DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

SEQUENTIAL BIOFUEL PRODUCTION FROM WASTE FRUITS

by
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İZMİR

SEQUENTIAL BIOFUEL PRODUCTION FROM WASTE FRUITS

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by
İlknur HACIOĞLU

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M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"SEQUENTIAL BIOFUEL PRODUCTION FROM WASTE FRUITS"** completed by **İLKNUR HACIOĞLU** under supervision of **ASSOC. PROF. DR. SERPİL ÖZMIHÇI** 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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SEQUENTIAL BIOFUEL PRODUCTION FROM WASTE FRUITS

ABSTRACT

The utilization of waste fruits in energy generation is a remarkable process that is a solution to energy crisis. In this study, dry fruits containing mycotoxins were evaluated for biohydrogen production by batch dark fermentation. Edible and waste fruits were used to determine the effect of mycotoxin on hydrogen production potential. 55.65 mL and 81.35 mL of hydrogen was produced with raisins and dry figs with mycotoxin, respectively. In edible raisins and dry figs, maximum cumulative hydrogen volume was found as 180.41 mL and 266.9 mL, respectively. Mycotoxin had a negative effect. Acidic pretreatment did not contribute to hydrogen production volume. However enhanced hydrogen production yields were obtained compared with the one without pretreatment.

Microwave pretreatment with different application times and power, was applied to dry figs for mycotoxin removal. Maximum total sugar concentration and total aflatoxin removal were obtained at 400W power, and 10 minutes. The optimum substrate concentration in the production of biohydrogen from dry figs with microwave pretreatment at 400W was 59 g L^{-1}, where the cumulative hydrogen volume was 161.9 mL and the yield was 207.5 mL H_2 g^{-1} total sugar.

Different microoganism concentrations were tested to enhance biohydrogen production potential with a substrate concentration of 59 g L^{-1}. The hydrogen production potential was enhanced with 1 g L^{-1} organism concentration. The liquid phase of the hydrogen production experiments obtained at the optimal conditions were subjected to methane generation. The maximum cumulative volume was 25.14 mL and the yield was 1.25 mL CH_4 g^{-1} TVFA.

Keywords: Biohydrogen, mycotoxin, dry fruit, microwave, dark fermentation

ATIK MEYVEDEN ARDIŞIK BİYOYAKIT ÜRETİMİ

ÖZ

Atık meyvelerin enerji üretiminde kullanımı, enerji krizine çözüm olabilecek dikkat çekici bir prosestir. Bu çalışmada, mikotoksin içeren kuru meyveler kesikli karanlık fermantasyon ile biyohidrojen üretiminde değerlendirilmiştir. Mikotoksinin hidrojen üretim potansiyeli üzerindeki etkisini belirlemek için yenilebilir ve atık meyveler kullanılmıştır. Mikotoksinli kuru üzüm ve kuru incirden sırasıyla 55.65 mL ve 81.35 mL hidrojen üretilmiştir. Yenilebilir kuru üzüm ve kuru incirde maksimum kümülatif hidrojen hacmi sırasıyla 180.41 mL ve 266.9 mL olarak bulunmuştur. Mikotoksin, biyohidrojen üretimi üzerinde olumsuz bir etkiye sahiptir. Asidik ön muamele, hidrojen üretim hacmine katkıda bulunmamıştır. Ancak, ön muamelesiz duruma kıyasla hidrojen üretim veriminde artış sağlanmıştır.

Kuru incirlerde mikotoksin giderimi için farklı uygulama süreleri ve gücünde mikrodalga ön işlemi uygulanmıştır. Maksimum toplam şeker konsantrasyonu ve toplam aflatoksin giderimi 400W güçte ve 10 dakikalık uygulama süresinde elde edilmiştir. 400W'da mikrodalga ön işlemi ile kuru incirden biyohidrojen üretiminde optimum başlangıç substrat konsantrasyonu 59 g L^{-1} olup, kümülatif hidrojen hacmi 161.9 mL ve verim 207.5 mL H_2 g^{-1} toplam şeker olarak bulunmuştur.

59 g L^{-1} substrat konsantrasyonunda biyohidrojen üretim potansiyelini artırmak için farklı mikroorganizma konsantrasyonları test edilmiştir. 1 g L^{-1} organizma konsantrasyonunda hidrojen üretim potansiyeli artmıştır. Ayrıca en uygun substrat ve organizma konsantrasyonlarında biyometan üretilmiştir. Deneylerin sonunda, 1.25 mL CH_4 g^{-1} TVFA üretim verimi ile maksimum 25.14 mL biyometan üretilebilmiştir.

Anahtar kelimeler: Biyohidrojen, mikotoksin, kuru meyve, mikrodalga, karanlık fermantasyon

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CHAPTER 1
INTRODUCTION

1.1 Preface/Description of the Problem

Fossil fuel is the main energy source globally and its overuse to meet the demands of the ever-increasing human population will lead to its depletion and pose a serious threat to energy security (Bhatia et al., 2021). It is predicted that the main oil and natural gas exporters of the Middle East countries will be unable to meet total energy needs in the next 50 years (Shields-Menard et al., 2018). Another concern is environmental issues such as global warming caused by fossil fuels and the release of greenhouse gases (CO_2, SO_2, NOx) emissions (Saratale et al., 2013). Renewable energy sources has received considerable attentions in recent years as they can overcome these problems. (Das & Veziroğlu, 2001). Solar energy, wind energy, geothermal energy and bioenergy such as bioethanol, biobuthanol, biomethane and biohydrogen have revealed great potential as being environmentally friendly and low carbon emitting energy. Among them, hydrogen is considered as the most promising alternative fuel due to its zero emissions to the environment and its high and efficient calorific value (122 kJ/kg) (Sharma et al., 2022).

Physical or conventional chemical methods in hydrogen production are both costly and involve processes that require high energy and temperature. For this reasons, biological methods in its production will lead to the use of substrates that will provide renewable carbon sources and an environmentally friendly production process will be realized. The biological methods used in the production of biohydrogen are biophotolysis, photofermentation, dark fermentation or integrated photo and dark fermentation (Maman et al., 2022). Contrary to photosynthetic methods, dark fermentation is more attractive than other biological processes due to its low light and energy requirement, high biohydrogen production rate and relatively abundant, renewable, cheap and highly biodegradable organic wastes to be used as substrates (Dzulkarnain et al., 2022) Substrates with high organic content such as agricultural wastes, lignocellulosic wastes, domestic solid wastes, kitchen wastes,

vegetable and fruit wastes have a high potential and can be used in the production of biohydrogen (Panin et al., 2020 ; Srivastava et al., 2021). Among these substrates, fruit waste attracts attention as an ideal substrate for biohydrogen production, due to its moisture content, high volatile solids content, high nutrient and sugar concentrations and biodegradability (Mahato et al., 2020).

Every year, a great amount of the fruit (more than 640 million metric tons) is wasted worldwide due to poor quality and inappropriate storage conditions (Mahato et al., 2020). There are many country-specific wastes that need to be evaluated for energy production affecting the food industry. For Türkiye, these fruits are raisins and dry figs. Contamination with mycotoxins in the export of these fruits causes economic losses. According to 2019/2020 data, Türkiye ranks first in the world with a production of 305.000 tons of raisins and 89.000 tons of dried figs (Türkiye Ministry of Agriculture and Forestry, 2022a; Türkiye Ministry of Agriculture and Forestry, 2022b). These waste materials can be converted into high value-added products such as biofuel production. However, the use of raw materials contaminated with mycotoxins in biofuel production may adversely affect the course of anaerobic degradation and production efficiency due to the complex and stable structures of mycotoxins (Ferrara et al., 2021; Tacconi et al., 2018).

Pretreatment methods including frying, dry heat treatment, radiation, ozone, ammonia, hydrogen bisulfite, hydrogen peroxide, chemical treatment with citric acid or lactic acid, enzymatic or microbiological processes in mycotoxin degradation have been tried many times. The water-assisted microwave (MW) application, which has attracted attention in recent years, has the advantage of decreasing the decomposition temperature of the mycotoxin and increasing the decomposition rate. Here, water absorbs microwave energy and generates the heat required for decomposition (Farag et al., 1996; Pluyer et al., 1987; Zhang et al., 2020, 2021).

The end products of a conventional anaerobic decomposition of biomass are methane and carbon dioxide. The process provide an efficient energy cycle from biomass to the gaseous fuel. The concept of hydrogen production under anaerobic

condition emerged a new biotechnological approach by separating the hydrogenic (hydrolysis and acidogenesis) and methanogenic stages under controlled conditions. This approach enhances biohydrogen and biomethane generation. In the acidogenesis phase, volatile fatty acids (acetic acid, butyric acid, lactic acid) and hydrogen are produced from hydrolyzed organic materials. The products produced during acidogenesis are reduced to hydrogen by obligate hydrogen producing bacteria called acetogen or homoacetogen. Then the non-gaseous products of hydrogen fermentation (organic acids) serve as substrates for the methanogenic consortium (Detman et al., 2017; Mari et al., 2020; Mitraka et al., 2022).

Evaluation of food waste in energy production is an important issue. Fruit wastes are remarkable because they have high sugar concentration. However, the sugar content of these wastes must be easily solved into the fermentation liquid and the mycotoxin must be separated from the fruit before fermentation. The organic content of waste fruits with mycotoxin can be evaluated for biohydrogen and biogas production. Biohydrogen can be obtained by acetogenic-anaerobic bacteria and methane production by methanogens.

CHAPTER 2
LITERATURE SURVEY

2.1 Hydrogen as a Clean Energy

Hydrogen is the element with the lowest density (relative density of 0.0695 with respect to air). It is an odorless, tasteless, and colorless gas. The hydrogen market is growing by up to 10% each year and is projected to exceed $191.80 billion in 2024. Hydrogen fuel cells are recognized as a promising technology for the future global energy supply. With a market share of 11% in 2025 and 34% in 2050, it is estimated that 36% and 69% of total global energy demand will be met from renewable sources, respectively. Hydrogen production technology will reduce the use of crude oil and coal to 40.5% and 36.7%, respectively, by 2030. Hydrogen is an alternative energy carrier and can be easily produced via electrolysis and converted back into electricity by fuel cell power plants. The use of hydrogen in the transportation system, both as a fuel in internal combustion engines and for fuel cell, has received very positive attention as an energy policy issue. The use of hydrogen does not contain toxic gas formation and CO_2 emission, its sole product is water vapor. Hydrogen powered vehicles significantly reduce exhaust emissions (Ahmed et al., 2021; Hosseini & Wahid, 2016).

Hydrogen is currently used as fuel for space shuttles in NASA's space program. Beside it is used in fuel cell to provide heat, electricity and drinking water to astronauts. Many automobile companies have also introduced cars and trucks that use fuel cells. The most common use of hydrogen in the future will be in the transportation. Hydrogen fuel cell vehicles can be run three times more efficiently than a gasoline-powered engine. The properties that make hydrogen more advantageous than other fuels are its flammability limits, ignition energy, explosion limits, flame speed, very low density and high diffusivity (Garland et al., 2012).

The product of biological ways hydrogen production is called 'biohydrogen' and unlike chemical or electrochemical processes, biomass is catalyzed by

microorganisms at ambient temperature and pressure (Kotay & Das, 2008). Fuel cell operation from biohydrogen is a sustainable and environmentally friendly method of electricity generation as it does not require any high energy input. The development of continuous and stable biohydrogen production from renewable sources is essential for commercial applications of fuel cells because hydrogen has such a high potential to generate electricity using a fuel cell. It can also be combined with biogas as fuel to ensure efficient combustion of fuel and to reduce exhaust gas emissions (Saravanan et al., 2021).

2.2 Biohydrogen Production Methods

About 90% of hydrogen is produced with conventional chemical methods, by the reactions of fossil fuels at high temperatures or high pressures. The product of these technologies is known as blue, gray or black H_2. Among all types of H_2, green hydrogen is closer to zero emissions and has a relatively low carbon footprint. Biohydrogen production as a green technology is a process in which microorganisms are used to produce hydrogen from a wide variety of biomass, where production takes place under mild conditions.

Wind, solar or hydroelectric assisted water electrolysis is included in green hydrogen production. It is a process in which water is electrically split into H_2 and O_2. However, for this electricity-based green hydrogen production to be implemented worldwide, it is a necessity to secure the continuous use of electricity. Biomass gasification, biophotolysis, dark fermentation, photofermentation, dry fermentation and combined dark and photofermentation are the others examples of green hydrogen production technologies. In gasification, a significant amount of energy is consumed even just for water evaporation from wet biomass. Photofermentation, dark fermentation and biophotolysis can be able to use wet biomass to produce green biohydrogen (Wang et al., 2023; Lee et al., 2022; Hosseini & Wahid, 2016)

Biohydrogen production can be carried out using carbohydrate-rich raw materials in mild conditions via fermentation. Organic wastes from food processing industries,

restaurant waste, household waste can be used (Manuel et al., 2022). Dark fermentation has some advantages over other biohydrogen production methods. First of all, the rate of dark fermentation is faster than that of photofermentation. The dark process is a stable and robust due to the functionality of the bacteria responsible for hydrogen production. A wide variety of substrates can be used compared to the photofermentative process (Lee et al., 2022).

2.2.1 Biophotolysis

Biophotolysis is associated with plant-type photosynthesis process. Like plants, some *Cyanobacteria* and green algae species can split water into H_2 and O_2 molecules by using sunlight through the catalytic activity of hydrogenase enzyme. Since hydrogenase activity is inhibited by oxygen, biophotolysis takes place under anaerobic conditions (Azwar et al., 2014; Kapdan & Kargi, 2006). This is a light dependent reaction. Biohydrogen production by microalgae through biophotolysis can occur in two ways as direct and indirect biophotolysis (Azwar et al., 2014).

Eukaryotic microalgae and prokaryotic cyanobacteria are capable of capturing atmospheric CO_2 and using their photosynthetic structures. In biophotolysis, light energy is absorbed by the photosystem of microalgae (PSI and PSII), and this energy is then transferred via the electron transport chain. Ferredoxin is reduced and electrons flow to the hydrogenase enzyme. (Nagarajan et al., 2017; Azwar et al., 2014).

In direct biophotolysis, light energy is directly converted to hydrogen by photosynthetic reactions via equations 2.1 and 2.2 below. In these reactions, the photosystem absorbs light and transports electrons to ferredoxin. This event causes the reduction of water. Ferredoxin in the reduced state moves electrons to the hydrogenase and hydrogen production is catalyzed by the conversion of a proton to the biohydrogen. Although the only reactant is water and it is a cheap method, the most important problem encountered in direct biophotolysis is that the oxygen produced by photolysis inhibits the hydrogenase enzyme activity (Melitos et al.,

2021; Li et al., 2022; Gürtekin, 2014)

$$2H_2O + \text{‘light energy’} \rightarrow 2H_2 + O_2 \quad (2.1)$$

$$2H^- + 2\text{Ferredoxin}^- \rightarrow H_2 + 2\text{Ferredoxin} \quad (2.2)$$

Indirect biophotolysis has been developed to overcome the oxygen sensitivity of hydrogen-producing enzymes in direct biophotolysis and to eliminate oxygen-related process restrictions. Cyanobacteria are capable of using CO_2 in the air as a carbon source and sunlight as an energy source. In indirect biophotolysis, electrons from water are used in the carbon fixation in Calvin cycle and then, it is stored in the form of carbohydrates to produce H_2. Here, hydrogen production by the two-step reactions is expressed in equations 2.3 and 2.4 (Ahmed et al., 2021).

$$12H_2O + 6CO_2 + \text{‘light energy’} \rightarrow C_6H_{12}O_6 + 6O_2 \quad (2.3)$$

$$C_6H_{12}O_6 + 12H_2O + \text{‘light energy’} \rightarrow 12H_2 + 6CO_2 \quad (2.4)$$

In studies on biohydrogen production by biophotolysis, hydrogen production rates in the range of 1.1 to 14.9 mL were obtained using green microalgae (*Chlamydomonas* sp., *Chlamydomonas reinhardtii*, *Platymonas subcordiformis*, *Chlorella sorokiniana* etc.) and cyanobacteria (*Anabaena* sp., *Arthrospira* sp., *Synechococcus* sp. etc.) (Berberoglu et al., 2008; Chader et al., 2009; Laurinavichene et al., 2006)

2.2.2 Photofermentation

Photofermentation is a fermentative conversion of VFAs into H_2 and CO_2 in the presence of light and under anaerobic conditions by the purple non-sulphur bacteria (PNS). PNS can convert the remaining VFAs in the hydrogenic effluent of dark fermentation to produced biohydrogen through the photofermentation process (Bhatia et al., 2021). However, in many recent studies, various wastes such as agricultural biomass, crude glycerol from biodiesel production and industrial wastes rich in organic substances have begun to be used as substrates in photofermentative hydrogen production (Mirza et al., 2013; Pott et al., 2013, 2014; Seifert et al., 2010a, 2010b). Brewery wastewater, dairy wastewater, fermented food waste, fresh olive mill waste, pulp an paper mill effluent, blackstrap molasses are also used as substrate (Melitos et al., 2021).

There are many studies using VFAs and industrial wastewater as substrates in photofermentation. These studies were summarized by Kapdan & Kargı, (2016) and different VFAs gave yields around 1.1 - 2.8 mol H_2 mol^{-1} substrate. Also, some literature using wastewaters as substrate was demonstrated in the review giving variuos hydrogen production yields and rates. Although the use of wastewater streams seems to be an advantage in this process, biohydrogen production by photofermentation has some limitations. For example, low hydrogen production rate, alkalinity of the fermentation medium, toxicity of high substrate concentration on bacteria, turbidity and color change that may occur during fermentation are issues that need to be overcome. Combined dark and photofermentation using co-cultures to avoid the inhibition caused by high organic acid concentration is among the topics that have been studied recently (Reungsang et al., 2018). In a study to solve the inhibition caused by alkalinity, olive mill wastewater (OMW) pretreated with clay was used as a substrate for photofermentative hydrogen production. Maximum hydrogen production of 31.5 L H_2 L^{-1} OMW was achieved. Here, coal is used to reduce the nitrogen content of the fermentation medium (Eroğlu et al., 2018).

2.2.3 Dark Fermentation

Dark fermentation is a process in which carbohydrate rich substrate is anaerobically broken down by hydrogen-producing obligate or facultative anaerobic organisms. Under anaerobic conditions, protons act as an electron acceptor, neutralizing the electrons obtained by the oxidation of organic substrates, resulting with hydrogen gas (Ghimire et al., 2015).

Since light penetration is not important in dark fermentation, a wide variety of colored substrates can also be used when compared to photofermentation. The selection of the organic substrate to be used as raw material in dark fermentation is an important parameter. Harsh pretreatment methods are generally not necessary as the sugar content in organic substrates containing simple sugars and disaccharides such as lactose or sucrose can easily diffuse into the fermentation medium. For example, starch-based cereals (barley, wheat, corn) or sugar containing crops (sugarcane, sugar beet, sorghum) contain easily fermentable sugars. On the contrary, materials with cellulosic structure that are resistant to chemical and biological processes definitely need an appropriate pretreatment prior to fermentation (Lukajitis et al., 2018).

Mixed cultures of various types of microorganisms are used in dark fermentation. Mixed cultures mainly consist of hydrogen producer, hydrogen consumer and non hydrogen producer bacteria. Therefore, an inoculum pretreatment that will inhibit hydrogen-consuming organisms while enriching hydrogen-producing organisms in culture is necessary to increase hydrogen yield (Magama et al., 2021). The volatile fatty acids (VFA's) are produced as by-products of dark fermentative biohydrogen production. These VFAs are commonly consist of acetate, butyrate, small fraction of propionate and lactate. Theoretically, 4 mol H_2 can be obtained for a mol glucose if only acetic acid is produced (Eker & Erkul, 2018; Ghosh & Roy, 2019; Tosuner et al., 2019).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2.5)$$

In practically, 4 mol H_2/mol glucose can't be obtained because the VFAs mixture contain other organic acids. Reaction equations showing hydrogen yields depending on the VFAs type are given below (Soares et al., 2020)

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \text{ (butyric acit pathway)} \quad (2.6)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \text{ (propionic acid pathway)} \quad (2.7)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH \text{ (lactic acid pathway)} \quad (2.8)$$

Carbohydrate-rich substrates have been widely used in the literature for biohydrogen production via dark fermentation. However, sustainable energy requires sustainable and renewable resources. For this reason, the use of waste as a substrate has been at the forefront in recent years (Ghimire et al., 2015). Pascualone et al., (2019) tried different substrate concentrations (5 to 25 g reducing sugars/L) of heat treated fruit and vegetable waste (FVW) and found a hydrogen production of 615 mL L^{-1} H_2 and a VHPR of 372.6 ml H_2 L^{-1} day^{-1} in mesophilic dark fermentation conditions. They also investigated the effect of heat treatment applied to the substrate on the production volume. They found that biohydrogen production with the heat treated substrate resulted in 2-fold higher hydrogen production. Magama et al., (2021) evaluated FVWs collected from restaurant garbage in the production of biohydrogen by dark fermentation. They used anaerobic sludge as inoculum and applied different pretreatments to the seed culture to increase H_2 yield. They reached a yield of 142.74 NmL g^{-1} VS H_2 with acidic pretreatment. In another study, biohydrogen production was carried out by dark fermentation under thermophilic and mesophilic conditions at substrate:inoculum ratios (SIR) ranging from 0.8 to 174 $VS_{substrate}$/ g $VS_{inoculum}$ from fruit wastes containing apple, lemon and melon. The highest cumulative total gas yield was found as 113 mL STP g^{-1} VS in mesophilic conditions. It has been reported that the hydrogen yield increases as the SIR value decreases (Cahyari et al., 2019). Ghimire et al. (2019), summarized the studies in which biohydrogen production was carried out by dark fermentation under different temperature and pH conditions using different types of substrates such as vegetable

waste, food waste, lignocellulosic waste, cheese whey, deoiled algae cake etc. These substrates resulted in hydrogen yields in the range of 24.8-310 mL H_2 g^{-1} VS^{-1}.

