eliminate methanogens. Then sludge was cultivated on a synthetic media. Initial cell concentration was $3\ g\ L^{-1}$. Experiments were carried out as fed-batch dark fermentation processes.

Wheat granules were ground and passed through in a sifter to obtain -200 mesh size wheat powder. The wheat powder solution was partly hydrolyzed and sterilized at 121°C by autoclave. pH was adjusted to 7.0. Na-thioglycolate ($200\ mg\ L^{-1}$) was added in order to provide anaerobic conditions (ORP value was -200 mV).

10, 20 and 30 L^{-1} wheat powder were used to feed solution. Feeding rate was 8.33 mL h^{-1} (200 mL d^{-1}). Wheat powder loading rates were 83.3, 166.6 and 249.9 mg WP h^{-1}.

0.5 L of heat treated and grown sludge was filled into fermenter. Initial glucose concentration was 4.75 ± 0.25 g/L. Before feeding, initial pH was adjusted to 7.0. pH and ORP were controlled and adjusted everyday. In addition, there was a control experiment under the same conditions without sludge inoculation.

They reported that residual substrate concentration decreased with time depending on the starch consumption. The highest results were obtained with the (4 g WP d^{-1} loading rate) 20 g L^{-1} feed wheat powder concentration after 4 days of operation. Cumulative hydrogen production was 3600 mL, hydrogen yield was 3.1 mol H_2 mol^{-1} glucose (465 mL H_2 g^{-1} starch), hydrogen production rate was 864 mL H_2 d^{-1}. The experiment with low concentration (10 g L^{-1}) was unsuccessful because of limited substrate. In the experiment, which was operated with high feed concentration (30 g L^{-1}), volatile fatty acids generated and inhibited the hydrogen production rate and yield.

De Gioannis et al., (2014) investigated the effects of the operating pH on hydrogen production by dark fermentation. They used two kinds of cheese whey as feed material. The first one, was pecorino cheese which was the product of mixture of cow and sheep milk, second one was mozzarella cheese which was only cow milk product. Either cheese whey, only or heat shock applied activated sludge and cheese whey mixture were used as biomass source in experiments. They utilized Clostridium species as microorganism and mesophilic batch reactors for fermentation. During the experiments, pH was controlled continuously. It's reported that the optimum pH depended on cheese whey type. In addition to pH, hydrogen production was also affected by substrate type and inoculum addition. Maximum hydrogen generation was reported as 171.3 NL H_2/kg TOC at pH 6.0 using heat shocked inoculum with cheese whey sample from mozzarella cheese production.

Baghchehsaraee et al., (2008) studied about the effect of heat pretreatment temperature on fermentative hydrogen production by dark fermentation using mixed culture. The researchers used two types of inocula. Activated sludge and anaerobically digested sludge. Sludges were dewatered and sieved through 2 mm screen. Experiments were operated in batch cultures. Heat treatments were applied at 65°C, 80°C, 95°C for 30 minutes in order to select hydrogen producing bacteria. Untreated inocula were utilized as control. The experiments were operated in 350 mL vials containing 120 mL media. In all vials the medium contained 10 g/L glucose as carbon source. As result, less amount of hydrogen was generated by untreated inocula. Lactic acid was produced as the main metabolite. Hydrogen production yields of 65°C pretreated anaerobically digested and activated sludges were 2.3 and 1.6 mol H_2/mol glucose respectively. The hydrogen production rates were 19.3 mL/h for activated sludge and 26.3 mL/h for anaerobic sludge. These were maximum values. They obtained maximum results at 65°C pretreatment. When treatment temperature increased from 65 to 95 °C, butyrate/acetate ratio increased from 1.5 to 2.4 and hydrogen production yield decreased approximately 15% at anaerobically digested sludge. The temperature increase from 65 to 95 °C at activated sludge resulted in suppression of hydrogen production by lactic acid formation. It's reported that temperature increase reduced species diversity.

Bao et al., (2013) studied on effects of substrate pretreatment and addition of metal ions (Fe^{2+} ,Mg^{2+}) or L-cysteine on biohydrogen production by dark fermentation. In this study, two pure bacterial strains were utilized as mixed cultures in a fermenter and starch hydrolysis and H_2 production processes were combined. Strain A1 produced amylase, so could starch hydrolysis and strain B1 produced hydrogen in experiments. Corn starch was used as substrate. Experiments showed that cumulative H_2 production increased from 838 to 1186 ml by boiling pretreatment of corn starch. Pretreatment affected the H_2 production rate also. That was much higher than untreated corn starch. When untreated corn starch was used the H_2 yield was 0.85 mol H_2/mol glucose but when pretreated corn starch was used the yield increased to 1.19 mol H_2/mol glucose. It's reported that H_2 production was accelerated by thermal pre-treatment, and metabolic pathways were altered. The maximum results were obtained with sole Fe^{2+} addition. Cumulative H_2 production

was 1928 mL, H_2 yield was 1.94 mol H_2/mol glucose. Sole L-cysteine addition resulted in 1.53 mol H_2/mol glucose H_2 yield. Combination of L-cysteine and metal ions resulted in lower H_2 production than sole L-cysteine but higher than the control. High to low ranking (for potential H_2 production) was; Fe^{2+} > L-cysteine > L-cysteine + Fe^{2+} > L-cysteine + Mg^{2+}. Gompertz equation fitted the cumulative H_2 production with pretreatment but not with element addition.

Wu et al., (2013) investigated the ozonation pretreatment effects on biohydrogen production from wheat straw. Batch dark fermentation method was used at experiments. A slurry mixture of cow manure and sediment was used as inoculum. Manure, sediment and water were mixed with 1:1:1 ratio and heated at 90 °C for 20 minutes then used as inoculum. Wheat straw lignin amount decreased by ozone treatment. Increase of ozone dose led the delignification increase. In addition, ozone treatment increased the reducing sugar yields too. Hydrogen production took place from both untreated and treated samples. Ozonation process was applied to sample 15, 30, 45 and 90 minutes. Results showed that hydrogen production increased 107%, 134%, 158% and 138% respectively compared to untreated sample. According to results, 90 minutes ozonation showed inhibitory effect on hydrogen production. The experiment results showed that ozonation pretreatment is technically feasible way to increase production on biohydrogen production from lignocellulosic biomass.

The effect of microwave irradiation of mixed culture on biohydrogen production from waste of sweet produced from Benicasa hispida was studied by Shinghal and Singh., (2014). Cow dung which was provided from a dairy was utilized as mixed microorganism source. In order to enrich the mixed culture in terms of anaerobes, the culture was grown in a batch anaerobic reactor for two weeks before using as inoculum. *Benincasa hispida* solid waste was used as substrate. Different powers (160, 320, 480, 640 and 800 W) were applied to mixed culture for 5 minutes. Experiments were performed in batch reactors and the results were compared with control reactor which was operated without inoculum treatment. The maximum hydrogen production yield was obtained at 320 W (5 minutes) pretreatment. The

hydrogen yield was 14 H_2 per mol soluble sugar. This study showed that the microwave irradiation power higher than 320 W deactivates hydrogen producing bacteria and decrease the biohydrogen production.

1.3 Scope and Objective

The major objective of this study is to investigate the biohydrogen production from sugar beet via dark fermentation. Many researchers investigated the hydrogen production from sugar beet. The difference of this study is using EX-FERM technique. It's aimed to provide maximum soluble sugar transfer to liquid and also usage of insoluble sugar in sugar beet by microorganism.

The detail of the objectives of this thesis can be summarized as follows;

- To investigate the effect of using different sugar beet concentrations,
- To investigate the effect of using different microorganism concentrations,
- To investigate the effect of sugar beet surface area on biohydrogen gas production potential by dark fermentation.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Dark Fermentation

2.1.1 Organisms

The mixed culture which was used in experiments was obtained from PAK-MAYA Baker's Yeast factory İzmir - TURKEY. The sludge of mixed culture was taken from acidogenic phase of anaerobic treatment plant. pH of sludge was adjusted to 5.9 by using sulphuric acid (10%). Sludge was boiled for two hours at 100ºC in order to eliminate the hydrogen consuming methanogenic bacteria and select spore forming hydrogen producing bacteria. The pH was controlled and kept constant at 5.9 by adding sulphuric acid during the heat treatment.

It's important to provide optimum C/N/P ratio (100/2/0.5) for microorganism growth. Therefore, nutrient media was prepared for anaerobic sludge. 20 ml molasses, 0.25 g $MgSO_47H_2O$, 0.435 g KH_2PO_4, 0.2 g L-cysteine, 0.25 g $FeSO_47H_2O$ and 0.869 g urea were mixed and completed to 1000 ml by adding distilled water. L-cysteine was responsible for ensuring anaerobic conditions. The pH was adjusted to range of 6.8 - 7.2 by adding sodium hydroxide. 25 ml heat treated anaerobic sludge and 225 ml nutrient media were filled in bottle. Thus, the cultivation media was obtained. The pH was 6.8 - 7.2 and ORP value was lower than -200 mV. This value showed that anaerobic conditions were provided. The bottle was closed with silicone stoppers. Argon gas was passed through in the bottle in order to remove the oxygen. The bottle was kept in the incubator at 37ºC for 18 hours. The same process was repeated after 18 hours. The same nutrient media was prepared and pH was adjusted to the range of 6.8 - 7.2. New cultivation media was obtained by using 25 ml cultivation media which was kept in incubator for 18 hours, and 225 ml nutrient media. pH of cultivation media was in the range of 6.8 - 7.8 and ORP value was lower than -200mV. Then, argon gas was passed through in the bottle. The

bottles were kept in the incubator at 37ºC for 18 hours. Then, the inoculum was ready to centrifuge process to obtain different amount of microorganism.

2.1.2 Preparation of Substrate

Sugar beet was used as carbon source in the experiments. Sugar beet was obtained from Konya Şeker factory. Experiments were operated as three sets. The sugar beets which were kept in the deep - freeze before use, were kept outside until they come to room temperature and dry. Then the sugar beets were cut and weighed for each set. At the first set effects of sugar beet amount were investigated and 0.54 cm^2, (total surface area) 5 g/L, 15 g/L, 30 g/L, 45 g/L and 60 g/L sugar beets were used. At the second set, effects of different amount of microorganisms were investigated and only 0.54 cm^2, 60 g/L sugar beet was used in each bottle. And at the third set, effects of surface area were investigated and sugar beets were used in different sizes. 0.06 cm^2, 0.54 cm^2, 1.5 cm^2, 2.94 cm^2, 6 cm^2 sized 60 g/L sugar beets were used.

2.1.3 Analytical Methods

2.1.3.1 Sampling Process

3 ml samples were taken from each bottle everyday and centrifuged at 8000 rpm for 15 minutes. The sample which was in the centrifuge tube was divided in liquid and solid phase. The clear liquid supernatants were used for total volatile fatty acid and total sugar analysis.

2.1.3.2 Total Sugar Analysis

Acid phenol spectrophotometric method was used for sugar analysis. All of the samples were centrifuged at 8000 rpm for 15 minutes before analysis. 1ml of the clear supernatant of centrifuged sample, 1 ml phenol solution and 5 ml concentrated sulphuric acid were mixed in glass tubes. Then they were kept in 25 - 30 ºC water bath for half an hour. The samples were analyzed by spectrophotometer (480 nm

wave length) in order to determine the sugar concentration by measuring the color difference. In addition to initial sugar concentrations, residual sugar concentrations were determined everyday during experiments.

