DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

BIOHYDROGEN GAS PRODUCTION FROM WASTE PAPER

by
Meltem SARP

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İZMİR

BIOHYDROGEN GAS PRODUCTION FROM WASTE PAPER

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by

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M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"BIOHYDROGEN GAS PRODUCTION FROM WASTE PAPER"** completed by **MELTEM SARP** under supervision of **ASSIST. PROF. DR. 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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BIOHYDROGEN GAS PRODUCTION FROM WASTE PAPER

ABSTRACT

Biological hydrogen production is considered as alternative technology for future. However, studies on hydrogen production via biological process, yield and formation rates of hydrogen gas are low. Therefore, the main fields of researches are to improve biohydrogen gas production by using different materials as substrate, recently.

The objective of this thesis to investigate hydrogen gas production potential from waste paper by dark fermentation and to find optimum substrate concentration and optimum microorganism concentration for hydrogen production from waste paper. For this purpose, biohydrogen gas production studies were carried out with using different substrate concentrations and different microorganisms concentrations. Heat treated anaerobic waste sludge was used as mixed microbial culture. Waste paper which was hydrolyzed in dilute acid at high temperature was used as substrate for this study. Fermentation process was carried on at 37 celsius degree in incubator. Total gas production, percentage hydrogen in total gas, hydrogen gas volume and sugar concentrations were monitored daily throughout the experiments. Total volatile fatty acid analysis was performed at the end of experiments. Hydrogen production rate, stationary phase and hydrogen formation potential were calculated by applying Gompertz equation on the cumulative hydrogen gas production datas. Calculations were performed with Statistica program.

In the end of experiments, highest cumulative volume of hydrogen gas concentration was obtained as 125.89 mL in the end of 120 hours with 20 gram per liter initial substrate concentration and 1 gram per liter initial biomass concentration. The sugar concentration above 20 gram per liter cause substrate and product inhibition. Maximum hydrogen yield was observed in 5 gram per liter total sugar concentration as 139.58 mL hydrogen per gram total sugar. Hydrogen yield

decreased with increasing initial sugar concentration due to inhibition of VFA and presence of furan derivatives resulting acid hydrolysis of waste paper.

Keywords: Biohydrogen, waste paper, dark fermentation, hydrolyze

ATIK KAĞITTAN BİYOHİDROJEN GAZI ÜRETİMİ

ÖZ

Biyolojik hidrojen üretimi gelecek için alternatif bir teknoloji olarak kabul edilmektedir. Ancak, biyolojik yöntemler ile hidrojen üretimi çalışmalarında, hidrojen gazı verimleri ve oluşum oranları düşüktür. Bu nedenle, son yıllarda substrat olarak farklı malzemeler kullanılarak biyohidrojen gazı üretimini geliştirmek amacı ile araştırmalar yapılmaktadır.

Bu tez çalışmasının amacı; atık kağıtlardan karanlık fermentasyon yolu ile hidrojen üretim potansiyelinin araştırılması ve atık kağıtlardan hidrojen üretimi için en uygun substrat konsantrasyonu ve mikroorganizma konsantrasyonunun belirlenmesidir. Bu amaçla farklı substrat konsantrasyonları farklı mikroorganizma konsantrasyonları kullanılarak biyohidrojen gazı üretimi çalışmaları yürütülmüştür. Karışık mikroorganizma kültürü olarak ısıl işlem görmüş anaerobik atık çamur kullanılmıştır. Bu çalışmada, yüksek sıcaklıkta seyreltilmiş asit ile hidrolize edilen atık kağıt substrat olarak kullanılmıştır. Fermentasyon işlemi 37 santigrat derecede inkübatörde yürütülmüştür. Toplam gaz üretimi, toplam gazda yüzde hidrojen miktarı, hidrojen gazı hacmi ve şeker konsantrasyonları deneyler boyunca günlük olarak izlenmiştir. Deneylerin sonunda toplam uçucu yağ asidi analizi gerçekleştirilmiştir. Hidrojen üretim hızı, durağan faz ve hidrojen oluşum potansiyeli, kümülatif hidrojen gazı üretimi verileri üzerinde Gompertz eşitliği uygulanarak hesaplanmıştır. Hesaplamalar “Statistica” programı ile yapılmıştır.

Deneylerin sonunda, en yüksek toplam hidrojen konsantrasyonu hacmi 125,89 mL olarak 120 saat sonunda litrede 20 gram ilk substrat konsantrasyonu ve litrede 1 gram mikroorganizma konsantrasyonu ile elde edilmiştir. Litrede 20 gram üzerindeki şeker konsantrasyonu, substrat ve ürün inhibisyonuna neden olmuştur. Maksimum hidrojen verimi, litrede 5 gram toplam şeker konsantrasyonunda her 1 gram toplam şeker için 139,58 mL hidrojen oluştuğu gözlenmiştir. Hidrojen verimi, uçucu yağ asiti

inhibisyonu ve atık kağıdın hidrolizi sonucunda elde edilen furan türevleri nedeniyle başlangıç şeker konsantrasyonun artması ile azalmıştır.

Anahtar kelimeler: Biyohidrojen, atık kağıt, karanlık fermentasyon, hidroliz

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CHAPTER ONE
INTRODUCTION

1.1 Biohydrogen Production via Dark Fermentation

The worldwide energy needs have been increasing exponentially. The reserves of fossil fuels have been decreasing. The combustion of fossil fuels has serious negative effects on the environment because of the greenhouse gas emissions. (Main greenhouse gases are carbon dioxide, methane, nitrous oxide, fluorinated gases). For these reasons, many researchers have been working on the exploration of new sustainable energy sources that could substitute fossil fuels. Hydrogen is considered as a viable alternative fuel of future. Because hydrogen is a clean fuel with high energy content of 122 kJ/g and can easily be used in fuel cells. Unavailability of hydrogen as a fuel in nature is a major problem. Therefore, inexpensive and environmental friendly hydrogen production method should be developed (Kapdan & Kargi, 2006).

Researchers are developing a wide range of technologies to produce hydrogen. Suresh et al., (2013) studied about comprehensive analysis and historical datas about hydrogen. According to their study almost the complete hydrogen production was based on fossil fuels. The details of the total hydrogen production is as follows: 49 % natural gas, 29 % liquid hydrocarbons, 18 % coal and 4 % electrolysis and others.

The main production technologies within hydrogen production systems are shown in Figure 1.1:

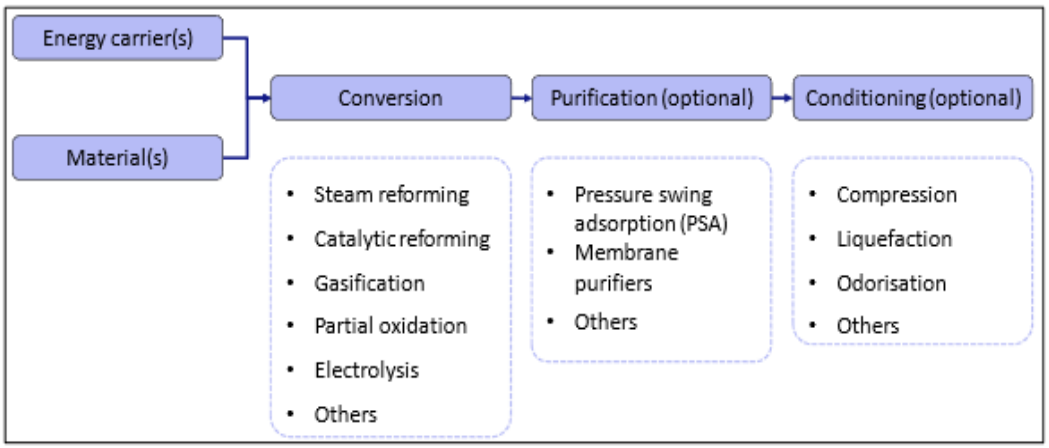

Figure 1.1 Different pathways of hydrogen production (Lazanovski et al., 2011)

In most of hydrogen production methods, certain amount of fuel is used. In hydrogen production via steam reforming method, water and natural gas are used under the high temperatures, slightly increased pressure and a catalyst. Catalytic reforming is only carried out in oil refineries where hydrocarbon molecules are arranged to produce specialised high-octane fuel. Partial oxidation is a chemical reaction where only part of the fuel is oxidised due to the fact that the fuel-air mixture is sub-stoichiometric. It can be done with almost all kinds of hydrocarbons. In electrolysis method, water is split into hydrogen and oxygen with electric current. After the hydrogen is produced in the conversion step, often it undergoes a purification step. The use of conversion and purification is determined mainly by the intended end use of the hydrogen (Lozanovski et al., 2011).

However these hydrogen production methods are not completely friendly for the environment. Because, these processes are based on fossil fuel and high energy consumption. The petroleum reserves of the world are depleting. Therefore, instead of fossil fuel origin hydrogen production methods, biological hydrogen production methods should be developed and used instead.

Generally, hydrogen production via biological methods are carried out at ambient temperature and pressure, hence less energy-more intense than chemical or electrochemical ones. Bio hydrogen can be produced by many different taxonomic

and physiological types of microorganisms. Recently various biological processes are used for bio hydrogen production. Among the various processes comprising the biological production of hydrogen, direct bio photolysis, indirect bio photolysis, dark fermentation, photo fermentation and hybrid reactor system are important.

Table 1.1 shows different biological hydrogen production processes with their advantages:

Table 1.1 Different biological hydrogen production processes with their advantages (Nath & Das, 2004a)

Process	General reactions and broad classification of microorganisms used	Advantages
Direct biophotolysis		
	$2\ H_2O + light = 2\ H_2 + O_2$ Micro-alge	**(i)** Can produce H_2 directly from water and sunlight **(ii)** Solar conversion energy increased by 10–folds as trees, crops
Indirect biophotolysis		
	$6\ H_2O + 6\ CO_2 + light = C_6H_{12}O_6 + 6\ O_2$ $C_6H_{12}O_6 + 2\ H_2O = 4\ H_2 + 2\ CH_3COOH + 2\ CO_2$ $2\ CH_3COOH + 4\ H_2O + light = 8\ H_2 + 4\ CO_2$ Overall reaction: $12\ H_2O + light = 12\ H_2 + 6\ O_2$ Microalgae, cyanobacteria	**(i)** Can produce H_2 from water **(ii)** Has the ability to fix N_2 from atmosphere
Dark fermentation		
	$C_6H_{12}O_6 + 6\ H_2O = 12\ H_2 + 6\ CO_2$ Fermentative bacteria	**(i)** It can produce H_2 all day long without light **(ii)** A variety of carbon sources can be used as substrates. **(iii)** It produces valuable metabolites such as butyric, lactic, and acetic acids as by products **(iv)** It is anaerobic process, so there is no O_2 limitation problem
Photo-fermentation		
	$CH_3COOH + 2\ H_2O + light = 4\ H_2 + 2\ CO_2$ Purple bacteria, Microalgae	**(i)** A wide spectral light energy can be used by these bacteria. **(ii)** Can use different waste materials like distillery effluents waste, etc.
Hybrid reactor system (Combined dark and photo-fermentation)		
	Stage 1 $C_6H_{12}O_6 + 2\ H_2O = 4\ H_2 + 2\ CH_3COOH + 2\ CO_2$ **Stage 2** $CH_3COOH + 2\ H_2O + light = 4\ H_2 + 2\ CO_2$ Fermentative bacteria followed by anoxygenic phototrophic bacteria (PNS)	Two stage fermentation can improve the overall yield of hydrogen.

Direct biophotolysis is carried out dissociation the water to hydrogen and oxygen molecules under sun light and in the presence of the micro algae. It uses the same process as plants and algae photosynthesis. But, it adapts them for the hydrogen gas production, instead of carbon containing biomass. Green algae possess enzymatic system, genetic system, metabolic system, and electron transport system for hydrogen gas production. Algae synthesize and activate enzymes in anaerobic incubation in the dark. One of the important enzyme to hydrogen production is hydrogenase and hydrogenase activity is extremely oxygen sensitive. This is the major bottleneck of direct biophotolysis. Direct biophotolysis requires entire production area to be enclosed in photobioreactor, which is able to both produce and capture H_2 and O_2. But it's not economically and not practical because of required to large volumes and large area to H_2/O_2 mixtures. Another limitations of direct biophotolysis are low light conversion efficiency and gas separation. Although direct biophotolysis is an attractive hydrogen production process in theory, when these limitations are considered, it should be developed further (Nath & Das, 2004a).

Hydrogen production process via direct biophotolysis is shown in Figure 1.2:

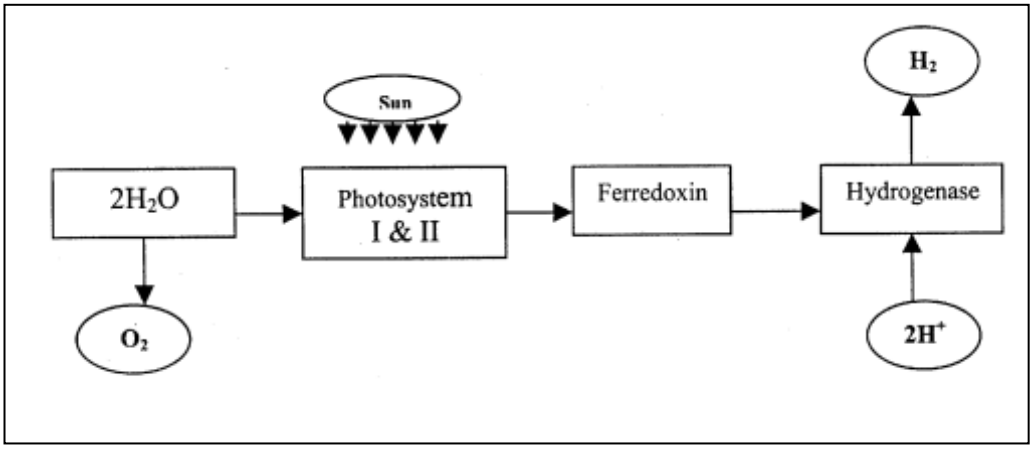

Figure 1.2 Direct biophotolysis (Nath & Das, 2004a)

In indirect biophotolysis; photosystem II uses the sunlight energy in photosynthesis for extract electrons from water molecules.Electrons released upon the oxidation of water are transported to the Fe-S protein ferredoxin on the reducing side of photosystem I. The hydrogenase, in the algae chloroplast stroma, accepts

electrons from reduced ferredoxin and donates them to two protons to generate one H_2 molecule. Some of the important factors of biophotolysis are, the need of light for hydrogen evolution, nitrogenase, oxygen sensitivity, and lower hydrogen evolution vs acetylene reduction. The cultivation of cyanobacteria in nitrate-free media under air and CO_2, followed by incubation in light under argon and CO_2, atmosphere, has rapidly become standard, since it results in immediate hydrogen production. Localization of nitrogenase in heterocysts provides an oxygen-free environment and the ability of heterocystous cyanobacteria to fix nitrogen in air. Hydrogenase and nitrogenase inhibitors are used in an attempt to screen for aerobic hydrogen evolution potential. It has been observed that these inhibitors allow for hydrogen to be released from aerobic cultures in amounts similar to those in argon (Nath & Das, 2004a).

The limitation of indirect biophotolysis is oxygen sensitivity of the hydrogen-evolving enzyme systems.

Hydrogen production via indirect biophotolysis process is shown in Figure 1.3:

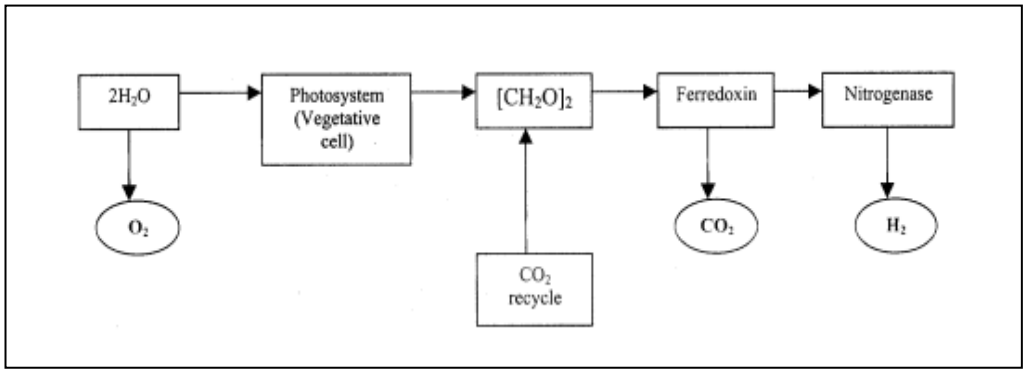

Figure 1.3 Indirect biophotolysis (Nath & Das, 2004a)

Photofermentation is carried out by photosynthetic bacteria. Because photosynthetic bacteria possess capacity to produce hydrogen through the action of their nitrogenase system. Photosynthetic bacteria breaks down organic substrats to produce hydrogen under illumination and in inert anaerobic atmosphere. Various organic acids and carbohydrates can be used as the substrate. Under different light sources, hydrogen production efficiency of photosynthetic bacteria varies. Producing

hydrogen by photosynthetic bacteria via photofermentation possess some advantages. The advantages of the hydrogen production via photofermentation by photosynthetic bacteria is as follows: High theoretical conversion yields, lack of O_2 evolving activity, ability to use wide spectral light energy and ability to consume organic substrates derived from wastes in association with wastewater treatment (Hallenbeck & Benemann, 2002; Nath & Das, 2004a;).

The main bottleneck of photobiological hydrogen production system is, large surface area is needed to collect light. Construction of a photobioreactor with a large surface/volume ratio for direct absorption of sunlight is expensive (Nath & Das, 2004a).

Hydrogen production by photosynthetic bacteria process is shown in Figure 1.4:

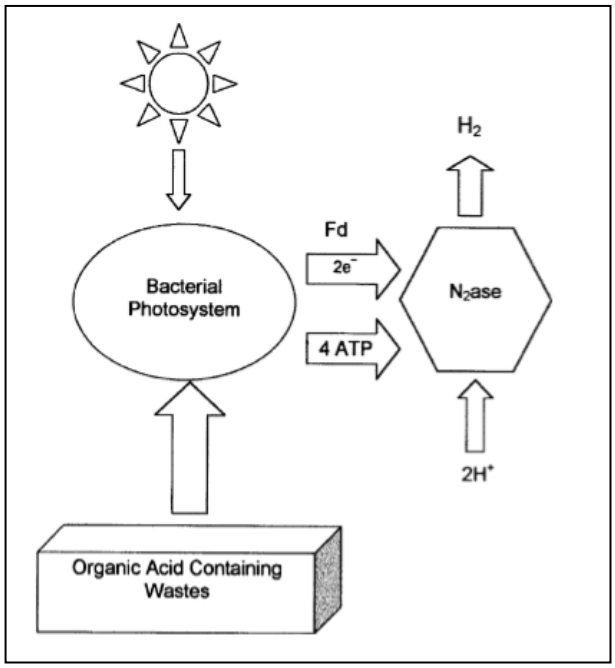

Figure 1.4 Schematic of hydrogen production by photosynthetic bacteria (Hallenbeck & Benemann, 2002)

Dark fermentation carried out in anoxic or anaerobic conditions. Carbohydrates are used for fermentation process. Mainly glucose is preferred as carbon source. Because, it give rise to acetic and butyric acids together with hydrogen. In hydrogen

production via fermentation process, pyruvate, the product of glucose catabolism, is oxidized to acetyl-CoA. Acetyl-CoA is converted to acetyl phosphate and results in the generation of ATP and the excretion of acetate. Pyruvate oxidation to acetyl-CoA requires ferredoxin (Fd) reduction. Reduced Fd is oxidized by hydrogenase which generates Fd and releases electrons as molecular hydrogen (Hallenbeck & Benemann, 2002; Nath & Das, 2004a).

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd(ox)} \rightarrow \text{Acetyl} - \text{CoA} + 2\text{Fd(red)} + CO_2 \qquad (1.1)$$

$$2\text{Fd(red)} \rightarrow 2\text{Fd(ox)} + H_2 \qquad (1.2)$$

The main end products of the dark fermentative hydrogen production are acetate and butyrate. Although acetate is the desired end product for high yield of formation, butyrate could be the dominant organic acid or more abundant in the organic acid mixture depending on fermantation conditions. Theoretically, when the end-product is acetic acid, 4 mol H_2 can be produced per 1 mol glucose consumed. But, when butyric acid is the end-product, 2 mol of H_2 per mole of glucose is obtained. (Bartacek et al., 2007).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \Delta G^o = -206 \text{ kJ} \qquad (1.3)$$

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2\,CH_2CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad (1.4)$$

Propionate is an end product lower the production of hydrogen. Propionic acid formation consumes 1 mol of H_2 per mole of propionic acid formed (Argun & Kargi, 2011). The other factors that causes decreasing in hydrogen generation is the presence of homoasetogens. These organisms consume hydrogen for acetic acid production. Therefore, the theoretical yield of 4 mole H_2/ mole glucose can not be realized in practice (Argun et al., 2009).

Figure 1.5 shows the conversion of renewable biomass into hydrogen via fermentation.