2.3 Feedstock for Biohydrogen Production

Feedstocks to produce biofuels are classified according to their origin as first, second, third and fourth generation feedstocks. First generation substrates are produced using sucrose or starch-rich food source biomass such as wheat, barley, corn, rice, potatoes, sweet sorghum, canola, soybean, sunflower, sugar beet, sugarcane. Second generation feedstocks are based on non-food biomass, namely lignocellulosic material, including agricultural and forestry waste and trees grown for energy production (Honarmandrad et al., 2022). Third generation raw materials use micro and macralgae as raw materials, which attract attention with their richness in carbohydrates (Maliha & Abu-Hijleh, 2022). Fourth generation substrates are classified as algae used for different purposes and the residual can be used for further production (Shokravi et al., 2021)

Feedstocks used in energy production should not compete with food resources and arable land used for food production. Therefore, it is advantageous to use second generation ones such as corn cobs, rice bran, wheat straw, wheat bran, grass, sawdust, poplar leaves, waste parts of fruits and vegetables etc. (Cheng et al., 2011). Although 3rd generation raw materials provide a production without sacrificing arable land and food resources with their high carbohydrate and fat contents, mass production of microalgae in an open field and their collection and concentration from a dilute cultivation medium is quite costly. For this reason, many studies in the literature have focused on first and second generation raw materials (Taşkan et al., 2022; Cheng et al., 2011).

In different studies on biohydrogen production from a wide variety of lignocellulosic wastes such as corn stover, wheat straw, wheat bran, sweet sorghum, rice bran, food wastes using mixed cultures as inoculum gave hydrogen yields in the range of 0.98 to 3 mol H_2 mol^{-1} hexose and hydrogen production volumes of 212 mL

(Datar et al., 2007; Liu & Cheng, 2010; Fan et al., 2006; Kongjan & Angelidaki, 2010; Shi et al., 2010; Kim et al., 2008).

Pretreatment of substrates before fermentation is an important issue in biohydrogen production. Especially for substrates in cellulose and lignin structure, a pretreatment that can be applied chemically, physically or enzymatically is essential. Because, organisms used in hydrogen production can only use simple sugars directly as carbon sources. Similarly, organic wastes, food wastes or wastewater cannot be used directly by organisms in hydrogen production without any pretreatment (Cheng et al., 2011; Chozhavendhan et al., 2020). Abubackar et al., (2019) obtained 27.19 NmL H_2 g^{-1} VS biohydrogen yield from autoclave pretreated vegetable and fruit wastes using dry fermentation method.

In fermentative hydrogen production, substrate dependent variables such as substrate:inoculum ratio and initial substrate concentration, as well as the pretreatment to be applied to the substrate, strongly affect the hydogen production yield. Along with these variables, various parameters such as inoculum type, operating pH and temperature, reactor operating mode, hydraulic retention time reveal the process sensitivity (Cappai et al., 2018). In a study in which peach pulp was used as a substrate through batch dark fermentation, the highest yield of 123.2 mL H_2 g^{-1} TOC was produced with 46% hydrogen in the gas phase at 5% (v/v) inoculum concentration. The highest hydrogen production rate (35.6 mL H_2 h^{-1}) was obtained with 10% activated sludge concentration (Argun & Dao, 2017). In another study, Argun & Dao, (2018) used peach pulp as a substrate to observe the effect of substrate inhibition on biohydrogen production yield and found the highest hydrogen production yield as 180 mL H_2 g^{-1} COD and hydrogen formation rate as 6.44 mL H_2 h^{-1} in the case where the initial substrate concentration was 34.16 g TS L^{-1}. At concentrations up to 34.16 g TS L^{-1}, there was an increase in yield and rate due to substrate limitation, while a decrease was observed in both parameters due to substrate inhibition at higher concentrations. The effect of different initial concentrations of wheat flour on biohydrogen production by dark fermentation from heat-treated sludge was investigated, and in the experiments performed between

10.69 and 35.14 g L^{-1} glucose concentrations, the highest cumulative hydrogen volume was obtained as 2379.6 mL at the highest substrate concentration. However, the highest yield of 1.9 mol H_2 mol^{-1} glucose was achieved even at 10.69 g L^{-1} sugar concentration, probably due to substrate inhibition (Han et al., 2015).

2.4 Biohydrogen Production from Waste Materials

The use of pure carbohydrates in hydrogen production is expensive. During the hydrolysis of carbohydrates, simple sugars such as glucose, sucrose, and hexose are formed. For example, rice, wheat and potato slurry are a high source of carbohydrates. Cellulose and lignocelluloses are the most abundant biopolymers available from fruits and vegetables. Dark fermentation uses a wide variety of waste materials to produce hydrogen, including organic wastes such as food and household waste, wastewater and industrial process waters. Among them, municipal solid waste is a very attractive raw material due to its easy availability and high organic content. The organic fraction of municipal solid waste includes food waste and kitchen waste (Yadav et al., 2018; Chozhavendhan et al., 2020).

Potential wastes used in biohydrogen production can be classified as agricultural wastes containing lignin and cellulose, organic fractions of municipal solid wastes (OFMSW), industrial wastewater rich in carbohydrates and waste sludge of wastewater treatment plant (Sekoai & Daramola, 2017; Kapdan & Kargı, 2006).

2.4.1 Agricultural Waste

The cellulose and hemicellulose content in the substrates included in this waste group must be hydrolyzed to carbohydrates before fermentation. For this purpose, grinding followed by mechanical or chemical delignification is recommended (Kapdan & Kargı, 2006). On the other hand, the pretreatment applied reduces the crystallinity of macromolecular cellulose, breaks it down into glucose that microorganisms can use directly, and reduces hemicellulose to monosaccharides (Zheng et al., 2022). In order to evaluate the wastes on biohydrogen production,

many studies have been carried out on lignocellulosic biomass consisting of agricultural wastes such as plant stalks, forestry wastes, grass, sawdust, unused parts of plants such as straw and bark (Chozhavendhan et al., 2020; Li & Chen, 2007; Datar et al., 2007; Pakarinen et al., 2008).

Fan et al. (2006) produced 68.1 mL H_2 g^{-1} TVS maximum cumulative hydrogen yield at 126.5 hours from wheat straw waste pretreated with HCl. They have achieved a 136-fold increase compared to the wheat wastes that it was not pretreated. The maximum hydrogen production rate of 10.14 mL H_2 g^{-1} TVS h^{-1} was obtained. In another study, 57 mL of H_2 g^{-1} VS biohydrogen produced from corn stalks pretreated with 0.5% NaOH, resulting in a 19-fold increase in yield compared to the untreated material. When the acid pretreatment was combined with the heat pretreatment, the maximum biohydrogen yield was obtained as 150 mL H_2 g^{-1} VS (Zhang et al., 2007). Similar results were obtained when steam explosion was applied to the corn straw, with a yield increasing from 9 mL H_2 g^{-1} raw corn straw to 68 mL H_2 g^{-1} when pretreatment application was compared (Li & Chen, 2007). In a study in which 8% NaOH and heat treatment at 100°C for 10 minutes were applied in combination to cotton plant waste, 17.1 mL H_2 g^{-1} VS was obtained with a 4-fold increase in yield compared to untreated waste (Ghasemian et al., 2016). Zhang et al. (2021) obtained 27.34 mL H_2 g^{-1} TS maximum hydrogen yield from corn cobs by microwave application at 772 W for 5 minutes. There was an 80% increase in the yield. As can be seen, when no pretreatment is applied to the lignocellulosic substrates, the yield is limited to 0.5 to 16 mL H_2 g^{-1} VS. The main limiting factor in biohydrogen production is the pretreatment step effecting on hydrogen production potential. Many different pretreatment methods applied to lignocellulosic feedstock give a different production potential. Therefore, the pretreatment step should be evaluated well to enhance the production yield and rate (Banu et al., 2020).

2.4.2 Industrial Wastewater

Industrial wastewater includes wastewater from sugar refineries (sugarcane vinasses, tequila vinasses etc.), plastic industry, cereals, olive mill, palm oil mill, cheese whey, brewery, baker's yeast, dairy industry, paper mill and beverage processing industry. These industries produce large amount of wastewater with high sugar and starch content. Thus, it constitutes an attractive raw material source that supports biochemical dark fermentation production by acid-producing bacterial species such as *Clostridium* and *Bacillus* (Sekoai & Daramola, 2017; Jayabalan et al., 2019; Kumar et al., 2015; Azbar et al., 2009).

Industrial wastewater contains toxic compounds such as phenols, benzenes, halogenated aliphatics that can inhibit hydrogen production. The costly and time-consuming chemical treatment methods required to eliminate these compounds prior to fermentation limit the use of these substrates in biohydrogen production. (Moreno-Andrade et al., 2015).

In biohydrogen production using different microorganism consortia from paper mill wastewater, 5.29 mmol H_2 g^{-1} $COD_{initial}$ was obtained with mixed culture and 1.22 mmol H_2 g^{-1} $COD_{initial}$ hydrogen yield was obtained when paperboard mill sludge was used. The optimum inoculum:substrate ratio was found to be 6 when mixed culture was used. It was argued in this study that the inoculum substrate ratio (ISR) directly affects the growth pattern of the microorganism, that high inoculum concentration prevents hydrogen production as a result of the biodegradation of the substrate by excessively increasing nutrient consumption, and high ISR leads to insufficient food for the microorganism. On the other hand, low ISR increased the production of acetic acid, which supports H_2 production. (Farghaly et al., 2015; Dechrugsa et al.,2013).

Brewery (Pachiega et al., 2019), beverage (Sivagurunathan & Lin, 2020), dairy industry (da Silva et al., 2019), palm oil mill effluent (Maaroff et al., 2018), alcohol industry wastewater (Poontawegeratigar et al., 2012) were used as a substrate in the

production of biohydrogen, and production yields in the range of 1.19 to 3.76 mol H_2 mol^{-1} sugar were achieved.

2.4.3 Waste Sludge from Wastewater Treatment Plant

Waste sludge occurred in wastewater treatment plants contains large amounts of carbohydrates and proteins that can be used in energy production. Anaerobic digestion of excess sludge can be accomplished in two steps. In the first stage organic matter will be converted to organic acids and in the second stage, organic acids will be used in hydrogen gas production using photo-heterotrophic bacteria. Hydrogen yields of 0.6 mol kg^{-1} $COD_{initial}$ and 1.2 mg H_2 g^{-1} COD were reported when waste sludge was used as substrate (Kapdan & Kargı, 2006).

2.4.4 Organic Fraction of Municipal Solid Waste

Municipal solid wastes consist of plastic, glass, metal, paper/cardboard, wood, kitchen and food wastes. These wastes are generated as a result of domestic, industrial or commercial activities. In 2011, approximately 2 billion tons of municipal solid waste was generated worldwide. It is estimated that this amount will reach to 9.5 billion tons in 2050 (Vyas et al., 2022).

The organic fraction of municipal solid waste (OFMSW) consists of food, fruit, vegetable, kitchen or garden waste generated by households, restaurants and small businesses. Statistical data show that in Türkiye nearly 60% of municipal solid wastes is consisted of organic wastes (Kuleyin & Nalkıran, 2020). These wastes have high carbonhydrate content, high humidity and anaerobic biodegradability. Yeshanew et al. (2018) evaluated OFMSW, which consists of various kitchen, food, paper and garden wastes, in the production of biohydrogen and biomethane through a two-stage anaerobic digestion system. They produced 41.7 mL of H_2/gVS_{added} biohydrogen by dark fermentation. The volatile fatty acid-rich effluent of biohydrogen production has been evaluated in methane production. Methane yield of 300 mL of CH_4/g $COD_{removed}$ was obtained. Biohydrogen production from OFMSW under mesophilic

conditions was investigated by Lay et al. (1999). At 0.4 g OFMSW/g biomass conditions, the pretreated mixed bacterial culture showed high hydrogen activity (125 mL/g^{-1} TVS h^{-1} and SHPR: 43 mL/g^{-1} VSS h^{-1}).

2.5 Food Waste as Raw Material for Biohydrogen Production

Food waste is an abundant resource that can be exploited for hydrogen production via anaerobic digestion. In addition, properties such as water content, nutrient values, volatile matter composition, particle size and biodegradability of food waste allow significant hydrogen production. In addition, the availability and cheap cost of food waste makes hydrogen production commercially feasible for electricity generation or transportation (Kapdan & Kargi, 2006). Animal waste, fruit and vegetable waste, bakery waste, kitchen waste, grain waste and dairy industry waste are evaluated in the category of food wastes (Yasin et al., 2013).

In a study of biohydrogen production from ground waste wheat, the substrates were pretreated with acid at pH:3. The highest hydrogen yield and specific hydrogen production rate (SHPR) were 1.46 mol H_2 mol^{-1} glucose and 83.6 mL H_2 g^{-1} cell h^{-1}, respectively, at an initial substrate concentration of 10 g L^{-1}. The highest SHPR (1221 mL H_2 g^{-1} cell h^{-1}) was obtained when a biomass concentration of 0.28 g L^{-1} (Sagnak et al., 2011). Yasin et al. (2013) revealed different studies evaluating food waste in biohydrogen production. Mixed cabbage, potatoes, chicken and lard wastes, apple, grape and pear wastes, bread wastes and pineapple wastes were used for mesophilic biohydrogen production in giving a range of hydrogen production yield of 1.3 - 2.1 mol H_2 mol^{-1} glucose. Panin et al. (2020) used food wastes for biohydrogen production via dark fermentation. The yields varied between 24-152 mL H_2 g^{-1} volatile solid. Han et al. (2016) produced 5850 mL of H_2 by batch dark fermentation from food waste hydrolyzate at an initial sugar concentration of 24.35 g/L. They obtained 245.7 mL H_2 g^{-1} glucose yield (1.97 mol hydrogen mol^{-1} glucose). The decrease in sugar concentration from 24.35 g/L to 2.12 g/L during fermentation and the fact that this happened in parallel with the increase in CHF showed that food waste supports the organisms by having the necessary nutrients for

biohydrogen production.

2.5.1 Fruit and Vegetable Waste

According to the Sustainable Development Goals, the generation of food waste, such as fruit and vegetable waste (FVW), needs to be significantly reduced by the end of 2030. The European Commission also highlights different action plans to reduce food waste. Reducing FVW production from the food industry and their evaluation for different commercial applications is a crucial area of intervention. In addition, food waste contains rich organic fraction for sustainable hydrogen production. FVW can be an alternative substrate to replace conventional carbon based substrates such as glucose, sucrose and fructose. Moreover, successful results have been obtained in many studies investigating the use of FVW with a wide variety of organic substrates such as digested sludge, swine manure, and fish waste (Dwivedi et al., 2020).

Grape must, which is a by-product of the alcohol industry, was used as a substrate in the production of biohydrogen and the hydrogen yield was obtained through endogenous fermentation as a 3.6 L H_2 $L^{-1}_{reactor}$ (François et al., 2021). Orange, grape, apple, banana and melon wastes were evaluated in biohydrogen production. The highest biohydrogen yield was obtained as 513 mL H_2 g^{-1} VS when 20% orange was used in the fruit mixture (Akinbomi & Taherzadeh, 2015). Dwivedi et al. (2020), studied on biohydrogen production at different FVW:microorganism ratios. The highest cumulative hydrogen volume was obtained as 125 mL. Also the highest hydrogen yield was achieved when the ratio of FVW to organism was 1. FVW are very suitable raw materials for producing bio-based energy carriers such as biogas, bioethanol and biohydrogen.

When fruit and vegetable wastes are used as a substrate in the anaerobic digestion process, it can be inhibitory on the methanegic consortium due to the accumulation of volatile fatty acids. Different studies evaluate the effects of VFA inhibition. In one study it was observed that high VFA concentrations inhibited anaerobic digesting

bacteria and archeae. They revealed that 900 mg L^{-1} propionic acid concentration inhibit methanogens (Franke-Whittle et al, 2014). Jiang et al (2018) utilized food wastes and pig manure with dry co-digestion and found that the threshold of VFA inhibition concentrations ranged between 16.5-18 g L^{-1}.

Fruit wastes are very suitable substrates for energy production, as seen in many studies in the literature. For this reason, the evaluation of waste fruits in biohydrogen production is one of the subjects that should be studied.

2.6 Mycotoxins

Mycotoxins are secondary metabolites produced by fungi belonging to *Aspergillus, Penicillium and Fusarium* species, which contaminate food and have toxic effects on human health. Fungus are part of the natural microflora of stored products. However, mycotoxin production is highly dependent on the composition of the product and the storage conditions, especially temperature and humidity. Although there are many types of mycotoxins identified, 5 of them are particularly noteworthy in terms of economical loss caused by contamination on foods. These are aflatoxins, deoxynivalenols, fumonisins, ochratoxins and zearalenones (Ukwuru et al., 2017). Major mycotoxins cause contamination of plant-derived foods, cereals, corn, soybeans, etc. In addition, it can be found in animal-derived products such as eggs, meat, milk and dairy by-products in varying concentrations due to transport from animals consuming contaminated feed, and may cause bioaccumulation in the food chain (Xu et al., 2022).

The most common mycotoxins that form naturally in agricultural products are ochratoxin (OTA) and aflatoxin (AF). Aflatoxins are primarily produced by *Aspergillus* species. 18 different aflatoxins have been identified. Its main members are AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2. AFM1 and AFM2 are compounds found in milk and dairy products that are hydroxylation products of AFB1 and AFB2. Among them, AFB1 is the most produced and most toxic and has been classified as a Group 1 carcinogen by the International Agency for Research on

Cancer. Dried fruits can be contaminated with aflatoxin, OTA or kojic acid but the common mycotoxin seen in dry figs are aflatoxins. It is characterized by a bright greenish yellow fluorescent appearance under UV light. On the other hand, in dried fruits such as raisins, sultanas and currants, the cause of concern is ochratoxin (Alkadi & Altal, 2019; Trucksess & Scott, 2008; Xu et al., 2022)

The maximum total concentration of aflatoxin in dried fruits permitted by the European Union is 10 µg kg^{-1} and the maximum concentration of OTA allowed for direct consumption in dried vine fruits is 10 µg kg^{-1} (Celiktas, 2015; Trucksess & Scott, 2008). Since these wastes, which are rich in carbohydrates, cannot be consumed as food, it is important to transform them into a product with high added value. There is no study in the literature on the production of hydrogen from dried fruits contaminated with mycotoxins. However, there are studies on the production of different energy sources. Celiktas & Soner (2015) obtained 5.9 g L^{-1} ethanol at 25 g L^{-1} reducing sugar concentration from aflatoxin-contaminated figs in their study.

The use of mycotoxin-contaminated raw materials in biogas production may adversely affect the course and quality of anaerobic digestion, and thus the biogas yield, due to the complex and stable structures of mycotoxins. There are studies expressing that high mycotoxin concentrations interfere with the microbial consortium. Corn contaminated with AFB1 was evaluated in biogas production. While it was observed that AFB1 concentration at the level of 25 µg kg^{-1} had no effect on biomethane yield; it was concluded that 100 µg kg^{-1} AFB1 contamination caused inhibition of the process through organic acid accumulation. Therefore, it is thought that mycotoxin has a negative effect on the anaerobic digestion process, mycotoxin removal should be attempted to prevent the reduction of production efficiency (Ferrara et al., 2021; Tacconi et al., 2018).

2.6.1 Pretreatment for Aflatoxin Degradation

Physical, chemical or biological methods can be applied for detoxification of food contaminated with aflatoxin. Examples of physical methods are mechanical

separation, heat, cooking, roasting, density separation, irradiation, ultrasound, solvent extraction, adsorption and microwave etc. Chemical degradation of aflatoxin is through chlorine, hydrogen peroxide, ozone, ammonia, alkali and acids. Chlorine, hydrogen peroxide, and ozone are considered less satisfactory, and bisulfite is a low-efficiency application with the possibility of toxic epoxide formation (Herzallah et al., 2008; Pluyer et al., 1987)

Although thermal methods for aflatoxin removal have been tried in many studies in the literature, since the thermal decomposition temperature of AFB1 is close to 300 °C, it remains stable up to its melting point when dry heating is applied (Yazdanpanah et al., 2005). In a study, AFB1 and AFG1 concentrations decreased by 58.8% and 64.5%, respectively, in peanut seeds roasted at 140°C for 40 minutes. After 25 minutes of application at 150°C, 68.5% and 73.3% removal were achieved, respectively (Ogunsanwo et al., 2004). Roasting at 150°C for 120 minutes resulted in 95% removal of AFB1 in aflatoxin-contaminated peanuts (Yazdanpanah et al., 2005).

Water-assisted microwave pretreatment (WMT) is a remarkable method applied in recent years for aflatoxin degradation. Here, water molecules contribute to the degradation of aflatoxin. It decreases the decomposition temperature by opening the lactone ring of AFB1. Water creates an excellent environment for absorbing microwave energy and generating heat. Different temperatures and treatment times were applied in MWT trials performed at an initial concentration of 0.1 mg L^{-1} AFB1. It was found that the higher the microwave power, the higher the application temperature. According to the results obtained, 17%, 46%, 62% and 74% of AFB1 were decomposed at 110 °C, 120 °C, 130 °C and 140 °C, respectively, with WMT applied for 30 minutes. In the aflatoxin removal study on corn, a serious decrease in AFB1 concentration occurred after 14 minutes at 500W microwave power. After 36 minutes of application, 58.6% removal was obtained. In the aflatoxin removal study on corn, a serious decrease in AFB1 concentration occurred after 14 minutes at 500W microwave power. After 36 minutes of application, 58.6% removal was obtained. On the other hand, aflatoxin was not degraded significantly at microwave power less than 400W. Aflatoxin degradation at 700W power was found to be the highest

56.8%. Increasing the power of the microwave reduces the treatment time by increasing the application temperature in a shorter time (Zhang et al., 2020; Zhang et al., 2021).

2.7 Sequential Biohydrogen and Biomethane (Biohythane) Production

The term ‘hythane’ was coined in the early 90's by Hydrogen Component Inc., a company that conducted several studies on the feasibility of using a mixture of CNG (compressed natural gas) and hydrogen as an internal fuel. They have shown that due to the lean burn of the hydrogen and CNG mixture, harmful gas emissions released to the environment are reduced and energy efficiency is preserved. This mixture contains 20% hydrogen by volume. The use of this mixture does not require storage and no modifications are required in CNG engines. As a result, the company patented this blend and 'Hythane' was born (Bolzonella et al., 2018).