2.1.3.3 Total Volatile Fatty Acid Analysis

Analytical kits (Spectroquant, 1.01763. 0001, Merck, Darmstadt, Germany) were used for total volatile fatty acid analysis. Spectophotometric readings were carried out via PC spectrometer (WTW Photolab S12). Total volatile fatty acid analyses were performed for each bottles at the end of all the sets.

2.1.3.4 Total Organic Carbon Analysis

Total organic carbon analyses were carried out via Shimadzu TOC analyzer.

2.1.3.5 Total Gas Volume Measurements

Total gas volume measurements were carried out everyday. Water displacement method was used for these measurements by using a solution containing sulphiric acid (2%) and NaCl (10%).

2.1.3.6 H_2 Gas Measurements

Samples were taken from head space of the bottles by using gas tight glass syringes. The measurements were carried out via gas chromatograph (HP Agilent 6890) everyday. The column was Alltech, Hayesep D 80/100 6" × 1/8" × 085". The carrier was nitrogen gas (30 ml min^{-1}) for these measurements and the head pressure was 22 psi. Oven temperature was 35 ºC and temperatures of injection, detector, and filament were 120 ºC, 120 ºC, 140 ºC respectively.

2.1.3.7 pH Measurements

pH measurements were carried out everyday by using pH meter. pH was adjusted to range of 6.8 - 7.2 by using 3M NaOH solution everyday during the experiments.

2.1.3.8 ORP Measurements

ORP measurement were carried out everyday by using ORP meter. In general ORP values were between -200 and -400 mV.

2.1.3.9 Calculations

The following equation were used for calculation of cumulative hydrogen gas volume :

$$V_{H2,\,i} = V_{H2,\,i-1} + V_{W,\,i} \times {}^{1}C_{H2,\,i} + V_{G,\,i}\,({}^{1}C_{H2,\,i} - {}^{2}C_{H2,\,i-1}) \qquad (2.1)$$

$V_{H2,\,i}$; cumulative hydrogen gas volume (CHV, (ml)) calculated for the i^{th} measurement,

$V_{H2,\,i-1}$; CHV (ml) calculated for the previous measurement of i^{th} ,

$V_{W,\,i}$; total gas volume measured by the water displacement method (mL) for the i^{th} measurement,

${}^{1}C_{H2,\,i}$; the concentration of hydrogen gas (%) in the total gas for the i^{th} measurement,

$V_{G,\,i}$; the head space volume of the bottle for the i^{th} measurement,

${}^{2}C_{H2,\,i-1}$; the concentration of hydrogen gas (%) in the head space of the bottle after argon gas was passed through for the $i\text{-}1^{th}$ measurement.

Ideal gas law equation was used to calculate the moles of cumulative hydrogen. Equation;

$$PV = nRT \tag{2.2}$$

P ; pressure of the gas (1 atm),

V ; cumulative hydrogen gas volume (L),

n ; the mole of cumulative hydrogen gas volume (mol),

R ; the ideal, or universal, gas constant, 0.082 (L atm / mol K),

T ; temperature of the gas (Kelvins ; 273 K = 0.00 ºC).

The temperature of the hydrogen gas was considered as room temperature (25 ºC).

The moles of total sugar (glucose) consumed by microorganisms during fermentation was calculated via following equation:

$$n = \frac{(TS_f - TS_0) \times V_0}{MW_{C_6H_{12}O_6}} \tag{2.3}$$

n; the moles of total sugar consumed (mol),

TS_f; final total sugar concentrations of the fermentation medium (g/L)

TS_0; initial total sugar concentrations of the fermentation medium (g/L),

V_0; the initial fermentation volume (L)

$MW_{C_6H_{12}O_6}$; 180 g/mol

The yield of hydrogen gas production was calculated via equation below:

$$Y = \frac{\Delta H_2}{\Delta S} \tag{2.4}$$

Y; hydrogen gas yield (mol H_2/mol glucose)

$\Delta \mathbf{H_2}$; the moles of cumulative hydrogen gas volume (mol)

$\Delta \mathbf{S}$; the moles of total sugar (glucose) consumed (mol) calculated

In this thesis, cumulative hydrogen volume versus time data were correlated with Gompertz Equation. Constants were determined by regression analysis via STATISTICA. Gompertz Equation is shown below (Han & Shin, 2004).

$$H_{(t)} = P\, exp\left\{-exp\left[\left(\frac{R_m \times e}{P}\right)(\lambda - t) + 1\right]\right\} \quad (2.5)$$

$H_{(t)}$; Cumulative hydrogen gas volume (ml) at any time t,

P; the maximum potential hydrogen formation (ml),

R_m; the maximum rate of hydrogen formation, HPR (ml/h),

λ ; duration of the lag phase (h),

e; 2.718,

t; time (h).

2.2 Experiments

In this study, experiments were performed in three sets. The effects of different parameters on biohydrogen production from sugar beet were investigated during the experiments. The experiments were operated as batch dark fermentation experiments. The EX-FERM technique was used. And a control bottle was prepared for each set.

A cultivation media was prepared for the microorganisms. The cultivation media which was centrifuged at 8000 rpm for 15 minutes and subjected to total suspended solid (TSS) analysis was grown in the incubator. Microorganism concentration was determined by TSS analysis. TSS analysis is performed as follows; filter paper (Whatman GF/C) is kept for 24 hours at 105 ºC oven to remove all the moisture in it. Then it is cooled to room temperature in desicator for 2 hours and weighed. It is placed on a vaccum device, then certain amount of sample was put on the filter. When all the water was vaccumed by device, the filter is taken on a watch glass and waited at 105 ºC in an oven again for 24 hours. Then it is cooled to room temperature in desicator for 2 hours and weighed again.

The microorganism concentrations was calculated via following equation during experiments;

$$x = \frac{(\text{last weight of the filter paper} - \text{first weight of the filter paper})}{\text{The volume of filtered sample (mL)}} * 1000 \quad (2.6)$$

(x: microorganism concentration)

According to result of TSS analysis, sufficient amount of cultivation media was centrifuged. The supernatant was removed and the solid part was used as inoculum. The cultivation media which was explained was prepared again for each set.

At the first set, the effect of sugar beet amount (substrate concentration) was investigated. Sugar beet surface area and microorganism concentration were kept constant. At first, the nutrient media was prepared. TOC analysis result showed that

sugar beet contains 40% carbon. So it was estimated that 60 g sugar beet contains 24000 mg carbon. Thus, the optimum C/N/P ratio of 100/2/0.5 for microorganism was estimated as 24000/480/120 and provided by using 1.043 g/L urea as nitrogen source, 0.522 g/L KH_2PO_4 as phosphorus source, 0.05 g/L $MgSO_4$, 0.025 g/L $FeSO_4$ and 0.1 g/L L-cysteine. L-cysetine was an important ingredient. Because, its function was to provide anaerobic conditions for fermentation. Then this nutrient media mixture was completed to 1000 ml by adding distilled water.

The sugar beets were allowed to dry and warm to room temperature before chopping process. All the sugar beets had 0.54 cm^2 total surface area before the weighing process. This size was chosen in order to prevent inhibition effect of sugar consumption from solid sugar beet surface. The bottles were filled with nutrient media, water and sugar beet mixture according to sugar beet concentration. The nutrient media was prepared considering the carbon content of 60 g/L sugar beet. So 150 ml nutrient media was filled into bottle with 60 g/L sugar beet. It was calculated that 75 ml nutrient media was necessary for carbon content of 30 g/L sugar beet and it was completed to 150 ml by adding 75 ml distilled water. According to this calculation method all the experiment bottles were prepared by using 9 g, 6.75 g, 4.5 g, 2.25 g, 0.75 g sugar beets, nutrient media and distilled water. In addition to these bottles, a control bottle was prepared by using 0.75 g sugar beet, without microorganism. 3ml samples were taken from each bottles and centrifuged at 8000 rpm for 15 minutes for the initial sugar concentration measurement after 15 minutes. After all adjustments, microorganism addition was carried out. The microorganism concentrations were determined by total suspended solid analysis. The cultivation media was centrifuged and according to the results of TSS analysis, 1 g/L microorganism was placed on all the bottles as inoculum except the control bottle. Volatile fatty acid formation causes to decrease of pH value. The pH values were adjusted to range of 6.8 - 7.2 by using NaOH solution and the ORP values were controlled and confirmed that between -200 mV and -400 mV. Negative values of ORP indicate the anaerobic conditions during the experiments. After pH and ORP measurements, all the bottles were plugged by using valve. Out of the valves were covered. All the spaces of the valves and where the bottle and valves were connected were filled with cold silicone. When the silicone was completely dried and didn't

allow gas output, the argon gas was passed through the bottles in order to remove the oxygen inside. After all these, the bottles were ready for incubation. Incubator temperature was 37 ºC. The bottles were kept in incubator for 24 hours. Incubator provided dark media and optimum temperature for fermentation during the experiments. The total gas measurements, hydrogen gas measurements, residual sugar analysis, ORP measurements and pH adjustments were performed everyday. The gas output ended at the end of 7 days. So, the set was terminated. Total organic carbon analysis (TOC) and total volatile fatty acid analysis (TVFA) were performed for each bottle at the end of the set. TOC analysis was applied on remaining sugar beets in the bottles. According to H_2 gas production results, the highest result was obtained at 60 g/L sugar beet.

At the second set, the effect of microorganism concentration was investigated. According to results of the first set, 0.54 cm^2, 60 g/L sugar beet was used as substrate in this set. The same nutrient media was prepared as in the first set in order to provide optimum C/N/P ratio of 100/2/0.5. Then the sugar beets which were dried and warmed to room temperature, were chopped and weighed. Sugar beets were filled into bottles. The carbon content of 60 g sugar beet was considered, while preparing the nutrient media. 150 ml nutrient media was added in each bottle. Then, the cultivation media was centrifuged at 8000 rpm for 15 minutes. As explained above, 0.25 g/L, 0.5 g/L, 1 g/L, 1.5 g/L and 2 g/L microorganisms were obtained by calculating the concentration of microorganism according to the TSS analysis in this set. A control bottle was prepared by using 60 g/L sugar beet and 150 ml nutrient media without microorganism. After addition of all the ingredients, 3 ml samples were taken from all of the bottles and centrifuged at 8000 rpm for 15 minutes for initial sugar analysis. pH was adjusted to range of 6.8 - 7.2 by using 3M NaOH solution. ORP values were checked in order to confirm the anaerobic conditions and it was seen that ORP values were between -200 mV and -400 mV. The bottles were plugged by using valves. All the spaces and where the valve and bottle connected were covered with cold silicone to prevent gas output. When the silicone dried and hardened, argon gas was passed through the bottles to remove the oxygen. All the bottles were kept in incubator for 24 hours. ORP and pH monitored daily. Total gas measurements, H_2 gas measurements, residual sugar analysis and pH adjustments

were performed everyday. The gas output ended at the end of 7 days and the set was terminated. Total organic carbon analysis (TOC) and total volatile fatty acid analysis (TVFA) were carried out for each bottle at the end of the set. TOC analysis was applied on remaining sugar beets in the bottles. According to H_2 gas production results, the highest result was obtained at 1 g/L microorganism concentration.