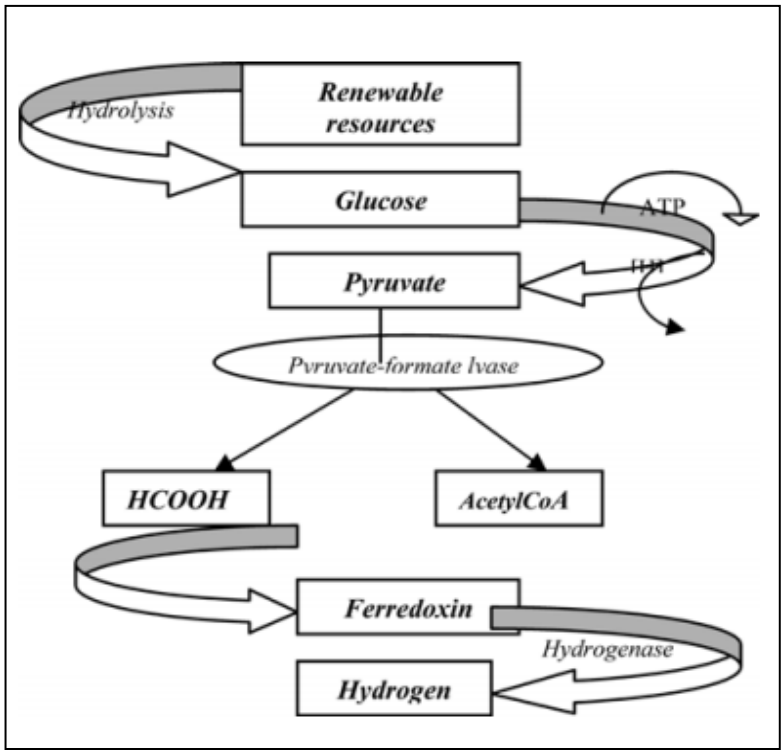

Figure 1.5 Schematic pathway for conversion of renewables to hydrogen via fermentation (Nath & Das, 2004b)

Dark fermentation is relatively simple process for hydrogen production. Because, it can be carried out by a wide variety of microorganisms and can be used wide variety substrates, including refuse and waste products. Moreover, fermentative hydrogen production generally proceeds at a higher rate and does not rely on the availability of light sources (Classen et al., 1999; Nath & Das, 2004a).

The idea of hybrid reactor system is based on combine to dark and photofermentation systems. Thus, organic acids and CO_2 which are produced in dark fermentation, can be converted to hydrogen by photosynthetic bacteria under light via photo fermentation. This increases the yield of hydrogen production. The limitation of this system is light collection as in photofermentation (Nath & Das, 2004a).

Within these varieties of biological hydrogen production processes, dark fermentation is very effective method. It can be operated and produce hydrogen continuously without the need for light. In dark fermentation, there is no O_2

limitation problem, because it is anaerobic process. Studies show that hydrogen production using the dark fermentation process, has a higher stability and better efficiency than other biological hydrogen production methods. The dark fermentation process has less operational cost and simple control. Other advantage of the dark fermentation process is, various organic sources and various microorganisms can be used as substrate in an effective way. And also it produces valuable metabolites such as butyric, lactic and acetic acids as by products.

In this thesis due to the many advantages of the dark fermentation process, dark fermentation method was chosen for hydrogen production.

There are variety microorganisms for hydrogen production. These microorganisms are divided into 3 group: anaerobes (Clostridia, methylotrophs, rumen bacteria, Methanogenic bacteria, archaea), facultative anaerobes (Escherichia coli, Enterobacter, Citrobacter) and aerobes (Alcaligenes, Bacillus) (Bergey et al., 1994; Nandi & Sengupta, 1998).

Hydrogen can be produced from pure microorganism cultures and mixed microorganism cultures. Mixed cultere is more advantageous than pure culture. Because, pure microorganism cultures can easily be contaminated with hydrogen consuming bacteria (Liu, 2008). In addition, mixed microorganism cultures can be easily obtained from waste water treatment plant or solid wastes.

Some environmental impacts are important for microorganisms activity. And microorganism activities affect the fermentation efficiency. The most environmental impacts for fermentation efficiency are temperature, pH, organic loading rate (OLR), hydraulic retention time (HRT) and Carbon/Nitrogen ratio.

Temperature is one of the important parameter for hydrogen production via fermentation. Temperature has effects on hydrogen production rate and hydrogen production efficiency. Increasing of temperature generally improve hydrogen production rate. However, sudden increase and sudden fall in temperature has

negative impact on hydrogen production. Sudden increase in temperature impedes hydrogen production and sudden fall in temperature decreases the hydrogen concentration. There isn't exact optimum temperature for hydrogen production by dark fermentation. Because, there are several kind of microorganisms can utilized in dark fermentation (Mohammadi et al., 2012; Wang & Wan, 2009). However, hydrogen producing by dark fermentation can be operated in microorganisms can categorized in four main groups: mesophilic, thermophilic, extreme thermophilic and hyperthermophilic. Optimum temperatures of mesophilic, thermophilic, extreme thermophilic and hyperthermophilic are 25-40°C, 40-65°C, 65-80°C, >80°C respectively (Levin et al., 2004).

The extreme thermophilic process is more advantageous than mesophilic and thermophilic processes. Extreme-thermophilic conditions have much higher hydrogen production rate and hydrogen production yield. At exteme-thermophilic condition (70°C), hydrogen yield reached the theoretical maximum of 4 mole hydrogen per mole glucose, where the ones at mesophilic and thermophilic conditions were normally less than 2 mole hydrogen per mole glucose (Van Niel et al., 2002). Also, high temperature minimizes the contamination by hydrogen consumers such as methanogens, solventogens (Levin et al., 2004).

pH is the key environmental factor for hydrogen production via dark fermentation. pH level has an effect on enzyme activity in microorganisms. It directly affects the hydrogenase metabolism (especially acidogens) and their activities. The initial pH of substrate is special importance in fermentative hydrogen processes as it directly affects the hydrogen yield. Researchers made extensive study to find the optimum pH on the hydrogen production via dark fermentation. They found different results under different environmental conditions and substrate types. Thus, there is no exact agreement on the optimum pH in fermentative hydrogen production (Mohammadi et al., 2012). Most of the published reports found the maximum hydrogen yield to be in the range of pH 5.5–6.0 (Chong et al., 2009; Fang & Liu, 2000; Tang et al., 2008; Wang & Wan, 2009).

Organic loading rate is one of the most significant parameters. It extensively studied to investigate the effects of various substrate loadings. Organic matter is utilized by microorganisms to produce hydrogen and it is measured by OLR (g/(Lday)) which represents the magnitude of chemical oxygen demand (COD) (g/L) fed within a certain period. OLR range is important to obtain optimal microbial growth and hydrogen production. However, the higher OLR does not necessarily lead to higher hydrogen yield. Optimum OLR depend of the reactor configuration, types of wastewater, inoculums, range of OLR applied, temperature, pH, etc. Therefore, there is no definitive optimum OLR in fermentative hydrogen production. For instance, Lin et al.,(2009) found the maximum hydrogen yield at an optimum OLR 120 g COD/(Lday), while Ozmihci & Kargi, (2011) reported 1.93 g COD/(Lday) as the optimum OLR (Mohammadi et al., 2012).

Hydraulic retention time (HRT) is the time period for a specific volume of liquid to be retained in the working volume of the reactor, and is in inverse relation to food to microorganisms ratio (F/M). Therefore, too long or too short HRT will result in unfavorable metabolic activities of the microorganisms. In dark fermentation processes without sludge pre-treatment and pH adjustment, for instance where OLRs are high and/or HRTs are low, the methanogenic activities will be inhibited due to the higher acidogenic rate. A wide range of HRT from 0.25 to 60 h was investigated with various organic substrates such as glucose, sucrose, starch, and in a few cases with organics available in real wastewaters. And researchers found different optimum HRT for their study. Optimum HRT depend on types of microorganisms and substrats, design of reactors, and other environmental factors. Therefore, there is no exact optimum HRT. For instance, Kim et al., (2008) found the maximum hydrogen yield at optimum HRT 36 h, while Show et al. reported 0.5 has the optimum HRT (Mohammadi et al., 2012).

The carbon/nitrogen (C/N) ratio is also important factor for dark fermentation process. C/N ratio is related to microbial growth and their activity. The optimum C/N value is important for improve microorganisms activities and hydrogen production. With regards to inoculum and substrate sources, the optimum C/N ratio value in a

particular anaerobic hydrogen process is expected to vary among different systems. Thus, there is no exact optimum C/N ratio for fermentative hydrogen production (Mohammadi et al., 2012).

Biomass is a viable renewable resource which includes agricultural residues, energy crops and industrial waste. This can be used for the production of power, heat and biofuels. In particular, energy crops can be converted into biofuels either as solid bio-feedstock via thermochemical routes or as carbohydrate substrates for biological conversion via fermentation processes. The use of microorganisms for biological hydrogen production via fermentation of plant carbohydrates has been recently attracting attention.

In dark fermentation, microrganisms need high carbon containing substrate for their metabolism, growth and to produce biogas such as hydrogen. In this case, pure sugars such as glucose, sucrose and lactose can be used as substrate, easily. Because, microorganisms can use pure sugar directly for their metabolic activities. But, the use of pure sugar is not economically convenient. Also, pure sugar can be used as food source. Therefore, if pure sugar will be used as a substrate, it will be lead of food to fuel competition. This causes, instead of pure carbonhydrates, the high organic content, biodegradable and easily and economically available biomasses should be used as the substrate.

Researchers have widely investigated to find new substrates for fermentative hydrogen production. They have studied on different kinds high organic containing, carbohydrate-rich materials. Also, starch containing and cellulosic food and agricultural industry wastes and waste waters, sewage sludge can be preferred. Because, the use of waste materials as substrate to fermentative hydrogen production is environmentally friendly and economically way. Therefore, researchers have studied on a lot of kind of carbohydrate-rich materials. Some of these carbohydrate-rich materials; sewage sludge, barley straw, corn stalks, sugar beet, wheat, sugar beet pulp, sweet corn, algae, cheese whey, olive mill, bakers yeast. These carbonhydrate-rich materials constitute substrates for first generation bio-fuels. The main issue for

first generation bio fuels, is the use of land for fuel. The land usage is important for the cost of food commodities and food security. Due to limitation of the raw materials, developments of second-generation biofuels are necessary. Second generation biofuels are made from lignocellulosic materials, which needs pre-treatment applications to use as substrate. Lignocellulosic materials are good substrate for hydrogen production via dark fermentation. Because, lignocellulosic materials are high carbon containing materials and also they are most abundant renewable biological resource. It constitutes a major portion of agricultural and forest wastes and industrial effluents such as pulp/paper and etc.

Lignocellulosic materials are composed of carbohydrate polymers (cellulose, hemicellulose), and an aromatic polymer (lignin). These carbohydrate polymers contain different sugar monomers (six and five carbon sugars) and they are tightly bound to lignin. The fermentable sugar is locked in lignin, cellulose and hemicellulose. The problem is to extract useful sugar content. Extracted sugars can then be fermented to hydrogen in the same way as first generation biofuel production.

Figure 1.6 shows that scheme of biohydrogen gas production from agricultural wastes containing starch or cellulose and wastewater.

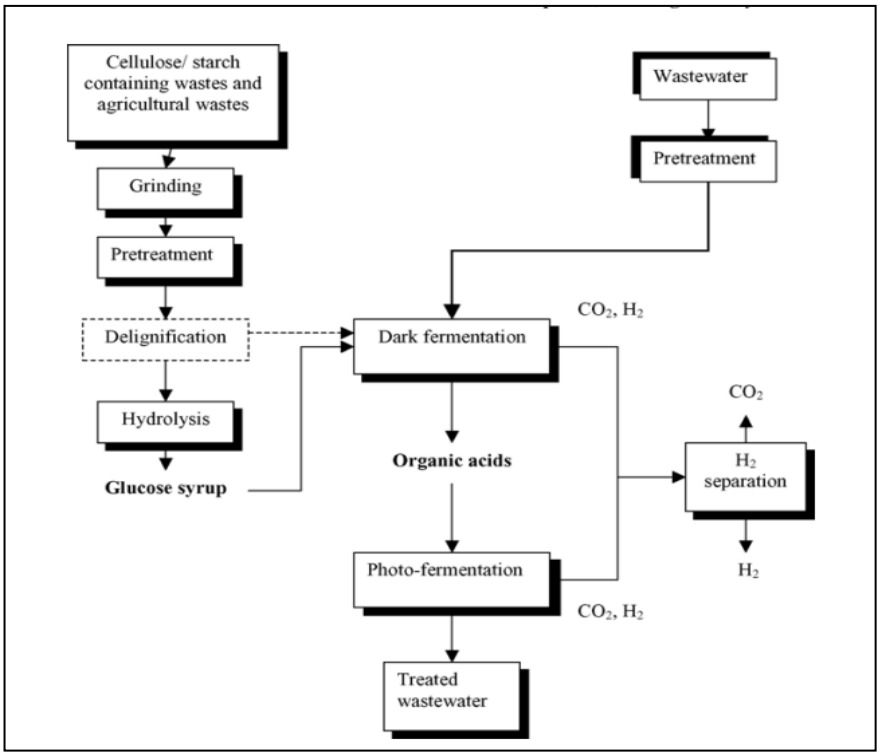

Figure 1.6 Scheme of biohydrogen gas production from agricultural wastes containing starch or cellulose and wastewater (Kapdan & Kargı, 2006)

1.2 Literature Review

Various kind of carbohydrate-rich materials have been investigated for fermentative hydrogen production. Also researchers have invastigated hydrogen production via dark fermentation under different environmental conditions.

Panagiotopoulos et al., (2009; 2010); Ma et al., (2011); Ozmihci & Kargi, (2011); Ferreira et al., (2013) and Kim et al., (2013) studied on first generation biofuels which are obtained from the sugars and vegetable oils found in crops.

Panagiotopoulos et al., (2009), studied on hydrogen production from sugar beet root is sugar-rich biomass. They had pre-treatment for their biomass to produce fermentable substrates from biomass. To pre-treatment of sugar beet roots, mechanical extraction of sucrose were used. At mechanical extraction of sucrose, firstly sugar beet was placed in glass beakers and demineralised water was poured on

top of the biomass and the beakers were closed and placed in a hot waterbath for 2 h. The content of the beakers was then manually separated to a fraction of biomass and a liquid fraction. And than the biomass was pressed.

Panagiotopoulos et al., (2010), used C.Saccharolyticus as microorganism to dark fermentative hydrogen production. They cultivated C. Saccharolyticus in anaerobic serum bottles (118 mL) in culture volumes of 20 mL at extrem thermophilic temperature 72^{o}C. They used batch reactors for fermentation. Batch fermentations under controlled conditions were performed in a jacketed 2 L bioreactor at a working volume of 1 L. The pH was controlled at approximately 6.8 (measured at room temperature) by automatic addition of 2 N NaOH. Cultures were continuously stirred at 350 rpm and sparged with nitrogen at 7 L/h. Normal growth of C. saccharolyticus on the sugar beet extract was achieved in batch fermentations under pH and hydrogen pressure controlled conditions, at an initial sucrose concentration of 10 g/L. Consumption of sucrose in fermentations was almost complete after 26 h. The main products of the fermentations were hydrogen, acetate and carbon dioxide. Maximum hydrogen production rate was approximately 12.8 mmol/(L·h). Hydrogen yields of 3.0 mol/mol hexose were observed in fermentations of sugar beet extract.

Panagiotopoulos et al., (2010), also studied with pure sucrose. They studied with pure sucrose as substrate under same fermentation condition, same microorganisms and same initial sucrose consentration (10 g/L). As a result a maximum hydrogen production rate of approximately 12.1 mmol/(L·h) was determined and hydrogen yields of 2.9 mol/mol hexose were observed in fermentations of sucrose.

Ma et al., (2011), studied on bio-hydrogen production from cornstalk wastes by dark fermentation. They used mix microorganism culture for dark fermentation. They obtained mix culture from anaerobic sludge, originating from a local natural river. The sludge was pre-heated at 100 °C for 15 min to inhibit the bioactivity of methanogens and other pathogenic microbes. Raw cornstalks to be used in the batch experiments were ground by a vegetation disintegrator after drying and then sieved down to 80-mesh size. Ma et al.,(2011) investigated the effect of different substrate

consentration and different initial pHs. They found optimum conditions for fermentative hydrogen production for their experimental study. In their study optimum enzymatic temperature is 50 °C, optimum enzymatic time is 72 h, optimum initial pH is 7.0 and optimum substrate concentration is 10 g/L. In their study, under the optimum condition, the maximum cumulative H_2 yield was 141.29 mL/g CS (cornstalk, or 164.48 mL/g TS, total solid, TS = 0.859 $W_{\text{dried cornstalk}}$), with an average H_2 production rate of 12.31 mL/g.h CS

Ozmihci & Kargi, (2011), studied on dark fermentative bio hydrogen production from ground wheat starch solution. They used Clostridium butyricum-NRRL 1024 and Clostridium pasteurianum-NRRL B-598 microorganisms. Mixed culture were used with an initial biomass ratio of 1/1. The highest hydrogen formation rate (280 mL/d), volumetric hydrogen formation rate (1857 mL H_2/L·d) and VFA concentration were obtained with a substrate loading rate of 5.52 g/d at HRT = 6 h. The highest hydrogen yield was obtained at 1.38 g/d substrate loading rate.

Ferreira et al., (2013), studied on bio hydrogen production from micro algae via dark fermentation. Micro algae biomass was used as a substrate for dark fermentation by *C.* Butyricum. Algae biomass has high starch content. Therefore, it's good substrate for dark fermentation. In their study, fermentation was conducted in an incubator at 37 °C, 150 rpm for 48 h. The hydrogen yield obtained was 7.3 g/kg of micro algae dried biomass (3.65 mmol H_2/g biomass).

Kim et al., (2013), studied about bio hydrogen production from sewage sludge. They used swage sludge as substrate. The substrate sewage sludge was obtained from a gravity sludge thickener line at the wastewater treatment plant. They used mix microorganisms culture. Mix culture is obtained from anaerobic digester sludge. Anaerobic digester sludge was taken from the wastewater treatment plant. In order to inactivate hydrogen-utilizing bacteria and harvest hydrogen-producing bacteria, the digested sludge was heat treated at 100°C for 15 min. They investigated pretreatment of sewage sludge effect on hydrogen production. As a result of their experiments they found that pretreated (30 min) sludge presented an optimal condition, resulting

in maximum hydrogen yield (25.1 mL H_2/g-VS) and the highest hydrogen content (60.0%). In theire experiments the initial pH was adjusted to 7.0 with 4 N NaOH and 4 N HCl during the hydrogen fermentation process.

Saratale et al., (2008), made intensive literature study on second generation biohydrogen production which are made from lignocellulosic materials. Table 1.2 shows that biohydrogen production performance using different kinds of lignocellulosic substrates with different kinds of microorganism culture via dark fermentation.

Table 1.2 Biohydrogen production performance using different kinds of lignocellulosic substrates (Saratale et al., 2008)

H_2 Producer	**Cellulosic Substrate**	**Temp.^oC**	**Initial pH**	**H_2 yield**
Mixed culture anaerobic	Microcrystalline	37	7.0	2.18 mmol/H_2 cellulose
Mixed cultue	Cellulose	55	6.5	102 mL H_2/g cellulose
Dried mixed sludge	Corn stover biomass	35	5.5	2.84 and 3.0 at neutral and acidic pretreatment
Heat-treated anaerobically digested sludge	Fodder maize Chicory fructo oligosaccharides	35	5.2	62.4 mL/g dry matter of fodder maize 218 mL/g Chicory fructo oligosaccharides
Heat-treated of anaerobically digested sludge	Prennial ryegrass (Lolium perenne)	35	5.2	75.6 mL H_2/g dry matter wilted perennial ryegrass 21.8 mL H_2/g dry matter of fresh perennial ryegrass
Anaerobic cow dung microflora	Cellulose, 3 g/L	55	7.0	2.8mmol H_2/g cellulose
Clostridum thermocellum JN4 and Thermoanaerobacterium Thermosaccharolyticum GD17	Microcrystalline cellulose, 10 g/L corn stalk powder 0.5% corn cob powder 0.5%	60	7.0	10 mmol H_2/g cellulose 16 mmol/L corn stalk powder 20.4 mmol/L corn cob powder
Individual strains clostridium pasteurionum	Hydrolyzed carboxymethyl cellulose, 10 g/L	35	7.0	1.09 mmol H_2/g glucose
Rominoccus albus	Sorghum extract, 3 g/L Sorghum stalks, 3 g/L Sorghum residues, 3 g/L	37	6.4	14.5 mmol H_2/g glucose 17.5 mmol H_2/g glucose 14.4 mmol H_2/g glucose
Thermotaga neapolitano	Cellulose, 5 g/L CMC, 5 g/L Glukose, 3 g/L Xylose, 3 g/L	75 36	7.0 6.8	1.07 mmol H_2/g cellulose 3.37 mmol H_2/g cellulose 14.6 mmol H_2/g substrate 16.1 mmol H_2/g substrate
Clostridium sp.strain No.2	Avicel hydrolysate, 3g/L Xylan hydrolysate, 3 g/L			19.6 mmol H_2/g substrate 18.6 mmol H_2/g substrate

According to Table 1.2, maximum hydrogen production yield was observed as 102 mL H_2/g cellulose which study was used mixed culture as biomass and cellulose as substrate.

These studies shows that biohydrogen can be produced from various kinds of lignocellulosic biomass in effective way.