Biohythane is a gas product obtained from the breakdown of organic materials in a two-stage anaerobic digestion process. It consists of a mixture of hydrogen and methane, where the hydrogen concentration can range from 10% to 30% (v/v). It can be produced in two consecutive stages. This process usually consists of biohydrogen production by dark fermentation followed by a second anaerobic digestion stage that produces methane (Bolzonella et al., 2018).

The two-stage anaerobic fermentation process in biohythane production is based on the functions between acidogens and methanogens, which differ in their physiological structures and nutritional needs, and their sensitivity to environmental conditions. In the first step, the substrate is fermented to H_2, carbon dioxide and VFAs by acidogens with an optimum pH of 5-6 and a hydraulic retention time (HRT) of 1-3 days. In the second step, VFA, lactic acid and alcohols remaining in the H_2 fermentation medium are converted to CH_4 and CO_2 by methanogens in the pH range of 7-8 and under 10-15 days of HRT (Thong et al., 2018).

Hydrogen and methane production studies were carried out from organic waste

such as olive pulp, which cause important environmental problems, and the effect of substrate concentration was investigated for olive pulp with high organic matter content. Chemical oxygen demand (18432.1 mg/L), total carbohydrate (6.08 mg/L), pH (6.50), temperature (35°C) and alkalinity parameters were calculated for the substrate used in the study. The highest total biogas production from olive pulp was measured as 90.04 mL at 50 g/L substrate concentration during 10 days of waiting time. Considering the hydrogen production from olive pulp, it was seen that the highest yield was 3.51 mL in 10 days of waiting time and the most suitable concentration to obtain this value was 50 g/L substrate (Türkmenler et al., 2018). In a study evaluating vegetable waste, 512.3 mL of biogas and 68.4 mL of H_2 were produced by using powdered broccoli at pH:7 and 35°C with a working volume of 70 mL and a stirring speed of 120 rpm. While the onion was in powder form, 593.7 mL of biogas and 214.7 mL of H_2 were obtained. On the other hand, 244.3 mL of biogas and 49.6 mL of H_2 were produced in powdered potatoes (Panin et al., 2020).

Thong et al, (2018) compiled studies on biohythane production. Wheat straw hydrolysate was used for biohythane production by *Caldicellulosiruptor saccharolyticus* with maximum H_2 production rate of 5.2 L H_2/L·d and methane production rate of 2.6 L CH_4/L·d. Biohythane production yields of food waste obtained as 205 mL H_2/g VS and 464 mL CH_4/g VS. Biohythane production of palm oil mill effluent (POME) was achieved as 201 mL H_2/g COD and 315 mL CH_4/g COD for hydrogen and methane, respectively.

2.8 Objectives and Scope of This Study

The utilization of waste to energy production is a need of the new world to cope with the energy crisis. The objective of this study is to investigate the biohydrogen production from mycotoxin-contaminated dry fruits. Biomethane production volume was evaluated with the rich of VFAs residual fermentation media of biohydrogen production.

The aims in the study can be listed as follows:

- To determine the effect of mycotoxin on biohydrogen production volume, biohydrogen production yield and specific biohydrogen production rate.
- To select the most significant parameters such as treatment period and MW power in water assisted microwave pretreatment of mycotoxin contaminated dry figs for percent total mycotoxin removal and total sugar.
- To determine the initial substrate concentration that gives the highest biohydrogen production volume and production yield from hydrolyzed dry figs under optimal microwave pretreatment conditions.
- To evaluate the effect of microorganism at the optimum initial substrate concentration determined in the previous step.
- To determine the feasibility of biomethane production from residual biomass of biohydrogen production in the final stage.

CHAPTER 3
MATERIAL AND METHODS

3.1 Substrate Preparation

Waste dry fig and raisin used in the experiments were obtained from Aegean Exporters Association and Orka Tarım Ltd, respectively. Based on the literature 100 gram of dry fig specie associated with our study contain 48 g total sugar, 3.3 g protein, 9.8 g dietary fiber, 2.03 mg Fe, 162 mg Ca, 830 mg K, 59 mg Na (Arvaniti et al., 2019) and 100 gram of raisin contain around 70 g total sugar, 3.07 g protein, 3.7 g dietary fiber, 1.88 mg Fe, 50 mg Ca, 749 mg K, 11 mg Na, 101 mg P in 100 grams (Khiari et al., 2018; Alnuwaiser, 2017). The composition analysis of the fig and raisin used in the study was in parallel with the literature resulting in that 43-55.8 g total sugar and 61-71.4 g total sugar in 100 grams, respectively. Moisture content was found as 16.5% for dry fig and 10.9% for raisin. The mycotoxin in dry fig were 69± 9.12 µg kg^{-1} aflatoxin B1, 5.99±0.64 µg kg^{-1} aflatoxin B2, 84.86±11 µg kg^{-1} aflatoxin G1, 5.79±0.9 µg kg^{-1} aflatoxin G2 (analysis method; R-Biopharm with a recovery of 100%±3) and raisin contained 16.8±2.3 µg kg^{-1} of ochratoxin (analysis method; VICAM with a recovery of 98.74%).

The effect of mycotoxin on hydrogen production was investigated by using the substrate with and without pretreatment. 16.66 g L^{-1} dry figs and raisins was chopped into small pieces to reduce the particle size. Then it was blended to obtain a slurry. Acid and heat pretreatment was applied to that slurry by using H_2SO_4 under the hydrolysis conditions as pH=2 and T=90°C for 30 min. Control experiments containing glucose and fructose as simple sugar representing mycotoxin free substrate were conducted in parallel to the experiments with contaminated fruits. In the experiments two different groups of substrates and two different sub-groups were used. The first group included mycotoxins and the second group of substrates were without mycotoxins. These groups wer used with and without acidic pretreatment. The set of experiments are explained in Figure 3.1. Table 3.1 summarizes the substrate types, pretreatment applied or not in the experiments.

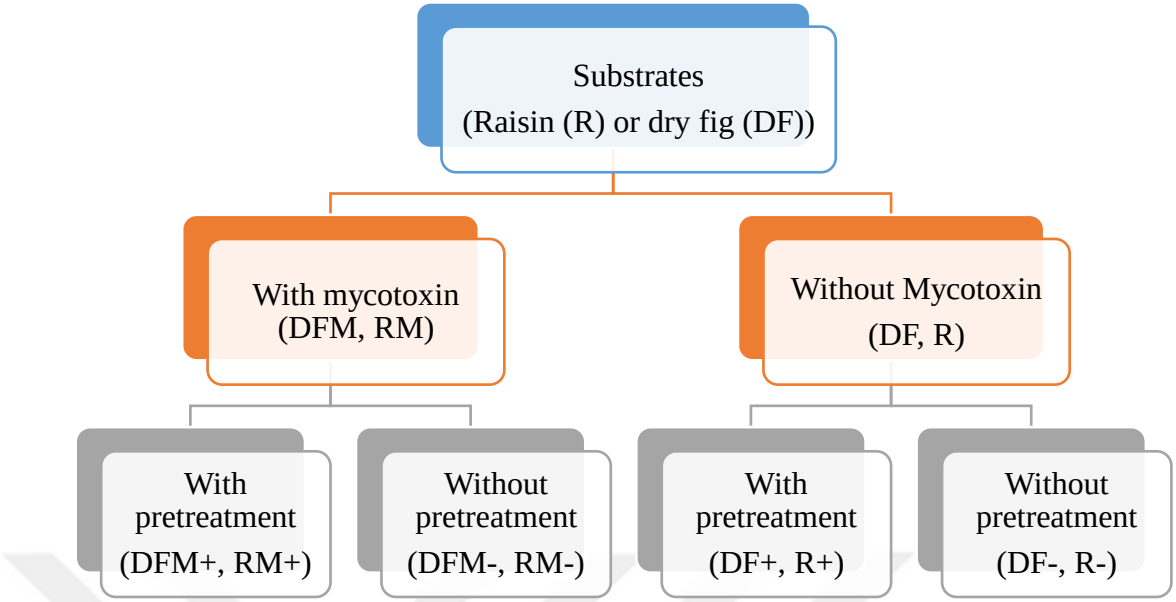


Figure 3.1 The schematic description of the experimental set up

Table 3.1 Substrate classification and abbreviations depending on the presence of mycotoxin and pretreatment application

Abbreviation	Substrate	Acidic Pretreatment
DFM-	Dry fig with mycotoxin	-
DFM+	Dry fig with mycotoxin	+
RM-	Raisin with mycotoxin	-
RM+	Raisin with mycotoxin	+
DF-	Dry fig without mycotoxin	-
DF+	Dry fig without mycotoxin	+
R-	Raisin without mycotoxin	-
R+	Raisin without mycotoxin	+
G	Glucose	-
F	Fructose	-

In the second stage of the experiment, dry fig was the sole substrate and water assisted microwave pretreatment was applied for mycotoxin removal and hydrolysis purposes. The variables were 250W, 350W, 400W, 500W, 600W, 700W, 800W MW power and 0 to 60 min duration. Dry fig concentration was 100 g L^{-1}. The figs were chopped again into small pieces to obtain a slurry of substrate.

3.2 Organism

Mixed culture was used as the organism in the experiments. For this purpose anaerobic sludge was taken from anaerobic wastewater treatment plant's acidogenic phase of PAKMAYA Yeast Company in İzmir. The sludge was boiled at pH:5 for 1 hour in order to select the spore forming hydrogen producing bacteria from the bacterial consortium in the mixed culture. The heat pretreated anaerobic sludge was inoculated into a growth media containing 10 g L^{-1} glucose, 2.8 g L^{-1} K_2HPO_4, 3.9 g L^{-1} KH_2PO_4, 0.25 g L^{-1} $MgSO_4$, 10 g L^{-1} peptone, 0.6 g L^{-1} yeast extract and 0.1 g L^{-1} L-cysteine for activation of the hydrogen producers. The culture was incubated at 37°C for two days and then used as inoculums. The biomass concentration used in the experimental bottles was 1.66 g L^{-1}. Serum bottles prepared for fermentation and microorganism cultivation were capped with silicone stoppers to obtain gas tight environment. Anaerobic conditions were obtained by passing nitrogen gas through the head space of the bottles for 10 minutes prior to fermentation (after inoculation of microorganism to the fermentation media).

3.3 Experimental Set-up

3.3.1 Biohydrogen Production by Dark Fermentation - Mycotoxic Effect

In batch dark fermentation experiments, 500 mL serum bottles (Isolab-Germany Boro 3.3) with 300 mL working volume were used to test biohydrogen production from waste substrates containing mycotoxins and simple sugars without mycotoxins. The substrate types were summarized in Table 3.1. The initial substrate concentration was 16.66 g dry weight L^{-1}. The total sugar concentration measured before fermentation was in the range of 9.22 - 12 g L^{-1} for substrate groups with or without mycotoxins, while it was in the range of 11.78 to 14.56 g L^{-1} for raisins with and without mycotoxins. Positive and negative control bottles containing glucose and fructose were also run in parallel to all experiments.

The fermentation media contained 2.8 g L^{-1} K_2HPO_4, 3.9 g L^{-1} KH_2PO_4, 0.25 g L^{-1}

$MgSO_4$, 10 g L^{-1} peptone, 0.6 g L^{-1} yeast extract and 0.1 g L^{-1} L-cysteine. The initial pH of the fermentation media was adjusted to 7.2 by 10 M NaOH.

Sterilization of the fermentation media before inoculation was achieved by autoclave treatment at 121°C for 15 minutes. Silicone stoppers and caps were used for sealing in test bottles. Anaerobic conditions were provided by passing nitrogen gas from the bottles for 10 min. 10% (v/v) inoculation was applied. The bottles were incubated in a static temperature-controlled incubator at 37°C and mixed manually several times a day.

3.3.2 Microwave Treatment for Mycotoxin Removal

Mycotoxin containing dry figs were pretreated with microwave at different microwave power between 250W and 800W and at different treatment periods (0-60 minutes) in order to observe the effect of microwave application on mycotoxin removal and to determine the conditions in which the maximum sugar concentration is obtained before fermentation. Experiments were carried out at an initial substrate concentration of 100 g $_{substrate}$ L^{-1}. For this purpose, 5 grams of DFs were slurried in 50 mL of distilled water.

The conditions that gave the highest total sugar concentration and percent of mycotoxin degradation were selected as the parameters of the microwave application before fermentation in the next experiments.

3.3.3 Production of Biohydrogen and Biomethane

The effects of different initial substrate concentrations on biohydrogen production volume and yield were investigated. 11.8- 29.5- 59- 88.5 and 118 g $_{wet\ substrate}$ L^{-1} initial substrate concentrations were used. In experiments carried out at a working volume of 100 mL (with total volume of 310 mL), approximately 50-60 mL of distilled water was added to the bottles prior to fermentation and pretreated at the optimum microwave power and application time selected. Then, nitrogen gas was

passed through the autoclaved media at 121°C for 15 minutes. The flasks were incubated at 37°C on the rotary mixer assembly and analyzed for 14 days. Contrary to the static incubator used in the production of dark biohydrogen in the first part, the reason for using the mixer system here is that a denser substrate concentration is used in less fermentation broth and therefore optimum mixing is tried to be achieved.

Biohydrogen production was carried out by adding different concentrations of organisms (0.5- 1, 1.5- 2, 2.5 g L^{-1}) to the fermentation media containing the initial substrate concentration giving the maximum biohydrogen production yield and cumulative hydrogen volume. Mixed culture bacteria were cultivated in a 2 L volume of glucose-containing fermentation medium in order to adjust the organism concentrations in the flasks. At the end of the 2 day incubation, the medium was centrifuged. The pellet was collected as a result of centrifugation, and the bacteria were concentrated in the minimum volume of growth medium. Concentrated organism concentration was calculated by the suspended solid method. The total suspended solids method is a conventional method developed to determine the biomass in activated sludge (Yücesoy et al., 2012). Concentrated stock organism concentration was measured as 18.6 g L^{-1}. The desired organism concentrations (0.5- 1, 1.5- 2, 2.5 g L^{-1}) were obtained by making dilutions from the stock organism culture.

Biomethane production was realized by inoculating untreated anaerobic sludge into the fermentation medium where biohydrogen production was carried out in the determined optimum substrate and organism concentrations. Biomethane production was carried out from the time that the volumetric biohydrogen production was fixed.

3.4 Analytical Methods

At certain intervals, as daily, 5 mL of liquid samples were taken from all bottles and centrifuged at 8000 rpm for 15 min. After centrifugation, total sugar concentration, pH and TVFA and simple sugar analysis were performed in the clear supernatant. The acid-phenol spectrometric method was used for total sugar

concentration analysis (Dubois et al., 1956). In this method, 1 mL of sample, 1 mL of 5% phenol solution and 5 mL of pure H_2SO_4 were mixed in a glass tube and cooled to room temperature for 20 minutes. Absorbance values of the samples were measured at 480 nm. The pH of the fermentation medium were performed with the aid of probe (WTW Scientific, Germany). The pH of the fermentation medium was maintained around 7.

Simple sugar composition and TVFA concentrations were determined by using a high performance liquid chromatograph (HPLC, Agilent 1100) with Aminex HPX-87H column (9 µm x 300 x 7.8 mm). Mobile phase was 5 mM sulphuric acid with a flow rate of 0.6 mL min^{-1}. Glucose and fructose concentrations were detected by RID at 50^{o}C. TVFA was detected by UV detector at 220 nm.

Gas samples were taken daily from the head space of the bottles with a gas-tight glass syringe. Hydrogen concentration was measured by using a gas chromatograph (Agilent 6890 N-GC). Column was Alltech, Hayesep D 80/100 6 in. x 1/8 in. x 0.85 in. Carrier gas was nitrogen with a flow rate of 30 mL min^{-1}. Head pressure was 22 psi. Temperatures of the oven, injection, detector and filament were 35°C, 120°C, 120°C, 140°C, respectively. The total gas volume was measured by the water displacement method using a solution containing 2% H_2SO_4 and 10% NaCl.

3.5 Theoretical Background

Equations used in the thesis for biohydrogen and biomethane are given below. Cumulative Hydrogen Gas Volume (CHF), Hydrogen Production Yield ($Y_{P/S}$), Spesific Hydrogen Production Rate (SHPR), Gompertz Model and its constants were calculated for biohydrogen while Cumulative Methane Formation, Methane Production Yield ($Y_{P/S}$), Spesific Methane Production Rate (SMPR) was calculated for methane production.

- Cumulative Hydrogen/Methane Gas Volume (Eker & Erkul, 2018)

$$V_{H_2,i} = V_{H_2,(i-1)} + V_w C_{H_2,i} + V_{G,i} + C_{H_2,i} - V C_{H_2,(i-1)} \quad (3.1)$$

$V_{H_2,i}$: Cumulative H_2 or methane volume calculated at the i^{th} measurement time (mL)

$V_{H_2,i-1}$: Cumulative H_2 or methane volume calculated at the $(i-1)^{th}$ measurement time (mL)

V_W : The total gas volume measured by the water displacement method (mL)

$V_{G,i}$: Head space gas volumes of the bottle for the i^{th} measurement time (mL)

$V_{G,i-1}$: Head space gas volumes of the bottle for the $(i-1)^{th}$ measurement time (mL)

$C_{H_2,i}$, $C_{H_2,i-1}$: Percent H_2 or methane for the i^{th} and $(i-1)^{th}$ measurement time

- Hydrogen or Methane Production Yield (Y)

$$Yp/s = \frac{\Delta H2}{\Delta S} \qquad Yp/s = \frac{\Delta CH4}{\Delta S} \quad (3.2)$$

Y p/s= mL H_2 or methane g^{-1} substrate or mol H_2 or methane mol^{-1} substrate

ΔH_2, ΔCH_4 = H_2 or methane production (mLor mol)

ΔS= Consumed total sugar (gram or mol)

- Volumetric Hydrogen Production Rate (VHPR)

$$VHPR = \frac{P}{Vo\ t} \quad (3.3)$$

P: Maximum potential of hydrogen formation

V_o = Initial Liquid Volume (L)

t: Time (h)

- Specific Hydrogen/Methane Production Rate (SHPR, SMPR)

$$SHPR, SMPR = \frac{Rm}{V\ o\ X\ o} \quad (3.4)$$

R_m: Maximum rate of hydrogen or methane (ml h^{-1})

V_o = Initial Liquid Volume (L)

X_o= Initial Biomass Concentration g X L^{-1}

Cumulative hydrogen formation also calculated with Gompertz equation in Equation (3.4). The Gompertz equation has the following form (Han & Shin, 2004).

$$H(t) = P \times \exp\left(-\exp\left[\frac{Rm \times e}{P}(\lambda - t) + 1\right]\right) \quad (3.5)$$

CHF: Cumulative hydrogen (mL H_2)

t: Time

P: Maximum potential of hydrogen formation

R_m: Maximum rate of hydrogen (ml h^{-1})

λ: Lag phase time (h)

e: 2.718

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Determination of the Effect of Mycotoxin on Biohydrogen Production by Dark Fermentation from Dry Fruits

Mycotoxin contaminated dry fig and raisin substrates were subjected to biohydrogen production via dark fermentation using anaerobic sludge as microorganism. The initial substrate concentration was 16.66 g dry weight L^{-1} (5 g dw in 300 mL broth) in all experimental media. In order to compare the effect of mycotoxin in this part, biohydrogen production was also carried out from mycotoxin-free (edible) dry figs and raisins in parallel with mycotoxin-containing substrates.

Time dependent cumulative hydrogen formation (CHF) of the previously expressed substrates in Table 3.1 are shown in Figure 4.1. As seen in Figure 4.1, fermentation takes place slowly in substrates containing mycotoxins, and hydrogen production is almost fixed at the 209^{th} hour. In fermentation medium containing edible dry fruits, high volumes of hydrogen were produced, and hydrogen production was maximized at 137^{th} hour for raisins and at the 160^{th} hour for dry figs. It can be concluded that, mycotoxin negatively affected hydrogen production and also extended the fermentation time.

Figure 4.2 gives the maximum CHF value of the tested substrates. The highest CHF was found to be 265 mL in (DF-) and the lowest CHF was found to be 55.65 mL in (RM-). Regardless of the mycotoxic effect biohydrogen production was significantly higher in DF than in R. The presence of mycotoxin (DFM– and DFM +) resulted in a 3.27- fold decrease in CHF compared to DF without mycotoxin (DF– and DF +). Mycotoxin increased the fermentation period and had a negative impact on hydrogen production.

CHF was not significantly increased with pretreated DF or R with mycotoxin (DFM +, RM +). Moreover, CHF decreased by 33%, in pretreated edible R (R–). This fact has been mainly explained as acid pretreatment may cause the formation of undesirable compounds such as furfural and 5-HMF, which inhibit fermentation (Argun et al, 2008). Glucose and fructose were also tested with the waste fruits to evaluate the effects of mycotoxin and pretreatment on biohydrogen production potential. The CHF obtained from edible DF was very close to fructose, and the CHF produced with mycotoxin-containing bottles was lower than fructose and glucose, which showed a clear negative effect of mycotoxin on biohydrogen production.