At the third set, the effects of sugar beet surface area were investigated. According to results of the first and seconds sets, 60 g/L sugar beet and 1 g/L microorganism were used. The same nutrient media was prepared as in the first and second sets in order to provide optimum C/N/P ratio of 100/2/0.5 for microorganisms. Sugar beets which were dried and warmed to room temperature, were chopped in different sizes. There were five different surface areas. 0.06 cm^2, 0.54 cm^2, 1.5 cm^2, 2.94 cm^2 and 6 cm^2. Then 150 ml nutrient media was added in all the bottles. The cultivation media was centrifuged at 8000 rpm for 15 minutes and 1 g/L microorganism was added to bottles by calculating the concentration of microorganism according to the result of TSS analysis. A control bottle was prepared by adding 0.06 cm^2, 60 g/L sugar beet and 150 ml nutrient media without microorganism addition. After adding all the ingredients to each bottle, 3 ml samples were taken from all of the bottles and centrifuged at 8000 rpm for 15 minutes for initial sugar analysis. pH value was adjusted to range of 6.8 - 7.2 by using 3M NaOH solution. ORP measurements were carried out and confirmed that the values were between -200 mV and -400 mV. The bottles were plugged by using valves. All the spaces were filled and covered around the valves by using cold silicone. The spaces, where the bottle and valve connects, were covered also in order to prevent gas output from the bottles. When the cold silicone dried and hardened enough, argon gas was passed through. So oxygen was removed from all the bottles. The bottles were kept in incubator for 24 hours. ORP and pH values were monitored everyday. Total gas measurements, H_2 gas measurements, residual sugar analysis and pH adjustments were performed everyday. The gas output ended at the end of 7 days. The set was terminated. Total organic carbon analysis (TOC) and total volatile fatty acid analysis (TVFA) were carried out for each bottle at the end of the set. TOC analysis was applied on remaining sugar beets in the bottles.

According to hydrogen gas production results, the highest result was obtained at 0.06 cm^2 surface area.

CHAPTER THREE

RESULTS AND DISCUSSIONS

In this thesis study, experiments were carried out as three sets. At the first set, the effects of different sugar beet concentrations were investigated. After first set, according to the results, the effects of microorganism concentrations were investigated by using the sugar beet concentration which provided the highest hydrogen gas production. At the third set, the effects of sugar beet surface area were investigated by using the most efficient amount of sugar beet and microorganism concentration.

3.1 Effects of Substrate Concentration on Cumulative Hydrogen Gas Production

Effects of substrate concentration on cumulative hydrogen gas production were investigated at the first set and the results of experiments are shown in following graphics. Experiments were carried out in 250 ml serum bottles. There were six experiment bottles one of them was control bottle. 0.54 cm^2 sized 5 g/L, 15 g/L, 30 g/L, 45 g/L and 60 g/L sugar beets were used in this set. Microorganism concentrations were kept constant at 1 g/L at all the bottles.

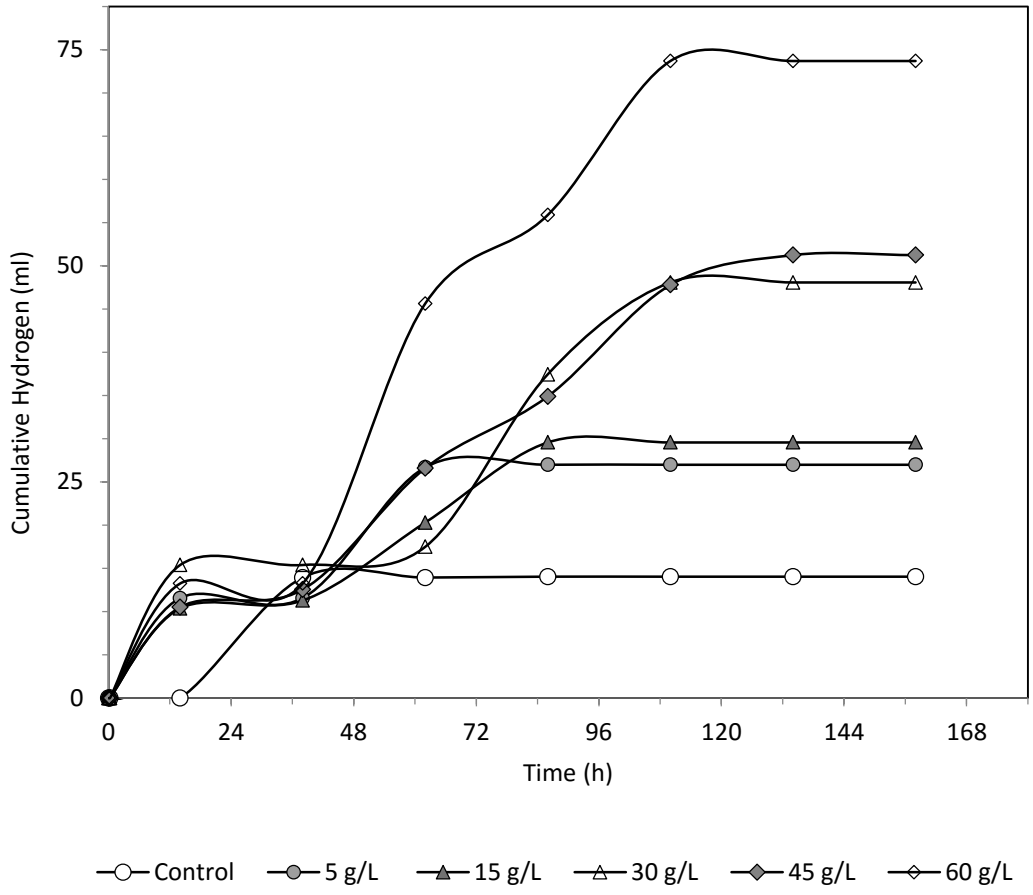


Figure 3.1 Variation of cumulative hydrogen gas volume depending on time at the first set.

The variation of the cumulative hydrogen gas volume from different concentration of sugar beet depending on time was shown in Figure 3.1.

According to the graphic, cumulative hydrogen gas volumes increased depending on time and terminated at 158 hours at all the bottles. The highest hydrogen production efficiency was obtained at 60 g/L sugar beet (73.68 ml). The lowest cumulative hydrogen production was obtained from 5 g/L sugar beet (26.98 ml). Insignificant hydrogen production in control bottle shows that the sterile conditions broke down. These results showed that hydrogen production increases by using high concentration of sugar beet. High concentrations provide more nutrient for microorganisms. Microorganisms can provide more hydrogen.

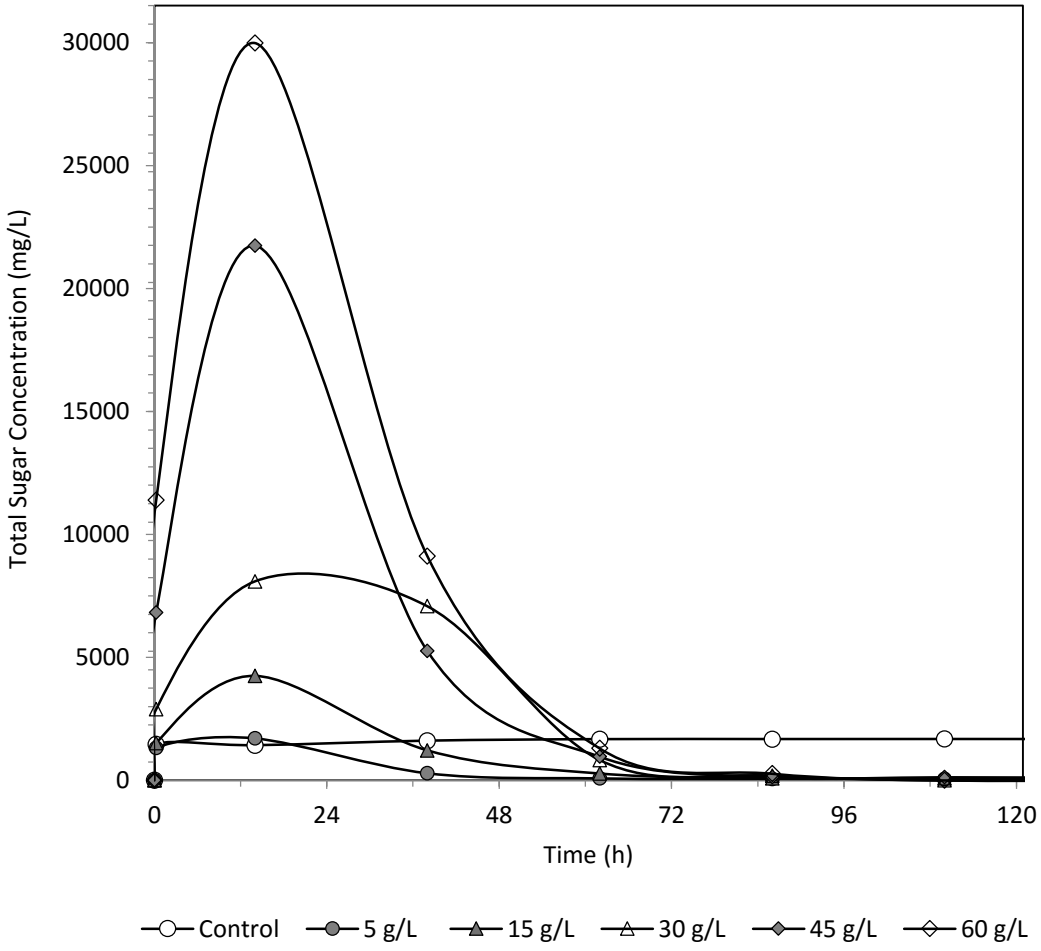


Figure 3.2 Variation of total sugar concentration depending on time at the first set.

Figure 3.2 shows variation of the total sugar concentrations in liquid phase depending on time. The sugar (sucrose) dissolved in the liquid and consumed by microorganisms also during the experiments. Microorganisms consume the sugar from the solid surface of the sugar beets at the same time. Microorganisms start to consume the sugar during sugar transfer. Firstly, sugar concentration increased, then sugar transfer slowed down, consumption rate increased and sugar concentrations decreased at the graphic. According to the graphic, the maximum sugar concentration levels in the liquid phase were reported between 12 - 24 hours for all the bottles. After 20 hours, decrease of the sugar transfer from solid sugar beet to liquid phase

and consumption of the sugar by microorganisms resulted in low sugar levels in the liquid phase.

Control bottle contains 5 g/L sugar beet, but maximum total sugar concentration is lower than the bottle containing 5 g/L sugar beet. This insignificant difference arise from the sugar which is in solid phase of sugar beet and the corruption of the sterile condition. The sugar concentration in control bottle is nearly stable. Because, there is no microorganism addition in bottle. The results overlap with cumulative hydrogen production results.