Researchers have investigated optimum conditions for bio hydrogen production. Therefore they have been studied on different organic loading rates, hydraulic retention times, temperatures, pHs and C/N ratios. Researchers found different optimum conditions for their own experiments. Reasons of differences are, they used different substrate, reactors design and microorganism types. Mohammadi et al., (2012) made intensive literature study on these conditions.

Table 1.3 shows that maximum hydrogen production rates (HPR) and hydrogen production yields on different organic loading rates (OLR) according to Mohammadi et al., (2012)'s study.

Table 1.3 The effect of OLR on dark fermentative hydrogen production

Type of reactor	Substrate	Substrate Range of OLR (g COD/ (Lday))	Optimum OLR (gCOD/ (Lday))	Maximum H_2 production	
				HPR	H_2 yield
Batch					
	Sucrose	10–40	20	–	4.00 mmol H_2/gCOD
	Sucrose	5–30	20	0.234mol H_2/(Lday)	–
	Dairy wastewater	2.4–4.7	3.5	70.7mol H_2/($kgCOD_R$ day)	–
	Chemical wastewater	6.3–7.9	6.3	13.44mol H_2/($kgCOD_R$ day)	–
	Glucose	3–18	9	–	9.53 mmol H_2/gCOD
	Food waste	20.7–41.4	41.4	0.019mol H_2/(Lday)	–
	Raw starch	10–25	10	–	9.05 mmol H_2/gCOD
	Gelatinized starch	10–25	10	–	9.47 mmol H_2/gCOD
	Hydrolyzed starch	10–25	25	–	10.4 mmol H_2/gCOD
	Ground wheat powder solution	0.87–4.4	1.93	0.018mol H_2/(Lday)	–
	Glucose	5–90	5	–	2.14 mmol H_2/gCOD
Continuous					
	Glucose	1.1–11.2	11.2	0.214mol H_2/(Lday)	–
	Glucose	2.5–20	10	1.75mol H_2/($kgCOD_R$ day)	–
	Cheese processing wastewater	5–14	14	–	3.12 mmol H_2/gCOD
	Glucose	4–30	22	–	9.27 mmol H_2/gCOD
	Sucrose	20–160	120	–	9.88 mmol H_2/gCOD
	Cheese processing wastewater	21–47	35	–	9.00 mmol H_2/gCOD
	Glucose	6.5–42.8	25.7	–	14.58 mmol H_2/gCOD

Table 1.3 shows a wide range of OLR investigation with utilizing either batch or continuous reactors with various organic substrates such as glucose, sucrose, starch, and few cases with organics available in real wastewaters. As shown, there is no definitive range or optimum OLR in fermentative hydrogen production. This phenomenon could be due to the diverse environmental/operating conditions or ranges of the variables applied in the studies.

According to Table 1.3, maximum H_2 production rate had been achieved at the study which had the organic loading rate between 2.4 and 4.7 g COD/(Lday), as 70.7 mol H_2/(kg COD_R day). In that study, dairy wastewater was used as substrate in batch reactor. Also maximum hydrogen yield had been achieved between 6.5 and 42.8 g COD/(Lday) organic loading rate as 14.58 mmol H_2/g COD. In that study, glucose was used as substrate in continuous reactor.

Table 1.4 shows maximum hydrogen production rates (HPR) and hydrogen production yields on different hydraulic retention times (HRT) according to Mohammadi et al., (2012)'s study.

Table 1.4 The effect of HRT on dark fermentative hydrogen production

Type of reactor	**Substrate**	**Range of HRT (h)**	**Optimum HRT (h)**	**Maximum H_2 production**	
				H_2 yield	HPR
Batch					
	Food waste	24–42	36	3.32 mmol H_2/$gVSS_{added}$	–
Continuous					
	Glucose	0.5–4	1	6.19 mmol H_2/gCOD	–
	Glucose	3–30	25	7.39 mmol H_2/gCOD	–
	Glucose	0.25–2	0.5	0.08 mmol H_2/g VSS	–
	Sucrose	2–6	6	9.88 mmol H_2/gCOD	–
	Glucose	1–24	2	–	7.2 L H_2/(Lday)
Olive oil mill effluent		7.5–60	14.5	–	200 L H_2/(Lday)
Cheese processing wastewater		1–5	3	22 mmol H_2/gCOD	–
Primary sewage biosolids		18–48	24	21.86 L H_2/kg VS	–
	Starch	3–48	6	–	4 L H_2/(Lday)

A wide range of HRT from 0.25 to 60h was investigated utilizing mainly continuous reactors with various organic substrates such as glucose, sucrose, starch, and in a few cases with organics available in real waste waters. Table 1.4 also shows there is no definitive range or optimum HRT in fermentative hydrogen production .

This phenomenon could be due to the diverse environmental /operating conditions or ranges of the variables applied in the studies.

According to Table 1.4, maximum H_2 production rate had been achieved at the study which had the hydraulic retention times between 7.5 and 60 h, as 200 L H_2/(Lday). In that study, olive oil mill effluent was used as substrate in continuous reactor. Also maximum hydrogen yield had been achieved between 1 and 5 h hydraulic retention times as 22 mmol H_2/g COD. In that study, cheese processing wastewater was used as substrate in continuous reactor.

Table 1.5 shows maximum hydrogen production rates (HPR) and hydrogen production yields on different temperatures according to Mohammadi et al. (2012)'s study.

Table 1.5 The effect of temperatures on dark fermentative hydrogen production

Type of reactor	Substrate	Range of temperature (^oC)	Optimum temperature (^oC)	Maximum H_2 production	
				H_2 yield	HPR
Batch					
	Sucrose	30–45	40	3.27 mmol H_2/gCOD	–
	Glucose	33–41	41	8.69 mmol H_2/gCOD	–
	Rice slurry	37–55	37	14.18 mmol H_2/gCOD	–
	Sucrose	30–40	35	–	5.88 L H_2/(Lday)
	Glucose	20–44	37	6.98 mmol H_2/gCOD	–
	Xylose	30–55	40	14.9 L H_2	–
	Glucose	25–55	40	0.06 mmol H_2/gCOD	–
	Sucrose	40–80	60	13.18 mmol H_2/gCOD	–
	Starch	37–55	55	1.44 mmol H_2/g starch	–
	Cattle wastewater	30–55	45	13.52 mmol H_2/gCODR	–
	POME	30–55	37	–	6.70 L H_2/(Lday)
	Glucose	35–55	55	15.10 mmol H_2/gCOD	–
	Glucose	37–55	37	11.35 mmol H_2/gCOD	–
Continuous					
	Esterification wastewater	15–25	25	150 ppm H_2	–
	Sucrose	30–45	40	9.00 mmol H_2/gCOD	–
	Xylose	30–55	50	7.29 mmol H_2/gCOD	–

(POME, palm oil mill effluent)

Table 1.5 shows various ranges of temperatures investigation for fermentative hydrogen production. The maximum H_2 production attained for each study is also given in this table. A wide range of temperature from 15 to 80^{o}C was investigated utilizing mainly batch reactors with various organic substrates. As shown in table 1.5, there is also no definitive range or optimum temperature in fermentative hydrogen production and sometimes the achieved results disagree with each other. For instance, study ,which was used sucrose as substrate, found the maximum H_2

yield at optimum temperature 60^{o}C, while study ,which was used esterification wastewater as substrate, reported 25^{o}C as the optimum temperature.

Table 1.6 shows maximum hydrogen production rates (HPR) and hydrogen production yields on different pHs according to Mohammadi et al.(2012) 's study.

Table 1.6 The effect of pHs on dark fermentative hydrogen production

Type of reactor	**Substrate**	**Range of pH**	**Optimum pH**	**Maximum H_2 production**	
				H_2 yield	HPR
Batch					
	Sucrose	4.7–6.3	5.5	8.58 mmol H_2/gCOD	–
	Sucrose	4.5–6.5	5.5	0.05 mmol H_2/gCOD	–
	Rice slurry	4.0–7.0	4.5	14.18 mmol H_2/g starch	–
	Sucrose	5.0–7.0	6.0	–	8.83 L H_2/(Lday)
	Sucrose	5.5–7.0	6.0	–	5.71 L H_2/(Lday)
	Cattle wastewater	4.5–7.5	5.5	14.80 mmol H_2/gCODR	–
	POME	5.0–8.5	5.5	11.43 mmol H_2/gCODR	–
	Fresh water pound sludge	6.5–8.0	7.0	2.71 mol H_2/mol substrate	–
	Molasses	5.0–8.0	6.5	8.3 L H_2/L	–
	Carbohydrate-rich organic waste	4.9–6.7	4.9	5.87 mmol H_2/gCOD	–
Continuous					
	Glucose	4.0–7.0	5.5	10.94 mmol H_2/gCOD	–
	Sucrose	3.4–6.3	4.2	8.39 mmol H_2/gCOD	–
	Sucrose	6.1–9.5	7.0	3.73 mmol H_2/gCOD	–
	Glucose	4.7–5.9	5.0	9.35 mmol H_2/g TVS	–

Table 1.6 lists various ranges of pH investigated by the other researchers and also the obtained optimum pH values. The maximum H_2 production attained for each study is also given in Table 1.6. A wide range of pH values from 3.7 to 9.5 was

investigated using batch and continuous reactors with synthetically formulated medium and real wastewaters as substrate. As shown in Table 1.6, there exists no exact agreement on the optimum pH in fermentative hydrogen production. For example, optimum pH was obtained at 7 when fresh water pound sludge was used as substrate, while optimum pH was 4.9 in the other work that carbohydrate-rich organic waste was used as substrate.

Table 1.7 shows maximum hydrogen production rates (HPR) and hydrogen production yields on different C/N ratio according to Mohammadi et al., (2012) 's study.

Table 1.7 The effect of C/N ratio on dark fermentative hydrogen production

Inoculum	**Substrate**	**Range of C/N ratio**	**Optimum of C/N ratio**	**Maximum H_2 yield**
Activated sludge	Sucrose	40–130	47	11.13 mmol H_2/g COD
Anaerobic sludge	POME	45–95	74	11.52 mmol H_2/g starch
Anaerobic sludge	Wheat powder	20–200	200	6.33 L H_2/L substrate
Cattle dung and sludge	Sucrose	30–100	50	0.275 L H_2/g sucrose

Table 1.7 shows the results of optimization of C/N ratio value in maximizing hydrogen generation from different substrates in batch reactors. With regards to inoculum and substrate sources, the optimum C/N ratio value in a particular anaerobic hydrogen process is expected to vary among different systems. For example, appropriate level of C/N value was obtained at 74 when anaerobic sludge and POME (palm oil mill effluent) were used as seed and substrate, respectively, while this ratio was 47 in the other work that applied activated sludge and sucrose as seed and substrate, respectively. Thus, there is no global optimum for C/N ratio value with respect to fermentative hydrogen production.

In this thesis, waste paper was chosen as substrate for dark fermentative hydrogen production. Paper is a kind of lignocellulosic material. Bio conversion of

lignocellulosic biomass includes hydrolysis of cellulosic materials to reducing sugars and producing of hydrogen and higher valuable products via fermentation (Saratale et al., 2008). Paper has high cellulose content (%85-99). Therefore, it is good substrate for bio conversition. On the earth, annual biosynthesis of cellulose by both land plants and marina algae occurs at a rate of tonnes per annum equivalent to more than four times the world's yearly totally consumption (Saratale et al., 2008).

Up to now, researchers investigated on wide range of waste materials as substrate via dark fermentation. Also, waste paper was used as feedstock for bioethanol production. But, reports on utilization of waste paper as substrate in biohydrogen production are limited. The using of waste papers as substrate has some advantages. Firstly, paper has high cellulose content. Therefore it is good substrate for dark fermentation. Secondly, reuse provide sustainablity. Sustainability is important because all the choices we pursue and all the actions that we make today will affect everything in the future. And natural resources are being depleted rapidly. So, reuse is important to reduce the use of natural resources. And thirdly, the using of foods as substrate for hydrogen production can create a food– fuel competition. And also the using of waste food can create food-fuel competition too. Because, waste foods are used as animal feed. However, the using of waste paper as substrate won’t create a food-fuel competition.

In this thesis, firstly waste paper was pre treated. The aim of pre treatment, to get rid of lignin and hemicellulose, reduce crystallinity cellulose and increase surface area of materials to improve formation of sugars (Saratale et al., 2008). Figure 1.7 shows schematic description of pretreatment on lignocellulosic materials.

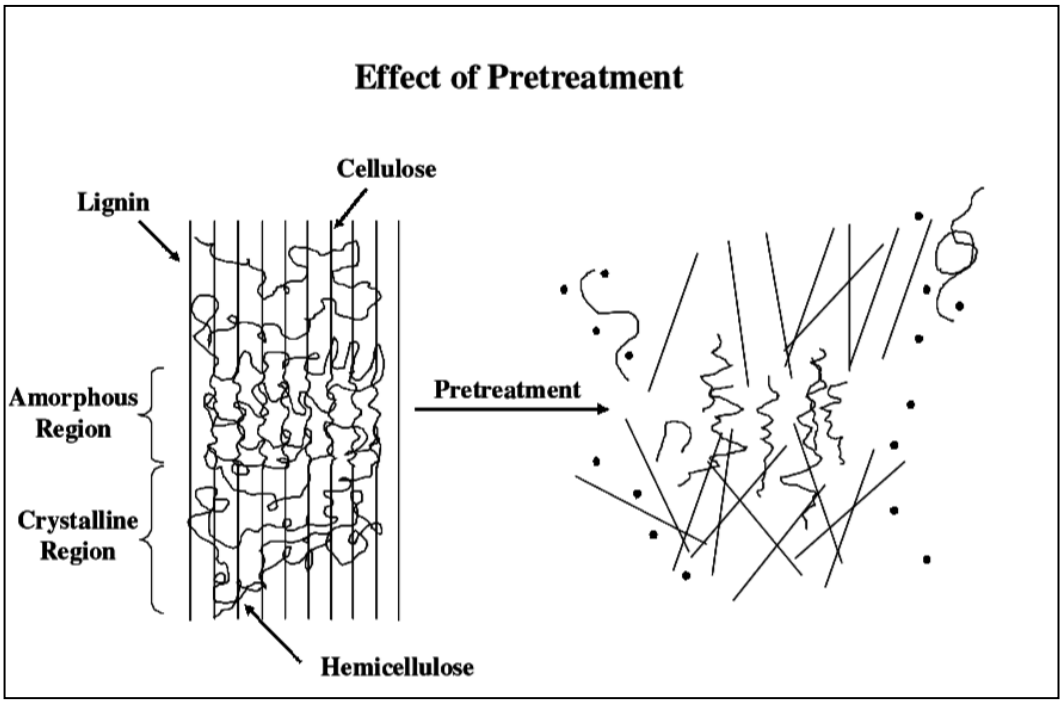

Figure 1.7 Schematic description of pretreatment on lignocellulosic materials (Mosier et al., 2005)

Several methods have been used as pretreatment of lignocellulosic materials. Figure 1.8 shows these pretreatment methods.

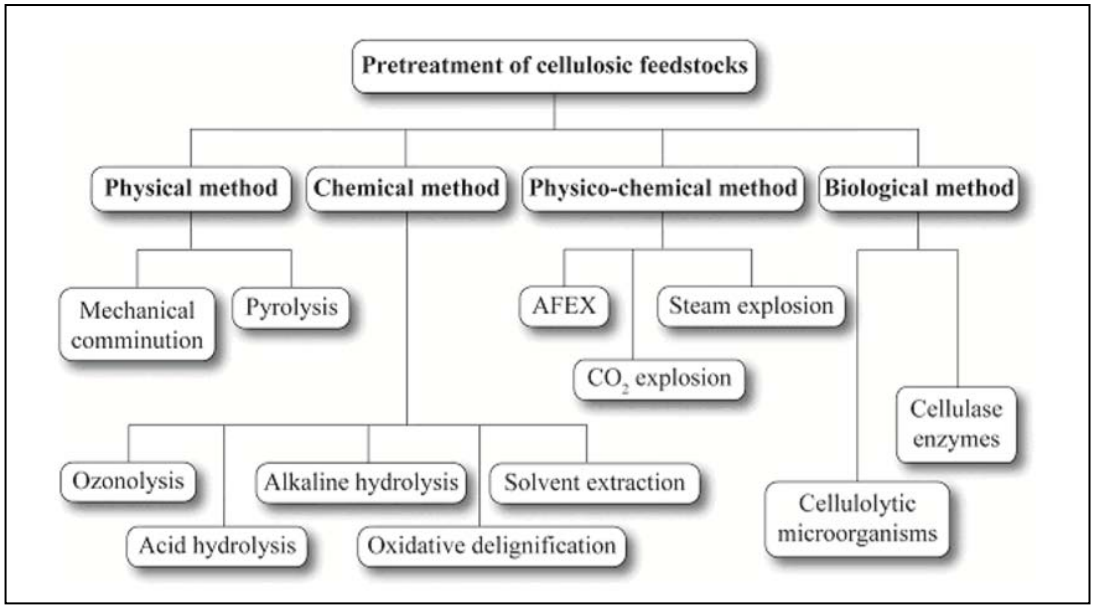

Figure 1.8 Methods for pretreatment of lignocellulosic materials (Saratale et al., 2008)

Physical pretreatment (mechanical comminution and pyrolysis) found to be effective in breaking down cellulose crystallinity but requires more cost for power and gives all the three major compounds in one product stream (Saratale et al., 2008).

Steam explosion, CO_2 explosion and ammonia fiber explosion (AFEX) are phsico-chemical pretreatment methods. Among the these phsico-chemical pretreatment methods, steam explosion is recognized as one of the cost-effective pretreatment process for hardwoods and agricultural residues. But hemicellulose is partially hydrolyzed in steam explosion and low-level fermentable sugar and inhibitory by-products formation can be observed after process. High sugar yields and low-level toxic compound can be obtained at AFEX but it requires high capital costs (Saratale et al., 2008).

Biological pretreatment of cellulose is carried out by cellulolytic microorganisms or catalyzed by cellulose enzyme complex (Saratale et al., 2008). There are limited numbers of bacteria and fungi, which have enzymes, can breakdown of cellulose and lignin. The enzymatic process of the cellulose by native biomass is low (yield <%20) because of its complex structure. On the other hand, polymeric hemicellulose can be hydrolyzed by enzyme or weak acid/base solutions.

Chemical pretreatment methods (ozonolysis, acid hydrolysis, alkaline hydrolysis, oxidative delignification and solvent extraction) are also effective pretretment procedures but requires high energy and chemicals (Saratale et al., 2008).

In this thesis, acid hydrolysis, which is a type of chemical pretreatment method, was chosen as pretreatment method. Because, acid hydrolysis is one of the effective method and common technology for biogass production (Saratale et al., 2008; Zheng et al., 2014). Acid hydrolysis provides; increase of accessible surface area, solubilization of hemicellulose, alteration of lignin structure and formation of furan derivatives (furfural/hydroxymethylfurfural (HMF)) (Zheng et al., 2014). Furan derivatives affect cell growth and respiration. HMF is considered less toxic than furfural. Phenolic compounds can produced from degradation of lignin. They have a

considerable inhibitory effect and are more toxic than furfural. The formation depends on temperature and residence time. The formation of inhibitors can be avoided by keeping the process temperature and residence time as low/short as possible. The effect of inhibitor compounds on biohydrogen formation yield depends on various factors such as the sugar concentration, the type of organism, concentration of inhibitors and their interaction.

In acid hydrolysis can be conducted either under concentrated acid (e.g. 30–70%) and low temperature (e.g. 40 °C) or under dilute acid (e.g. 0.1%) and high temperature (e.g. 230 °C). Both inorganic and organic acids, including sulfuric acid (H_2SO_4), hydrochloric acid (HCl), nitric acid (HNO_3), phosphoric acid (H_3PO_4), acetic acid, and maleic acid, have been used for dilute acid pretreatment, with H_2SO_4 being the most commonly used. Although concentrated acid is highly effective on cellulose hydrolysis, it is extremely toxic, corrosive, and dangerous, and requires expensive materials, such as specialized non-metallic materials or alloys, for reactor construction. In addition, the acid must be recovered after biomass treatment for economic reasons, as it is an energy and cost intensive process. Therefore, dilute acid is favored over concentrated acid for lignocellulosic biomass pretreatment (Zheng et al., 2014). Dilute sulfuric acid is mixed with biomass to hydrolyze hemicellulose to xylose and other sugars. The acid is mixed or contacted with the biomass, and the mixture is held at temperatures of 160–220 °C for periods ranging from minutes to seconds (Mosier et al., 2005).

In theory, enzymatic hydrolysis provides better yields as compared to acid hydrolysis. However the complex structure of cellulose is resistant to enzyme.

In this thesis dilute acid hydrolysis was performed under high temperature as a pre-treatment method due to its advantages.

In this study mix microorganism culture was used for dark fermentation. Mix culture was obtained from acidogenic phase of the anaerobic treatment plant of the PAK MAYA Bakers Yeast Company in Izmir, Turkey . The sludge was pre-heated

to selection of spore forming anaerobic bacteria and removal of methanogens that consume hydrogen gas. Clostridial species found in mixed heattreated anaerobic acidogenic culture can degrade carbohydrates, organic acids, alcohols and aromatic compounds. Fermentation was conducted in an incubator at 37°C. pH was adjusted to 7.0 every day, during the hydrogen fermentation process.

1.3 Objectives and the Scope

Hydrogen gas is an environmental friendly fuel, therefore hydrogen is considered as a viable alternative fuel of future. Researchers have studied on hydrogen production via different methods. And studies have shown that the most environmentally friendly hydrogen production methods are biological methods. One of the most advantageous method of these biological methods is bio hydrogen production by dark fermentation.