The time-dependent variation of the initial total sugar concentration in substrates with and without mycotoxins is given in Figure 4.3. The highest initial total sugar concentration was 14.6 g L^{-1} in R- medium and the lowest was 9.2 g L^{-1} in DFM-

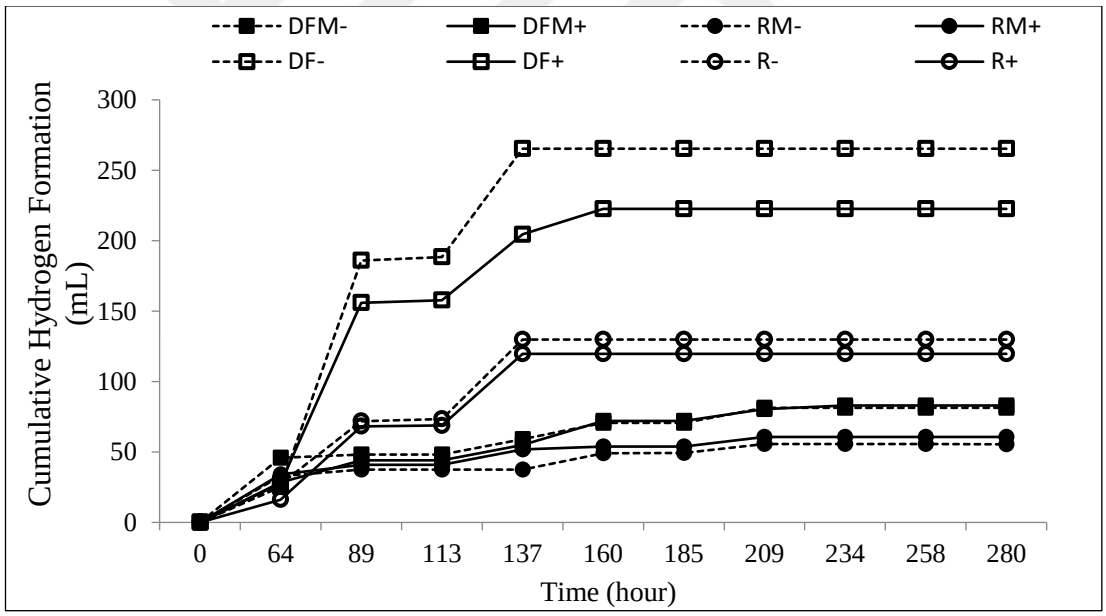


Figure 4.1 Time-dependent variation of cumulative hydrogen formation of different tested substrates with and without mycotoxins

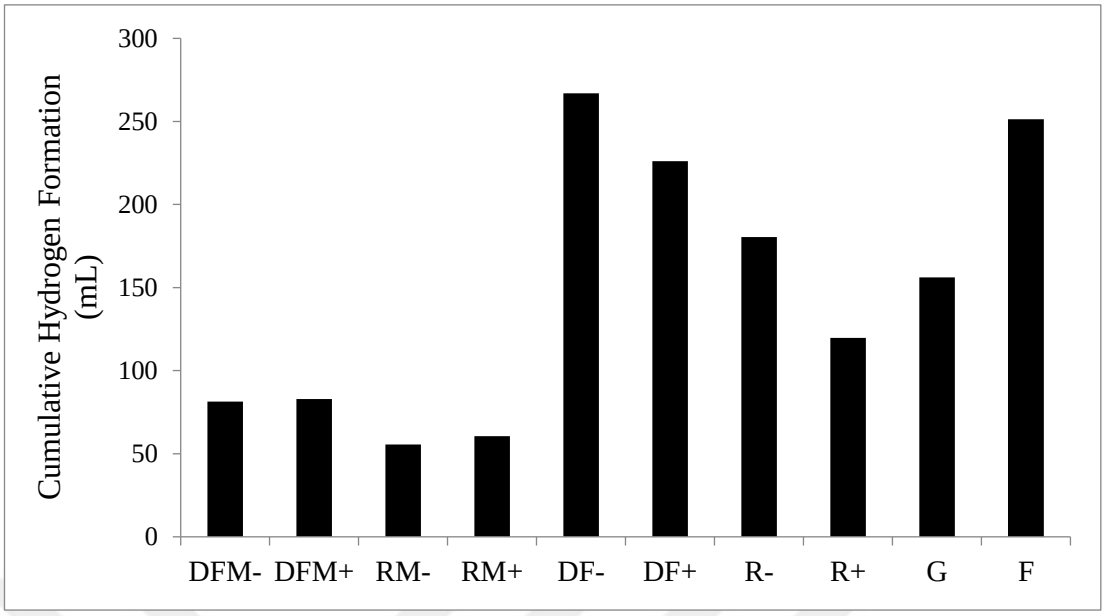


Figure 4.2 Maximum cumulative hydrogen formation of experimental substrates

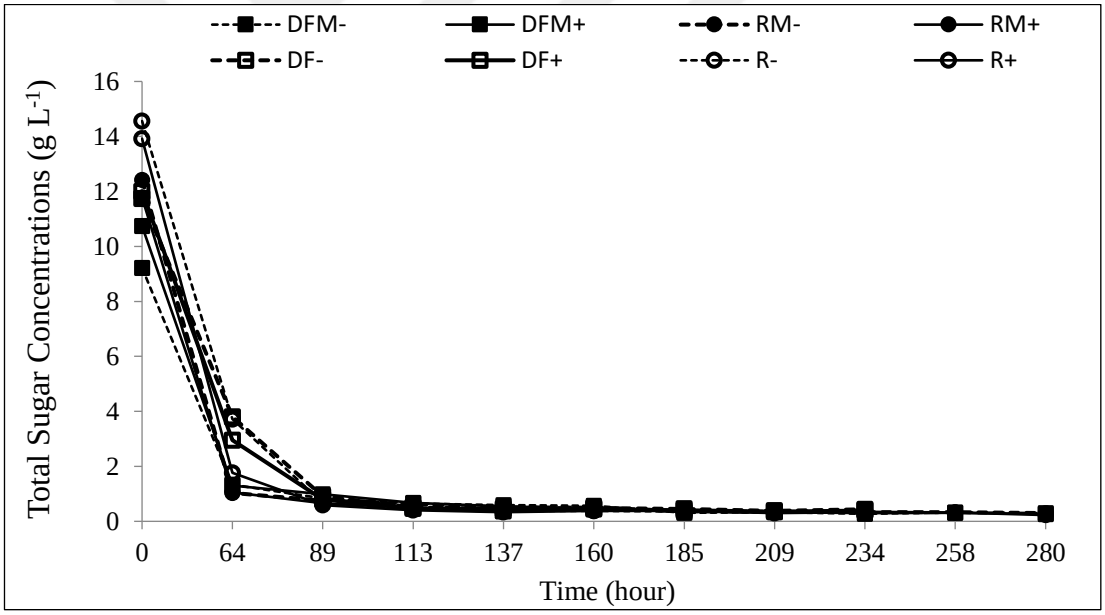


Figure 4.3 Variation of total sugar concentrations with time in experimental fermentation media

As seen in Figure 4.3, nearly 90% of the initial total sugar concentration was consumed in the first three days of fermentation in all tested bottles. This result is parallel with the cumulative hydrogen volume which reached to a maximum value at 89 hours of fermentation. The sugar consumed by the organism is converted into hydrogen via the organic acid production metabolic pathway.

The variation of pH in the fermentation broth in parallel with sugar consumption is given in Figure 4.4. Due to the consumption of sugar by the organism and the formation of organic acid, sharp decreases in pH were observed in the early days of fermentation. At pH values below 5, hydrogenase activity was inhibited and non-hydrogen-producing pathways, including lactic acid production, become dominant (Baldi et al., 2019). Therefore, the pH was monitored daily and when it was below 6.5, it was adjusted to a range of 6.5 – 7 with NaOH addition. This is a critical step in achieving the desired yield in hydrogen production.

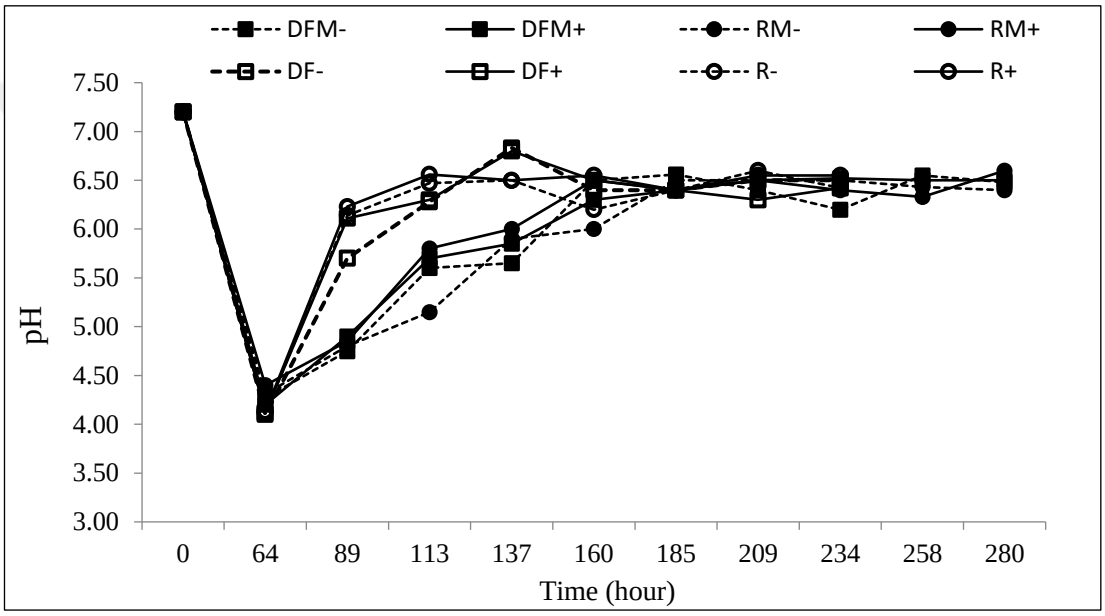


Figure 4.4 Variation of pH with time for different substrates

The sugar monomers, which are hydrolyzed in the fermentation liquid before fermentation, are converted into volatile fatty acids such as butyric acid, acetic acid, lactic acid, propionic acid by acidogenic bacteria. Final volatile fatty acid (VFA) concentrations are depicted in Figure 4.5 for each experimental bottles. The predominant organic acids were acetic and butyric acid. Lactic and propionic acid concentrations were not included to the total volatile fatty acid calculations as they were at negligible levels.

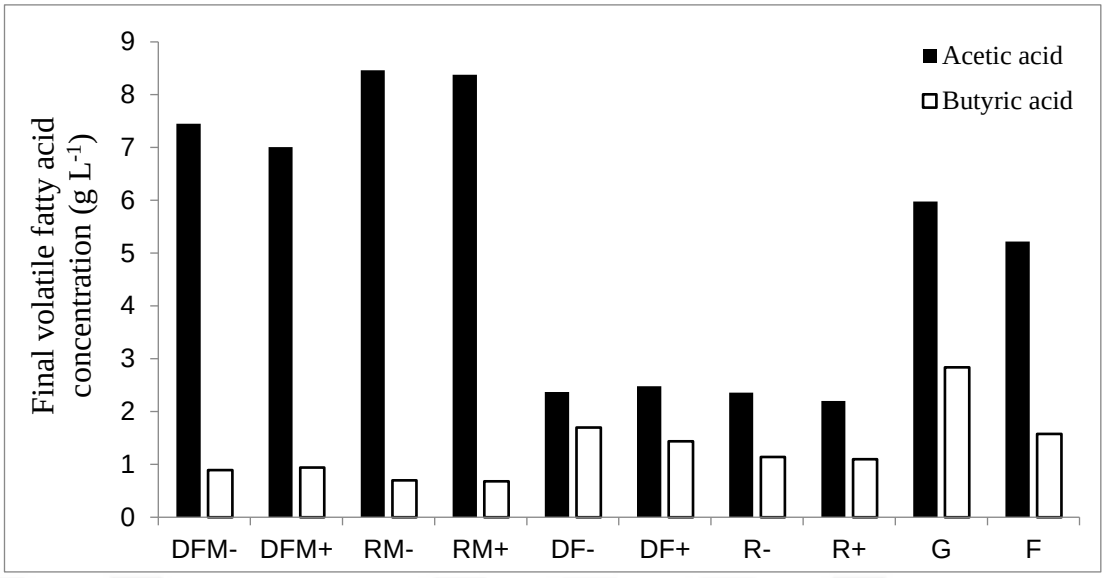


Figure 4.5 Final acetic acid and butyric acid concentrations in the various experimental media

It is evident in Figure 4.5 that the final total volatile fatty acid (TVFA) concentrations in the fermentation media of mycotoxin containing substrates were found to be higher than the production from edible fruits. Final TVFA concentrations for DFM-, DFM+, RM- and RM+ were 8.34, 7.95, 9.16 and 9.06 g L^{-1}, respectively. On the other hand, the TVFA concentration was found to be approximately 2 times lower in the mycotoxin-free substrates. TVFA concentrations in fermentation media with these substrates range from 3.3 to 4 g L^{-1}. It can be concluded that high TVFA production in mycotoxin containing substrates leads to inhibition on biohydrogen production. In fact, literature studies indicate that TVFA concentrations higher than 2.5 g L^{-1} lead to product inhibition on the biohydrogen production pathway (Argun et al., 2008; Eker & Sarp, 2017). Also, the ratio of acetic acid produced in mycotoxin-containing substrates to butyric acid varied between 7.5 and 12. In these bottles, acetic acid was predominantly produced and caused product inhibition. Butyric acid and acetic acid production was very close to each other in edible substrates. For example, the final acetic and butyric acid concentrations were found as 2.37 and 1.7 g L^{-1}, respectively, in the DF- substrate, where CHF was the highest. These results indicate that acetic and butyric acid ratios in these bottles can effect biohydrogen production.

Hydrogen production yield values were calculated according to the total sugar consumed and the total TVFA produced for experimental substrates are given in Figure 4.6. Similar trend to CHF indicated in Figure 4.2 is also observed. The substrates without mycotoxin gave higher yields than the fermentation media including mycotoxin. The highest biohydrogen yield was obtained with DF- which was 79 mL H_2 g^{-1} total sugar and also 219 mL H_2 g^{-1} TVFA. DF+ produced the second highest hydrogen yield when compared with the other wastes substrates. The yields achieved with DFM's were 30.5 and 26.6 mL H_2 g^{-1} total sugar in raw and pretreated DFM, respectively. It can be observed that pretreatment did not have a significant effect on yields. Even it caused a decrease in yield on mycotoxin-free substrates. However, there were some positive effects of pretreatment on yields which will be explained in detail in Section 4.2. As seen in CHF, fig yielded higher hydrogen yield results than raisins on both total sugar and TVFA basis. Therefore, mycotoxin removal will be attempted only on dried figs in the next phases of the study.

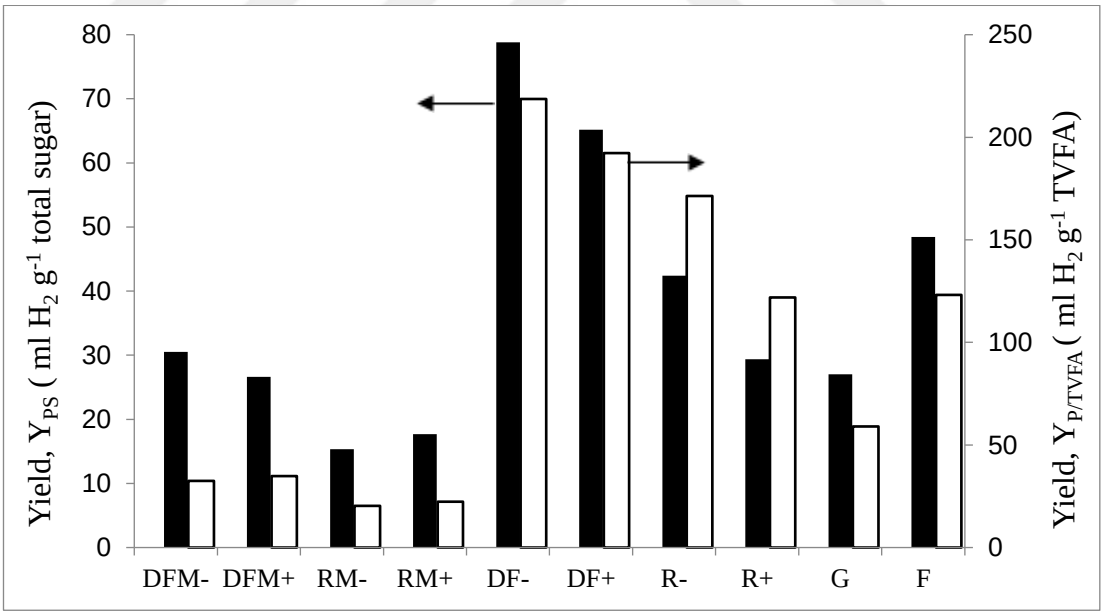


Figure 4.6 Variation of biohydrogen yields based on total sugar and TVFA with tested substrates

Table 4.1 gives the Gompertz equation constants and SHPR values for all substrate types used in the experiments. Gompertz equation was used with CHF data to determine the constants by using TIBCO Statistica program. The highest hydrogen

formation rate (R_m=4.1 mL h^{-1}) and hydrogen formation potential (P = 266 mL) were obtained with DF- substrate. While the P value for fructose from the control bottles was 249.8 mL, this value was 266 mL for the DF- substrate. But the hydrogen production rate of DF- is twice that of the fructose containing control group. In addition, it is about 4 times higher than the glucose containing control group. This shows that dry figs are suitable for hydrogen production. However, the presence of mycotoxins in these substrates reduced the P value in the range of 64 to 71% regardless of pretreatment. Similarly, the reduction in hydrogen production potential due to the presence of mycotoxins in raisin-containing substrates ranged from 54 to 74%.

The highest specific hydrogen production rate (SHPR) was obtained with DF- as 42.3 mL H_2 g^{-1} cellmass h^{-1}. SHPR decreased nearly 6 times with dry fig with mycotoxin, when compared with edible dry fig.

Table 4.1 Gompertz equation constans for hydrogen production

Substrates	λ (h)	P (mL)	R_m (mL h^{-1})	SHPR (mL H_2 g^{-1} cellmass h^{-1})
DFM-	26.2	77	0.7	7.3
DF-	42.6	266	4.1	42.3
RM-	49.8	46.3	2.4	25.1
R-	43.6	180.8	2.6	27.1
DFM+	33.6	81	0.7	6.7
DF+	40.1	222.8	3.3	34.2
RM+	36.8	55.9	0.9	8.88
R+	41.1	121.9	1.4	14.4
G	36.3	168.6	0.9	9.9
F	80.6	249.8	2	80.6

4.2 Evaluation of the Necessity of Acidic Pretreatment in Biohydrogen Production from Dry Fruits with or without Mycotoxins

The aim of pretreatment is to achieve an increased sugar concentration in the fermentation media and remove mycotoxins by destroying the rigid structure of them causing inhibition effects on organism activity. The mycotoxins as aflatoxin and ochratoxin were analyzed before and after pretreatment. The initial concentrations were given in materials and methods part. The pretreatment efficiencies of the mycotoxins were 30.1, 31.5, 17, 18.1, 40.2% for aflatoxin B1, aflatoxin B2, aflatoxins G1, aflatoxins G2 and OTA, respectively.

Figure 4.7 shows the time-dependent variation of CHF for pretreated and untreated dry figs with and without mycotoxins. Regardless of pretreatment, mycotoxin containing dry fig (DFM-, DFM+) had lower CHF than edible dry figs (DF-, DF+). CHF was obtained as 266.9 mL in the absence of pretreatment (DF-), it was found as 226.14 mL in the DF+ substrate.

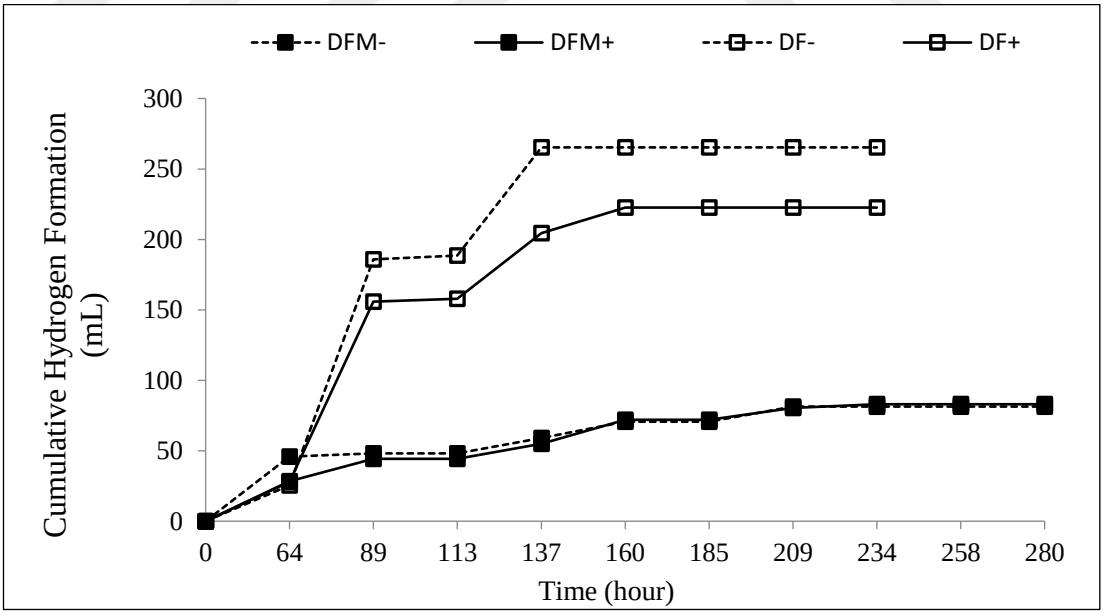


Figure 4.7 Time-dependent variation of CHF from acidic pretreated and untreated waste and edible dry figs.