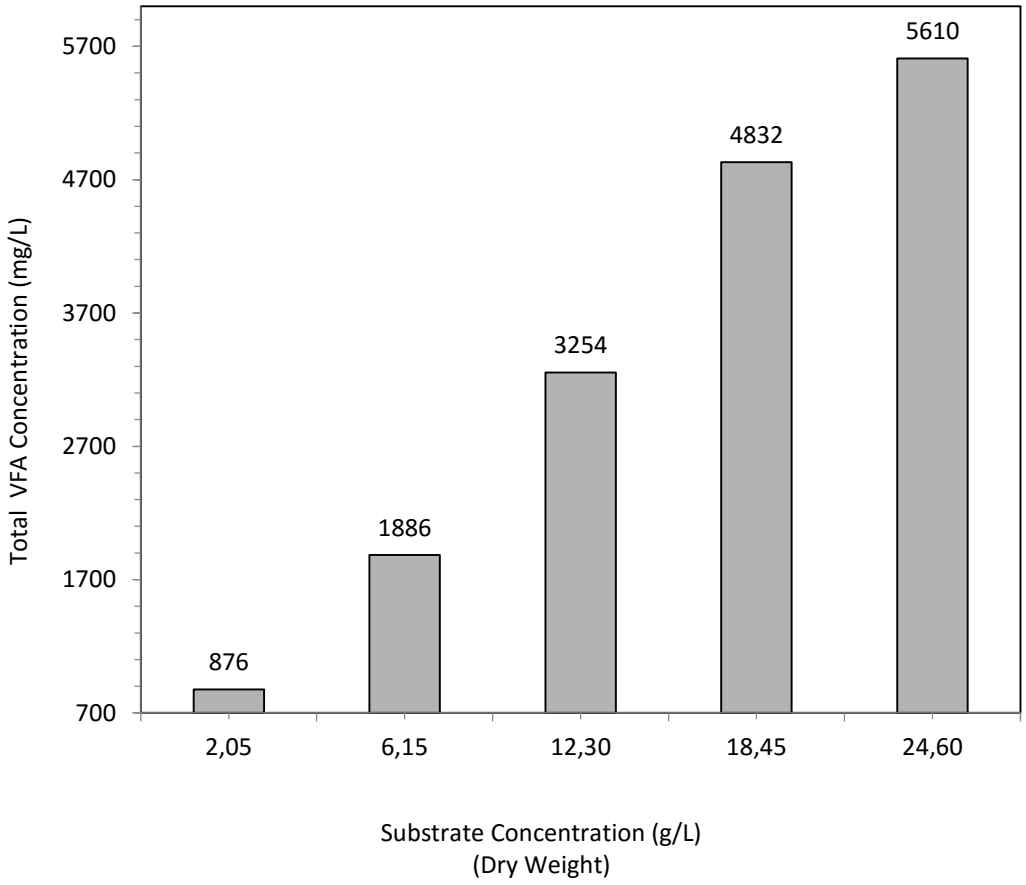


Figure 3.3 Total volatile fatty acid (TVFA) concentrations for various substrate concentrations at the end of 158 hours at the first set.

The sugar content of liquid phase is converted to volatile fatty acids, CO_2, biomass and H_2, during dark fermentation process. Hydrogen gas is one of by-product of volatile fatty acid formation. The final TVFA concentrations vary parallel with hydrogen production. High final TVFA concentrations show high cumulative hydrogen gas formation. TVFA production depends on active microorganism amount, initial organic substrate concentration and pH level of dark fermentation process. Organic acid types related with pH level of fermentation media. On the other hand, volatile fatty acid generation increases with existence of great deal of *Clostridum sp.* culture. CO_2 and H_2 (generated during dark fermentation) could be converted to acetic acids by homoasetogens without hydrogen gas production.

Final total volatile fatty acid (TVFA) concentrations in liquid phase at the end of 158 hours are shown in graphic above. According to graphic TVFA concentrations increase with increasing sugar beet concentration (dry weight). According to Figure 3.3 the cumulative hydrogen production results overlap with TVFA results. Maximum TVFA value is 5610 mg/L and obtained at 24.6 g/L (dry weight) sugar beet. Minimum TVFA value is 876 mg/L and obtained at 2.05 g/L (dry weight) sugar beet.

Table 3.1 Gompertz equation constants for various sugar beet concentrations

Sugar Beet Concentration (g/L)	**P(mL)**	**R_m (mL/h)**	**λ (h)**	**R^2**
5	22.53	18.11	1.78	0.84
15	31.20	0.38	0.99	0.98
30	56.95	0.44	3.61	0.97
45	57.77	0.50	9.11	0.99
60	78.61	0.93	16.36	0.99

Table 3.1 shows the Gompertz constants for various sugar beet concentrations which were calculated by STATISTICA program. The highest cumulative hydrogen gas was obtained from the 60 g/L of initial sugar beet concentration but the highest hydrogen production rate (R_m) was calculated as 18.11 ml/h while initial sugar beet concentration was 5 g/L. The hydrogen production potential increased with the increasing substrate concentration. The highest cumulative hydrogen production was obtained at 60 g/L concentration. The highest lag phase was obtained at 60 g/L sugar beet. This result shows that microorganism activity was inhibited by high sugar concentration.

3.2 Effects of Microorganism Concentrations on Cumulative Hydrogen Gas Production

Effects of microorganism concentrations on cumulative hydrogen gas production and the results of the third set were investigated in second set. For this aim, six bottles were prepared including a control bottle without microorganism addition. 0.25 g/L, 0.5 g/L, 1 g/L, 1.5 g/L and 2 g/L microorganisms were used by calculating the concentration of microorganism in this set. Experiments were conducted in 250 ml serum bottles. Effects of microorganism concentrations on cumulative hydrogen gas production and the results were explained in following graphics.

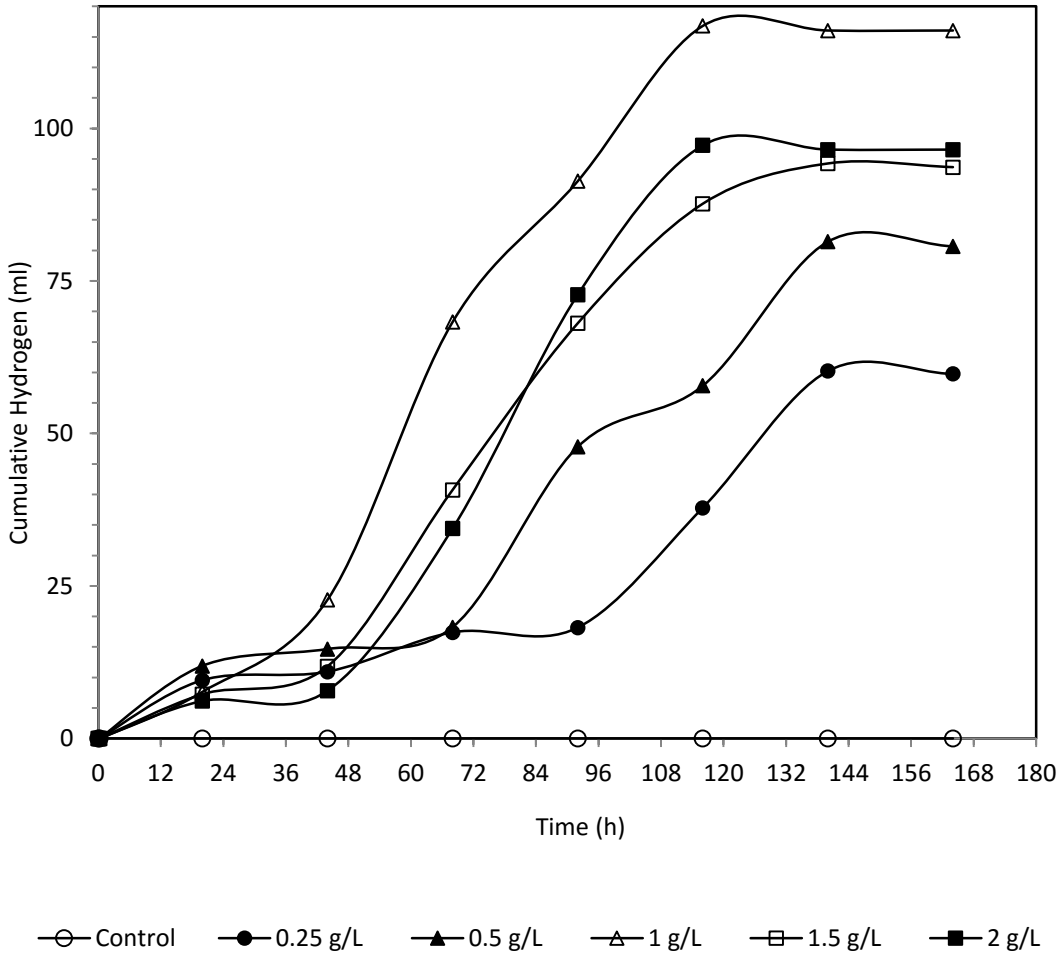


Figure 3.4 Variation of cumulative hydrogen production depending on time at the second set.

The change of the cumulative hydrogen gas volume from different concentrations of microorganism depending on time was shown in graphic above.

According to Figure 3.4, cumulative hydrogen gas volumes increased depending on time and terminated at the end of 164 hours at all the bottles. The highest hydrogen production efficiency was obtained from the bottle which contains 1 g/L microorganism (116.05 ml). The lowest cumulative hydrogen production was obtained from 0.25 g/L microorganism (59.76ml). There is no production at the control bottle. The results show that the optimum microorganism concentration is 1 g/L. Maximum TFVA formation was seen at 2 g/L microorganism containing bottle, excessive formation of TVFA showed inhibitory effect on hydrogen production.

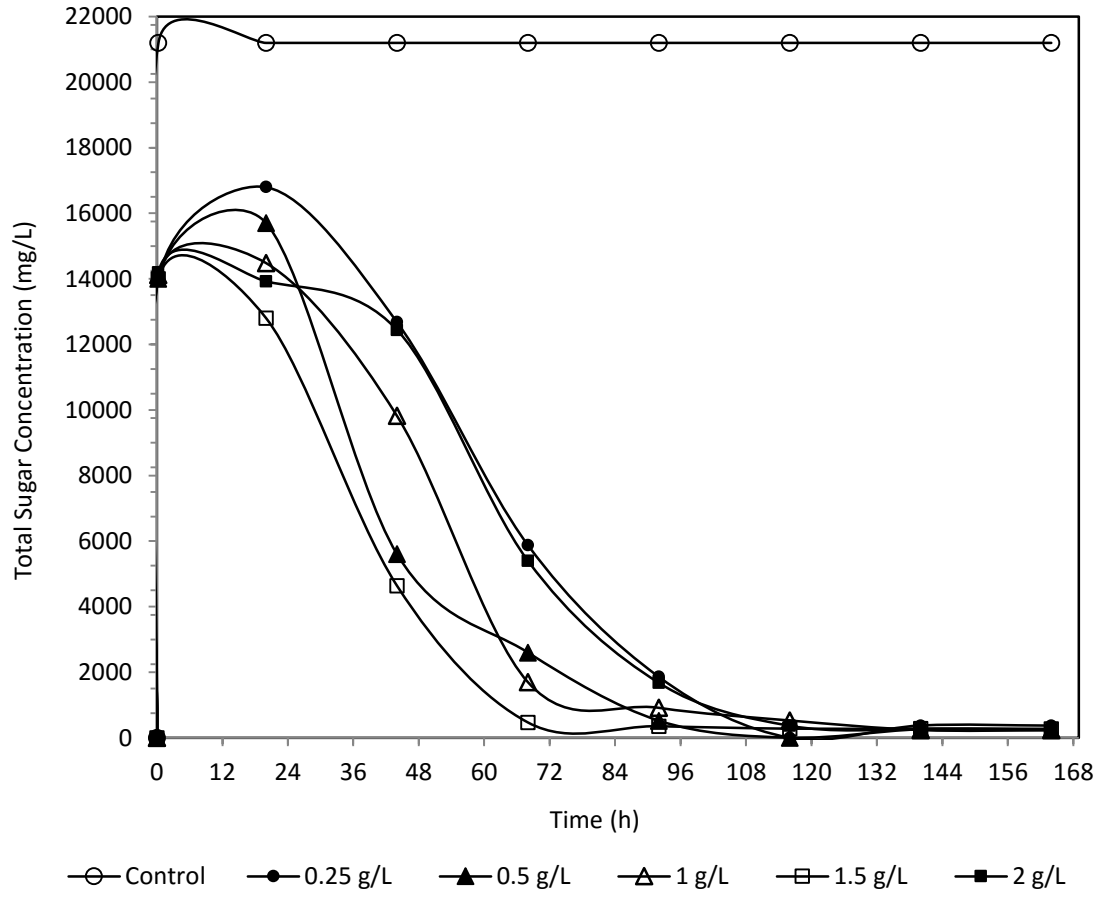


Figure 3.5 Variation of total sugar concentration depending on time at the first set.