In dark fermentation microorganisms convert carbon source to hydrogen. Therefore, for dark fermentation, carbon rich materials are needed as substrate to microorganisms. And waste paper is high carbon content material. Also the using of waste paper as substrate has different advantageous. Using of waste materials provide to reduce the use of natural resources. And the using of waste paper as substrate won’t create a food-fuel competition.

Since, waste paper that has the appropriate structure for the biohydrogen production and up to now there are limited number of study on biohydrogen production from waste paper, in this thesis biohydrogen production from waste paper by dark fermentation is investigated. The main aims of this thesis is to investigate hydrogen gas production potential from waste paper by dark fermentation and to find optimum substrate concentration and optimum microorganism concentration for hydrogen production from waste paper. In order to achieve this aim, the scope of the study was as follows:

- Investigation of pretreatment methods for mix microorganisms culture and waste paper.

- Preparation of medium for provide to required for C/N ratio to microorganisms.

- Investigation to find optimum conditions on hydrogen gas production potential from waste paper by dark fermentation, the effect of using different sugar concentration for hydrogen production and the effect of using different amount of microorganisms.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Raw Material

Mixed culture of microorganisms, which used in this experiment, was obtained from the acidogenic phase of the anaerobic treatment plant of the PAK MAYA Bakers Yeast Company in Izmir, Turkey.

Waste paper was used as carbon source in this experiment. The size of waste paper was reduced by using a grinding to obtain very small fine particles (60 mesh size) and increase the surface area. Composition of waste paper feedstock is office paper and cardboard.

The using of waste paper as carbon source directly by microorganisms is difficult. Therefore, acid hydrolisis was performed to waste paper.

2.2 Hydrolysis of Waste Paper

Acid hydrolysis is the effective method to degradation of cellulose. Therefore acid hydrolysis was performed to waste paper. Using dilute sulfuric acid increases the reaction rate and the hydrolyzed cellulose yield. And using dilute acid with high temperature is increased the efficiency of cellulose hydrolysis. (Kazan, 2006) Therefore in this experiment dilute sulfuric acid was used with high temperature.

Firstly, dilute acid solution was prepared. 28 mL sulfuric acid was completed to 1 L with distilled water in a bottle. 100 g waste paper was weighed on an electronic precision scales. In 1 liter bottle, 100 g waste paper was completed to 1 L with dilute acid solution. In this experiment, 7 bottle waste paper-dilute acid mixture was prepared in this manner to obtain a sufficient amount of substrate. To obtain good paper-dilute acid mixture, bottles were stirred with the mechanical mixer for 30 minutes. pH of waste paper-dilute acid mixtures was 2.2.

The prepared acidic mixtures was placed in autoclave for hydrolysis under high temperature. Hydrolysis was carried out in the autoclave at 121^{o}C and 90 minutes. After autoclaving, liquids in bottles were collected by filtration in a 2 liter glass bottle. Thus, cellulose degradation has occurred and carbon source is available to be used by the microorganisms.

Collected carbon containing liquid, which in the 2 liter bottle, was boiled and water was evaporated. Because, first sugar concentration of substrate is not too high. One of the aim of this study is to determine an optimal sugar concentration for the production of hydrogen from waste paper. And to obtain high sugar concentration, provides carrying out the experiments in a wide range of sugar concentration. After evaporation, high sugar concentration carbon source was obtained.

2.3 Fermentation

2.3.1 Microorganism Cultivation

In order to inactivate hydrogen-utilizing bacteria and to obtain spore forming hydrogen producing bacterial culture, heat treatment was applied to sludge at 100°C for 2 hours when pH is equal to 5.9. After that heat treated, when the sludge came to the room temperature, 25 mL treated sludge and 225 mL media was mixed in bottle. To prepare the media; 60 g glucose, 2.61 g of urea, 1.3 g KH_2PO_4, 0.25g $MgSO_4$ and 0.1 g L-cysteine, was completed in 1 liter with distilled water. Two bottles of sludge-media mixture was prepared. pH of the sludge-media mixture was adjusted between 6.8 to 7.2 in the bottles. Argon gas was passed through the bottles and than the bottles were plugged with gas-tight silicone stoppers with needle. Needle was used to exhaust the gas which produced by the microorganisms. These bottles were kept in the incubator for 24 hours. After incubation fresh media was prepared. The fresh media content was same as first one. pH of media was adjusted between 6.8 to 7.2. Taking some amount of mixture from bottles which were in the incubator and they were mixed with freshly prepared media in new bottles. 4 new bottles were prepared as 25 mL of sludge-media mixture with 225 mL of fresh media. pH of

mixtures were adjusted between 6.8 to 7.2, again. Argon gas was passed through the bottles and than the bottles were plugged with gas-tight silicone stoppers with needle. These 4 bottles were kept in the incubator for 18 hours. After 18 hours, microorganisms were ready for fermentation.

2.3.2 Media Preparation

Hydrolyzed waste paper was used as carbon source for microorganisms. Carbon is the most important parameter for microorganisms activity. However, microorganisms need nitrogen and phosphorus as well as carbon. Therefore, to ensure that activity of the microorganism, carbon, nitrogen and phosphorus containing media was prepared. For this media, hydrolyzed waste paper was used as carbon source. And as nitrogen and phosphorus source, chemical component was used.

In this study, carbon /nitrogen/ phosphorus ratio is 100/2/0.5. $(NH_4)_2SO_4$ was used as nitrogen source and KH_2PO_4 was used as phosphorus source.

Firstly, pH of hydrolyzed waste paper was adjusted between 6.8 to 7.2. Then, sugar concentration was measured with a spectrophotometer. According sugar concentration, to provide of 100/2/0.5 ratio, $(NH_4)_2SO_4$ and KH_2PO_4 was added into media. And 0.050 g/L $MgSO_4$, 0.025 g/L $FeSO_4$ was added to media for microorganisms activity. 0.1 g/L L-cysteine was added to media for provide anaerobic condition. To obtain good media mixture, bottle were stirred with the mechanical mixer for 1 hour. After stirring, pH of media was adjusted 7.0. And media was ready for fermentation.

2.3.3 Fermentation Process

Aims of this study are to determine optimum sugar concentration and optimum amount of microorganisms concentration for hydrogen production from waste paper. Therefore, two different sets of experiments were performed. In the first set of these

sets, the amount of microorganisms was kept constant and used different amount of sugar concentration. And the second set of experiments, sugar concentration was kept constant, the amount of microorganisms was changed.

The first set of experiments, constant concentration of microorganisms were determined 1 ± 0.08 g/L for each bottle. Suspended solids analysis was performed to determine the amount of microorganisms. Suspended solid analysis were made for microorganisms which were grown in an incubator for 18 hours. As a result of suspended solids analysis, necessary amount of sludge-media mixture was determined to obtain of 1 g/L of microorganisms. For each bottle, sufficient amounts of sludge-medium mixture, which were cultivated for 18 hours in an incubator, was put on centrifuge tubes. These tubes were centrifuged at 8000 rpm for 30 minutes. After centrifuging the tubes, water was poured and remaining at the bottom solid microorganisms were used for fermentation.

In the first set, different sugar concentrations were obtained by fermentation medium, which was diluted with different amounts of distilled water.

Batch dark fermentation experiments were carried out in 0.25 L 6 serum bottles (Isolab-Germany) for each sets. One bottle was control bottle, for each set. In the control bottle, only diluted fermentation medium, which had same sugar concentration as lowest sugar concentration bottle, was placed for first set. For other 5 bottles, media, which include different sugar concentration, and 1 g/L microorganisms were placed. Total amount of material for each bottle was adjusted to 0.15 L. Each bottles of pH were adjusted between 6.8 to 7.2. Each bottle is sealed with gas valve. The periphery of the valve and caps were covered with silicone to do not miss gases. Argon gas was passed through the bottles to provide anaerobic conditions in the bottles. And finally, bottles were placed in to 37^{o} C incubator. During fermentation, the pH of medium was adjusted 6.8 and oxygen reduction potential (ORP) of the medium was adjusted -350 ± 25 mV, to provide suitable conditions for microorganisms. 0.1 g/L of Na-thioglycolate was added to the bottles and argon gas passed from the head space of the bottles to maintain anaerobic

conditions. pH of the medium decreased from 6.8 to 5.0±0.5 due to VFA formation and was adjusted between 6.8 ±0.3 during fermentation with 10 M NaOH, every day. ORP values varied between -200 and -300 mV.

Same fermentation procedure were used for second set. But bottles had different amount of microorganisms and constant concentration of sugar. Optimum sugar concentration was choosen from first set.

2.4 Analytical Methods

2.4.1 Suspended Solids Analysis

Suspended solids analysis method is based on gravimetric measurement basis. According to this method, retained by the standard glass fiber filter and dried at 103-105°C residue indicates the suspended solids. Difference between the total solid content to total dissolved solids values are acceptable as the value of total suspended solid.

The filter paper placed on a watch glass and is kept in an oven at 103°C for 24 hours. After, the filter paper is removed from the oven, it is cooled in a desiccator for 2 hours. After filter paper is weighed out at accurately scales, it is placed in the suspended solids vacuum device. A certain amount of sample homogeneously put and is filtered with the help of the suspended solid vacuum device. And than, the filter paper placed on a watch glass and is kept in an oven at 103°C for 24 hours again. After oven, filter paper is cooled in a desiccator for 2 hours and weighed out at accurately scales.

Microorganism concentration is calculated by the following equation.

$$x = \frac{(\text{last weight of the filter paper} - \text{first weight of the filter paper})}{\text{The amount of filtered sample (mL)}} * 1000 \qquad (2.1)$$

(X= Microorganism concentration)

2.4.2 Sampling

3 mL samples removed with injection syringe from the each bottles everyday. They were centrifuged at 8000 rpm for 30 minutes and the clear supernatants were used for analysis.

2.4.3 Total Sugar Analysis

Total sugar concentrations were determined by the acid-phenol spectrometric method. Using of phenol-sulfuric acid reaction, a method has been developed to determine submicro amounts of sugars and related substances. (Dubois et al., 1956)

2.4.4 Total Gas Volume Measurement

The total gas volume produced was determined by water displacement method every day. To determination of total gas volume produced sulfuric acid (2%) and NaCl (10%) containing solution was used.

2.4.5 H_2 Gas Measurements

Hydrogen gas was sampled from the head space of the bottles by using gas-tight glass syringes. Hydrogen gas concentration in the gas phase was determined with gas chromatograph (HP Agilent 6890). The column was Alltech, Hayesep D 80/100 6" × 1/8" × 085". Nitrogen gas was used as carrier with a flow rate of 30 mL/min and the head pressure was 22 psi. Temperatures of the oven, injection, detector, and filament were 35^{o}C, 120^{o}C, 120^{o}C, 140^{o}C, respectively.

2.4.6 pH and ORP Measurements

pH and Oxidation Reduction Potential (ORP) of the fermentation medium were monitored by using a pH meter and ORP meter with relevant probes (WTW Sci.,

Germany). pH was adjusted between 6.8 and 7.2 with 10 M NaOH and/or 5 M H_2SO_4 for every day.

ORP measures an aqueous system's capacity to either release or accept electrons from chemical reactions. ORP values characterize a system's relative state for gaining or losing electrons (Bier et al., 2009). Organisms activities has a direct correlation between ORP. In this study, ORP values varied between −100 and −300 mV, in general. These values provide a suitable environment for the activity of microorganisms.

2.4.7 Total Volatile Fatty Acid Analysis

Volatile fatty acids (VFAs) are a class of short-chain carboxylic acids generally defined as having six or less carbon atoms per molecule. These acids are known to be important intermediates in the terminal steps of the anaerobic decomposition of organic matter. VFAs are produced by 'acid-forming' bacteria during the initial steps of decomposition, i.e. the dissimilation of carbohydrates, amino acids, and long-chain fatty acids (Sansone & Martens, 1981).

TVFA analyses were carried out by using analytical kits (Spectroquant, 1.01763. 0001, Merck, Darmstadt, Germany) and a PC spectrometer (WTW Photolab S12).

2.5 Calculations

The produced total volume of gas, was determined using gas liquid displacement system with based on the principle of combined vessels. The liquid phase consists of a mixture of 2% H_2SO_4 and 10% NaCl. The cumulative hydrogen gas production was determined by the following equation (Logan et al., 2002):

$$V_{H2,\,i} = V_{H2,\,i\,-1} + V_W\,C_{H2,i} + V_{G,i}\,C_{H2,i} - V_{G,i-1}\,C_{H2,\,i-1} \qquad (2.2)$$

Where $V_{H2,\ i}$ and $V_{H2,\ i-1}$ are the volumes of cumulative hydrogen (mL) calculated after the i^{th} and the previous measurement; V_W is the total gas volume measured by the water displacement method (mL); $C_{H2,i}$ is the concentration of H_2 gas in the total gas measured by the water displacement method (%); $V_{G,i}$ and $V_{G,i-1}$ are the volumes of the gas in the head space of the bottle for the i^{th} and the previous measurement (mL); $C_{H2,i}$ and $C_{H2,\ i-1}$ are the percent H_2 in the head space of the bottle for the i^{th} and the previous measurement.

The following generalized gas equation was used to calculate the mole number of cumulative hydrogen.

$$PV = nRT \tag{2.3}$$

Where n is mmol H_2 gas, P= 1 atm, V= Cumulative total hydrogen gas volume (mL), R= 0.082 (L atm / mol K) , T= Temperature in Kelvin (K)

In batch fermentations, cumulative hydrogen versus time data were correlated with the Gompertz equation and the constants were determined by regression analysis with Statistica. The Gompertz equation has the following form (Han & Shin, 2004):

$$H(t) = P * \exp\left\{-\exp\left[\frac{Rm*e}{P}\ (\lambda - t) + 1\right]\right\} \tag{2.4}$$

Where H is the cumulative hydrogen (mL H_2) at any time t; P is the maximum potential hydrogen formation (mL); Rm is the maximum rate of hydrogen formation (mL/h), λ is duration of the lag phase, „e" is 2.718 and „t" is time (h). The coefficients of the Gompertz equation were determined by regression analysis using the experimental data.

Hydrogen formation yield and specific hydrogen production rate (SHPR) are important parameters indicating the effectiveness of fermentation. The yield was calculated by using the following equation.

$$Y = CHF / V_0 (S_0 - S) \quad (2.5)$$

Where Y is the hydrogen gas yield (mL H_2/g TS or mol H_2/mol glucose); CHF is the cumulative hydrogen gas formation (mL); V_o is the initial fermentation volume (L); S_0 and S are the initial and final total sugar concentrations (g/L).

The SHPR (mL H_2/g biomass.h at certain temperature and 1 atm) were calculated with using the following equation,

$$R_x = R_m / V_o X_o \quad (2.6)$$

Where R_m is the volumetric hydrogen formation rate as calculated from the Gompertz equation (mL H_2/h); Vo is the initial volume of the fermentation broth (L) and X_o is the initial biomass concentration (g biomass/L).

CHAPTER THREE
RESULTS AND DISCUSSIONS

Hydrogen production from waste paper via dark fermentation study was carried out in two sets with batch reactors. Each sets contained 6 experiment bottles. In the first set of this study, the amount of microorganisms was kept constant and used different amount of sugar concentration. The aim of this set to determine the optimum substrate concentration for hydrogen production from waste paper. And in the second set of this study, sugar concentration was kept constant, as determinded in first set, and used different amount of microorganisms. As a result of this thesis the optimum sugar concentration and microorganism concentration was determined for hydrogen production from waste paper via dark fermentation.

In this study, waste paper was used as carbon source. The using of waste paper as carbon source directly by microorganisms is difficult. Therefore, acid hydrolysis was performed to waste paper under high temperature.

3.1 Hydrolysis of Waste Paper and Hydrogen Production via Dark Fermentation

Hydrolysis of waste paper was performed as explained “Hydrolysis of Waste Paper” part. In 1 liter bottle, 100 g waste paper was completed to 1 L with dilute sulfuric acid solution. In this experiment, 7 bottle waste paper-dilute acid mixture was prepared in this manner to obtain a sufficient amount of substrate. The prepared acidic mixtures was placed in autoclave for hydrolysis under high temperature. Hydrolysis was carried out in the autoclave at 121^{o}C and 90 minutes. After autoclaving, liquids in bottles were collected by filtration in a 2 liter glass bottle.

For the first set of this study, approximately 1700 mL liquid form of hydrolysed waste paper was obtained from 7 bottles of waste paper-dilute acid mixture. First sugar concentration was determined as approximately 30 g/L by the acid-phenol spectrometric method. Liquid form of hydrolysed waste paper was boiled and water

was evaporated. Because, first sugar concentration of substrate is not too high. And to obtain high sugar concentration, provides carrying out the experiments in a wide range of sugar concentration. After evaporation, approximately 1400 mL liquid form of hydrolysed waste paper was obtained and first sugar concentration was determined as approximately 45.5 g/L.

Firstly, 1400 mL liquid form of hydrolysed waste paper was sterilizaited in the autoclave at 90^{o}C and 30 minutes. The aim of sterilization is to inhibite microorganisms which can be lived in high sugar concentration liquid. After sterilization, pH was adjusted between 6.8 and 7.2 with NaOH. Initial pH of liquid form of hydrolysed waste paper was 2.2 from acid hydrolysis.

In this study, carbon (C) /nitrogen (N)/ phosphorus (P) ratio was determined as 100/2/0.5. To provide this C/N/P ratio, according to sugar concentration and amount of liquid hydrolysed waste paper chemical compounds were added in to media. Liquid hydrolysed waste paper was used as carbon source, $(NH_4)_2SO_4$ was used as nitrogen source and KH_2PO_4 was used as phosphorus source. $MgSO_4$ and $FeSO_4$ was added to media for microorganisms activity and L-cysteine was added to media for provide anaerobic condition. Media contained following amount of chemical compounds to provide C/N/P ratio for first set:

$(NH_4)_2SO_4 \rightarrow 5.46$ g
$KH_2PO_4 \rightarrow 1.246$ g
$MgSO_4 \rightarrow 0.07$ g
$FeSO_4 \rightarrow 0.035$ g
L-cysteine $\rightarrow 0.14$ g

These chemical compounds were added into 1400 mL liquid form of hydrolysed waste paper. To obtain good media mixture, bottle were stirred with the mechanical mixer for 1 hour. After stirring, pH of media was adjusted 7.0. And media was ready for fermentation.

To dark fermentation, microorganisms, which were grown in an incubator for 18 hours were used. Grown of microorganisms were explained in “Microorganism cultivation” part. After 18 hours, 40 mL of sludge-medium mixture, which were cultivated for 18 hours in the incubator, was put on centrifuge tube. This tube was centrifuged at 8000 rpm for 30 minutes. After centrifuging the tube, water was poured and remaining at the bottom solid microorganisms were used for suspended solid analysis. The result of this analysis amount of microorganisms, which are remained at the bottom of 40 mL centrifuge tube, were determined as 1 g/L.

For the first set, to provide 1 g/L microorganism amount, one 40 mL centrifuge tube was used for each bottle. For the each bottle, 40 mL sludge-medium mixture, which were cultivated for 18 hours in an incubator, was centrifuged at 8000 rpm for 30 minutes and water was poured and remaining at the bottom solid microorganisms were used for experiments.

For the second set, same procedure was carried out for hydrolysed of waste paper. Approximately 1700 mL liquid form of hydrolysed waste paper was obtained from 7 bottles of waste paper-dilute acid mixture. As a result of first sugar analysis, sugar concentration was determined as approximately 40 g/L. In the first set, optimum sugar concentration was determined as lower than 40 g/L. Therefore, in the second set, wasn’t performed the evaporation.

To provide C/N/P ratio as 100/2/0.5, chemical compounds were added in to media according to sugar concentration and amount of liquid hydrolysed waste paper. Media contained following amount of chemical compounds to provide C/N/P ratio for second set:

$(NH_4)_2SO_4 \rightarrow 6.48$ g
$KH_2PO_4 \rightarrow 1.48$ g
$MgSO_4 \rightarrow 0.085$ g
$FeSO_4 \rightarrow 0.043$ g
L-cysteine $\rightarrow 0.34$ g

In the second set, different amount of microorganisms were used for each bottles. Therefore, according to 40 mL of sludge-medium mixture was contained 1 g/L microorganisms, different amount of sludge-medium mixture was used for each bottles.

For each set, sufficient amount of media and microorganisms were placed in to each bottles. Total amount of material for each bottle was adjusted to 0.15 L. Each bottles of pH were adjusted between 6.8 to 7.2. Each bottle is sealed with gas valve as explained in "Fermentation Process" part. Argon gas was passed through the bottles to provide anaerobic conditions in the bottles. And finally, bottles were placed in to 37^{o}C incubator to start dark fermentation. During fermentation, the pH of a bottles were adjusted between 6.8 to 7.2 every 24 hours to provide suitable conditions for microorganisms.

3.2 The Effect Sugar Concentration on Production of Hydrogen Gas

To determine the sugar concentration effect and to find optimum sugar concentration on hydrogen production from waste paper via dark fermentation, different sugar concentrations were used.

For this set of experiments, 6 bottles were used. One bottle of these was used as control bottle. 5 bottles contained media, which was prepared for first set, and 1 g/L microorganisms. Control bottle contained only media. All bottles contained 0.15 L material. To obtained different sugar concentration, media was diluted with distilled water.