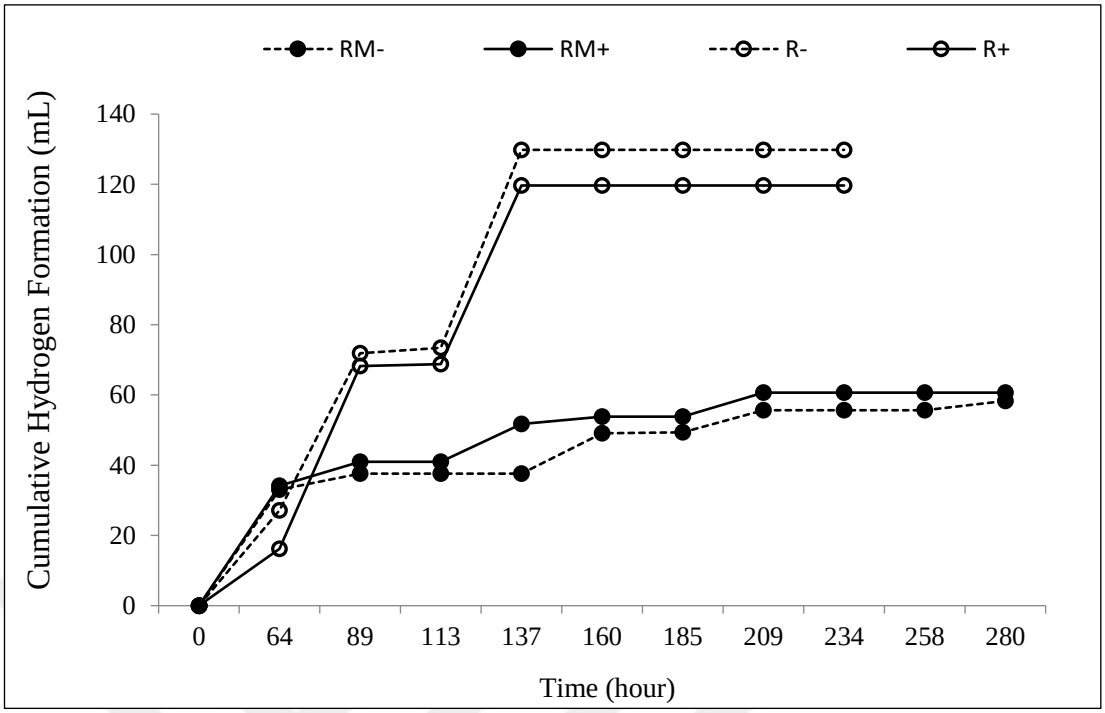


Figure 4.8 Time-dependent variation of CHF from acidic pretreated or untreated waste and edible raisins

In Figure 4.8, the time-dependent variation of CHF of pretreated or raw raisins with or without mycotoxins is indicated. The same trend compared to dry fig is valid for raisins. While the CHF calculated for RM- was 55.65 mL, it was found to be quite close, 60.67 mL for RM+. Pretreatment applied to RM did not significantly increased CHF. Moreover, pretreatment applied on edible raisins reduced CHF by nearly 33%. This fact is been explained as pretreatment may cause the formation of toxic compounds such as furfural and 5-HMF, which inhibit bacterial fermentation from edible dry fruits (Merklein et al., 2016).

For both fruit groups, it concluded that pretreatment in the presence of mycotoxin had no positive effect on the hydrogen production volume. Moreover, pretreatment of mycotoxin-free substrates reduced the hydrogen production volume.

At Table 4.2 to understand the effect of pretreatment depending on mycotoxin, the ratios of pretreated and unpretreated yields were evaluated. The yields of DFM- were 2.58 times lower than DF–, while the yields of DFM + were approximately 2.45-fold lower than DF+. These ratios showed that pretreatment decreased mycotoxic effects

in biohydrogen production. Same trends were also observed in raisin where a ratio of 2.76 in RM–/R– decreased to 1.66 in RM+ /R+. The yields obtained with DF– (218.66 mL H2 g^{-1} TVFA) and R– (171.43 mL H2 g^{-1} TVFA) were comparable with the literature using FW. In presence of mycotoxins in DFM– and RM–, the values were 6.7 and 8.46 times lower, respectively. In the presence of mycotoxins without pretreatment, biohydrogen production yields were nearly 37% of mycotoxin-free substrates. When pretreatment was applied, they increased to 41% of mycotoxin-free dried fig and 60% of mycotoxin-free raisin (Figure 4.6). Furthermore, SHPR decreased 5.86 times with DFM-, when compared with DF- which showed inhibition effect of mycotoxins. When pretreatment was applied to these substrates (DFM+, DF+) a 4.7-fold decrease is been observed, which is lower than unpretreated ones, showing a positive effect of pretreatment.

Table 4.2 Yield ($Y_{P/S}$: mL H_2 g^{-1} total sugar, mL H_2 g^{-1} TVFA) and SHPR ratios of substrates depending on the presence of mycotoxin and pretreatment application

	Ratios based on yields with total sugar	**Ratios based on yields with TVFA**	**Ratios based on SHPR**
DF-/DFM-	2.58	6.7	0.92
DF+/DFM+	2.45	5.53	0.81
R-/RM-	2.76	8.46	0.35
R+/RM+	1.66	5.01	0.53

The main facts of having lower biohydrogen volume in mycotoxin-containing dry fruits may be due to inhibitory effect of acidic pretreatment, shift of mechanism into different hydrogen-consuming end products because of mycotoxins or their by-products after pretreatment that may have toxic effects on hydrogen producing bacteria. To use these substrates efficiently for biohydrogen production, mycotoxin needs to be removed with methods not causing inhibitory factors. As a result, in the next experiments of the study, no acidic pretreatment will be applied to the substrate. Instead of, water assisted microwave pretreatment will be applied for mycotoxin degradation. In addition, only dry figs, which give the highest biohydrogen production volume and yield, were used as substrate in the next experiments.

4.3 Mycotoxin Removal by Water-Assisted Microwave Pretreatment

The maximum total sugar concentration, hydrolysis efficiencies (%) and the percentage of mycotoxin removal will be considered with water-assisted microwave application (250W to 800W) for enhancing biohydrogen production. The variation of total sugar concentration over time in different MW power applications is shown in Figure 4.9.

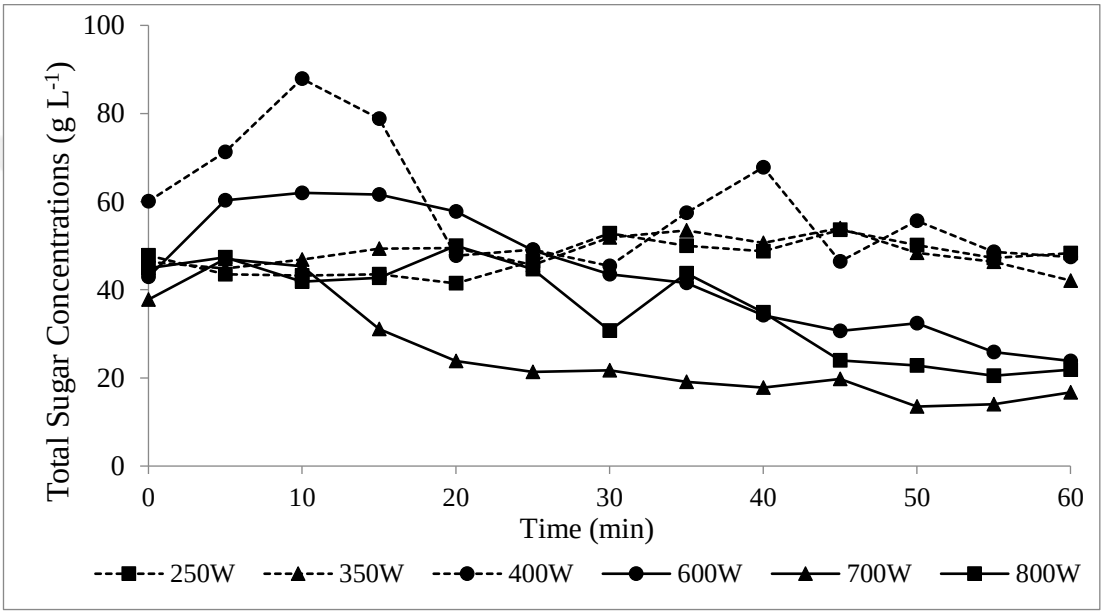


Figure 4.9 Time dependent variation of total sugar concentration depending on microwave power and exposure time

The total sugar concentration had not a significant increase with 250W, 350W MW power. The highest total sugar concentration was obtained as 87.9 g L^{-1} after 10 minutes of application at 400W microwave power. In applications with microwave power higher than 400W, the total sugar concentration tended to decrease as the application time increased. As can be clearly seen, the total sugar concentration tends to decrease after 20 minutes in the 400W to 800W MW power. This can be explained by the fact that overheating at high microwave power leads to sugar caramelization. However, the maximum total sugar concentration that could be reached at low microwave power such as 250W and 350W was limited to nearly 54 g L^{-1}.

The hydrolysis efficiencies (%) with different MW powers are shown in Figure 4.10. Accordingly, at 400W and 600W MW powers, the highest hydrolysis efficiencies were achieved with 46.2% and 44.3%, respectively. After 600W, there was a sharp decrease. Moreover, the sudden heating at 800W had a very low effect on the increase in the amount of sugar, even, causing the hydrolysis efficiency to remain lower than 250 W. However, a remarkable hydrolysis efficiency was not achieved at low microwave power.

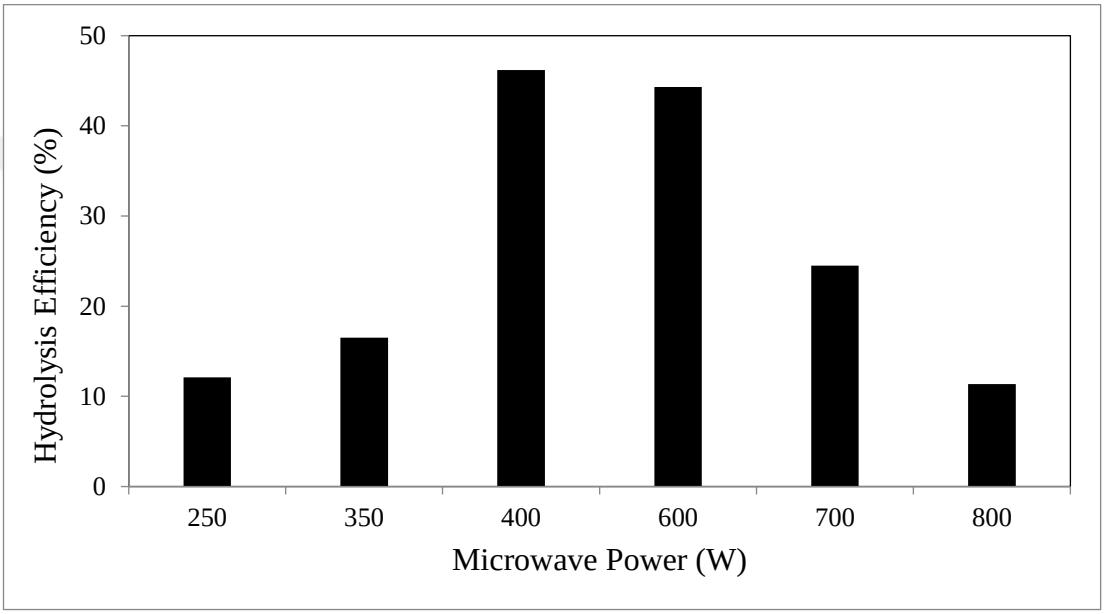


Figure 4.10 Hydrolysis efficiency with different microwave powers applied on mycotoxin containing dry figs

Significant increase in total sugar concentrations acchieved only at 400W and 600W power. In addition, the success to be achieved in mycotoxin removal will be decisive in the selection of the parameters of the microwave pretreatment. The selection of this parameter is very important since it is known that the presence of mycotoxins negatively affects the biohydrogen production process. The results of the aflatoxin concentrations obtained at the end of the application periods at 250W, 400W and 600W microwave powers are given in Table 4.3. The total aflatoxin amount of dry figs before mycotoxin removal is 166 ug kg^{-1} as mentioned before in the section 3.1. The total aflatoxin removal was found as 76.6%, 77.8% and 75.1% as a result of 250W, 400W and 600W microwave power application, respectively.

Table 4.3 AFB1, AFB2, AFG1, AFG2 and total aflatoxin concentrations obtained as a result of different microwave applications

Final Aflatoxin Concentration After Microwave Treatment (ug kg^{-1})	250W	400W	600W
Aflatoxin B1	26.97	25.48	21.04
Aflatoxin B2	11.93	11.44	4.95
Aflatoxin G1	0	0	11.92
Aflatoxin G2	0	0	2.58
Total Aflatoxin	**38.9**	**36.92**	**40.49**

Considering both the percent total aflatoxin removal and the highest percent increase in the total sugar concentration, in the experiments to be carried out after this stage, 400W microwave power will be applied to the dried fig substrates for 10 minutes as a pretreatment.

4.4 Effect of Initial Substrate Concentration on Biohydrogen Production from Microwave Treated Dry Figs

Dry figs that were pretreated with microwave were evaluated for biohydrogen production and prepared at different concentrations as 11.8 g L^{-1}, 29.5 g L^{-1}, 59 g L^{-1}, 88.5 g L^{-1} and 118 g L^{-1} to determine the effect of initial substrate concentration on hydrogen production yield and CHF.

In Figure 4.11, the time dependent variation of CHF obtained in experimental bottles using different initial substrate concentrations is given. The highest CHF was achieved as 161.9 mL at 380th hour in the fermentation medium containing 59 g L^{-1} substrate concentration. In parallel with increasing substrate concentrations up to 59 g L^{-1}, an increase in hydrogen production was observed. However, at higher concentrations, there is a decrease in hydrogen production probably due to substrate inhibition. The lowest CHF was found to be 80 mL at 118 g L^{-1}. At 118 g L^{-1}, CHF remained constant until the 333rd h. However, after the 333rd h, it increased from 57.39 mL to 80 mL. As the substrate concentration increased, the fermentation time was also prolonged.

There was a sharp increase in biohydrogen production within the first 21 hours of fermentation in experimental media containing 11.8, 29.5 and 59 g L^{-1} substrate concentrations. While the fermentation ended at 45 hours in the medium with 11.8 g L^{-1} dry figs, the end of fermentation in the experiment with 29.5 g L^{-1} was approximately 100 hours. In fermentation mediums containing low sugar concentration, the organism was able to use the sugar rapidly and convert it to biohydrogen with a higher production rate. In summary, at low substrate concentrations, fermentation ends within the first few days and CHF remains constant.

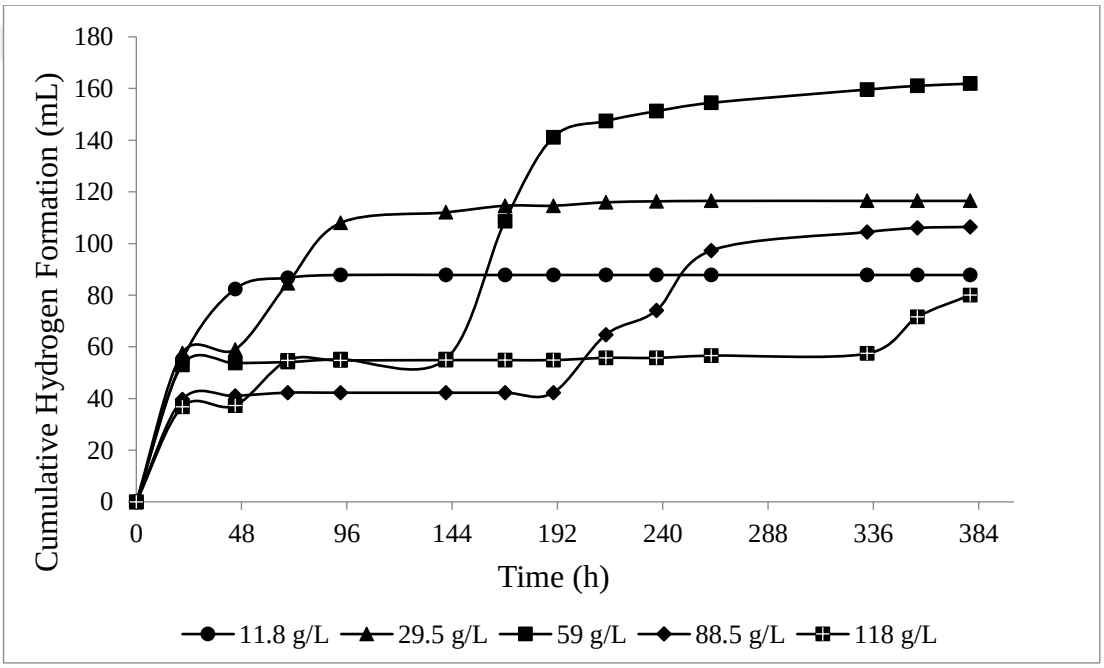


Figure 4.11 Cumulative hydrogen formation for treated dry figs at different initial substrate concentrations

In Figure 4.12, the change of sugar concentration over time is given. Sugar consumption lasted longer in media with high substrate concentration. Initial sugar concentrations in the media containing 11.8, 29.5, 59, 88.5 and 118 g L^{-1} substrate concentrations were 11.65, 22.3, 47.4, 79.8 and 85.1 g L^{-1}, respectively. At the highest substrate concentration (118 g L^{-1}), the sugar concentration in the medium could not be consumed even at the 380th hour of fermentation and 7 g L^{-1} sugar remained. The fluctuations observed in the total sugar concentration may be due to

the simultaneous use of sugar by the organism and sugar hydrolysis.

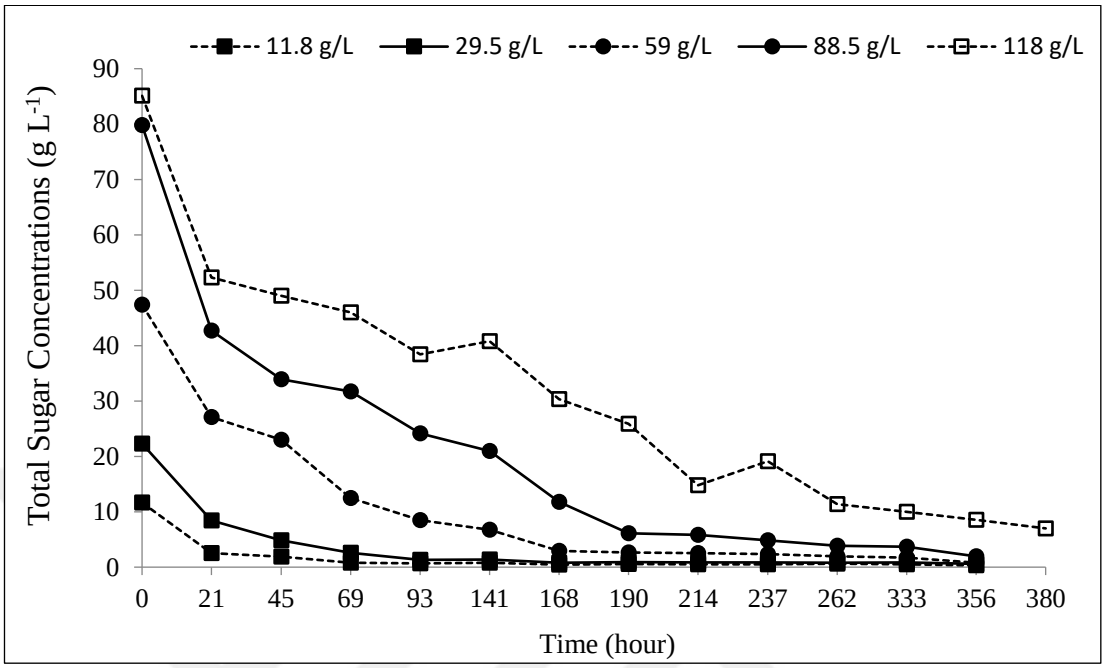


Figure 4.12 Time-dependent change of total sugar concentration in experimental bottles containing different substrate concentrations

Figure 4.13, Figure 4.14, Figure 4.15, Figure 4.16 and Figure 4.17 showed that the time dependent variations of volatile fatty acid concentrations (acetic acid, butyric acid, lactic acid) in experimental media containing initial substrate concentrations of 11.8, 29.5, 59, 88.5 and 118 g L^{-1}, respectively. Since propionic acid is negligible in all fermentation media, it is not included in the graphs. As the substrate concentration increased, the amount of lactic acid produced increased over time. The highest lactic acid concentration was obtained as 83.5 g L^{-1} at the 333 hours in the medium containing 118 g L^{-1} substrate. The low CHF obtained in this bottle may be associated with the high lactic acid accumulation. Despite this, the accumulation of lactic acid reached a maximum of 5.76 g L^{-1} in the medium of 11.8 g L^{-1}, and an increase in butyric acid and acetic acid concentration was observed simultaneously with its rapid consumption. In the same bottle, the final acetic acid concentration was 6.1 g L^{-1}, while the final butyric acid concentration was 3.44 g L^{-1}.