Figure 3.5 shows the change of total sugar concentration in the liquid phase depending on time. According to the EX-FERM technique, while sugar concentration is increasing, sugar consumption from liquid phase by microorganism comes true simultaneously. Microorganisms consume the sugar from solid surface of the sugar beets also.

According to Figure 3.5, despite of the sugar consumption of microorganisms, the maximum sugar concentration levels in the liquid phase were reported between 12 - 24 hours for all the bottles. After 20 hours, decrease of the sugar transfer from solid sugar beet to liquid phase and consumption of the sugar by microorganisms resulted in low sugar levels in the liquid phase.

There is no significant change in control bottle. Because there is no microorganism and production in it. This results overlap with cumulative hydrogen production results. The total sugar analysis results are different for all bottles although the sugar beet concentrations are the same. This case arises from the consumption of sugar. The maximum sugar level (16800 mg/L) was obtained from 0.25 g/L microorganism. Low microorganism concentration causes low sugar consumption thus high sugar concentration. The maximum total sugar level is 14480 at the bottle containing 1 g/L microorganism. The maximum cumulative hydrogen gas production was obtained from this bottle. The total sugar concentrations at the graphic show the sugar levels in the liquid phase. These results show that microorganisms consume sugar from solid surface of sugar beet also.

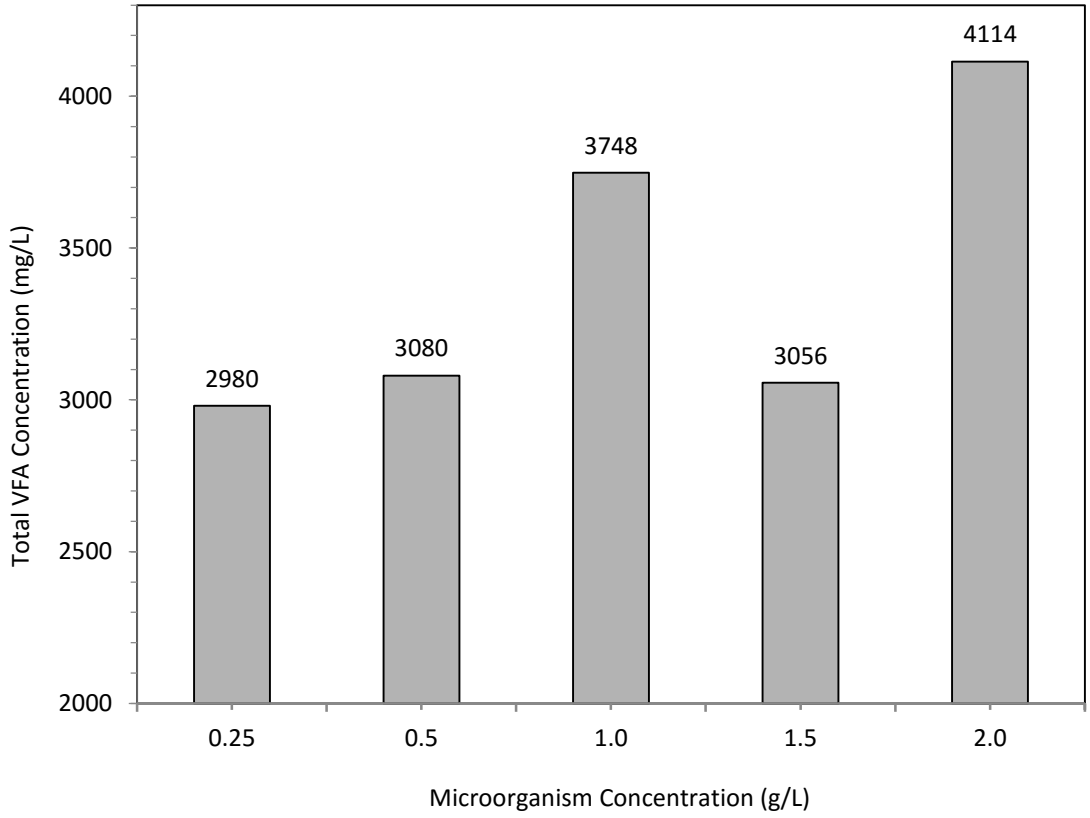


Figure 3.6 Total volatile fatty acid (TVFA) concentrations for various microorganism concentrations at the end of 164 hours at the second set.

Volatile fatty acid formation is one of results of H_2 production process. High hydrogen production results in high volatile fatty acid generation. According to Figure 3.6, minimum TVFA value is obtained from the bottle containing 0.25 g/L microorganism. Maximum TVFA value is obtained at 2 g/L microorganism. This result shows that TVFA formation showed inhibitory effect on hydrogen production. TVFA value at 1.5 g/L microorganism containing bottle, shows that microorganism consumed sugar and then consumed the volatile fatty acids.

Table 3.2 Gompertz equation constants for various microorganism concentrations.

Microorganism Concentration (g/L)	**P(mL)**	**R_m (mL/h)**	**λ (h)**	**R^2**
0.25	148.66	0.55	47.67	0.98
0.5	112.03	0.71	30.71	0.99
1	120.34	1.76	30.38	0.99
1.5	98.44	1.33	37.07	0.99
2	100.22	1.83	48.36	0.99

Table 3.2 shows the Gompertz constants for various microorganism concentrations which were calculated by STATISTICA program. Cumulative hydrogen production datas were correlated with Gompertz equation. The highest cumulative hydrogen gas was obtained at 1 g/L of initial biomass concentration and the highest hydrogen production rate was calculated as 1.83 ml/h when initial biomass concentration was 2 g/L. The results of potential hydrogen formation obtained at 1.5 g/L and 2 g/L of initial biomass concentration were almost same. The lag phase increased with increasing initial biomass concentration up to 0.5 g/L. Increases in biomass concentration resulted in lower hydrogen gas potential due to mass transfer limitation in flocks or consumption of hydrogen by homo acetogens.

3.3 Effects of Surface Area of Sugar Beets on Cumulative Hydrogen Gas Production

Surface area of substrate is one of important parameters that affecting hydrogen gas production. Surface area affects the sugar amount that dissolving in liquid and sugar consumption by microorganism. Effects of surface area on cumulative hydrogen gas production were investigated in the third set. For this aim, six bottles were prepared including a control bottle without microorganism addition. 0.06 cm^2, 0.54 cm^2, 1.5 cm^2, 2.94 cm^2, and 6 cm^2 surface areas were used in this set. Microorganism concentration (1 g/L) and sugar beet concentration (60 g/L) were stable in all the bottles. Experiments were conducted in 250 ml serum bottles. Effects of surface area of sugar beets on cumulative hydrogen gas production were explained in following graphics.

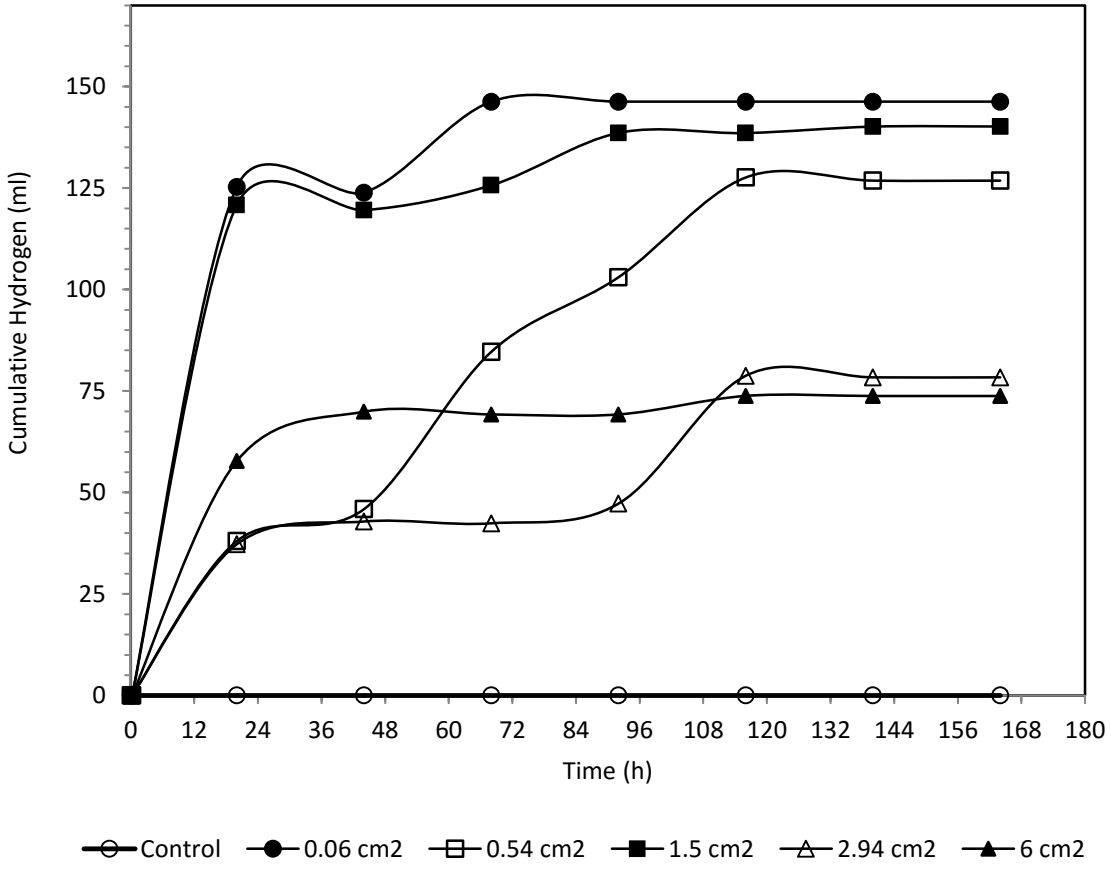


Figure 3.7 Variation of cumulative hydrogen production depending on time at the third set.

The variation of the cumulative hydrogen gas volume from different surface areas of sugar beet depending on time was shown Figure 3.7.

According to Figure 3.7, cumulative hydrogen gas volumes increased depending on time and terminated at the end of 168 hours. The highest hydrogen production efficiency was obtained from 0.06 cm^2 sugar beets (146.25 ml). The lowest cumulative hydrogen production was obtained from 6 cm^2 sugar beet containing bottle (73.75ml). There is no production in the control bottle. The results show that the optimum surface area for hydrogen production is 0.06 cm^2. Large surface area decreases the hydrogen production.