Initial sugar concentrations were adjusted approximately 45 g/L, 35 g/L, 20 g/L, 10 g/L and 5 g/L. 1 g/L microorganisms were added these 5 bottles. Control bottle contained approximately 5 g/L sugar concentrated media.

Initial pH of all bottles were adjusted between 6.8 and 7.2. Each bottle is sealed with gas valve. The periphery of the valve and caps were covered with silicone to do

not miss gases. Argon gas was passed through the bottles to provide anaerobic conditions in the bottles. And, bottles were placed in to 37^{o}C incubator to start dark fermentation. During fermentation, the pH of a bottles were adjusted between 6.8 to 7.2 every 24 hours with 10 M NaOH. Every day, for each bottles; total sugar analysis, total gas volume measurement and H_2 gas measurements were performed.

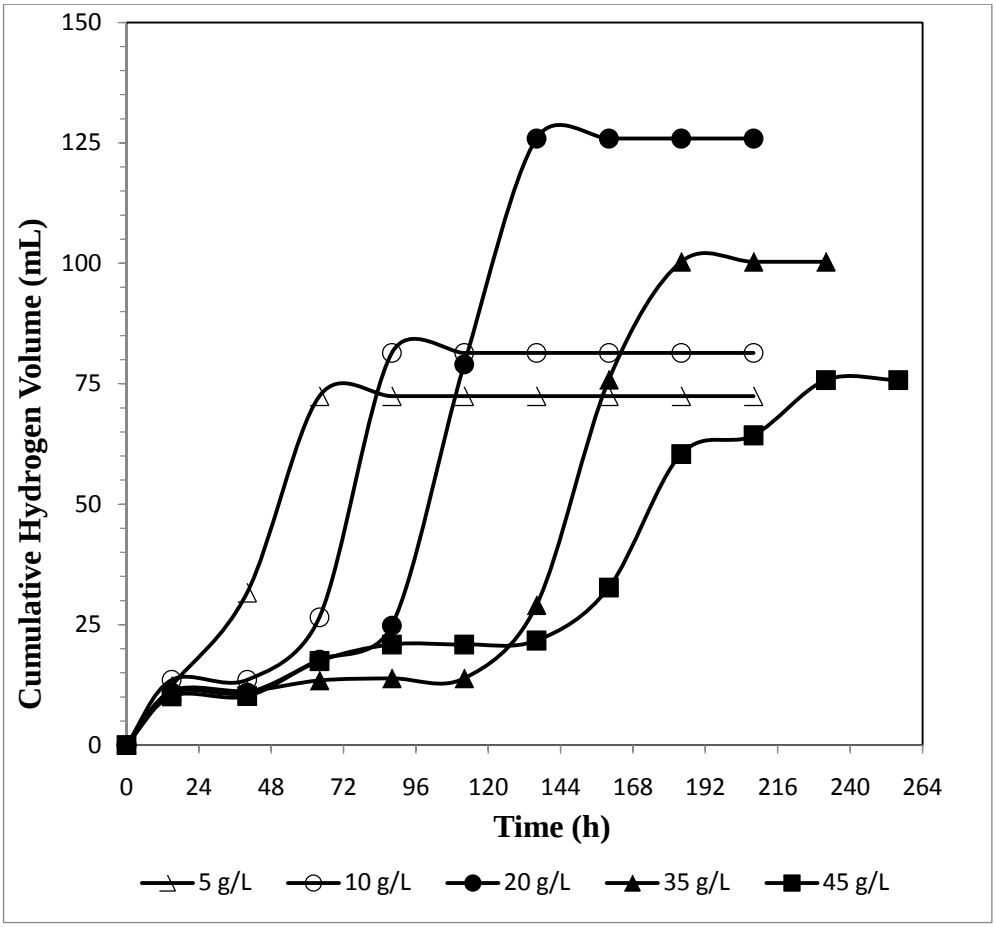

Figure 3.1 Time-dependent changes in the cumulative hydrogen production

Figure 3.1 shows time-dependent changes in the cumulative hydrogen production for each bottle. To reach the maximum level of cumulative hydrogen production varies according to the initial sugar concentrations. Cumulative hydrogen gas increased with increasing with time. Higher concentrations of sugar significantly affect the hydrogen production performance of culture resulting in decreasing in cumulative hydrogen gas volume. The end of experiment, 20 g/L initial substrate contained bottle was obtained highest cumulative volume of hydrogen gas

concentration (125.89 mL). 35 g/L initial substrate contained bottle was obtained 100.31 mL cumulative hydrogen. 10 g/L initial substrate contained bottle was obtained 81.41 mL cumulative hydrogen. 45 g/L and 5 g/L initial substrate contained bottles were obtained 75.76 mL and 72.43 mL cumulative hydrogen, respectively. These results indicate that the sugar concentration above 20 g/L cause substrate and product inhibition.

According to Figure 3.1, there is a direct correlation between the initial sugar concentration to reach a constant value of the cumulative hydrogen. In the microorganism contained bottles, the first sugar concentration 5 g/L bottle's cumulative hydrogen reached a constant value at the end of 64 hours, while, the first sugar concentration 45 g/L bottle's cumulative hydrogen reached a constant value at the end of 232 hours. The highest cumulative hydrogen value 125.89 mL was obtained at the end of 136 hours.

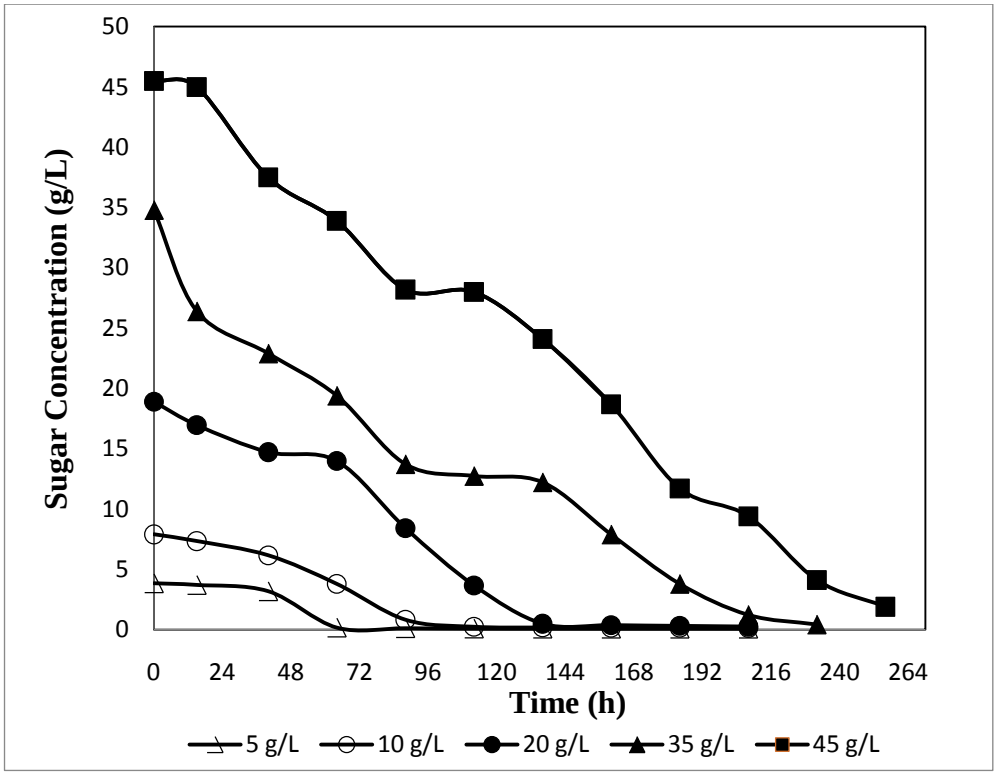

Figure 3.2 Time-dependent changes in the sugar concentration

Figure 3.2 shows, the change of substrate concentration depending on the time. Concentration of sugar decreased depend on the time in the graph, approximately 5 g/L initial sugar concentration contained bottle's substrate concentrations were fixed after 64 hours. Initial sugar concentration of approximately 10 g/L contained bottle's sugar concentration was fixed at the end 88 hours. Initial sugar concentration of approximately 20 g/L contained bottle's sugar concentration was fixed at the end of 136 hours. Approximately 35 g/L and 45 g/L initial sugar concentration contained bottles' sugar concentrations were fixed at the end of 232 and 256 hours, respectively.

The results indicate that the sugar concentration is inversely proportional to the cumulative hydrogen production. For instance, at the end of 136 hours, 20 g/L initial sugar concentration contained bottle's reached of minimum sugar concentration while, at the same time that bottle reached of maximum value of cumulative hydrogen, according to Figure 3.1 and Figure 3.2.

Figure 3.3, 3.4, 3.5, 3.6 and 3.7 shows, time-dependent changes in sugar concentration and cumulative hydrogen production for each bottle, separately.

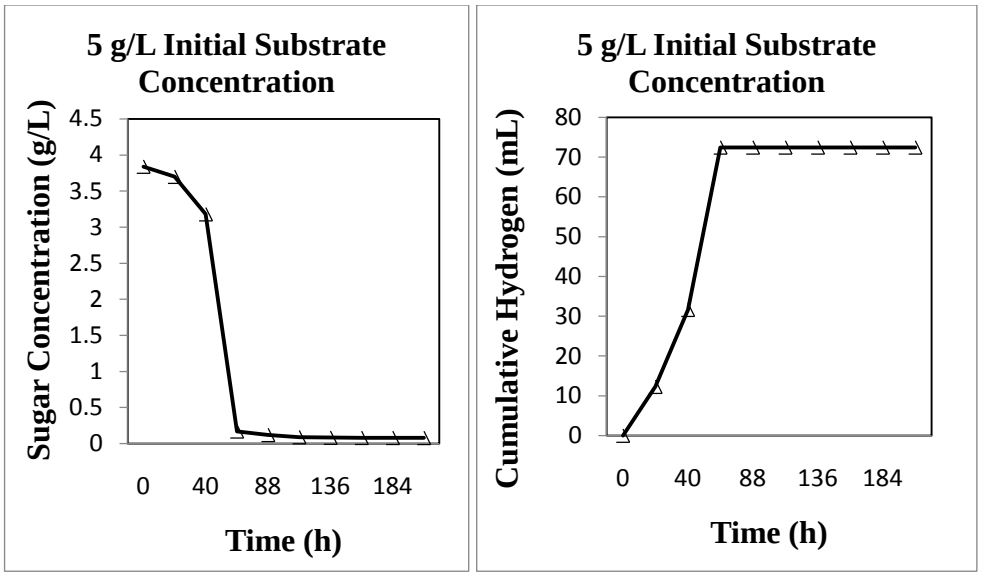

Figure 3.3 Time-dependent changes in sugar concentration and cumulative hydrogen production at 5 g/L initial substrate concentration

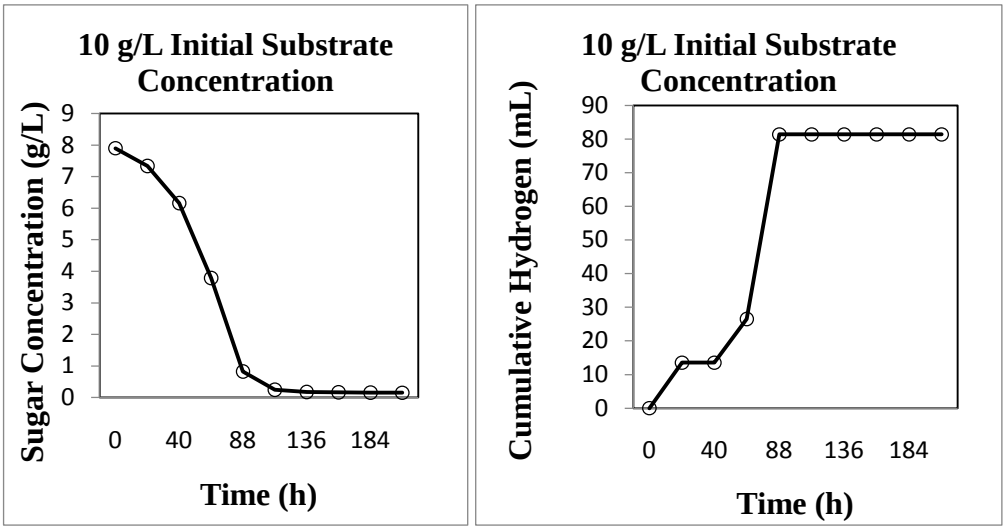

Figure 3.4 Time-dependent changes in sugar concentration and cumulative hydrogen production at 10 g/L initial substrate concentration

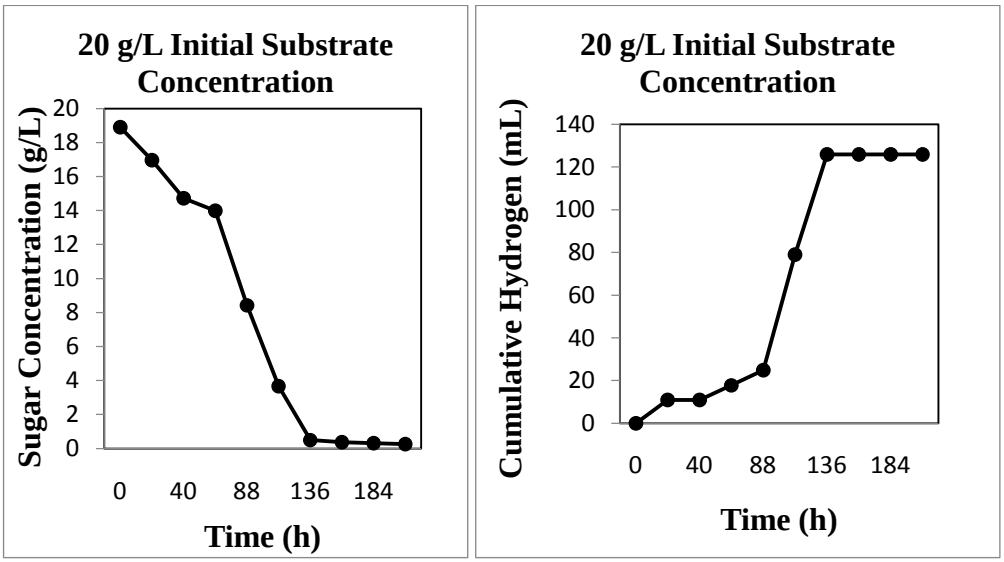

Figure 3.5 Time-dependent changes in sugar concentration and cumulative hydrogen production at 20 g/L initial substrate concentration

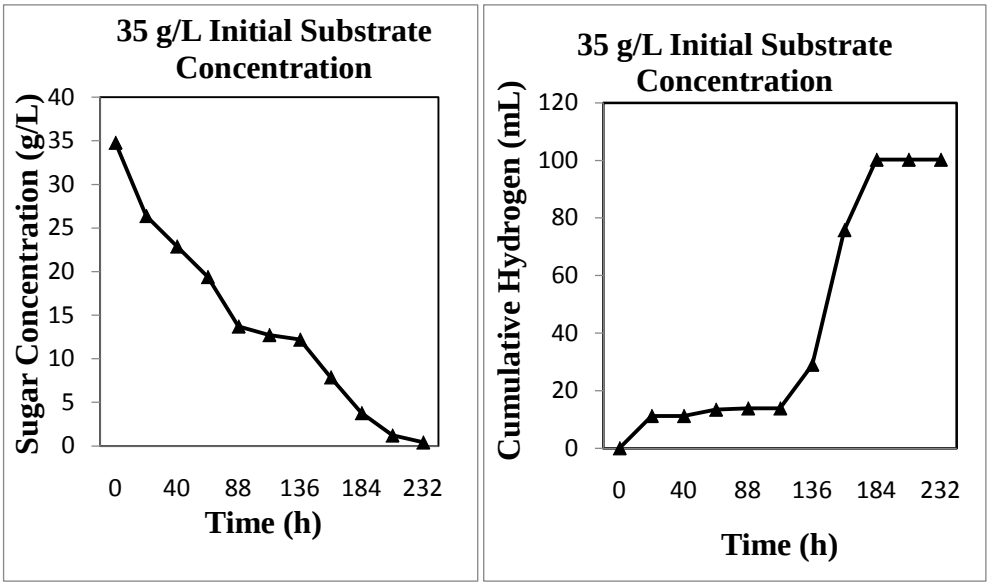

Figure 3.6 Time-dependent changes in sugar concentration and cumulative hydrogen production at 35 g/L initial substrate concentration

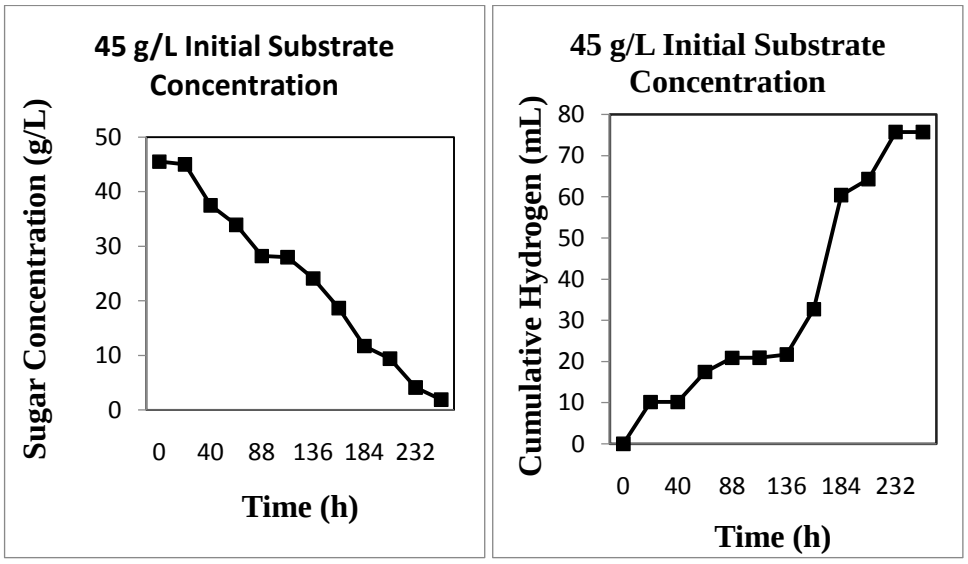

Figure 3.7 Time-dependent changes in sugar concentration and cumulative hydrogen production at 45 g/L initial substrate concentration

Table 3.1 Gompertz equation constants in the sugar concentration variation experiments

Sugar Concentration (g/L)	**P(mL)**	**R_m (mL/h)**	**λ (h)**	**R^2**
5	72.43	9.68	36.73	0.98
10	81.41	9.85	61.31	0.98
20	128.99	2.80	80.37	0.99
35	103.95	2.06	120.34	0.98
45	95.73	0.44	67.96	0.81

Table 3.1 shows the calculated Gomperzt constants. Constants were calculated by applying Gompertz equation on the cumulative hydrogen gas production datas. Calculations were performed with Statistica program. The highest cumulative hydrogen gas was obtained from the 20 g/L sugar concentration but the highest hydrogen production rate (R_m) was calculated as 9.85 mL/h while initial sugar concentration was 10 g/L. The calculated production rate of hydrogen, 35 and 20 g/L initial sugar concentration values were close. The lowest stationary phase(λ) was obtained from 5 g/L as 36.73 hours. The level of by-products, particularly furan derivatives after pre-treatment of waste paper may inhibit microorganisms at different initial sugar concentrations. As a result of presence of furan derivatives, the duration of lag phase increased with increasing hydrolyzed sugar as expected. The lowest lag phase obtained at low biomass/substrate ratio.

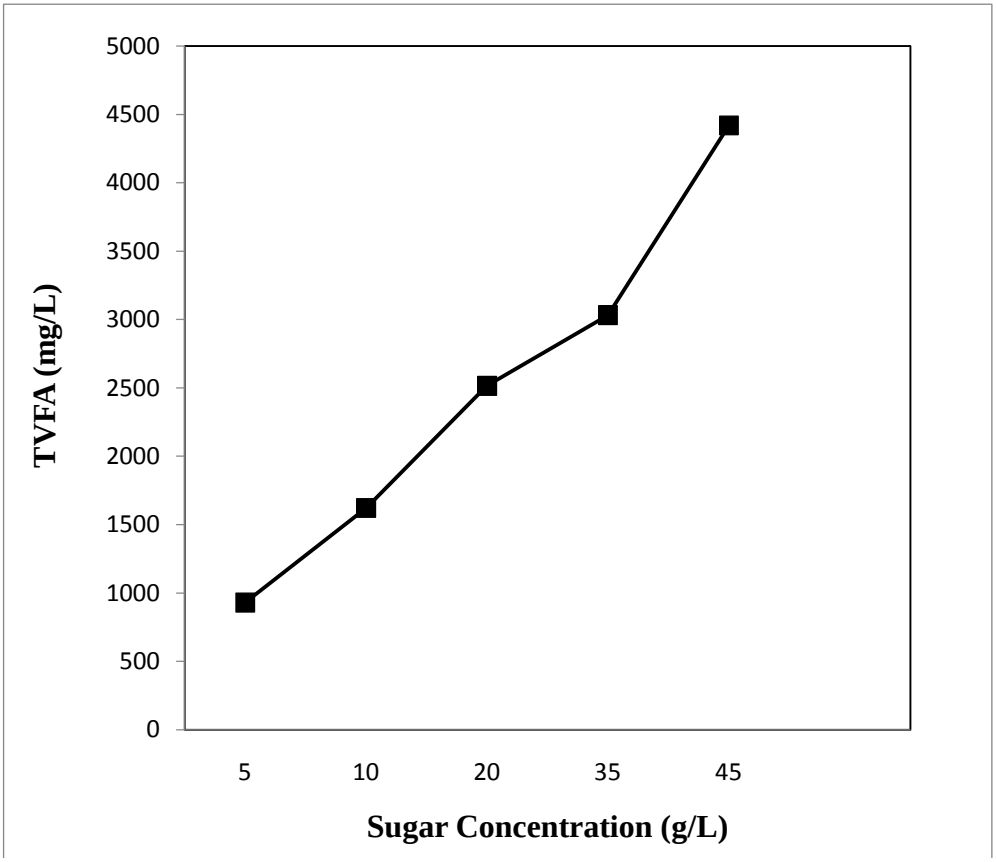

Figure 3.8 Changing of TVFA concentrations depend on varying concentrations of sugar

Figure 3.8 shows TVFA concentrations changes depend on the sugar concentration. TVFA concentrations decreased linearly with the sugar concentration. The end of experiment, the lowest concentration of TVFA (930 mg/L) was obtained from 5 g/L initial sugar concentration contained bottle, which had lowest initial sugar concentration. The highest TVFA concentration was obtained (4420 mg/L) from 45 g/L which had the highest initial sugar concentration.