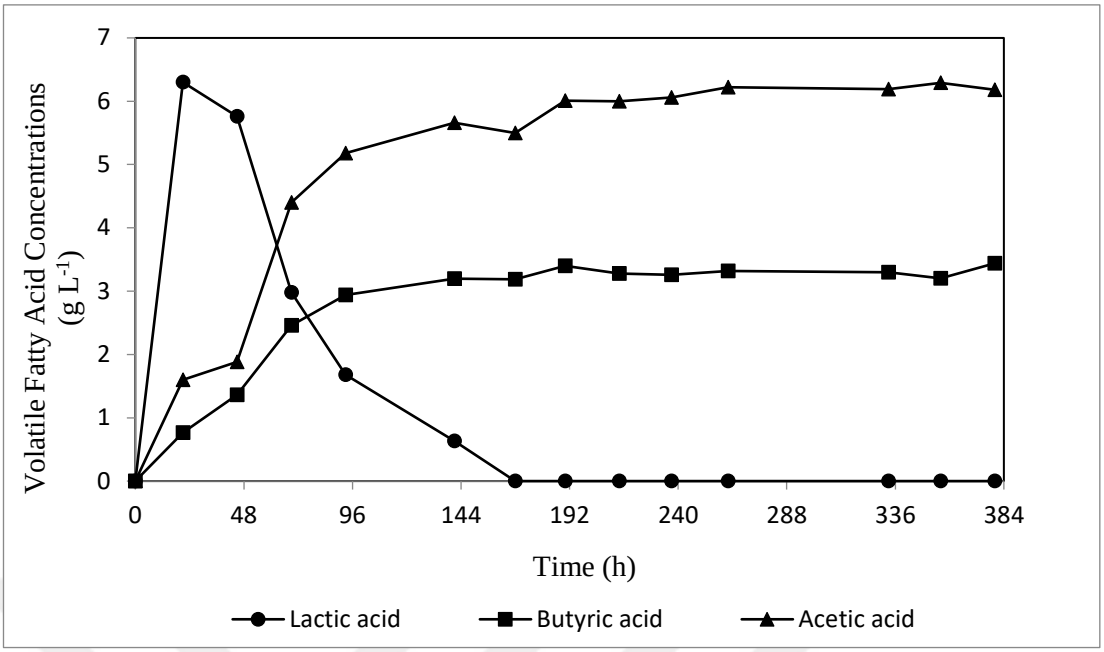


Figure 4.13 Time dependent change of volatile fatty acid concentration in the fermentation medium containing 11.8 g L^{-1} initial dry fig concentration

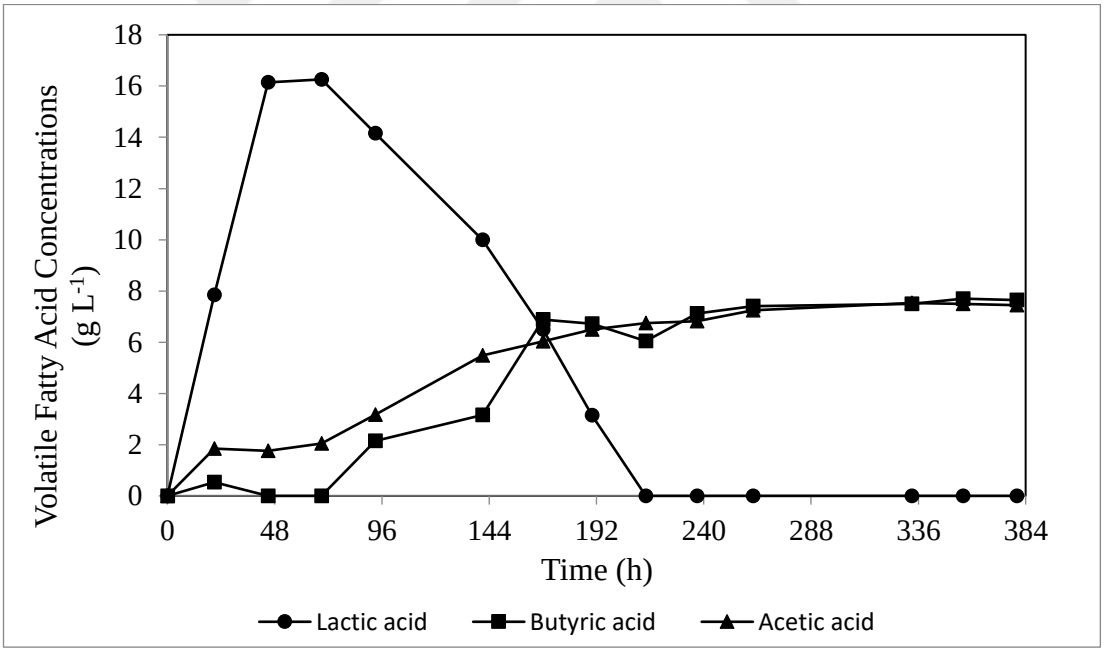


Figure 4.14 Time dependent change of volatile fatty acid concentration in the fermentation medium containing 29.5 g L^{-1} initial dry fig concentration

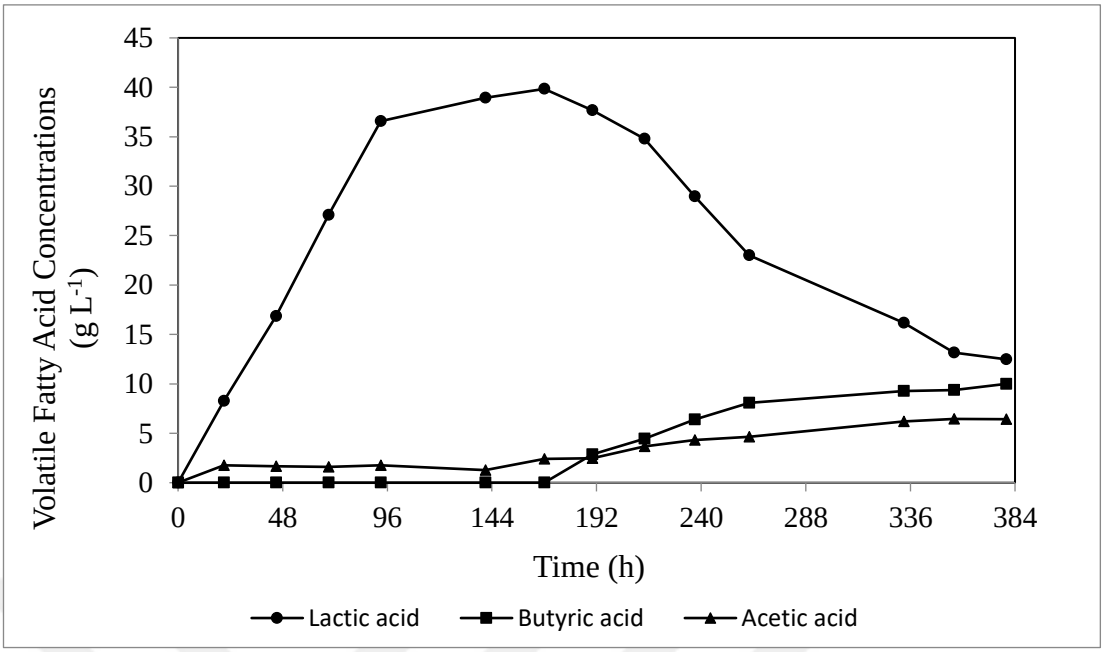


Figure 4.15 Time dependent change of volatile fatty acid concentration in the fermentation medium containing 59 g L^{-1} initial dry fig concentration

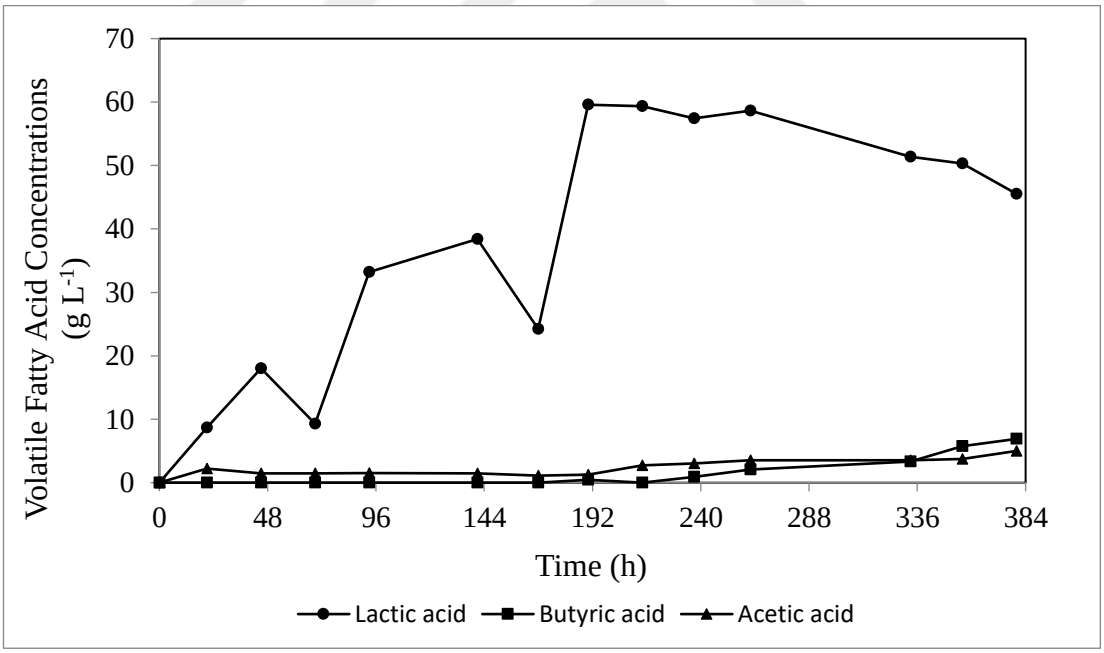


Figure 4.16 Time dependent change of volatile fatty acid concentration in the fermentation medium containing 88.5 g L^{-1} initial dry fig concentration

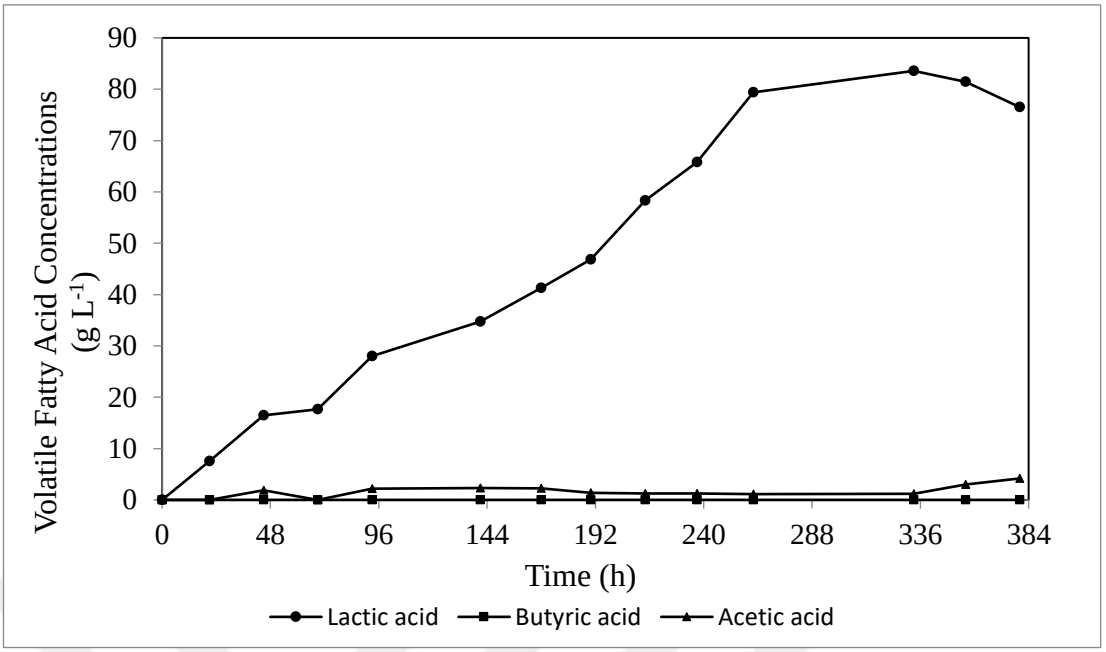


Figure 4.17 Time dependent change of volatile fatty acid concentration in the fermentation medium containing 118 g L^{-1} initial dry fig concentration

Lactic acid could be consumed after 9 days in a medium of 29.5 g L^{-1}. As the substrate concentration increases, lactic acid accumulation increases and it cannot be consumed at high substrate concentrations, suppressing the hydrogen production potential through its accumulation in the fermentation broth. The concentration of butyric and acetic acid in this bottle equalized after 384 hours and was found to be 7.5 g L^{-1}. Final acetic and butyric acid concentrations were 6.44 and 10 g L^{-1}, respectively, in a 59 g L^{-1} bottle which was the fermentation medium with the highest CHF. It has been determined that lactic acid accumulates in the broth and cannot be consumed. However, the accumulation is insignificant compared to the accumulation that occurs at higher substrate concentrations, and the inhibition on hydrogen production is less in the concentration of 59 g L^{-1}.

Considering all these results, it can be concluded that the reason for substrate inhibition at higher substrate concentrations is that bacterial metabolism is directed to the lactic acid production pathway and suppresses biohydrogen production over this. While lactic acid production is dominant, the organism does not follow the hydrogen production pathway, and the fact that acetic and butyric acid is produced in relatively lower concentrations confirms this opinion. Butyric acid was not formed at a

substrate concentration of 118 g L^{-1}. Acetic acid production was also limited as 4.18 g L^{-1}.

In Figure 4.18, yield values for substrates at different concentrations are expressed on the basis of mol H_2 mol^{-1} glucose. In addition, the yield calculated on the basis of the volume of hydrogen produced per gram of total sugar consumed are shown in Figure 4.19.

In parallel with the cumulative hydrogen formation, the maximum hydrogen production yield was found to be 207.5 mL H_2 g^{-1} total sugar in the experimental media containing 59 g L^{-1} substrate. The yield tends to decrease at higher concentrations. On the other hand, the highest yield was obtained as 1.39 mol H_2 mol^{-1} glucose, which is 1/3 of the theoretical yield. The lowest yield was obtained in the medium containing the highest initial substrate concentration (0.5 mol mol^{-1} glucose and 75.3 mL H_2 g^{-1} total sugar).

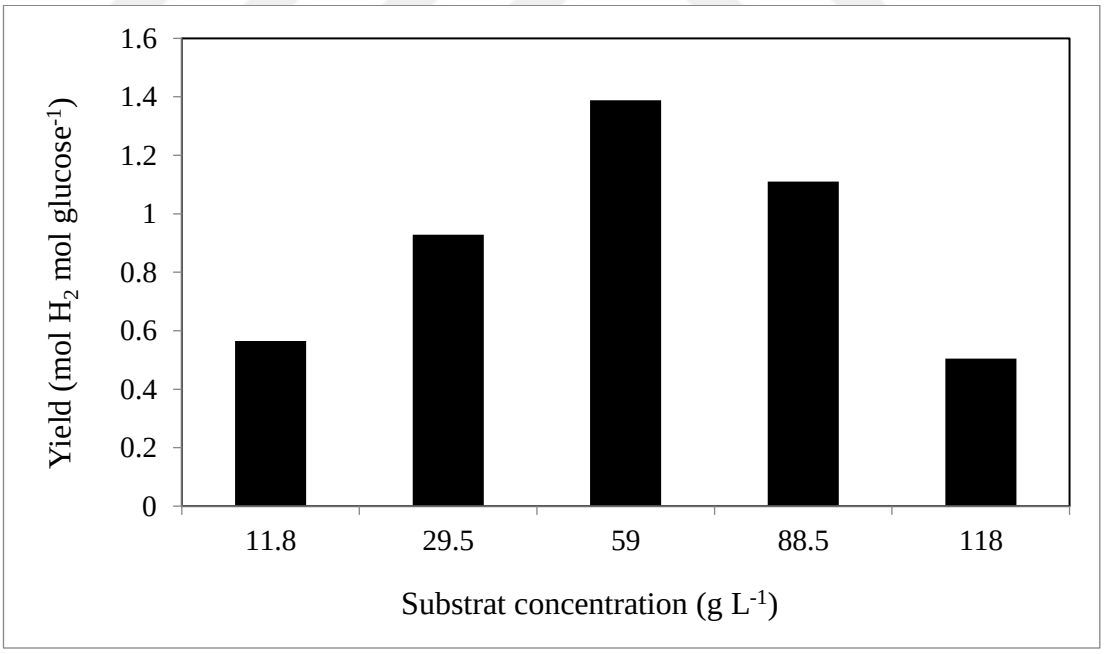


Figure 4.18 Yield values (mol H_2 mol^{-1} glucose) for dry fig at different initial substrate concentrations

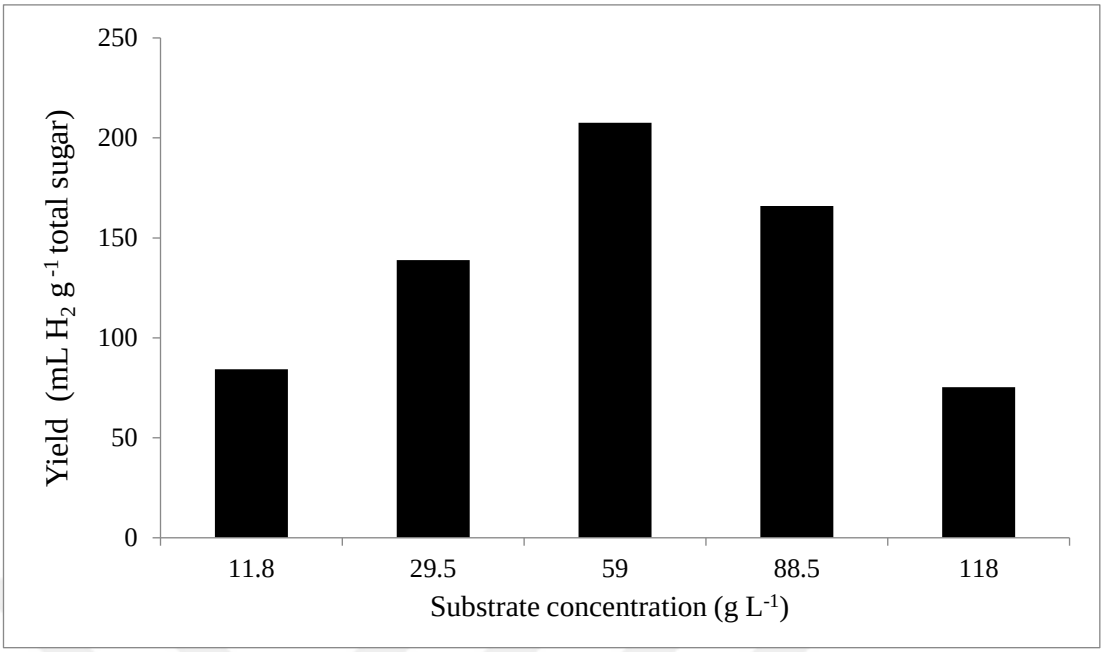


Figure 4.19 Yield values (mL H_2 g^{-1} total sugar) for dry fig at different initial substrate concentrations

Although CHF and biohydrogen production yield are two important parameters in evaluating the hydrogen production potential, the volumetric hydrogen production rates were also taken into account in the selection of the most suitable substrate concentration.

Figure 4.20 depicts the volumetric hydrogen production rates (VHPR) for the experimental bottles. The highest VHPR was achieved in the media with the lowest substrate concentration. The reason for this is that at low substrate concentrations, the organism consumes sugar rapidly due to its metabolism and can effectively perform hydrogen conversion via acetic and butyric acid pathways. At high sugar concentrations, the adaptation of the organism to the environment takes a long time, also the fermentation period is prolonged as sugar hydrolysis continues simultaneously.

The highest VHPR was observed as 18.3 mL H_2 L^{-1} h^{-1} in the 11.8 g L^{-1} media. The second highest VHPR was found in media containing 59 g L^{-1} substrate concentration (17.53 mL H_2 L^{-1} h^{-1}). The lowest VHPR was found to be 4.8 mL H_2 L^{-1} h^{-1} in the medium containing 118 g L^{-1} substrate concentration.

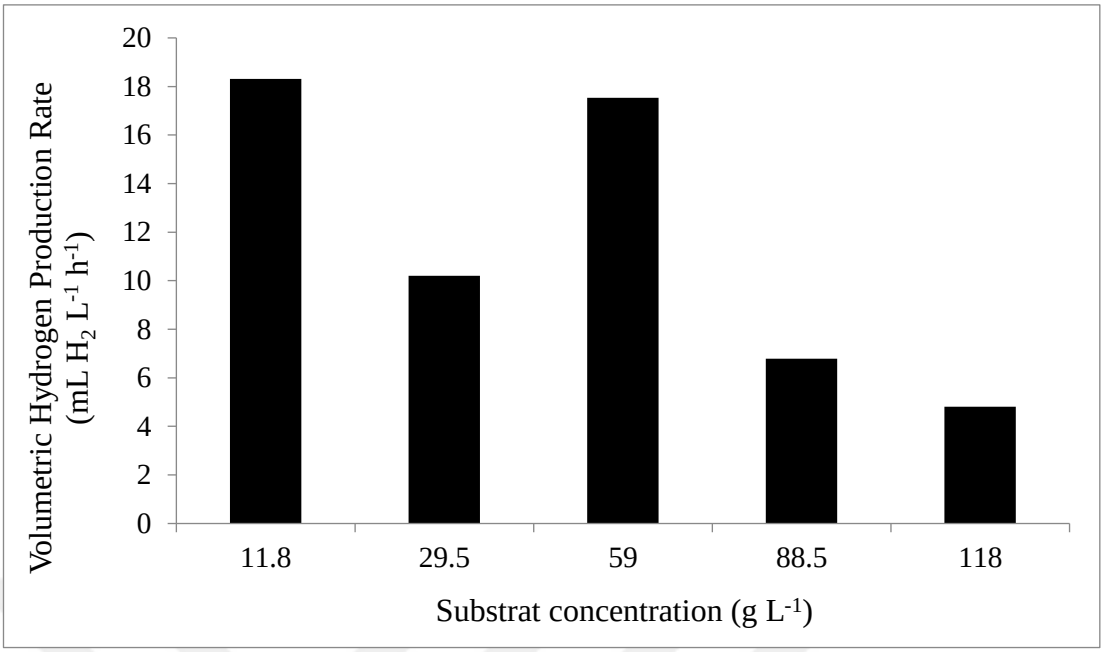


Figure 4.20 VHPR values in biohydrogen production from dry figs at different substrate concentrations

Considering the CHF, hydrogen yield and VHPR data, 59 g L^{-1} was chosen as the optimum initial substrate concentration and the next stages of the experiments were carried out with a substrate concentration of 59 g L^{-1}.

4.5 Effect of Microorganism Concentration on Biohydrogen Production from Dry Figs at Optimum Initial Substrate Concentration

The aim of this step is to determine the most suitable organism concentration in experimental media containing dry figs with optimized substrate concentration. For this purpose, dry figs at the initial substrate concentration of 59 g L^{-1}, which were treated for 10 minutes at 400W MW power were used as the substrate.

Heat treated anaerobic sludge was used as organism in all experiments. Different organism concentrations of 0.5, 1, 1.5, 2, 2.5 g L^{-1} were added to the fermentation broths. The variation of CHF over time in bottles containing different concentrations of organisms is given in Figure 4.21. The highest CHF was obtained as 438 mL in the media containing 2.5 g L^{-1} of organisms. This was followed by the 1 g L^{-1} with 421 mL. No significant difference was observed between the trials in terms of

hydrogen production.