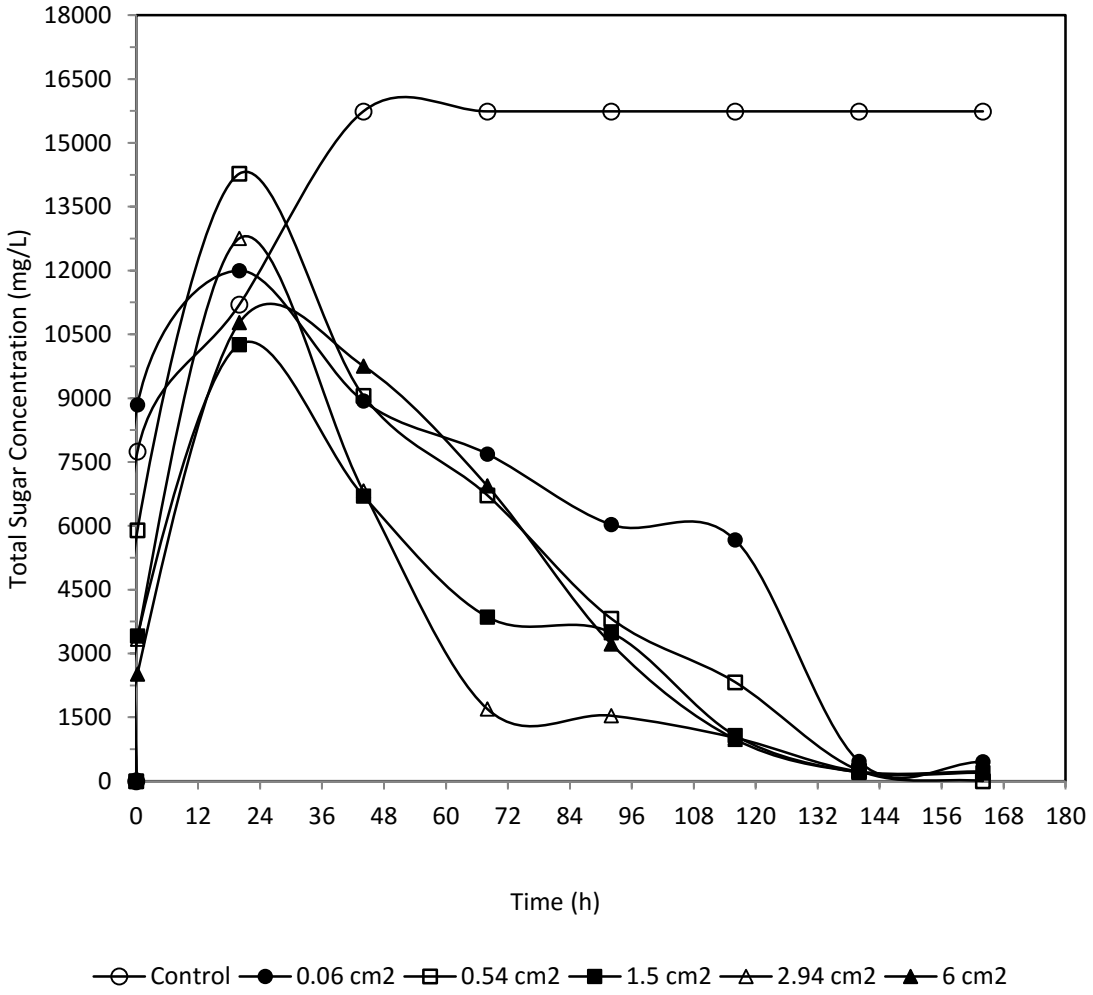


Figure 3.8 Variation of total sugar concentration depending on time at the third set.

Figure 3.8 shows the change of total sugar concentration in the liquid phase depending on time. According to Figure 3.8, the maximum sugar concentration levels in the liquid phase were reported between 18 - 20 hours. After 20 hours, decrease of the sugar transfer from solid sugar beet to liquid phase and consumption of the sugar by microorganisms resulted in low sugar levels in the liquid phase.

There is no significant change in control bottle. Because there is no microorganism and hydrogen production in the bottle. This result overlaps with cumulative hydrogen production results. The maximum total sugar level (14280 mg/L) was obtained from 0.54 cm^2 sugar beet. The minimum total sugar level was obtained from the bottle that contains 1.5 cm^2 sugar beet. The maximum and minimum sugar levels vary depending on transfer period of sugar to liquid. Maximum 12000 mg/L total sugar concentration was obtained from the bottle which contains 0.06 cm^2 sugar beet pieces. Because, there were a lot of surface for sugar transfer and this was advantageous condition for microorganisms. Microorganisms could reach and consumed the sugar easily. Thus, the high cumulative hydrogen production result was obtained at surface area of 0.06 cm^2.

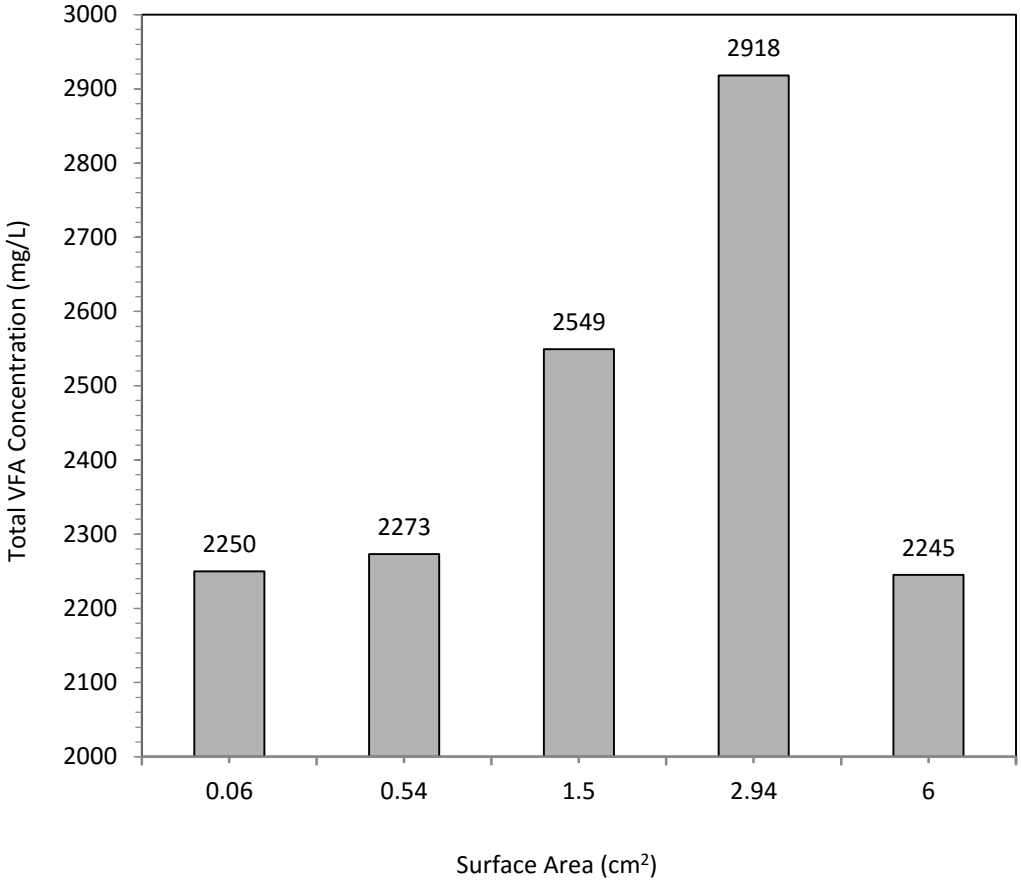


Figure 3.9 Total volatile fatty acid (TVFA) concentrations for various surface areas at the end of 158 hours at the third set.

Final TVFA concentrations are shown in Figure 3.9. The maximum TVFA concentration (2918 mg/L) was obtained from the bottle that contains 2.94 cm^2 sugar beet particles. Minimum TVFA concentration (2245 mg/L) was obtained from the bottle that contains 0.06 cm^2 sugar beet particles. The results show that volatile fatty acid formation showed inhibitory effect on hydrogen production at 1.5 cm^2 and 2.94 cm^2.

Table 3.3 Gompertz equation constants for various sugar beet surface areas.

Sugar Beet Surface Area (cm^2)	**P(mL)**	**R_m (mL/h)**	**λ (h)**	**R^2**
0.06	139.85	143.61	2.62	0.99
0.54	139.85	94.46	8.80	0.99
1.5	133.84	9.65	3.89	0.99
2.94	57.68	67.71	2.63	0.83
6	69.42	66.97	0.96	0.98

Table 3.3 shows the Gompertz constants for various sugar beet surface areas which were calculated by STATISTICA program. Cumulative hydrogen production datas were correlated with Gompertz equation. The highest hydrogen production potential values were calculated at 0.06 cm^2 and 0.54 cm^2 of surface area. The results of potential hydrogen formation obtained at 0.06 cm^2 and 0.54 cm^2 of sugar beet surface area were almost same. The maximum hydrogen formation rate was obtained from 0.06 cm^2 of sugar beet. The results show that microorganisms could reach to sugar readily into the sugar beet. The lowest lag phase was calculated as 0.96 h at 6 cm^2 of sugar beet. Lag phase decreases with increasing surface area except 0.06 cm^2. The smaller surface area provides high mass transfer. Therefore, high biohydrogen production can be obtained at smaller surface areas.

CHAPTER FOUR

CONCLUSIONS

Hydrogen gas is one of promising energy source because it's renewable and clean energy source with high energy content. But, the cost of production is an important negative factor for hydrogen production. Hydrogen production technologies must be improved. Thus, hydrogen production can be more competitive with the other clean resource. Biological methods are more advantageous than physical and chemical methods. H_2 gas can be produced by algae or bacteria as biologic.

Large amount of study shows that dark fermentation is one the preferable and advantageous way for biohydrogen production. The cost is lower than many other methods. It' doesn't require oxygen because of being anaerobic process and it doesn't require light too. Many species of biomass and substrate can be used in this method. Higher production rates can be obtained with dark fermentative production. The sources such as sugar beet and sugar cane are suitable for fermentation due to high amount of sugar content.

In this thesis, bio-hydrogen production from sugar beet via dark fermentation experiments were performed. Experiments were carried out as three sets. There were six bottles containing one control bottle in each set. A cultivation media was prepared for microorganism cultivation before each set. Pre-treatment was not applied to sugar beet. EX-FERM technique was used in the experiments. Extraction and fermentation processes occur simultaneously. While soluble sugar is passing to water, fermentation process is occurring in surface of sugar beet and liquid at the same time.

The effect of sugar beet concentration was investigated in the first set of experiments. Five different concentrations were prepared (control, 5 g/L, 15 g/L, 30 g/L, 45 g/L and 60 g/L). The control bottle contained 5g/L sugar beet. The microorganism concentration was kept constant at 1 g/L and sugar beet surface areas were kept constant at 0.54 cm^2. The highest hydrogen production efficiency was obtained from 60 g/L sugar beet containing bottle. The cumulative hydrogen gas

production is 73.68 ml. There was not observed significant hydrogen production in the control bottle. The cumulative hydrogen gas productions are 26.98 ml, 29.57 ml, 48.06 ml, 51.24 ml for 5 g/L, 15 g/L, 30 g/L and 45 g/L, respectively. The cumulative hydrogen production increase with increasing initial substrate concentration. The highest TVFA value (5610 mg/L) was obtained at 60 g/L of sugar beet. The lowest TVFA value (876 mg/L) was obtained at 5 g/L of sugar beet. The results are well matched with the results of cumulative hydrogen production. Because volatile fatty acids are by-product of hydrogen production. High TVFA production is a results of high hydrogen production. Gompertz constants were calculated by using Gompertz equation via Statistica program. The highest cumulative hydrogen gas was obtained from the 60 g/L of initial sugar beet concentration but the highest hydrogen production rate (R_m) was calculated as 18.11 ml/h while initial sugar beet concentration was 5 g/L. The hydrogen production potential increased with the increasing substrate concentration. The highest cumulative hydrogen production was obtained at 60 g/L concentration. The highest lag phase was obtained at 60 g/L sugar beet. This result shows that microorganism activity was inhibited by high sugar concentration.