Sugar compounds were converted to H_2, VFAs, CO_2 and biomass during dark fermentation. Final TVFA concentrations are closely related to hydrogen gas production since H_2 gas is a co-product of VFA formation. Final TVFA concentrations increased with increasing initial sugar concentrations. High TVFA production affect hydrogen production as expected due to product inhibition on microorganisms.

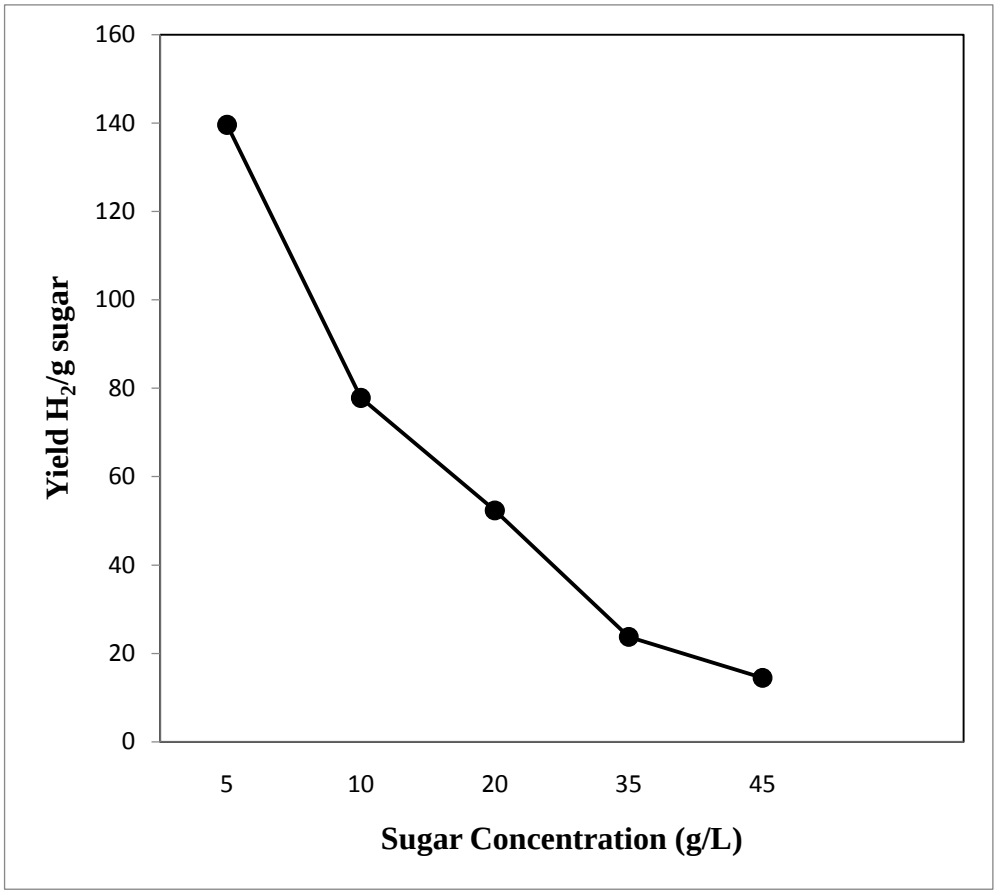

Figure 3.9 Variation of hydrogen production efficiency with the sugar concentration

Variation of the hydrogen production efficiency with the sugar concentration is shown in Figure 3.9. Maximum hydrogen production efficiency was observed in 5 g/L sugar concentration as 139.58 mL H_2/g sugar. Minimum hydrogen production efficiency was observed in 45 g/L sugar concentration as 14.48 mL H_2/g sugar. The highest amount of hydrogen produced 20 g/L sugar concentration's hydrogen production efficiency was observed as 52.34 mL H_2/g sugar.

Hydrogen yield decreased with increasing initial sugar concentration due to inhibition of VFA and presence of furan derivatives resulting acid hydrolysis of waste paper.

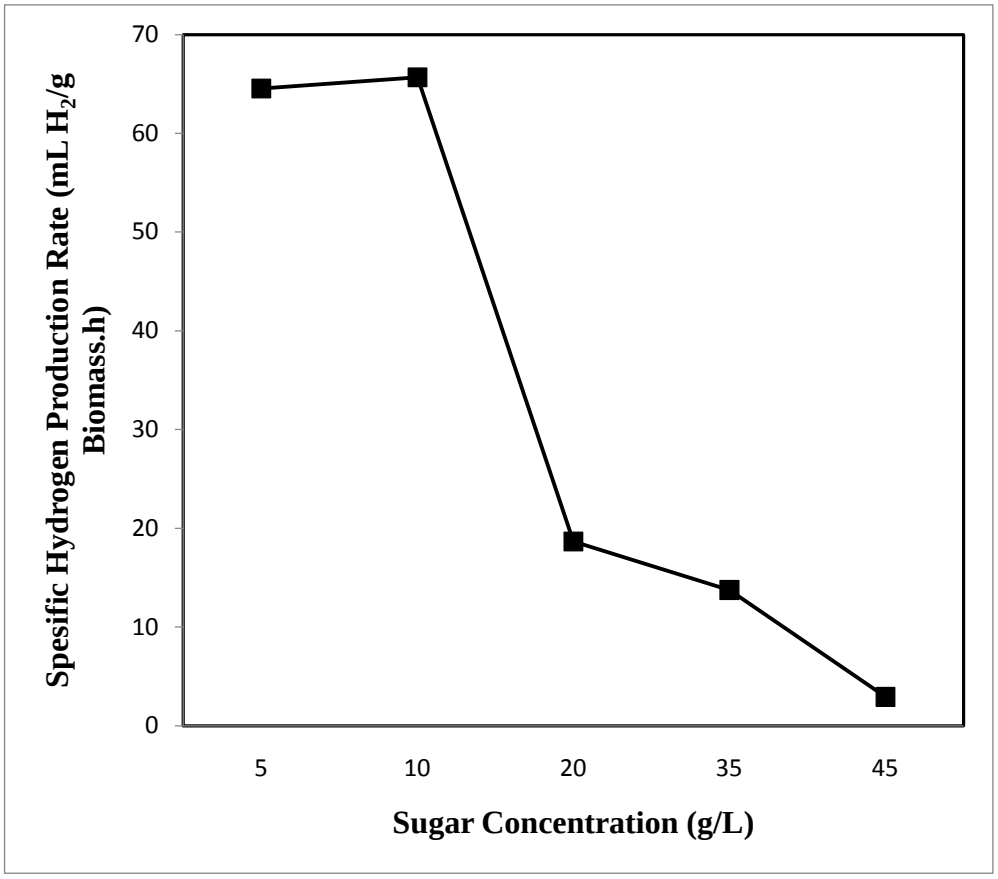

Figure 3.10 Specific hydrogen production rate depend on the sugar concentration

Figure 3.10 shows the specific hydrogen production rates depending on sugar concentration. The based on graphic, maximum hydrogen production rate (65.68 mL H_2/g Biomass.h) was observed from 10 g/L sugar concentration and minimum hydrogen production rate (2.93 mL H_2/g Biomass.h) was observed from 45 g/L sugar concentration.

3.3 The Effect of Biomass Concentration on Production of Hydrogen Gas

To determine the effect of biomass concentration and optimum biomass concentration on hydrogen production from waste paper via dark fermentation, sugar concentration was kept constant and different microorganisms amount were used.

In the first set, maximum cumalative hydrogen volume was obtained from 20 g/L bottle. Therefore, in this set sugar concentration was used as 20 ± 2.5 g/L for all bottles.

For this set of experiments, 6 bottles were used. One bottle of these was used as control bottle. 5 bottles contained media, which was prepared for second set and different amount of microorganisms. Control bottle contained only media. All bottles contained 0.15 L material. Different amount of sludge-medium mixture was used for each bottles, based on 40 mL of sludge-medium mixture was contained 1 g/L microorganisms.

Initial biomass concentrations were adjusted approximately 2 g/L, 1.5 g/L, 1 g/L, 0.5 g/L and 0.25 g/L. Media, which contained approximately 20 g/L sugar concentration, was added for whole bottles.

Initial pH of all bottles were adjusted between 6.8 and 7.2. Each bottle is sealed with gas valve. The periphery of the valve and caps were covered with silicone to do not miss gases. Argon gas was passed through the bottles to provide anaerobic conditions in the bottles. And, bottles were placed in to $37^{o}C$ incubator to start dark fermentation. During fermentation, the pH of a bottles were adjusted between 6.8 to 7.2 every 24 hours with 10 M NaOH. Every day, for each bottles; total sugar analysis, total gas volume measurement and H_2 gas measurements were performed.

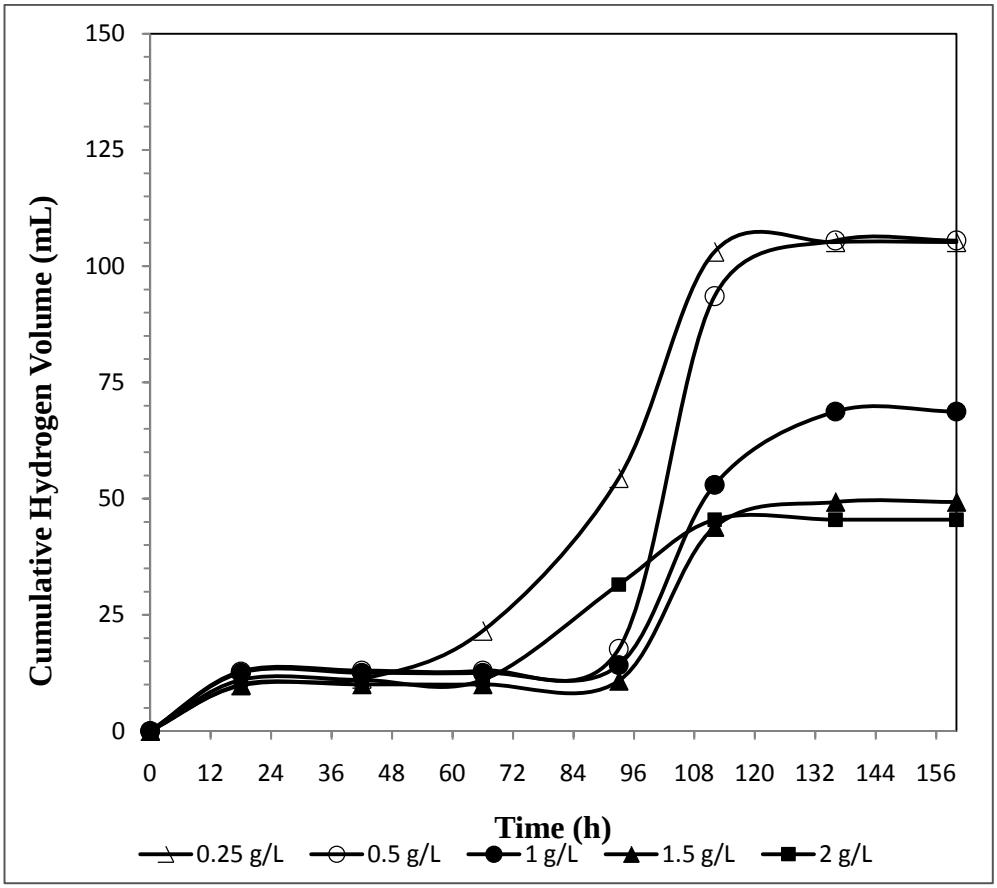

Figure 3.11 Time-dependent changes in the cumulative hydrogen production

Figure 3.11 shows, time-dependent changes in the cumulative hydrogen production for each bottle. The end of experiment, 0.5 g/L and 0.25 g/L initial biomass contained bottles were obtained highest cumulative volume of hydrogen gas concentrations (105.54 mL and 105.17 mL, respectively). 1 g/L initial biomass contained bottle was obtained 68.73 mL cumulative hydrogen. 1.5 g/L and 2 g/L initial biomass contained bottles were obtained 49.30 mL and 45.47 mL cumulative hydrogen, respectively.

Cumulative hydrogen gas production decreased with increasing initial biomass concentration. A decrease in cumulative gas may be due to consumption of hydrogen by biomass to produce acetic acid.

According to Figure 3.11 highest cumulative hydrogen volumes were obtained at the end of 136 hours from 0.5 g/L and 0.25 g/L initial biomass contained bottles. 1.5

g/L and 1 g/L initial biomass contained bottles reached the constant cumulative hydrogen value at the end of 136 hours too. 2 g/L initial biomass contained bottle reached the constant cumulative hydrogen value at the end of 112 hours.

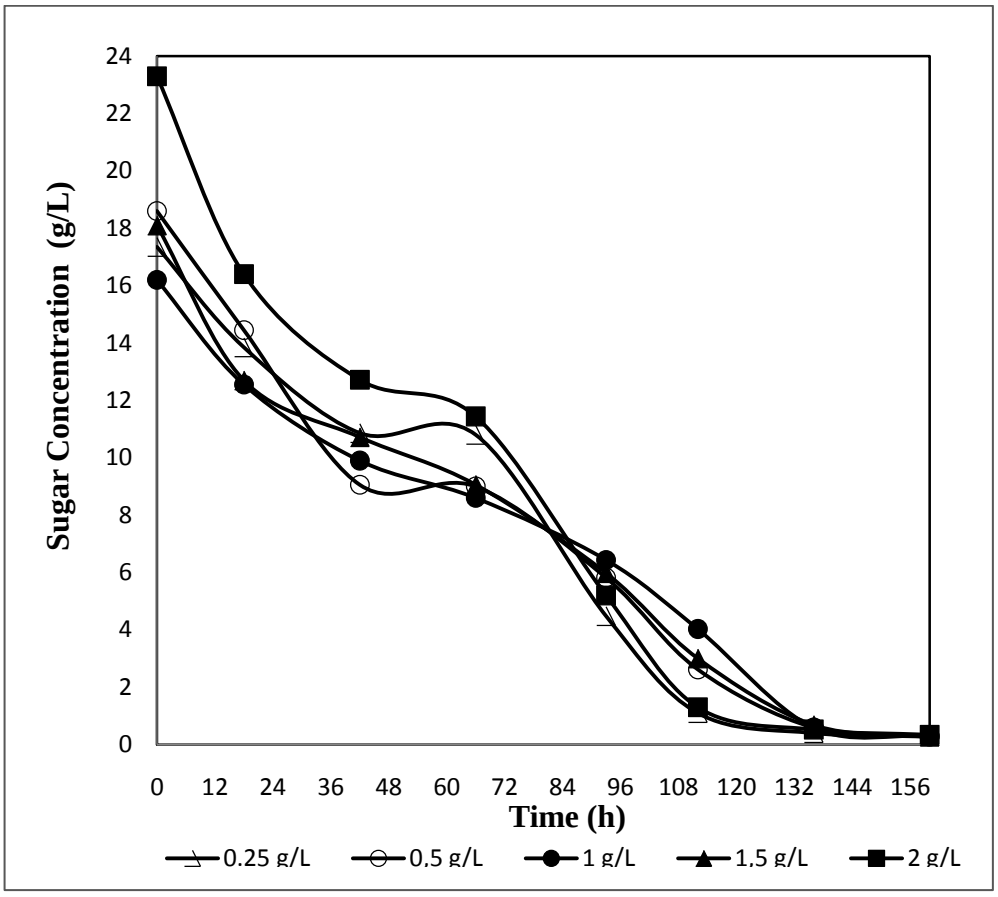

Figure 3.12 Time dependent changes of the sugar concentration

Figure 3.12 shows, the change of substrate concentration depending on the time. Concentration of sugar decreased depend on the time in the graph. In the all of the bottles,except the control bottle, sugar concentrations were almost exhausted at the end of 160 hours. The reason of this, initial sugar concentration of all bottles were approximately same.

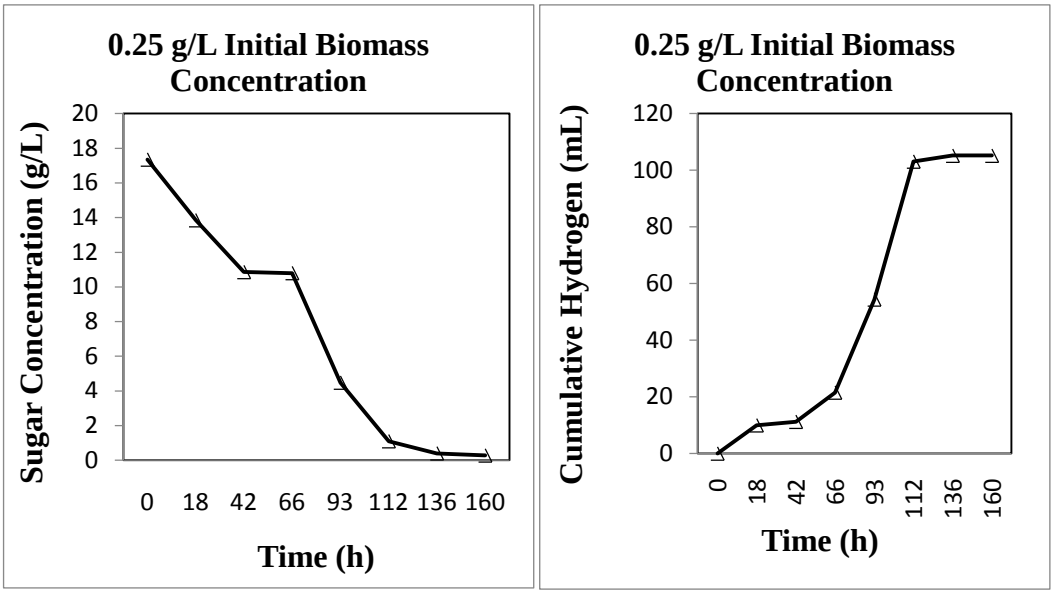

Figure 3.13 Time-dependent changes in sugar concentration and cumulative hydrogen production at 0.25 g/L initial biomass concentration

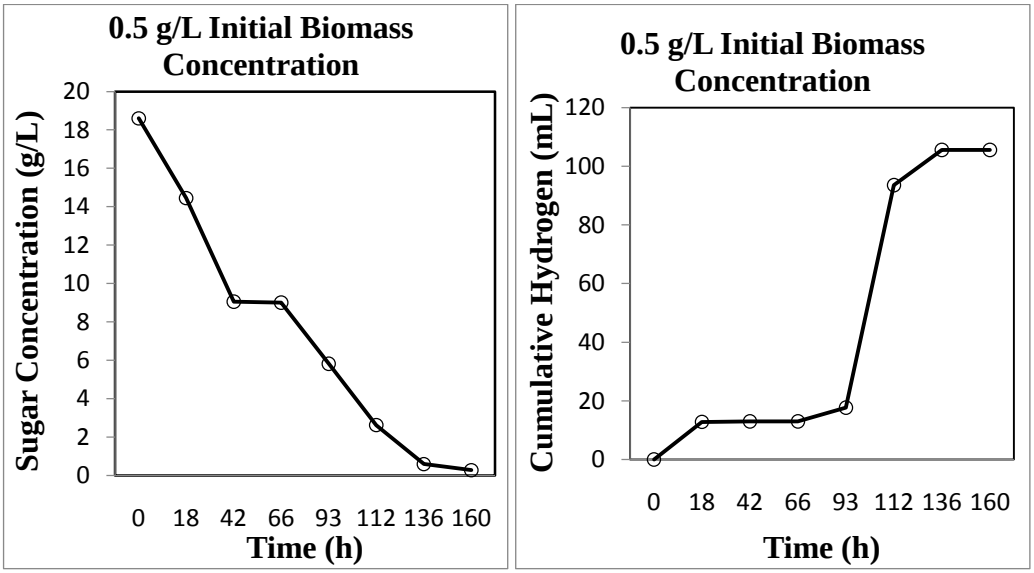

Figure 3.14 Time-dependent changes in sugar concentration and cumulative hydrogen production at 0.5 g/L initial biomass concentration