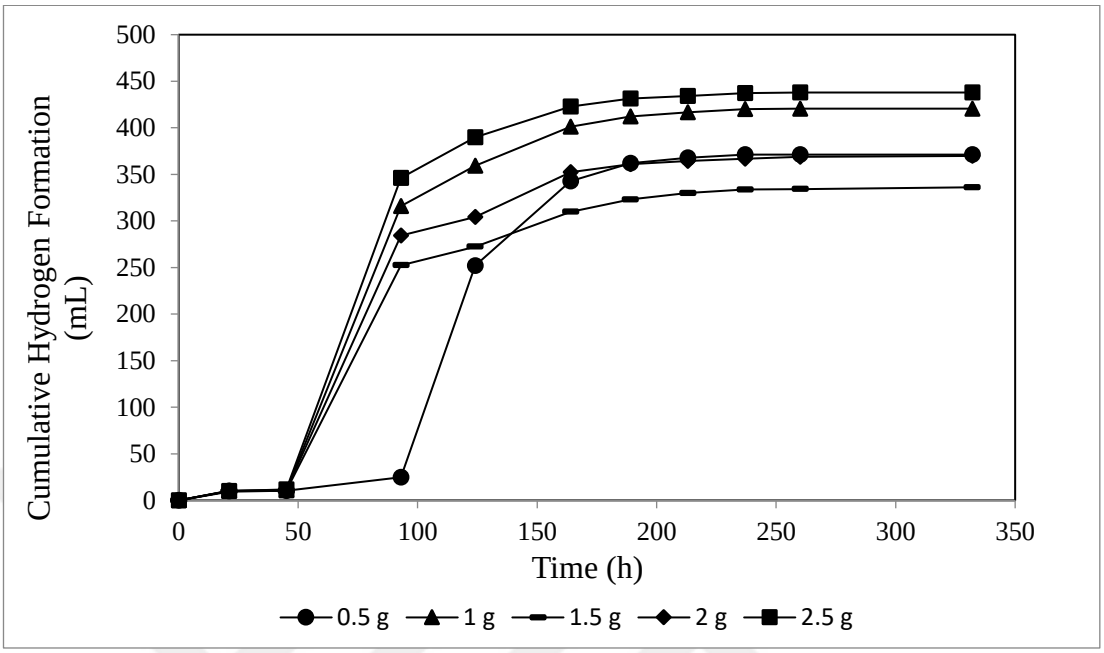


Figure 4.21 Change of CHF over time in bottles containing different concentrations of organisms

The variation of total sugar concentration over time is shown in Figure 4.22. At the lowest microorganism amount (0.5 g L^{-1}), the total sugar concentration decreased from 43.8 g L^{-1} to 13.4 g L^{-1} within the first 93 hours after fermentation started. However, the logarithmic increase in hydrogen production only occurred after 93 hours (Figure 4.21). This shows that at low organism concentration, bacteria use the sugar in the broth primarily for growth and adaptation to the high concentration of sugar in the media according to their cell density, rather than hydrogen production. Hydrogen formation in all remaining organism amounts increases linearly after the first 2 days of fermentation and production ends at approximately 164 to 189 hours (Figure 4.21). The amount of sugar in all media was almost consumed.

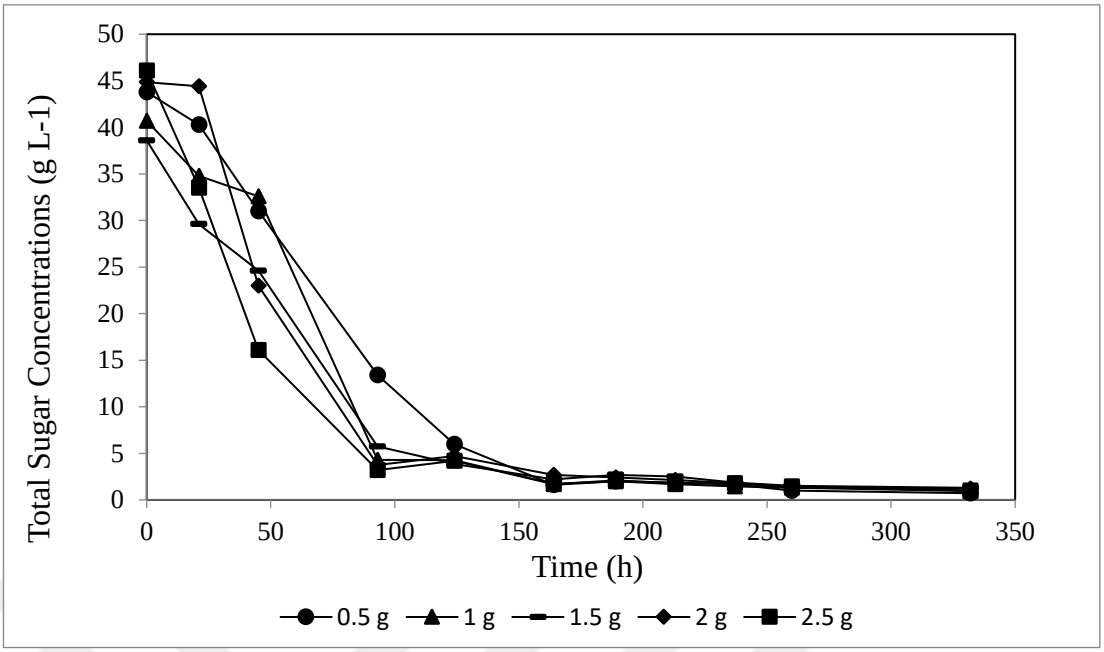


Figure 4.22 Change of total sugar concentrations over time in experimental bottles

The intermediate products expected to be converted on the hydrogen production pathway of the total sugar consumed are acetic acid and butyric acid. However, Figure 4.23, Figure 4.24, Figure 4.25, Figure 4.26 and Figure 4.27 shows that lactic acid accumulation is also present in these trials. Figure 4.23 shows the time-dependent variation of VFA concentrations in the media containing 0.5 g L^{-1} organisms. In Figure 4.24, change of VFA over time for 1 g L^{-1} bottle is given.

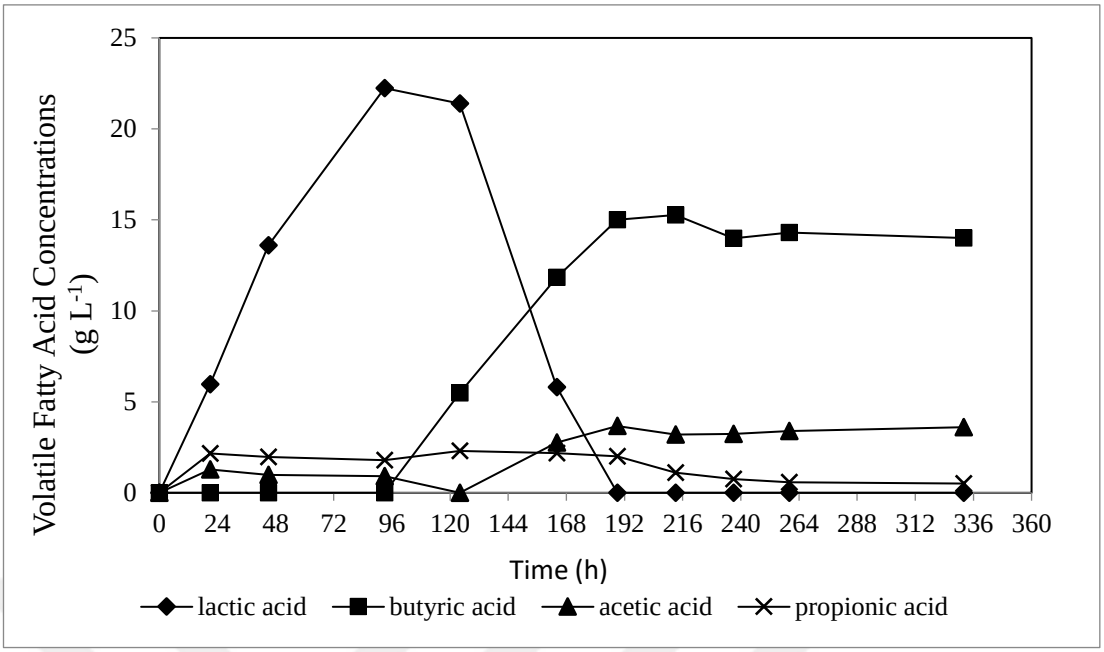


Figure 4.23 Time-dependent change of VFA concentrations (0.5 g L^{-1})

As can be seen in Figure 4.23, the amount of lactic acid in the media reached its peak value as 22.24 g L^{-1} at the 93rd hours. Parallel to this, butyric acid production occurred in this time interval and the butyric acid concentration reached its peak value as 15.27 g L^{-1} in 213 hours. The final acetic acid concentration was limited to 4.88 g L^{-1}.

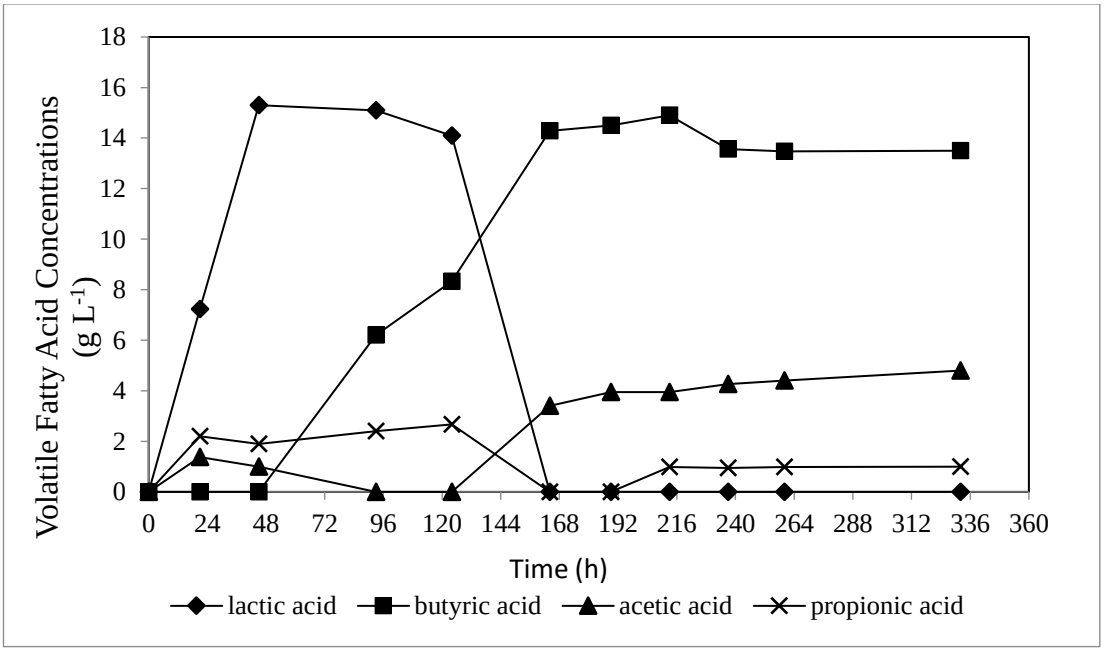


Figure 4.24 Time-dependent change of VFA concentrations (1 g L^{-1})

Similarly, lactic acid was produced in the media of 1 g L^{-1} in the first 124 hours, and after reaching its highest concentration as 14.1 g L^{-1}, it began to be consumed or transformed, and a parallel increase in the amount of acetic and butyric acid was observed.

Lactic acid accumulated in all bottles within the first 164 hours of fermentation and started to be consumed/converted in the last stages of fermentation, in parallel with the decrease in hydrogen production rate, and was completely depleted at the time of maximum hydrogen production. The increase in the amount of butyric acid in parallel with the consumption of lactic acid reveals that lactic acid may have been converted to butyric acid. Figure 4.25 shows VFA production over time in medium containing 1.5 g L^{-1} organisms. After the 120 hours, the lactic acid was rapidly depleted and simultaneously the amount of butyric acid increased 2-fold from 6.9 to 13.4 g L^{-1}. However, even during the time intervals of lactic acid production, there was an increase in CHF due to the presence of acetic and butyric acid in the media.

From batch dark fermentation studies in the literature, it is known that hydrogen-producing microbial consortia or pure cultures of *Clostridium butyricum* are capable of converting lactate and acetate to butyric acid. Lactic acid accumulation was found to be highest at the lowest organism concentration. This is related to the low amount of organisms trying to adapt to the environment of high sugar concentration (Detman et al., 2019; Tao et al., 2016)

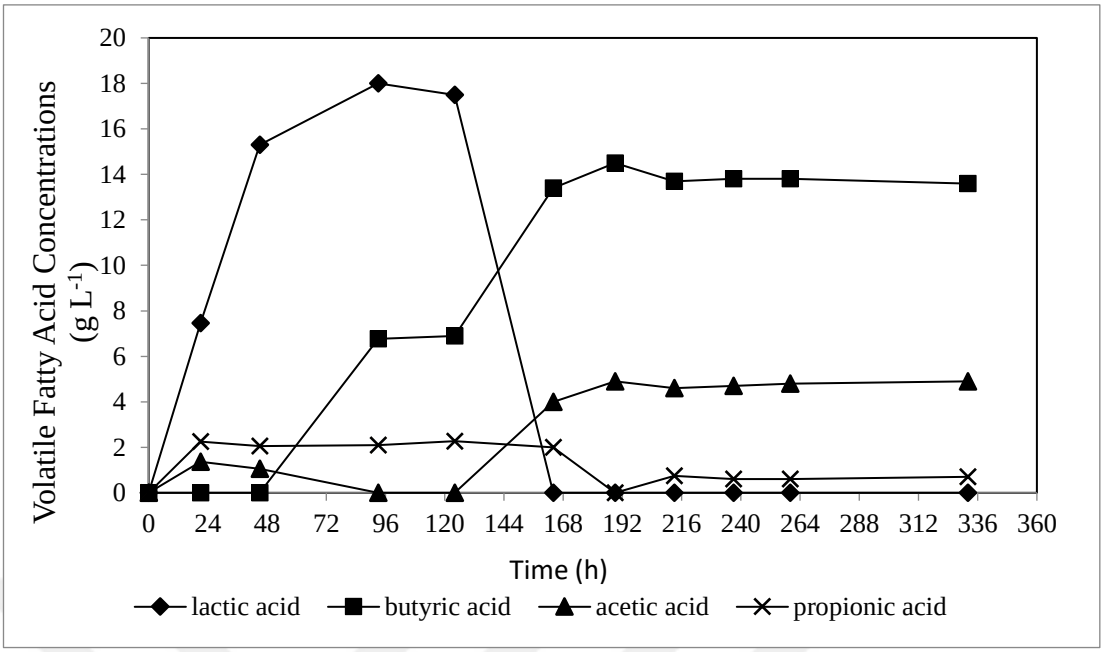


Figure 4.25 Time-dependent change of VFA concentrations (1.5 g L^{-1})

Figures 4.26 and 4.27 show the time dependent variation of VFA in media of 2 and 2.5 g L^{-1}, respectively. Considering all graphs, no linear correlation was found between CHF and lactic acid formation. Also, there is no relationship between lactic acid production and organism concentrations. However, it is noteworthy that the lag phase lasted 93 hours in the test broth containing the lowest concentration of organisms. It is also noteworthy that the highest amount of lactic acid accumulation is observed in this bottle (0.5 g L^{-1}) with the medium at a concentration of 2 g L^{-1}. Hydrogen production took place at later stages compared to other media. This is also not a desired phenomenon.

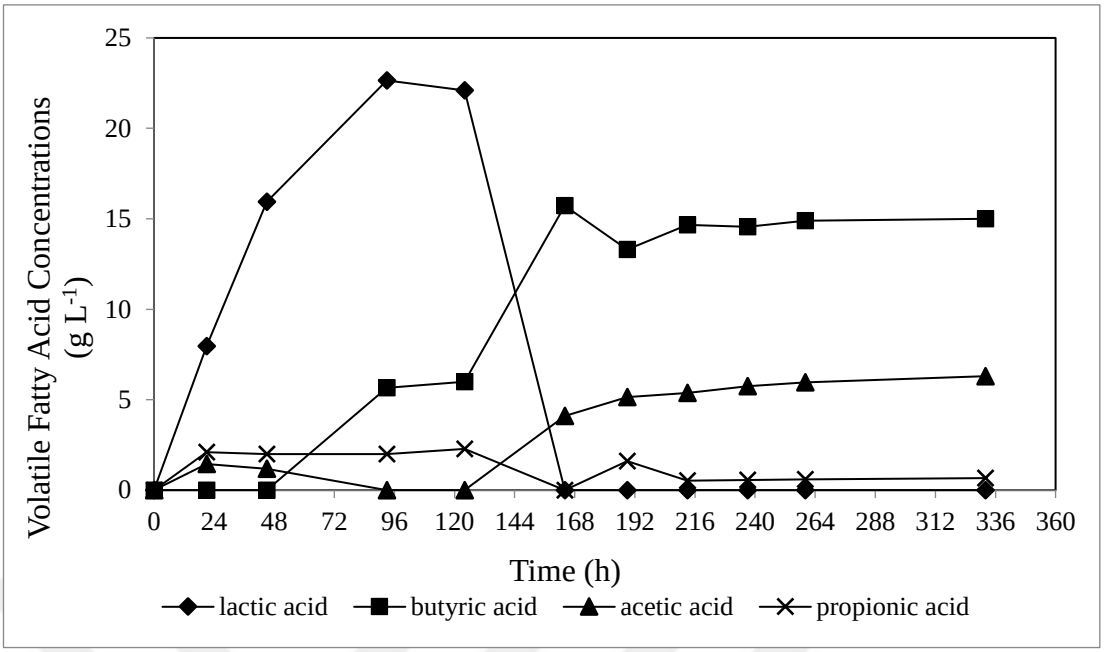


Figure 4.26 Time-dependent change of VFA concentrations (2 g L^{-1})

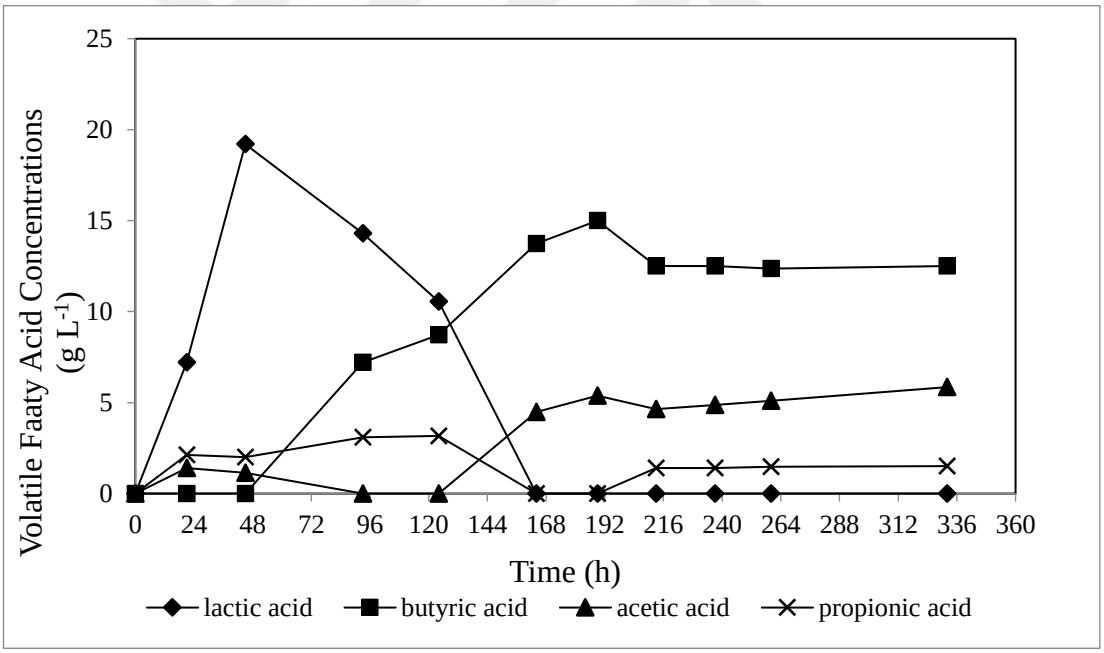


Figure 4.27 Time-dependent change of VFA concentrations (2.5 g L^{-1})

Biohydrogen production yields in different microorganism concentrations were given in Figure 4.28 and Figure 4.29. The results obtained in cumulative hydrogen production have also observed in hydrogen production yield. Accordingly, the highest yield was obtained as 0.69 mol H_2 mol^{-1} glucose (102.9 mL H_2 g^{-1} total sugar) in the experimental medium containing 1 g L^{-1} of organisms.