In the second set, the effect of microorganism concentration was investigated by using five different microorganism (0.25 g/L, 0.5 g/L, 1 g/L, 1.5 g/L and 2 g/L). Control bottle did not contain microorganism. Sugar beet was kept constant at 60 g/L based on first set results. Sugar beet surface area was kept constant at 0.54 cm^2. The highest hydrogen production result is 116.05 ml and was obtained from 1 g/L of microorganism concentration. The results show that the concentrations up to 1 g/L, hydrogen gas production was affected due to mass transfer limitation between intersection of solid and liquid. The cumulative hydrogen gas production was obtained as 59.76 ml, 80.69 ml, 93.65 ml, 96.54 ml at 0.25 g/L, 0.5 g/L, 1.5 g/L and 2 g/L respectively. Maximum TVFA value is obtained at 2 g/L of microorganism concentration. On the other hand, the maximum hydrogen gas production was observed at 1 g/L of microorganism concentration. The result shows that high TVFA generation inhibits hydrogen production. Gompertz constants were calculated by STATISTICA program. The highest cumulative hydrogen gas was obtained at 1 g/L

of initial biomass concentration and the highest hydrogen production rate was calculated as 1.83 ml/h. The results of potential hydrogen formation obtained at 1.5 g/L and 2 g/L of initial biomass concentration were almost same. The lag phase increased with increasing initial biomass concentration up to 0.5 g/L. Increases in biomass concentration resulted in lower hydrogen gas potential due to mass transfer limitation in flocks or consumption of hydrogen by homo acetogens.

The effect of surface area of sugar beet was investigated by changing sugar beet size between 0.06 cm^2 and 6 cm^2. Sugar beet concentration was kept constant at 60 g/L and microorganism concentration was kept constant at 1 g/L based on previous experiments. The highest hydrogen production efficiency was 146.25 ml and obtained at the bottle containing 0.06 cm^2 sugar beet. Hydrogen gas formation was not observed at control bottle. The results show that the optimum sugar beet size for hydrogen production is 0.06 cm^2. Sugar transfer to liquid depends on surface area of particle. Mass transfer limitation can be avoid by providing high surface area.

TVFA value is 2245 mg/L was obtained at 0.06 cm^2 surface area. Maximum TVFA value was obtained as 2918 mg/L at 2.94 cm^2 sugar beet containing bottle. Gompertz constants were calculated by STATISTICA program. The highest hydrogen production potential values were calculated at 0.06 cm^2 and 0.54 cm^2 of surface area. The results of potential hydrogen formation obtained at 0.06 cm^2 and 0.54 cm^2 of sugar beet size were almost same. The maximum hydrogen formation rate was obtained from 0.06 cm^2 sized sugar beet. The results show that microorganisms could reach to sugar readily into the beet. The lowest lag phase was calculated as 0.96 h at 6 cm^2 of sugar beet. Lag phase decrease with increasing sugar beet size except 0.06 cm^2. The smaller surface area provides high mass transfer. Therefore, using smaller surface areas can be resulted in high biohydrogen production.

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APPENDICES

ABBREVIATIONS

C/N	Carbon to nitrogen ratio
CHF	Cumulative hydrogen gas formation
HPR	Hydrogen production rate
HRT	Hydraulic retention time
ORP	Oxidation reduction potential
Rm	Maximum hydrogen formation rate
TS	Total sugar
TVFA	Total volatile fatty acid
VFA	Volatile fatty acid

RAW EXPERIMENTAL DATA

A.1 Raw Data for Effects of Sugar Beet Concentration

Table A.1.1 Raw data for control serum bottle for first set.

Control 5 g/L Sugar Beet		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0	0.00	H0	0.00	150	165	0	V0	0.00	0	
0	0.25	3	6.9		1466	0.00	H1	0.00	147	168	0	V1	0.00	0.00	
1	14	3	6.9		1426	0.00	H2	0.00	144	171	5	V2	0.00	0.00	
2	38	3	6.9		1610	8.00	H3	0.08	141	174	0	V3	13.92	0.56	
3	62	3	6.9		1672	3.40	H4	0.03	138	177	0	V4	13.92	0.56	
4	86	3	6.9		1675	3.40	H5	0.03	135	180	0	V5	14.02	0.56	
5	110	3	6.9		1678	3.30	H6	0.03	132	183	0	V6	14.02	0.56	
6	134	3	6.9		1675	3.00	H7	0.03	129	186	0	V7	14.02	0.56	TOC (mg/kg)
7	158	3	6.9	1250	1676	2.00	H8	0.02	126	189	0	V8	14.02	0.56	546112

Table A.1.2 Raw data for 5 g/L sugar beet containing serum bottle.

5 g/L Sugar Beet		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0	0.00	H0	0.00	150	165	0	V0	0.00	0	
0	0.25	3	6.9		1318	0.00	H1	0.00	147	168	0	V1	0.00	0.00	
1	14	3	4.4		1705	6.38	H2	0.06	144	171	10	V2	11.55	0.46	
2	38	3	5.3		285	4.90	H3	0.05	141	174	45	V3	11.55	0.46	
3	62	3	6.7		77	13.00	H4	0.13	138	177	5	V4	26.68	1.07	
4	86	3	6.9		57	12.60	H5	0.13	135	180	5	V5	26.98	1.09	
5	110	3	6.9		60	11.00	H6	0.11	132	183	5	V6	26.98	1.09	
6	134	3	6.9		62	9.00	H7	0.09	129	186	5	V7	26.98	1.09	TOC (mg/kg)
7	158	3	6.9	876	59	8.00	H8	0.08	126	189	5	V8	26.98	1.09	497771

Table A.1.3 Raw data for 15 g/L sugar beet containing serum bottle.

15 g/L Sugar Beet		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0	0.00	H0	0.00	150	165	0	V0	0.00	0	
0	0.25	3	6.9		1518	0.00	H1	0.00	147	168	0	V1	0.00	0.00	
1	14	3	4.1		4245	5.72	H2	0.06	144	171	10	V2	10.35	0.42	
2	38	3	4.3		1215	5.00	H3	0.05	141	174	40	V3	11.27	0.45	
3	62	3	5.9		274	9.00	H4	0.09	138	177	20	V4	20.30	0.82	
4	86	3	6.9		78	14.00	H5	0.14	135	180	0	V5	29.57	1.19	
5	110	3	6.9		77	11.00	H6	0.11	132	183	0	V6	29.57	1.19	
6	134	3	6.9		79	9.00	H7	0.09	129	186	0	V7	29.57	1.19	TOC (mg/kg)
7	158	3	6.9	1886	81	8.00	H8	0.08	126	189	0	V8	29.57	1.19	507934

Table A.1.4 Raw data for 30 g/L sugar beet containing serum bottle.

30 g/L Sugar Beet		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0	0.00	H0	0.000	150	165	0	V0	0.00	0	
0	0.25	3	6.9		2892	0.00	H1	0.000	147	168	0	V1	0.00	0.00	
1	14	3	4.0		8090	8.50	H2	0.085	144	171	10	V2	15.39	0.62	
2	38	3	4.3		7080	6.40	H3	0.064	141	174	20	V3	15.39	0.62	
3	62	3	4.7		830	6.10	H4	0.061	138	177	40	V4	17.49	0.70	
4	86	3	6.7		193	15.00	H5	0.150	135	180	25	V5	37.44	1.51	
5	110	3	6.9		116	19.00	H6	0.190	132	183	15	V6	48.06	1.93	
6	134	3	6.9		113	16.00	H7	0.160	129	186	10	V7	48.06	1.93	TOC (mg/kg)
7	158	3	6.9	3254	111	12.00	H8	0.120	126	189	0	V8	48.06	1.93	489819

Table A.1.5 Raw data for 45 g/L sugar beet containing serum bottle.

45 g/L Sugar Beet		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (saat)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0	0.00	H0	0.00	150	165	0	V0	0.00	0	
0	0.25	3	6.9		6830	0.00	H1	0.00	147	168	0	V1	0.00	0.00	
1	14	3	4.0		21740	5.80	H2	0.06	144	171	10	V2	10.50	0.42	
2	38	3	4.1		5260	5.00	H3	0.05	141	174	65	V3	12.53	0.50	
3	62	3	5.4		964	9.60	H4	0.10	138	177	60	V4	26.58	1.07	
4	86	3	6.9		135	11.00	H5	0.11	135	180	50	V5	34.89	1.40	
5	110	3	6.9		130	15.00	H6	0.15	132	183	35	V6	47.79	1.92	
6	134	3	6.9		115	15.00	H7	0.15	129	186	20	V7	51.24	2.06	TOC (mg/kg)
7	158	3	6.9	4832	116	10.00	H8	0.10	126	189	0	V8	51.24	2.06	497345

Table A.1.6 Raw data for 60 g/L sugar beet containing serum bottle.

60 g/L Sugar Beet		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0	0.00	H0	0.00	150	165	0	V0	0.00	0	
0	0.25	3	6.9		11396	0.00	H1	0.00	147	168	0	V1	0.00	0.00	
1	14	3	3.9		29980	7.32	H2	0.07	144	171	10	V2	13.25	0.53	
2	38	3	4.0		9120	5.70	H3	0.06	141	174	40	V3	13.25	0.53	
3	62	3	4.7		1300	15.00	H4	0.15	138	177	105	V4	45.63	1.84	
4	86	3	6.9		272	16.00	H5	0.16	135	180	50	V5	55.88	2.25	
5	110	3	6.9		173	20.00	H6	0.20	132	183	50	V6	73.68	2.97	
6	134	3	6.9		151	15.00	H7	0.15	129	186	25	V7	73.68	2.97	TOC (mg/kg)
7	158	3	6.9	5610	140	12.00	H8	0.12	126	189	25	V8	73.68	2.97	481049

A.2 Raw Data for Effects of Microorganism Concentration

Table A.2.1 Raw data for control serum bottle for second set.

Control 0.25 g/L Microorganism		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0.0	0.00	H0	0.00	150	165	0	V0	0.00	0.00	
0	0.25	3	6.9		21200	0.00	H0	0.00	147	168	0	V0	0.00	0.00	
1	20	3	6.9		21200	0.00	H1	0.00	144	171	0	V1	0.00	0.00	
2	44	3	6.9		21200	0.00	H2	0.00	141	174	0	V2	0.00	0.00	
3	68	3	6.9		21200	0.00	H3	0.00	138	177	0	V3	0.00	0.00	
4	92	3	6.9		21200	0.00	H4	0.00	135	180	0	V4	0.00	0.00	
5	116	3	6.9		21200	0.00	H5	0.00	132	183	0	V5	0.00	0.00	
6	140	3	6.9		21200	0.00	H6	0.00	129	186	0	V6	0.00	0.00	TOC (mg/kg)
7	164	3	6.9	1845	21200	0.00	H7	0.00	126	189	0	V7	0.00	0.00	488676

Table A.2.2 Raw data for 0.25 g/L microorganism contining serum bottle.

0.25 g/L Microorganism		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0.0	0.00	H0	0.000	150	165	0	V0	0.00	0.00	
0	0.25	3	6.9		13988	0.00	H0	0.00	147	168	0	V0	0.00	0.00	
1	20	3	3.9		16800	5.00	H1	0.050	144	171	20	V1	9.55	0.38	
2	44	3	4.5		12680	5.00	H2	0.050	141	174	25	V2	10.95	0.44	
3	68	3	5.1		5879	5.10	H3	0.051	138	177	120	V3	17.40	0.70	
4	92	3	6.9		1860	3.70	H4	0.037	135	180	85	V4	18.18	0.73	
5	116	3	6.9		1013.5	10.00	H5	0.100	132	183	80	V5	37.82	1.52	
6	140	3	6.9		373	15.60	H6	0.156	129	186	75	V6	60.23	2.42	TOC (mg/kg)
7	164	3	6.9	2980	370	16.00	H7	0.160	126	189	50	V7	59.76	2.41	397269

Table A.2.3 Raw data for 0.5 g/L microorganism containing serum bottle.