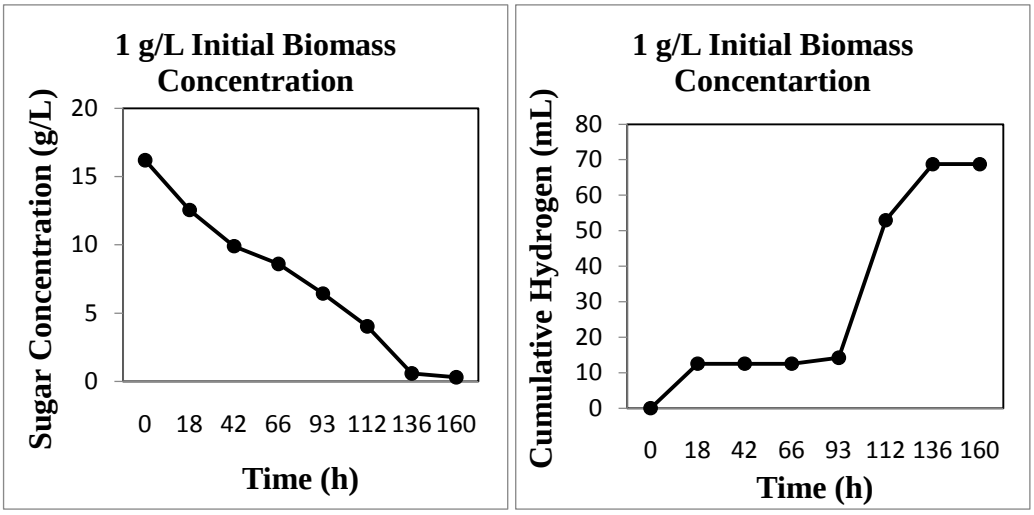

Figure 3.15 Time-dependent changes in sugar concentration and cumulative hydrogen production at 1 g/L initial biomass concentration

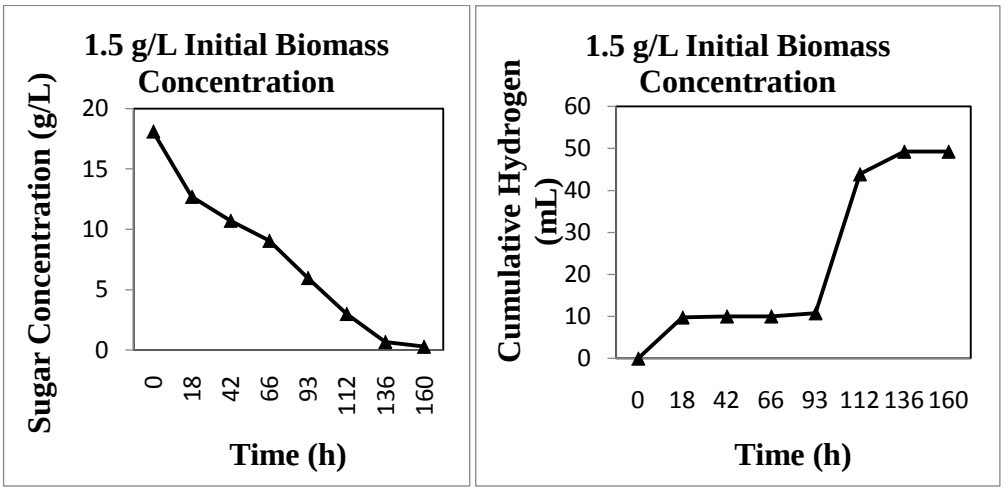

Figure 3.16 Time-dependent changes in sugar concentration and cumulative hydrogen production at 1.5 g/L initial biomass concentration

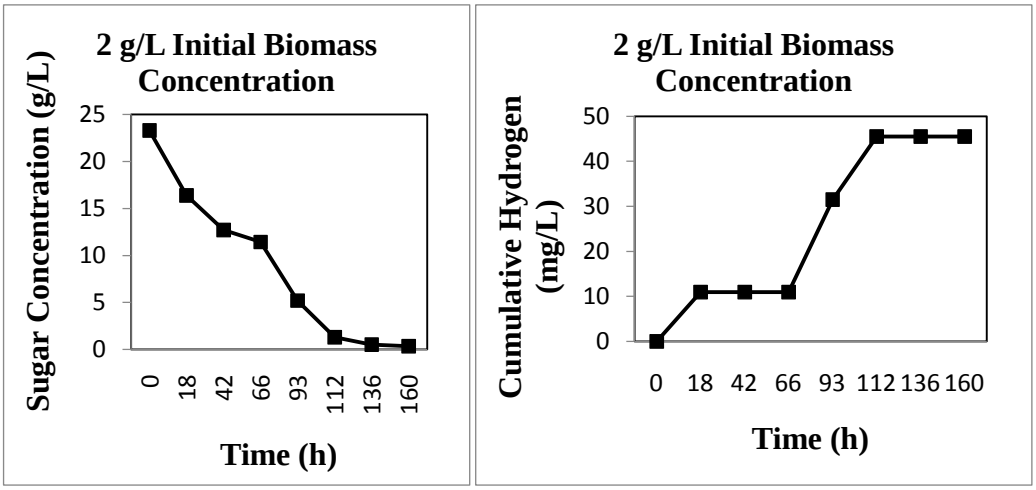

Figure 3.17 Time-dependent changes in sugar concentration and cumulative hydrogen production at 2 g/L initial biomass concentration

Figure 3.13, 3.14, 3.15, 3.16 and 3.17 shows, time-dependent changes in sugar concentration and cumulative hydrogen production for each bottle, separately.

Table 3.2 Gompertz equation constants in the biomass concentration variation experiments

Biomass Concentration (g/L)	**P(mL)**	**R_m (mL/h)**	**λ (h)**	**R^2**
0.25	105.18	7.12	85.30	0.95
0.50	105.76	5.49	90.04	0.98
1.00	69.72	2.40	87.32	0.96
1.50	64.81	0.59	53.36	0.95
2.00	50.52	0.64	40.89	0.96

Table 3.2 shows the calculated Gomperzt constants. Constants were calculated by applying Gompertz equation on the cumulative hydrogen gas production datas. Calculations were performed with Statistica program. The highest cumulative hydrogen gas was obtained from the 0.5 g/L and 0.25 g/L initial biomass concentration and the highest hydrogen production rate (R_m) was calculated as 7.12 mL/h while biomass concentration was 0.25 g/L. Also, the highest stationary

phase(λ) was obtained from 0.5 g/L biomass concentration as 90.04 hours. The lowest stationary phase(λ) was obtained from 2 g/L biomass concentration as 40.89 hours.

The lag phase increased with decreasing initial biomass concentration. The maximum potential hydrogen formation and hydrogen formation rate also increased with decreasing initial biomass concentration. Increases in biomass concentration resulted in lower hydrogen gas potential due to mass transfer limitation in flocks or consumption of hydrogen by homo acetogens.

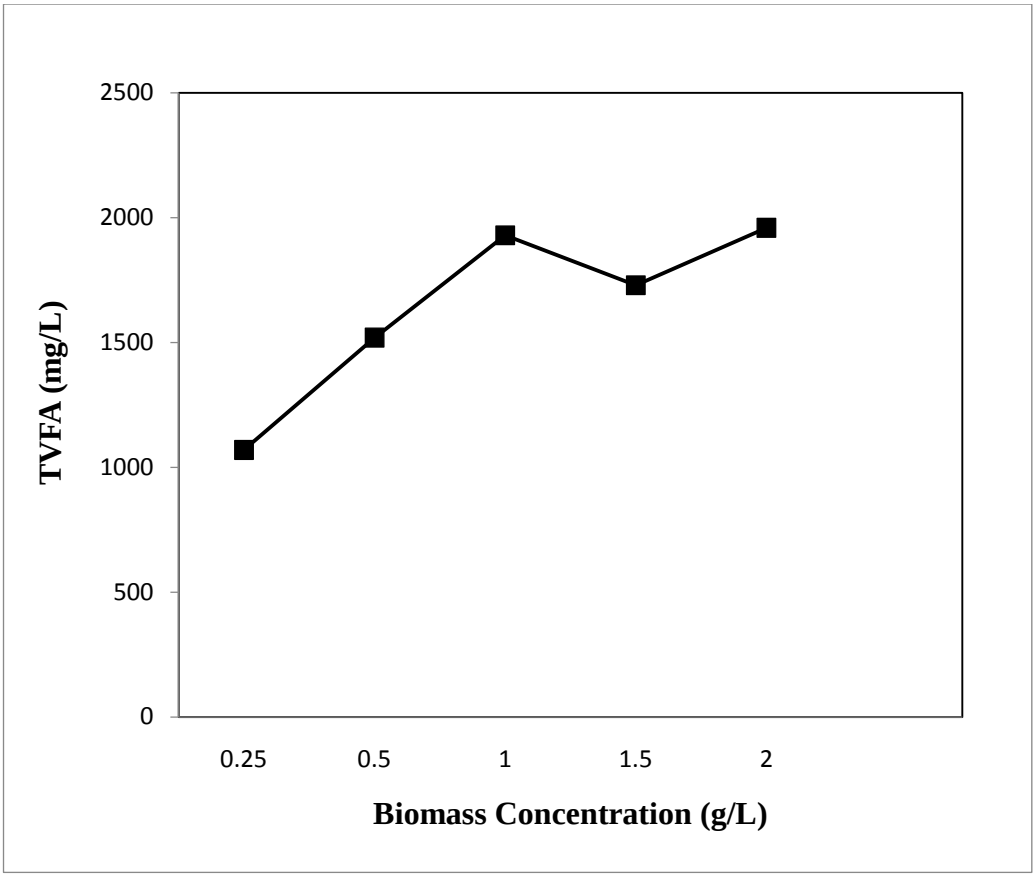

Figure 3.18 Changing of TVFA concentrations depend on varying concentration of biomass

Figure 3.18 shows TVFA concentrations changes depend on the biomass concentration. The end of experiment, the lowest concentration of TVFA (1070 mg/L) was obtained from 0.25 g/L initial biomass contained bottle, which had lowest initial biomass concentration. The highest TVFA concentrations were

obtained from 2 g/L and 1 g/L biomass contained bottles as 1960 mg/L and 1930 mg/L, respectively. Highest TVFA formation observed in highest biomass concentration as expected.

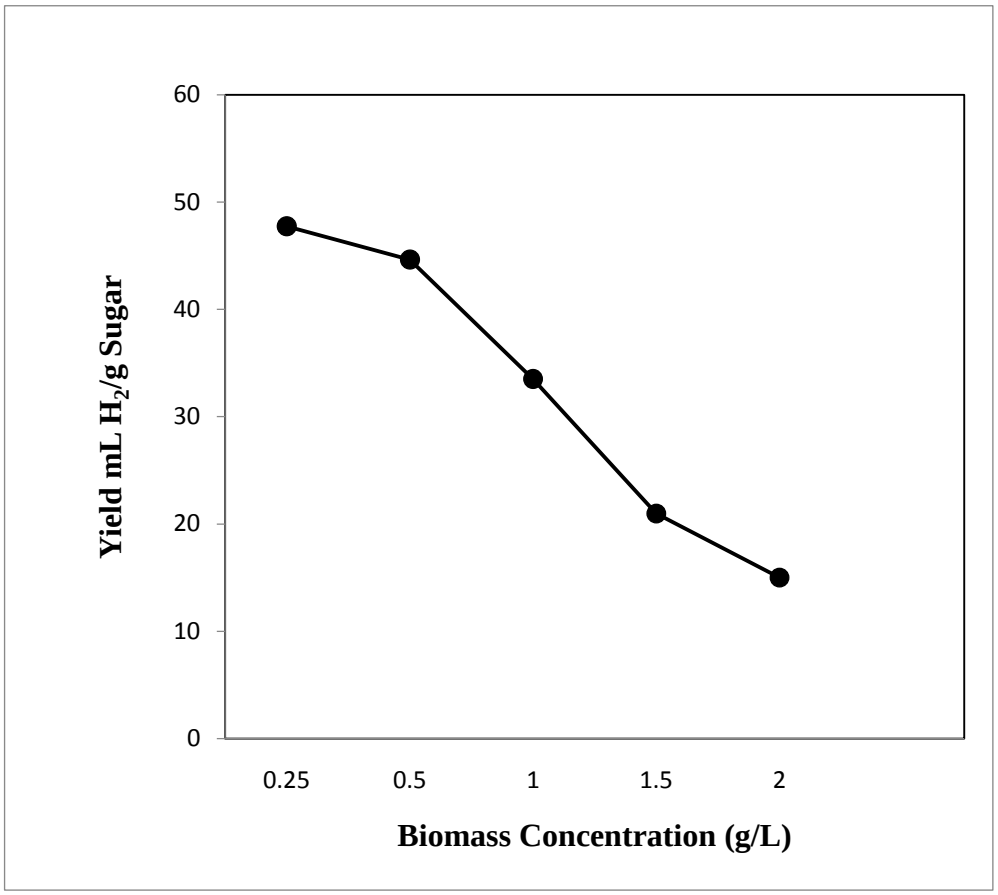

Figure 3.19 Variation of hydrogen production efficiency with the biomass concentration

Variation of the hydrogen production efficiency with the biomass concentration is shown in Figure 3.19. Maximum hydrogen production efficiency was observed in 0.25 g/L biomass concentration as 47.75 mL H_2/g sugar. Minimum hydrogen production efficiency was observed in 2 g/L biomass concentration as 15.01 mL H_2/g sugar. The highest amount of hydrogen produced by 0.5 g/L and 0.25 g/L biomass concentrations and maximum hydrogen production efficiencies were observed same bottles.

Hydrogen formation yield decreased with increasing biomass concentration due to inhibition of TVFA and consumption of H_2 by cells.

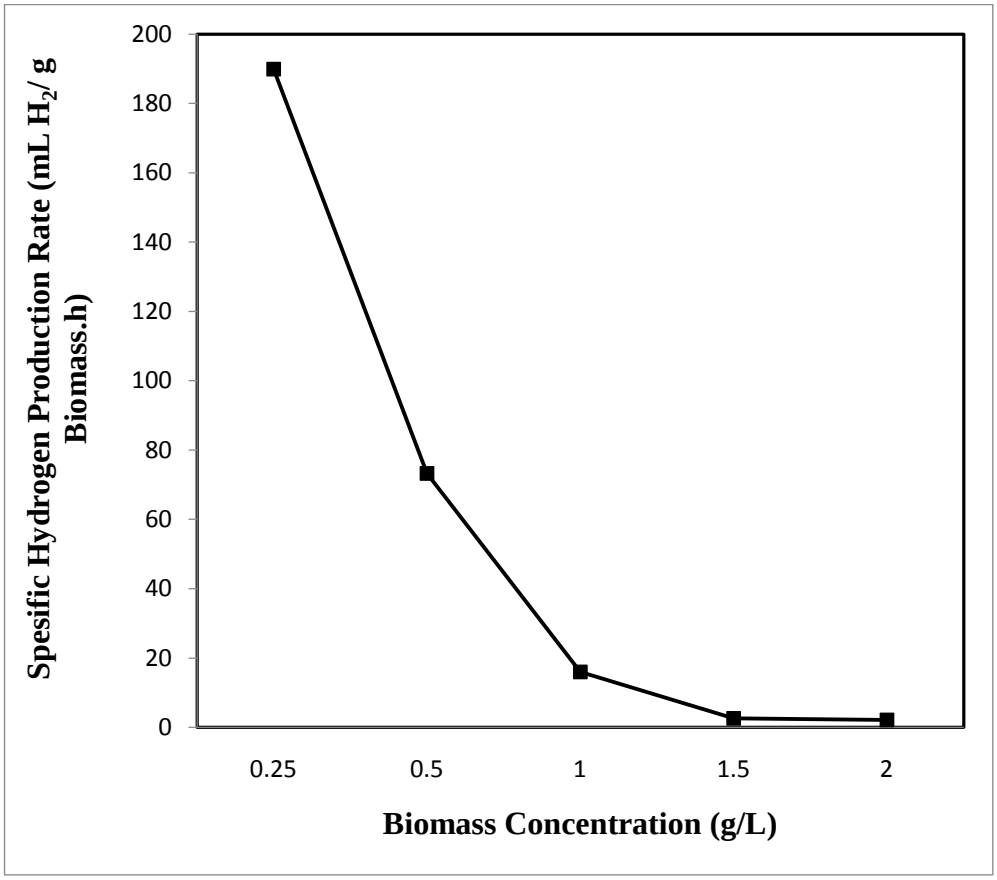

Figure 3.20 Specific hydrogen production rate depend on the biomass concentration

Figure 3.20 shows the specific hydrogen production rates depending on biomass concentration. The based on graphic, maximum hydrogen production rate (189.87 mL H_2/g Biomass.h) was observed from 0.25 g/L biomass concentration and minimum hydrogen production rate (2.13 mL H_2/g Biomass.h) was observed from 2 g/L biomass concentration.

CHAPTER FOUR
CONCLUSION

The fossil fuels reserves have been decreasing in the world and also the combustion of fossil fuels has serious negative effects on the environment. Therefore there is need to a new energy source. This new energy source should be renewable and sustainable. Hydrogen is considered as a viable alternative fuel of future. Because, hydrogen is a clean and high energy fuel.

Researchers have been working on various methods to produce hydrogen gas. Hydrogen production via biological methods are carried out at ambient temperature and pressure, hence less energy more intense than other hydrogen production methods. Therefore, hydrogen production by biological methods are more environmentally friendly than other methods. Studies have shown that, one of the most advantageous method of these biological methods is bio hydrogen production by dark fermentation. Dark fermentation is relatively simple process for hydrogen production. Because, it can be carried out by a wide variety of microorganisms and can be used wide variety substrates, including refuse and waste products. Moreover, fermentative hydrogen production generally proceeds at a higher rate and does not rely on the availability of light sources. And also, dark fermentation is an anaerobic process, so there is no O_2 limitation.

In this thesis bio hydrogen production from waste paper by dark fermentation is investigated. Paper is a kind of lignocellulosic material. Lignocellulosic materials are high carbon containing materials and most abundant renewable biological resource. Lignocellulosic materials constitutes a major portion of agricultural, forest wastes and industrial effluents such as pulp/paper, etc. Therefore, it is good substrate for bio conversition. Also, using of waste papers as substrate has some advantages. Reuse of waste paper provide sustainablity. Sustainability is important because all the choices we pursue and all the actions that we make today will affect everything in the future. Natural resources are being depleted rapidly. So, reuse is important to reduce the use of natural resources. Using of the sugars and vegetable oils found in crops, which are

substrate for first generation bio hydrogen, can create an issue for the use of land for fuel. The land usage is important for the cost of food commodities and food security. However, the using of waste paper as substrate won't create a food-fuel competition.

The main aims of this thesis is to investigate biohydrogen gas production potential from hydrolyzed waste paper by dark fermentation and to find optimum substrate concentration and optimum microorganism concentration.

The experiments were performed in two sets. Each set contains 6 bottles which one of them is control bottle. In both sets, acid hydrolysis at high temperature were performed for waste paper can be used easily as substrate by microorganisms.

To determine the optimum sugar concentration on hydrogen production from waste paper via dark fermentation, different sugar concentrations were used and microorganisms concentration was kept constant, for first set.

At the end of acid hydrolysis for the first set, sugar concentration was obtained as approximately 30 g/L. Liquid form of hydrolysed waste paper was boiled and water was evaporated to obtain high sugar concentration. After evaporation, first sugar concentration was determined as approximately 45.5 g/L. The media was prepared with obtained hydrolysis liquid so that C/N/P ratio was 100/2/0.5. Media with different sugar concentration and 1 g/L microorganisms were placed each bottle except for control bottle. Sugar concentrations in bottles were approximately, 45 g/L, 35 g/L, 20g/L, 10 g/L and 5 g/L. Control bottle included only approximately 5 g/L sugar concentration media. During the experiment, sugar analysis, total gas measurement, hydrogen analysis and pH measurements were performed everyday for all bottles.

For the first set of measurements was continued until sugar concentrations were fixed for each bottles. Therefore measurements of first set lasted approximately 256 hours. The end of experiment, 20 g/L initial substrate contained bottle was obtained highest cumulative volume of hydrogen gas concentration. 125.89 mL

cumulative volume of hydrogen gas was obtained in the end of 120 hours from this bottle. 45.5 g/L, 35 g/L, 10 g/L, 5 g/L initial substrate contained bottles were obtained 75.76 mL, 100.31 mL, 81.41 mL and 72.43 mL cumulative volume of hydrogen gas, respectively. Cumulative hydrogen formation increased with the initial substrate concentration and reached a maximum level at 20 g/L total sugar concentration. Higher total sugar concentrations resulted in a decrease in the yield due to substrate inhibition and by-products of hydrolysis of waste paper.

Gompertz constants were calculated by applying Gompertz equation on the cumulative hydrogen gas production datas. Calculations were performed with Statistica program. Highest hydrogen production rate (R_m) was calculated as 9.85 mL/h while initial sugar concentration was 10 g/L. The lowest stationary phase(λ) was obtained from 5 g/L as 36.73 hours. The level of by-products, particularly furan derivatives after pre-treatment of waste paper may inhibit microorganisms at different initial sugar concentrations. As a result of presence of furan derivatives, the duration of lag phase increased with increasing hydrolyzed sugar as expected. The lowest lag phase obtained at low biomass/substrate ratio.