0.5 g/L Microorganism		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0.0	0.00	H0	0.000	150	165	0	V0	0.00	0.00	
0	0.25	3	6.9		14000	0.00	H0	0.000	147	168	0	V0	0.00	0.00	
1	20	3	3.9		15700	5.92	H1	0.059	144	171	30	V1	11.90	0.48	
2	44	3	4.7		5600	3.31	H2	0.033	141	174	215	V2	14.65	0.59	
3	68	3	5.0		2600	3.64	H3	0.036	138	177	80	V3	18.25	0.73	
4	92	3	6.1		520	15.00	H4	0.150	135	180	60	V4	47.80	1.92	
5	116	3	6.9		262.5	16.60	H5	0.166	132	183	40	V5	57.82	2.33	
6	140	3	6.9		256	25.00	H6	0.250	129	186	30	V6	81.44	3.28	TOC (mg/kg)
7	164	3	6.9	3080	249	20.00	H7	0.200	126	189	10	V7	80.69	3.25	486418

Table A.2.4 Raw data for 1 g/L microorganism containing serum bottle.

1 g/L Microorganism		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0.0	0.00	H0	0.00	150	165	0	V0	0.00	0.00	
0	0.25	3	6.9		14120	0.00	H0	0.00	147	168	0	V0	0.00	0.00	
1	20	3	4.0		14480	4.00	H1	0.04	144	171	20	V1	7.64	0.31	
2	44	3	4.3		9820	9.00	H2	0.09	141	174	70	V2	22.76	0.92	
3	68	3	4.9		1700	19.00	H3	0.19	138	177	145	V3	68.28	2.75	
4	92	3	6.0		916	21.00	H4	0.21	135	180	90	V4	91.35	3.68	
5	116	3	6.9		530	25.00	H5	0.25	132	183	70	V5	116.8	4.70	
6	140	3	6.9		238	18.00	H6	0.18	129	186	20	V6	116.05	4.67	TOC (mg/kg)
7	164	3	6.9	3748	230	14.00	H7	0.14	126	189	20	V7	116.05	4.67	434207

Table A.2.5 Raw data for 1.5 g/L microorganism containing serum bottle.

1.5 g/L Microorganism		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0.0	0.00	H0	0.000	150	165	0	V0	0.00	0.00	
0	0.25	3	6.9		14000	0.00	H0	0.000	147	168	0	V0	0.00	0.00	
1	20	3	3.7		12800	3.80	H1	0.038	144	171	20	V1	7.26	0.29	
2	44	3	4.4		4636	3.90	H2	0.039	141	174	110	V2	11.84	0.48	
3	68	3	5.3		465	10.00	H3	0.100	138	177	180	V3	40.75	1.64	
4	92	3	6.4		350	15.00	H4	0.150	135	180	120	V4	68.05	2.74	
5	116	3	6.9		275	20.00	H5	0.200	132	183	50	V5	87.65	3.53	
6	140	3	6.9		278.4	20.00	H6	0.200	129	186	30	V6	94.25	3.79	TOC (mg/kg)
7	164	3	6.9	3056	270	10.00	H7	0.100	126	189	10	V7	93.65	3.77	383256

Table A.2.6 Raw data for 2 g/L microorganism containing serum bottle.

2 g/L Microorganism		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0.0	0.00	H0	0.000	150	165	0	V0	0.00	0.00	
0	0.25	3	6.9		14200	0.00	H0	0.000	147	168	0	V0	0.00	0.00	
1	20	3	3.3		13920	3.40	H1	0.034	144	171	10	V1	6.15	0.25	
2	44	3	4.3		12440	3.50	H2	0.035	141	174	40	V2	7.83	0.32	
3	68	3	4.8		5400	10.00	H3	0.100	138	177	150	V3	34.44	1.39	
4	92	3	6.0		1680	20.00	H4	0.200	135	180	100	V4	72.74	2.93	
5	116	3	6.9		3690	23.00	H5	0.230	132	183	80	V5	97.23	3.91	
6	140	3	6.9		248.5	18.00	H6	0.180	129	186	40	V6	96.54	3.89	TOC (mg/kg)
7	164	3	6.9	4114	241.4	11.00	H7	0.110	126	189	15	V7	96.54	3.89	417828

A.3 Raw Data for Effects of Size of Sugar Beet

Table A.3.1 Raw data for control serum bottle for third set.

Control 0.06 cm^2		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0.0	0.00	H0	0.00	150	165	0	V0	0.00	0.00	
0	0.25	3	6.9		7750	0.00	H0	0.00	147	168	0	V0	0.00	0.00	
1	20	3	6.9		11200	0.00	H1	0.00	144	171	0	V1	0.00	0.00	
2	44	3	6.9		15740	0.00	H2	0.00	141	174	0	V2	0.00	0.00	
3	68	3	6.9		15740	0.00	H3	0.00	138	177	0	V3	0.00	0.00	
4	92	3	6.9		15740	0.00	H4	0.00	135	180	0	V4	0.00	0.00	
5	116	3	6.9		15740	0.00	H5	0.00	132	183	0	V5	0.00	0.00	
6	140	3	6.9		15740	0.00	H6	0.00	129	186	0	V6	0.00	0.00	TOC (mg/kg)
7	164	3	6.9	1024	15740	0.00	H7	0.00	126	189	0	V7	0.00	0.00	516149

Table A.3.2 Raw data for 0.06 cm^2 sized sugar beet containing serum bottle.

0.06 cm^2 Sugar Beet		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/l)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0.0	0.00	H0	0.00	150	165	0	V0	0.00	0.00	
0	0.25	3	6.9		8844	0.00	H0	0.00	147	168	0	V0	0.00	0.00	
1	20	3	3.3		12000	48.00	H1	0.48	144	171	90	V1	125.28	5.04	
2	44	3	4.3		8934	27.00	H2	0.27	141	174	60	V2	123.84	4.98	
3	68	3	4.8		7691	27.00	H3	0.27	138	177	80	V3	146.25	5.89	
4	92	3	6		6030	14.00	H4	0.14	135	180	70	V4	146.25	5.89	
5	116	3	6.9		5672	20.00	H5	0.20	132	183	30	V5	146.25	5.89	
6	140	3	6.9		463	15.00	H6	0.15	129	186	25	V6	146.25	5.89	TOC (mg/kg)
7	164	3	6.9	2250	458	14.00	H7	0.14	126	189	20	V7	146.25	5.89	422814

Table A.3.3 Raw data for 0.54 cm^2 sized sugar beet containing serum bottle.

0.54 cm^2 Sugar Beet		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0.0	0.00	H0	0.00	150	165	0	V0	0.00	0.00	
0	0.25	3	6.9		5898	0.00	H0	0.00	147	168	0	V0	0.00	0.00	
1	20	3	3.7		14280	21.00	H1	0.21	144	171	10	V1	38.01	1.53	
2	44	3	4.4		9060	20.00	H2	0.20	141	174	45	V2	45.90	1.85	
3	68	3	5.3		6720	19.00	H3	0.19	138	177	210	V3	84.63	3.41	
4	92	3	6.4		3820	20.00	H4	0.20	135	180	80	V4	103.00	4.15	
5	116	3	6.9		2327	26.00	H5	0.26	132	183	50	V5	127.58	5.13	
6	140	3	6.9		248	20.00	H6	0.20	129	186	20	V6	126.80	5.10	TOC (mg/kg)
7	164	3	6.9	2273	240	17.00	H7	0.17	126	189	10	V7	126.80	5.10	436137

Table A.3.4 Raw data for 1.5 cm^2 sized sugar beet containing serum bottle.

1.5 cm^2 Sugar Beet		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0.0	0.00	H0	0.00	150	165	0	V0	0.00	0.00	
0	0.25	3	6.9		3414	0.00	H0	0.00	147	168	0	V0	0.00	0.00	
1	20	3	4		10260	43.00	H1	0.43	144	171	110	V1	120.83	4.86	
2	44	3	4.3		6700	22.00	H2	0.22	141	174	70	V2	119.54	4.81	
3	68	3	4.9		3860	16.00	H3	0.20	138	177	45	V3	125.66	5.06	
4	92	3	6.0		3490	23.00	H4	0.23	135	180	30	V4	138.56	5.58	
5	116	3	6.5		1074	20.00	H5	0.20	132	183	20	V5	138.53	5.58	
6	140	3	6.9		208	19.00	H6	0.90	129	186	15	V6	140.12	5.64	TOC (mg/kg)
7	164	3	6.9	2549	200	10.00	H7	0.10	126	189	10	V7	140.15	5.64	447208

Table A.3.5 Raw data for 2.94 cm^2 sized sugar beet containing serum bottle.

2.94 cm^2 Sugar Beet		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0.0	0.00	H0	0.00	150	165	0	V0	0.00	0.00	
0	0.25	3	6.9		3340	0.00	H0	0.00	147	168	0	V0	0.00	0.00	
1	20	3	3.9		12760	19.00	H1	0.19	144	171	25	V1	37.24	1.50	
2	44	3	4.7		6820	15.00	H2	0.15	141	174	80	V2	42.85	1.72	
3	68	3	5.0		1700	10.00	H3	0.10	138	177	70	V3	42.40	1.71	
4	92	3	6.1		1540	11.00	H4	0.11	135	180	25	V4	47.25	1.90	
5	116	3	6.7		1021	23.00	H5	0.23	132	183	40	V5	78.74	3.17	
6	140	3	6.9		229	6.60	H6	0.07	129	186	10	V6	78.35	3.15	TOC (mg/kg)
7	164	3	6.9	2918	230	5.00	H7	0.05	126	189	5	V7	78.35	3.15	465406

Table A.3.6 Raw data for 6 cm^2 sized sugar beet containing serum bottle.

6 cm^2 Sugar Beet		Sample Volume	Measurements				Hydrogen		V_{liquid}	V_{space}	$V_{t,gas}$	Cumulative Hydrogen		Cumulative Hydrogen	
Day	t (hour)	(ml)	pH	TVFA (mg/L)	Sugar (mg/L)	H_2 %			(ml)	(ml)	(ml)	(ml)		(mmol)	
0	0	0	6.9		0.0	0.00	H0	0.00	150	165	0	V0	0.00	0.00	
0	0.25	3	6.9		2522	0.00	H0	0.00	147	168	0	V0	0.00	0.00	
1	20	3	3.9		10780	23.00	H1	0.23	144	171	80	V1	57.73	2.32	
2	44	3	4.5		9760	22.00	H2	0.22	141	174	60	V2	69.88	2.81	
3	68	3	5.1		6940	14.00	H3	0.14	138	177	55	V3	69.22	2.79	
4	92	3	6.9		3230	11.00	H4	0.11	135	180	40	V4	69.22	2.79	
5	116	3	6.9		986	12.00	H5	0.12	132	183	20	V5	73.78	2.97	
6	140	3	6.9		211	10.00	H6	0.10	129	186	15	V6	73.75	2.97	TOC (mg/kg)
7	164	3	6.9	2245	213	9.00	H7	0.09	126	189	10	V7	73.75	2.97	442764