Maximum hydrogen production efficiency was observed in 5 g/L sugar concentration as 139.58 mL H_2/g sugar. Minimum hydrogen production efficiency was observed in 45 g/L sugar concentration as 14.48 mL H_2/g sugar. Hydrogen yield decreased with increasing initial sugar concentration due to inhibition of VFA and presence of furan derivatives resulting acid hydrolysis of waste paper.

In the end of first set experimets TVFA analysis was performed. According to TVFA analysis, the highest TVFA concentration was obtained (4420 mg/L) from 45 g/L initial sugar concentration containing bottle. In this bottle which has highest TVFA concentration, couldn't reached desired hydrogen production efficiency. The reason for this, high TVFA production affect hydrogen production as expected due to product inhibition on microorganisms.

To determine the optimum microorganism concentration on hydrogen production from waste paper via dark fermentation, different microorganism concentrations were used and sugar concentration was kept constant, for second set. In the first set, maximum cumalative hydrogen volume was obtained from 20 g/L bottle. Therefore, in this set sugar concentration was used as approximately 20 g/L for all bottles.

Initial biomass concentrations were adjusted approximately 2 g/L, 1.5 g/L, 1 g/L, 0.5 g/L and 0.25 g/L for second set. Media, which contained approximately 20 g/L sugar concentration, was added for whole bottles except for control bottle. Control bottle contained only media.

Measurements of second set lasted approximately 160 hours. Because, sugar concentrations were almost exhausted at the end of 160 hours. The end of experiment, 0.5 g/L and 0.25 g/L initial biomass contained bottles were obtained highest cumulative volume of hydrogen gas concentrations. 105.54 mL and 105.17 mL cumulative volume of hydrogen gas was obtained from 0.5 g/L and 0.25 g/L initial biomass contained bottles, respectively. 1 g/L initial biomass contained bottle was obtained 68.73 mL cumulative hydrogen. 1.5 g/L and 2 g/L initial biomass contained bottles were obtained 49.30 mL and 45.47 mL cumulative hydrogen, respectively. Cumulative hydrogen gas production decreased with increasing initial biomass concentration. A decrease in cumulative gas may be due to consumption of hydrogen by biomass to produce acetic acid.

The highest cumulative hydrogen gas was obtained from the 0.5 g/L and 0.25 g/L initial biomass concentration and the highest hydrogen production rate (R_m) was calculated as 7.12 mL/h while biomass concentration was 0.25 g/L. The highest stationary phase(λ) was obtained from 0.5 g/L biomass concentration as 90.04 hours. The lowest stationary phase(λ) was obtained from 2 g/L biomass concentration as 40.89 hours. The lag phase increased with decreasing initial biomass concentration. The maximum potential hydrogen formation and hydrogen formation rate also increased with decreasing initial biomass concentration. Increases in biomass

concentration resulted in lower hydrogen gas potential due to mass transfer limitation in flocks or consumption of hydrogen by homo acetogens.

Maximum hydrogen production efficiency was observed in 0.25 g/L biomass concentration as 47.75 mL H_2/g sugar. Minimum hydrogen production efficiency was observed in 2 g/L biomass concentration as 15.01 mL H_2/g sugar. Hydrogen formation yield decreased with increasing biomass concentration due to inhibition of TVFA and consumption of H_2 by cells.

The lowest concentration of TVFA (1070 mg/L) was obtained at 0.25 g/L of initial biomass concentration. TVFA reached the maximum level (1960 mg/L) when initial biomass concentration was 2 g/L. Highest TVFA formation observed in highest biomass concentration as expected

According to ultimate results of experiments; highest cumulative volume of hydrogen was obtained as 125.89 mL, highest hydrogen production rate was obtained as 9.85 mL/h and highest hydrogen production efficiency was obtained as 139.58 mL H_2/g sugar.

At the end of experiment, 125.89 mL cumulative hydrogen, which is highest cumulative hydrogen value, was obtained from 20 g/L initial substrate concentration and 1 g/L initial microorganism contained bottle in the first set. But, at the end of second set, 68.73 mL cumulative hydrogen volume was obtained from bottle which contained 20 g/L initial substrate concentration and 1 g/L initial microorganism. Despite two sets of experiments carried on same conditions, results are different. The reasons of this result is considered as the stock sludge kept in the fridge for a long time and for each set new sludge was used. Therefore microorganisms activities could be slowdown. Also using mixed culture can be lead to this different results.

Achieved results were observed to be comparable with earlier studies in literature.

The bottleneck of biohydrogen production from lignocellulosic materials; there are limited number of pretreatment methods has been reported as being potentially cost-effective methods in the literature. And also, by-products, which can inhibit microbial growth and metabolism, are released during the pretreatment processes. This affects the efficiency of hydrogen production. Therefore, commercialization of bio hydrogen production from lignocellulosic materials needs new researches and improvement of pre- treatment processes.

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APPENDICES

ABBREVIATIONS

AFEX	Ammonia fiber explosion
C/N	Carbon to nitrogen ratio
CHF	Cumulative hydrogen gas formation
COD	Chemical oxygen demand
HMF	Hydroxymethylfurfural
HPR	Hydrogen production rate
HRT	Hydrolic retantion time
ORP	Oxidation reduction potential
POME	Palm oil mill effluent
Rm	Maximum hydrogen formation rate
SHPR	Specific hydrogen production rate
TS	Total sugar
TVFA	Total volatile fatty acid
VFA	Volatile fatty acid

RAW EXPERIMENTAL DATA

A.1 Raw Data for Effects of Initial Sugar Concentration

Table A.1.1 Raw Data for 45 g/L Initial Sugar Concentration Content Serum Bottle

45 g/L		Sample Volume	Analysis				Hydrogen		V_{liquid}	V_{space}	Vt,gas	Cumulative Hydrogen		Cumulative Hydrogen
Date	t (h)	(mL)	pH	TVFA (mg/l)	Sugar (g/l)	H_2 %			(mL)	(mL)	(mL)	(mL)		(mmol)
0	0	0	6.9		45.50	0	H0	0	150	165	0	V0	0.00	0.00
1	15	3	5.26		45.00	5.7	H1	0.057	147	168	10	V1	10.15	0.41
2	40	3	5.35		37.50	3.3	H2	0.033	144	171	11	V2	10.15	0.41
3	64	3	4.91		33.90	6.5	H3	0.065	141	174	25	V3	17.44	0.70
4	88	3	4.63		28.20	6.8	H4	0.068	138	177	40	V4	20.89	0.84
5	112	3	5.65		28.00	5.3	H5	0.053	135	180	25	V5	20.89	0.84
6	136	3	5.8		24.10	5.1	H6	0.051	132	183	20	V6	21.70	0.87
7	160	3	6.07		18.68	9.2	H7	0.092	129	186	35	V7	32.70	1.32
8	184	3	6.39	2239	11.71	18	H8	0.18	126	189	60	V8	60.41	2.43
9	208	3	6.25	2354	9.40	17	H9	0.17	123	192	31	V9	64.30	2.59
10	232	3	6.53		4.11	18	H10	0.18	120	195	50	V10	75.76	3.05
11	256	3	6.81	4420	1.90	12	H11	0.12	117	198	40	V11	75.76	3.05

Table A.1.2 Raw data for 35 g/L initial sugar concentration content serum bottle

35 g/L		Sample Volume	Analysis				Hydrogen		V_{liquid}	V_{space}	Vt,gas	Cumulative Hydrogen		Cumulative Hydrogen
Date	t (h)	(mL)	pH	TVFA (mg/l)	Sugar (g/l)	H_2 %			(mL)	(mL)	(mL)	(mL)		(mmol)
0	0	0	6.9		34.80	0	H0	0	150	165	0	V0	0,00	0.00
1	15	3	5.21		26.40	6.3	H1	0.063	147	168	10	V1	11.21	0.45
2	40	3	5.22		22.90	4.9	H2	0.049	144	171	25	V2	11.21	0.45
3	64	3	4.72		19.40	5.6	H3	0.056	141	174	15	V3	13.42	0.54
4	88	3	4.7		13.72	5.3	H4	0.053	138	177	15	V4	13.85	0.56
5	112	3	5.93		12.74	4.7	H5	0.047	135	180	10	V5	13.85	0.56
6	136	3	6.27		12.22	11	H6	0.11	132	183	32	V6	29.04	1.17
7	160	3	6.17		7.89	23	H7	0.23	129	186	105	V7	75.84	3.05
8	184	3	6.41	2662	3.79	25	H8	0.25	126	189	80	V8	100.31	4.04
9	208	3	6.37	3032	1.22	20	H9	0.2	123	192	23	V9	100.31	4.04
10	232	3	6.65		0.42	14.4	H10	0.144	120	195	20	V10	100.31	4.04

Table A.1.3 Raw data for 20 g/L initial sugar concentration content serum bottle

20 g/L		Sample Volume	Analysis				Hydrogen		V_{liquid}	V_{space}	Vt,gas	Cumulative Hydrogen		Cumulative Hydrogen
Date	t (h)	(mL)	pH	TVFA (mg/l)	Sugar (g/l)	H_2 %			(mL)	(mL)	(mL)	(mL)		(mmol)
0	0	0	6.9		18.90	0	H0	0	150	165	0	V0	0.00	0.00
1	15	3	4.95		16.96	6.1	H1	0.061	147	168	10	V1	10.86	0.44
2	40	3	5.01		14.72	3.3	H2	0.033	144	171	15	V2	10.86	0.44
3	64	3	4.62		13.98	6.3	H3	0.063	141	174	25	V3	17.75	0.71
4	88	3	5.74		8.42	8.7	H4	0.087	138	177	30	V4	24.80	1.00
5	112	3	6.3		3.67	24	H5	0.24	135	180	110	V5	79.00	3.18
6	136	3	6.16		0.49	33	H6	0.33	132	183	90	V6	125.89	5.07
7	160	3	7.02		0.37	23	H7	0.23	129	186	10	V7	125.89	5.07
8	184	3	6.87	2515	0.31	20	H8	0.2	126	189	10	V8	125.89	5.07
9	208	3	6.8		0.25	16	H9	0.16	123	192	2	V9	125.89	5.07

Table A.1.4 Raw data for 10 g/L initial sugar concentration content serum bottle

10 g/L		Sample Volume	Analysis				Hydrogen		V_{liquid}	V_{space}	Vt,gas	Cumulative Hydrogen		Cumulative Hydrogen
Date	t (h)	(mL)	pH	TVFA (mg/l)	Sugar (g/l)	H_2 %			(mL)	(mL)	(mL)	(mL)		(mmol)
0	0	0	6.9		7.90	0	H0	0	150	165	0	V0	0.00	0.00
1	15	3	5.43		7.34	7.6	H1	0.076	147	168	10	V1	13.53	0.54
2	40	3	4.81		6.16	3.4	H2	0.034	144	171	11	V2	13.53	0.54
3	64	3	5.33		3.79	9.7	H3	0.097	141	174	20	V3	26.53	1.07
4	88	3	5.79		0.83	25	H4	0.25	138	177	110	V4	81.41	3.28
5	112	3	6.99		0.24	21	H5	0.21	135	180	0	V5	81.41	3.28
6	136	3	7.02		0.18	18	H6	0.18	132	183	5	V6	81.41	3.28
7	160	3	6.86		0.16	13	H7	0.13	129	186	10	V7	81.41	3.28
8	184	3	6.95	1621	0.16	12	H8	0.12	126	189	5	V8	81.41	3.28
9	208	3	7.04		0.15	11	H9	0.11	123	192	1	V9	81.41	3.28

Table A.1.5 Raw data for 5 g/L initial sugar concentration content serum bottle

5 g/L		Sample Volume	Analysis				Hydrogen		V_{liquid}	V_{space}	Vt,gas	Cumulative Hydrogen		Cumulative Hydrogen
Data	t (h)	(mL)	pH	TVFA (mg/l)	Sugar (g/l)	H_2 %			(mL)	(mL)	(mL)	(mL)		(mmol)
0	0	0	6.9		3.84	0	H0	0	150	165	0	V0	0.00	0.00
1	15	3	4.92		3.70	6.9	H1	0.069	147	168	10	V1	12.28	0.49
2	40	3	4.97		3.18	14	H2	0.14	144	171	50	V2	31.63	1.27
3	64	3	5.95		0.17	26	H3	0.26	141	174	75	V3	72.43	2.92
4	88	3	6.6		0.12	22	H4	0.22	138	177	0	V4	72.43	2.92
5	112	3	6.91		0.09	19	H5	0.19	135	180	3	V5	72.43	2.92
6	136	3	6.93		0.08	16	H6	0.16	132	183	10	V6	72.43	2.92
7	160	3	7.02		0.08	12	H7	0.12	129	186	10	V7	72.43	2.92
8	184	3	7.07	930	0.08	11	H8	0.11	126	189	5	V8	72.43	2.92
9	208	3	7.12		0.08	8	H9	0.08	123	192	2	V9	72.43	2.92

Table A.1.6 Raw data for control serum bottle

Control		Sample Volume	Analysis				Hydrogen		V_{liquid}	V_{space}	Vt,gas	Cumulative Hydrogen		Cumulative Hydrogen
Date	t (h)	(mL)	pH	TVFA (mg/l)	Sugar (g/l)	H_2 %			(mL)	(mL)	(mL)	(mL)		(mmol)
0	0	0	6.9		5.56	0	H0	0	150	165	0	V0	0.00	0.00
1	15	3	6.8		5.00	4.8	H1	0.048	147	168	0	V1	8.06	0.32
2	40	3	6.8		4.99	3.3	H2	0.033	144	171	10	V2	8.06	0.32
3	64	3	6.8		2.64	8.2	H3	0.082	141	174	25	V3	18.74	0.75
4	88	3	6.8		1.95	8.3	H4	0.083	138	177	10	V4	19.99	0.80
5	112	3	6.8		1.55	7.7	H5	0.077	135	180	1	V5	19.99	0.80
6	136	3	6.8		1.50	8.4	H6	0.084	132	183	5	V6	21.92	0.88
7	160	3	6.8		1.47	7	H7	0.07	129	186	5	V7	21.92	0.88
8	184	3	6.8	103	1.33	6.3	H8	0.063	126	189	8	V8	21.92	0.88
9	208	3	6.8		1.16	6.1	H9	0.061	123	192	1	V9	21.92	0.88

A.2 Raw Data for Effects of Initial Biomass Concentration

Table A.2.1 Raw data for 2 g/L initial biomass concentration content serum bottle

2 g/L		Sample Volume	Analysis				Hydrogen		V_{liquid}	V_{space}	Vt,gas	Cumulative Hydrogen		Cumulative Hydrogen
Date	t (h)	(mL)	pH	TVFA (mg/l)	sugar (g/l)	H_2 %			(mL)	(mL)	(mL)	(mL)		(mmol)
0	0	0	6.9		23.30	0	H0	0	150	165	0	V0	0.00	0.00
1	18	3	4.79		16.40	6.1	H1	0.06	147	168	12	V1	10.98	0.44
2	42	3	4.92		12.72	5.6	H2	0.06	144	171	12	V2	10.98	0.44
3	66	3	4.98		11.44	4.2	H3	0.04	141	174	30	V3	10.98	0.44
4	93	3	5.61		5.20	12	H4	0.12	138	177	55	V4	31.51	1.27
5	112	3	5.29		1.31	16	H5	0.16	135	180	40	V5	45.47	1.83
6	136	3	6.8		0.53	14	H6	0.14	132	183	8	V6	45.47	1.83
7	160	3	6.92	1960	0.35	12	H7	0.12	129	186	12	V7	45.47	1.83

Table A.2.2 Raw data for 1.5 g/L initial biomass concentration content serum bottle

1.5 g/L		Sample Volume	Analysis				Hydrogen		V_{liquid}	V_{space}	Vt,gas	Cumulative Hydrogen		Cumulative Hydrogen
Date	t (h)	(mL)	pH	TVFA (mg/l)	Sugar (g/l)	H_2 %			(mL)	(mL)	(mL)	(mL)		(mmol)
0	0	0	6.9		18.10	0	H0	0	150	165	0	V0	0.00	0.00
1	18	3	5.3		12.70	5.5	H1	0.06	147	168	10	V1	9.79	0.39
2	42	3	5.67		10.72	5.1	H2	0.05	144	171	15	V2	10.04	0.40
3	66	3	4.92		9.06	3.8	H3	0.04	141	174	10	V3	10.04	0.40
4	93	3	5.42		5.98	3.9	H4	0.04	138	177	12	V4	10.80	0.43
5	112	3	6.04		3.01	16	H5	0.16	135	180	70	V5	43.90	1.77
6	136	3	6.17		0.69	15	H6	0.15	132	183	45	V6	49.30	1.98
7	160	3	6.64	1730	0.29	12	H7	0.12	129	186	15	V7	49.30	1.98

Table A.2.3 Raw data for 1 g/L initial biomass concentration content serum bottle

1 g/L		Sample Volume	Analysis				Hydrogen		V_{liquid}	V_{space}	Vt,gas	Cumulative Hydrogen		Cumulative Hydrogen
Date	t (h)	(mL)	pH	TVFA (mg/l)	Sugar (g/l)	H_2 %			(mL)	(mL)	(mL)	(mL)		(mmol)
0	0	0	6.9		16.20	0	H0	0	150	165	0	V0	0.00	0.00
1	18	3	5.09		12.55	7	H1	0.07	147	168	11	V1	12.53	0.50
2	42	3	5.47		9.90	6	H2	0.06	144	171	11	V2	12.53	0.50
3	66	3	4.88		8.60	3.9	H3	0.04	141	174	8	V3	12.53	0.50
4	93	3	5.6		6.44	4.4	H4	0.04	138	177	15	V4	14.19	0.57
5	112	3	5.79		4.03	19	H5	0.19	135	180	65	V5	52.95	2.13
6	136	3	6.58		0.59	21	H6	0.21	132	183	55	V6	68.73	2.77
7	160	3	7.2	1930	0.30	14	H7	0.14	129	186	10	V7	68.73	2.77

Table A.2.4 Raw data for 0.5 g/L initial biomass concentration content serum bottle

0.5 g/L		Sample Volume	Analysis				Hydrogen		V_{liquid}	V_{space}	Vt,gas	Cumulative Hydrogen		Cumulative Hydrogen
Date	t (h)	(mL)	pH	TVFA (mg/l)	Sugar (g/l)	H_2 %			(mL)	(mL)	(mL)	(mL)		(mmol)
0	0	0	6.9		18.60	0	H0	0	150	165	0	V0	0.00	0.00
1	18	3	5.07		14.45	7.2	H1	0.07	147	168	10	V1	12.82	0.52
2	42	3	4.88		9.06	6.6	H2	0.07	144	171	15	V2	13.00	0.52
3	66	3	4.89		9.00	4.9	H3	0.05	141	174	12	V3	13.00	0.52
4	93	3	6.02		5.82	6.7	H4	0.07	138	177	20	V4	17.67	0.71
5	112	3	5.95		2.63	27	H5	0.27	135	180	145	V5	93.56	3.77
6	136	3	6.63		0.60	26	H6	0.26	132	183	50	V6	105.54	4.25
7	160	3	7.13	1520	0.28	18	H7	0.18	129	186	10	V7	105.54	4.25

Table A.2.5 Raw data for 0.25 g/L initial biomass concentration content serum bottle

0.25 g/L		Sample Volume	Analysis				Hydrogen		V_{liquid}	V_{space}	Vt,gas	Cumulative Hydrogen		Cumulative Hydrogen
Date	t (h)	(mL)	pH	TVFA (mg/l)	Sugar (g/l)	H_2 %			(mL)	(mL)	(mL)	(mL)		(mmol)
0	0	0	6.9		17.35	0	H0	0	150	165	0	V0	0.00	0.00
1	18	3	6.07		13.85	5.6	H1	0.06	147	168	10	V1	9.97	0.40
2	42	3	6.14		10.86	5.3	H2	0.05	144	171	30	V2	11.21	0.45
3	66	3	5.46		10.80	9.5	H3	0.1	141	174	30	V3	21.53	0.87
4	93	3	5.69		4.48	20	H4	0.2	138	177	70	V4	54.40	2.19
5	112	3	6.05		1.10	29	H5	0.29	135	180	110	V5	103.10	4.15
6	136	3	6.8		0.40	27	H6	0.27	132	183	18	V6	105.17	4.23
7	160	3	7.09	1070	0.28	20	H7	0.2	129	186	10	V7	105.17	4.23

Table A.2.6 Raw data for control serum bottle

Control		Sample Volume	Analysis				Hydrogen		V_{liquid}	V_{space}	Vt,gas	Cumulative Hydrogen		Cumulative Hydrogen
Date	t (h)	(mL)	pH	TVFA (mg/l)	Sugar (g/l)	H_2 %			(mL)	(mL)	(mL)	(mL)		(mmol)
0	0	0	6.9		17.90	0	H0	0	150	165	0	V0	0.00	0.00
1	18	3	6.8		16.30	7.3	H1	0.07	147	168	8	V1	12.85	0.52
2	42	3	6.8		12.58	6.9	H2	0.07	144	171	5	V2	12.85	0.52
3	66	3	6.8		11.60	6.7	H3	0.07	141	174	20	V3	14.05	0.57
4	93	3	6.8		7.69	14	H4	0.14	138	177	75	V4	37.67	1.52
5	112	3	6.8		7.26	14	H5	0.14	135	180	10	V5	39.49	1.59
6	136	3	6.8		6.58	12	H6	0.12	132	183	8	V6	39.49	1.59
7	160	3	6.8	620	6.42	11	H7	0.11	129	186	5	V7	39.49	1.59