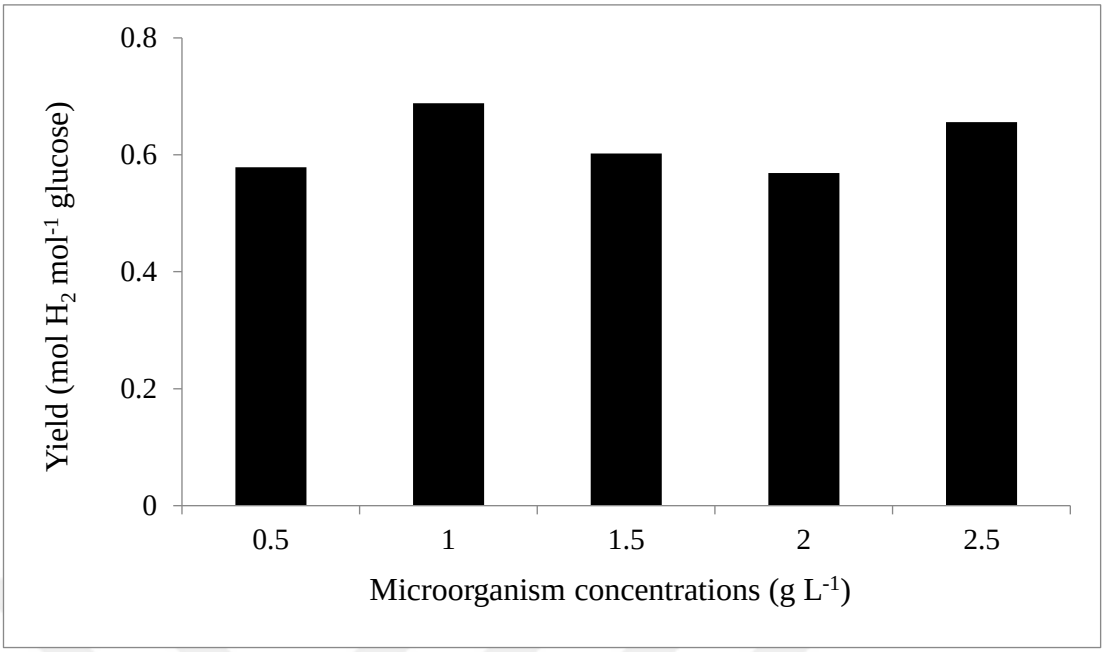


Figure 4.28 Yield in fermentation media using different concentrations of organisms, mol mol^{-1}

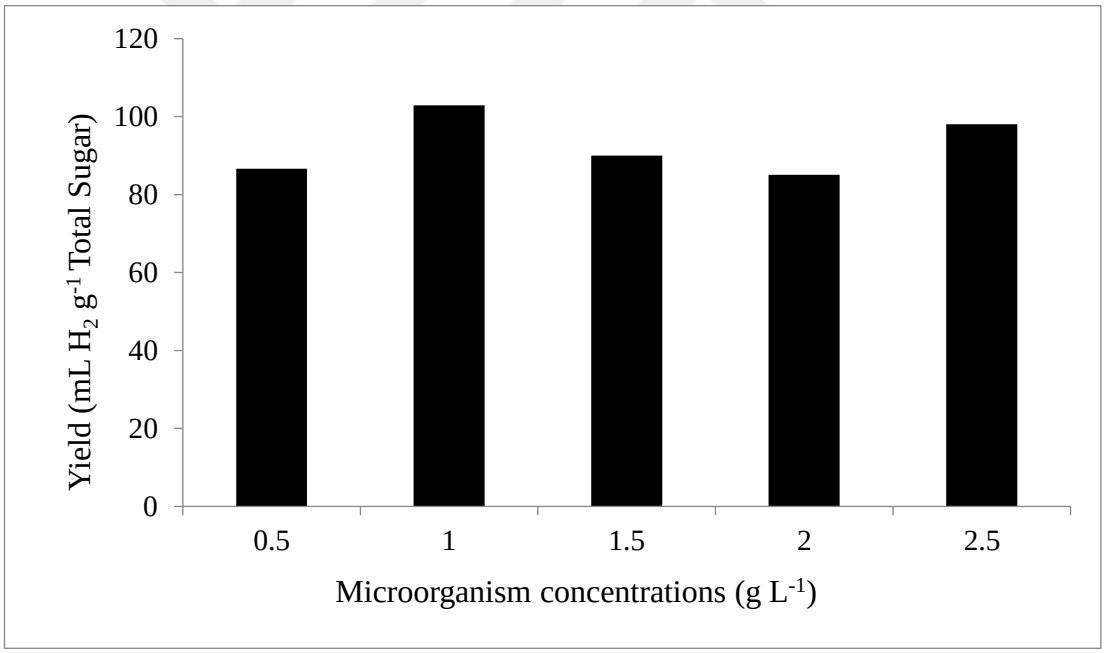


Figure 4.29 Yield in fermentation media using different concentrations of organisms, mL g^{-1} total sugar

The lowest yield was found as 0.57 mol mol^{-1} in experimental media containing 0.5 and 2 g L^{-1} organisms, where lactic acid accumulates at the highest concentration. However, the yield values obtained in experimental bottles are very close to each other.

Specific hydrogen production rates (SHPR), which is another important criterias in the selection of the most appropriate parameters in production, are given in Figure 4.30. As the organism concentration increased, a decrease in the SHPR was observed. The highest SHPR was found as 3.83 mL H_2 g^{-1} biomass $hour^{-1}$ in the fermentation media containing 0.5 g L^{-1} of organisms. The lowest SHPR was obtained as 0.9 mL H_2 g^{-1} biomass $hour^{-1}$ in the experimental media containing 2.5 g L^{-1} microorganism.

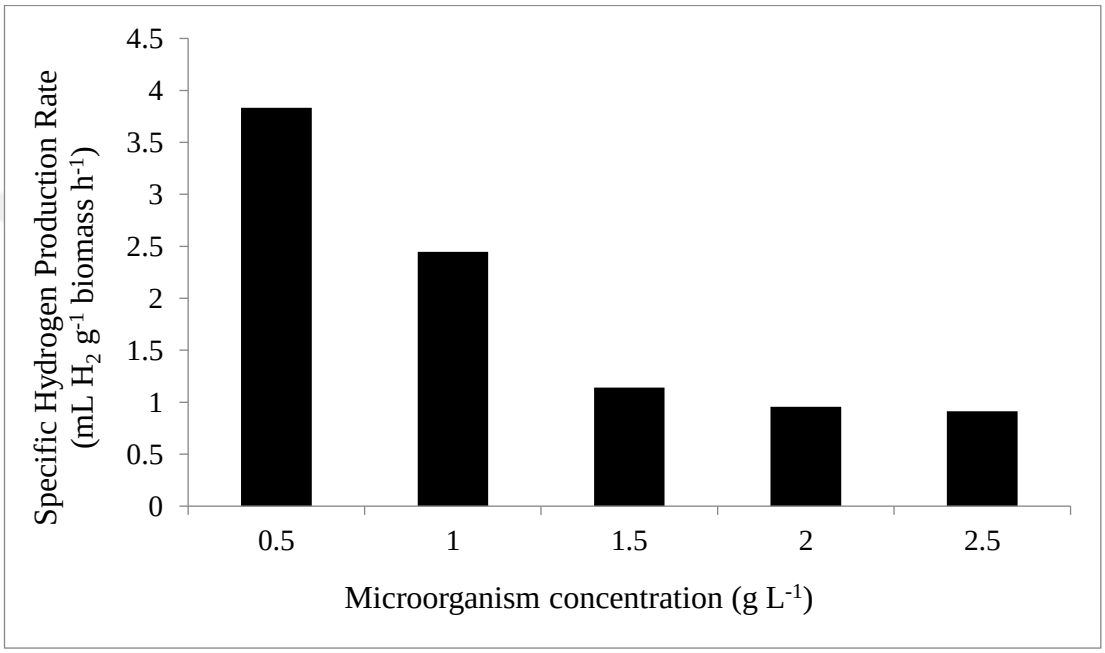


Figure 4.30 SHPR in fermentation media using different concentrations of organisms

In the light of all the data, when the yield and CHF values and SHPR are taken into account, the most appropriate organism concentration is 1 g L^{-1}. However, in the final stage, methane production was carried out directly by adding methanogen bacteria culture to the bottles where hydrogen production was carried out. For this purpose, production volumes will be compared by sequentially producing methane in all media where hydrogen production is carried out with different organism amounts.

4.6 Biomethane Production by Effluent of Biohydrogen Production

Biomethane production was carried out with the effluent of biohydrogen production. In this experiments, the initial dry fig concentration was 59 g L^{-1} containing in the range of 38.6 to 46 g L^{-1} total sugar. These experiments produced 17.45 to 20 g L^{-1} TVFA. Biomethane producing bacteria were inoculated into bottles in which the effect of organism concentration on biohydrogen production was evaluated.

Figure 4.31 shows the time-dependent change of cumulative methane production (CMF) from residual effluent of biohydrogen production. The highest CMF was obtained with 1.5 g L^{-1} organism concentration as 25.14 mL. This was followed by 1 g L^{-1} medium as 23.11 mL. Although the CMF values are very close to each other, the lowest methane production was obtained in the experimental bottles containing the lowest and highest concentrations of residual biomass organisms.

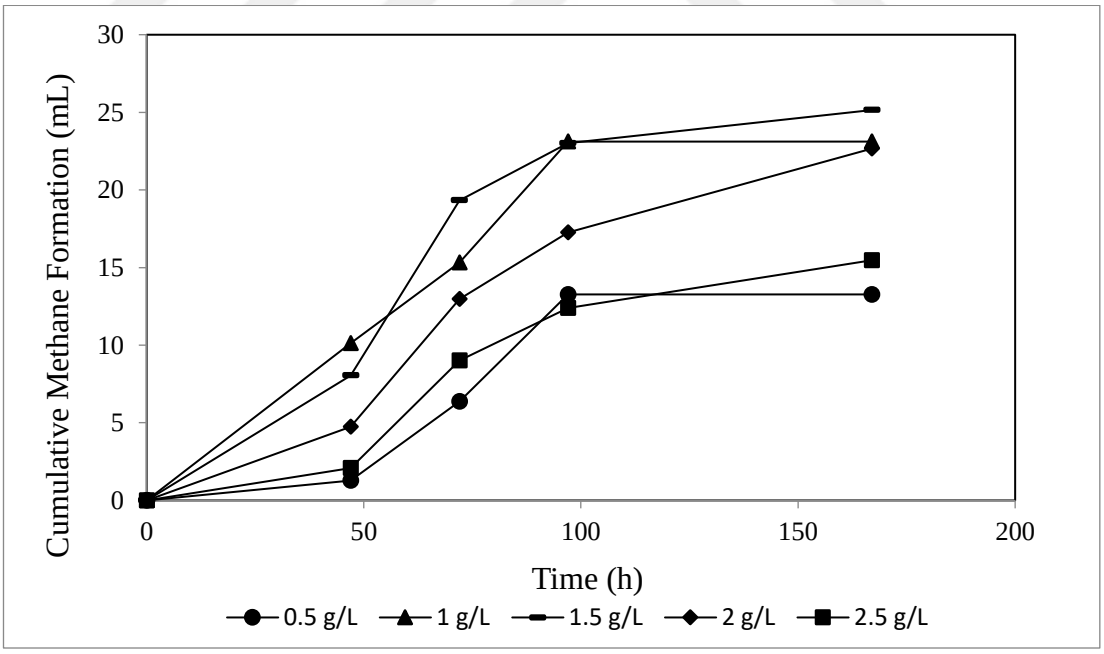


Figure 4.31 Time dependent variation of cumulative methane production from dark fermentation effluent at different organism concentrations

Methane formation is expected to occur with the use of volatile fatty acids released during biohydrogen production. Table 4.4 shows the variation in the

percentage of hydrogen and methane in the experimental bottles during the 4-day methane production. In the table, experimental media containing organism concentrations of 0.5, 1, 1.5, 2 and 2.5 g L^{-1} are referred to as Experimental Bottle 1, 2, 3, 4 and 5, respectively. While the hydrogen concentration in the fermentation media decreased over time, the methane percentage increased in all bottles during the fermentation period. This shows that methane production may have occurred through the depletion of hydrogen and its conversion to methane.

Table 4.4 Variation of methane and hydrogen percentage in experimental bottles during methane production

Experimental Bottle	**DAY 1**		**DAY 2**		**DAY 3**		**DAY 4**	
	H_2 (%)	CH_4 (%)	H_2 (%)	CH_4 (%)	H_2 (%)	CH_4 (%)	H_2 (%)	CH_4 (%)
1	13.3	0.5	10.3	2.46	4.52	5.23	2.6	6.48
2	14.5	4	9.42	5.75	2.28	9.06	1.3	10
3	10	2.98	2.94	7.52	0.28	8.94	0.28	9.6
4	7.1	1.87	3.43	5.13	0.3	6.9	0.28	8.64
5	3.1	0.9	2.34	3.85	0.58	5.25	0.28	6.5

In Figure 4.32, the time-dependent change of TVFA concentrations in experimental media is given. Accordingly, TVFA concentrations could not decrease below 14.6 g L^{-1}. This may be due to substrate inhibition. Lo et al (2011) reported that butyric acid causes substrate inhibition in photofermentative hydrogen production from dark fermentation effluents. In addition, the substrate utilization efficiency for butyric acid is found to be much lower than that of acetic acid and lactic acid, confirming that it causes substrate inhibition. In Figure 4.33, yields for methane production from dark fermentation effluent of biohydrogen production were given.

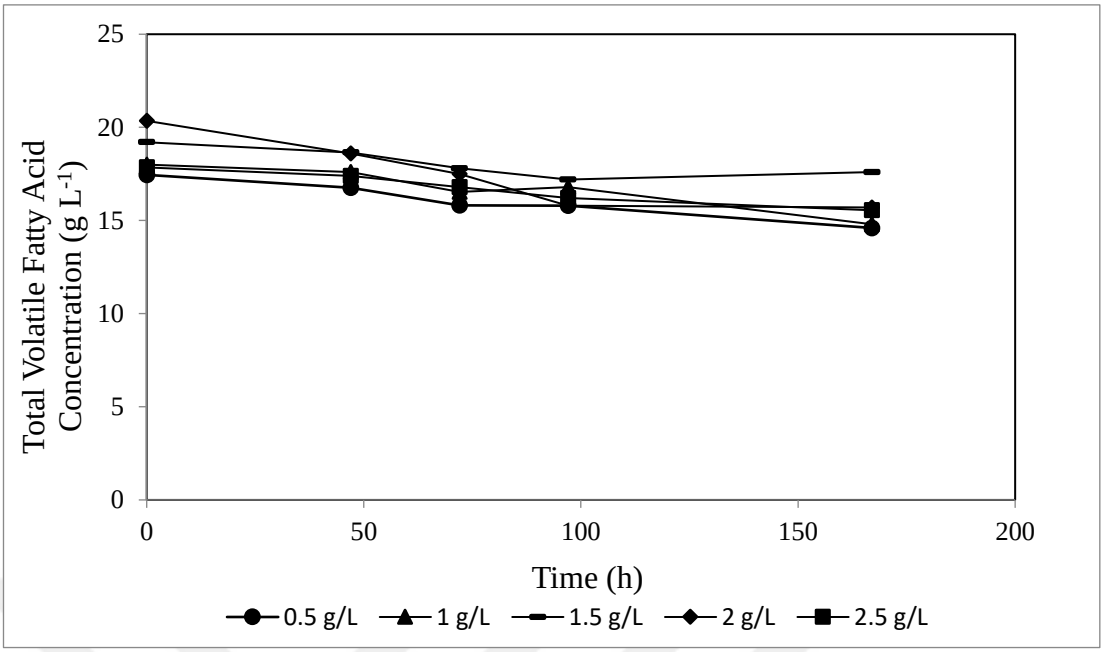


Figure 4.32 Change of total volatile fatty acid concentrations with time in the tested bottles

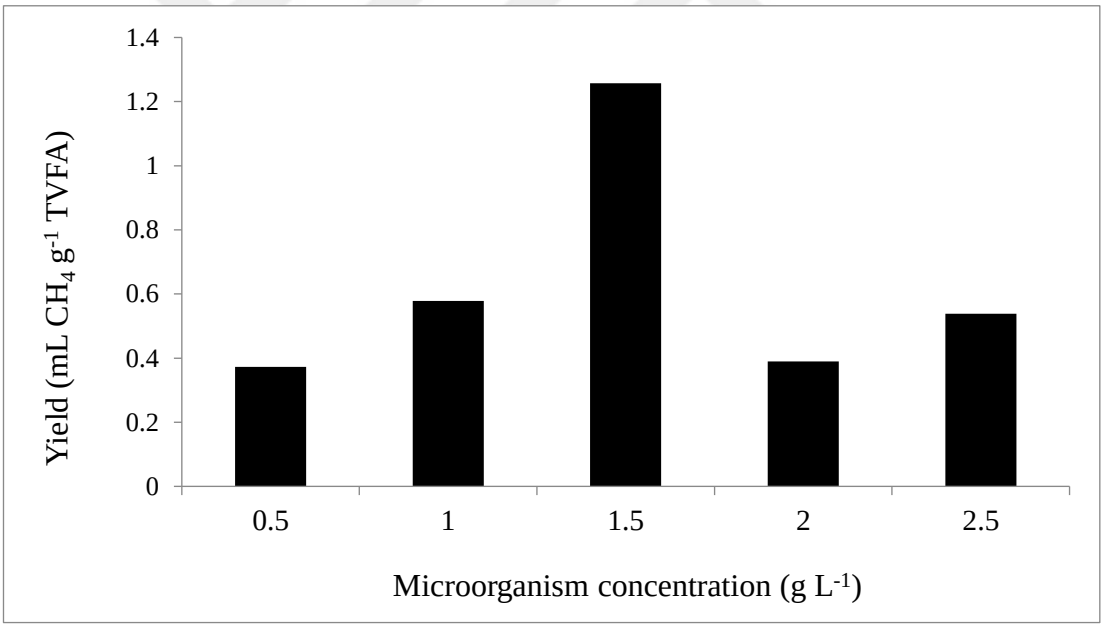


Figure 4.33 Methane production yields from dark fermentation effluent at different hydrogen producing organism containing media

The highest yield value was obtained as 1.25 mL CH4 g^{-1} TVFA in the medium containing 1.5 g L^{-1} hydrogen producing organism concentration. However, there was not a significant production potential in the tested bottles. TVFA were not consumed much over time (Figure 4.32). This shows that high TVFA concentrations

have an inhibitory effect on methanogens. This was explained by Jiang et al. (2018). They revelaed that above 18 g L^{-1} TVFA concentrations have an inhibitory effect on biogas production.

Figure 4.34 summarizes the outputs of sequential fuel production. Accordingly, an initial sugar concentration of 40.7 g L^{-1} was obtained by microwave treatment using 59 g L^{-1} dry fig substrate at a concentration of 1 g L^{-1} organism. 420.55 mL of hydrogen was produced with a yield of 0.69 mol mol^{-1}. The dark fermentation effluent contains 18 g L^{-1} TVFA. With methanogens inoculated into the medium, 22.7 mL of CMF could be obtained. The yield was 0.58 mL CH_4 g^{-1} TVFA.

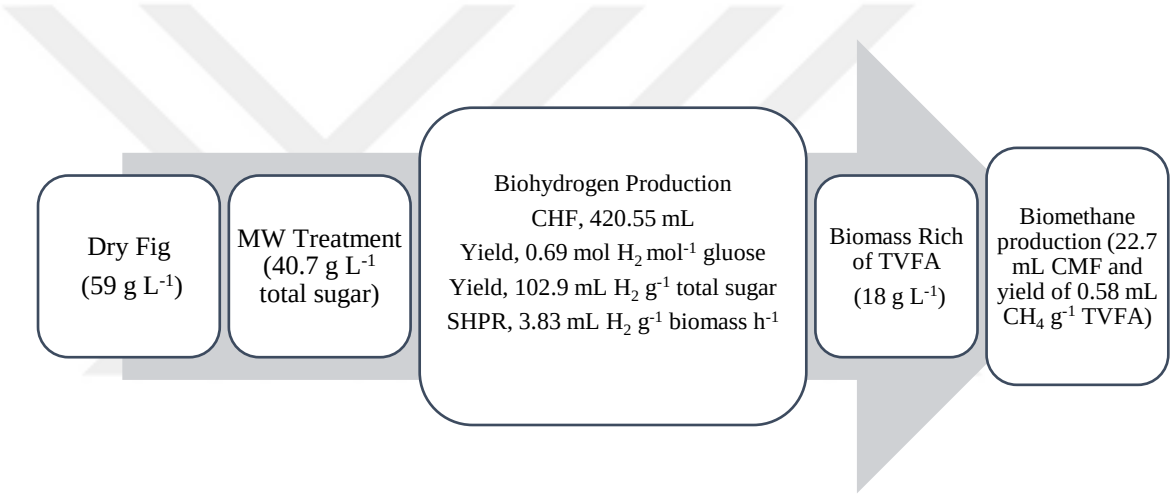


Figure 4.34 Overall sequential biohydrogen and biomethane production process

CHAPTER 5
CONCLUSION & FUTURE STUDIES

5.1 Conclusion

The aim of this study is to investigate the feasibility of converting dried fruits, which are abundant in our country, into a product with high added value, through the evaluation of mycotoxin-contaminated dried fruits in biohydrogen production by dark fermentation in batch mode. Anaerobic sludge containing a mixed consortium of bacteria was used as the microorganism and the experiments were carried out under mesophilic conditions.

It was found that mycotoxin had a negative impact on biohydrogen yield and production volume. In addition to the mycotoxic effect, the effect of the pretreatment applied before fermentation on the production potential was examined and it was concluded that acidic pretreatment did not have any contribution on the hydrogen production volume. In fact, acidic pretreatment on edible dried fruits used as a control group in the experiments caused a decrease in yield. This is due to the toxic effects of metabolites such as furfural and 5-HMF, which may occur during acidic pretreatment, on the microorganism. However acidic pretreatment had a significant positive effect on comparison of yield ratios when mycotoxin presence was observed.

At the initial substrate concentration of 16.66 g dw L^{-1}, the highest cumulative hydrogen volume was obtained as 265 mL in the experimental bottle containing dry figs without acidic pretreatment that was mycotoxin-free (DF-). Also the highest specific hydrogen production rate (42.3 mL H_2 g^{-1} cellmass $h^{-}1$) was obtained with DF- according to Gompertz Equation. Here, the organic acid composition and concentration directly affected the biohydrogen yield. TVFA concentration in the range of 7.95 to 9 g L^{-1} obtained in mycotoxin containing fruits caused product inhibition in biohydrogen production and caused a sharp decrease in yield an formation volume. In addition, the concentration of TVFA in mycotoxin-free fruits did not exceed 4 g L^{-1}. The highest yield in these fruits was obtained in DF- substrate

as 79 mL H_2 g^{-1} total sugar. The lowest cumulative hydrogen volume was obtained as 58 mL in raisins containing mycotoxins.

Experiments were continued with dry fig substrate as it gave higher hydrogen production potential. Since the negative effect of mycotoxin was clearly observed, water-assisted microwave application was applied to dried figs as a method of mycotoxin removal. The highest mycotoxin removal was obtained as 77.8% at 400W microwave power at 10 minutes of treatment. A higher aflatoxin removal percentage than the values obtained in different studies in the literature related to water-assisted microwave application was achieved in this study.

The most suitable parameters of mycotoxin removal method via microwave were applied to the substrates as a pretreatment before fermentation and biohydrogen production potentials were investigated at different initial substrate concentrations. In many studies in the literature, it is known that high substrate or initial sugar concentrations cause substrate inhibition and reduce yield. In the light of this information, it was expected to obtain similar results in our study. CHF showed a 2-fold reduction at concentrations higher than the initial substrate concentration of 59 g L^{-1}. While the highest CHF was obtained as 161.9 mL in the medium containing 59 g L^{-1}, the lowest CHF was obtained as 80 mL in the medium containing 118 g L^{-1} substrate concentration. In addition, the highest hydrogen yield (207.5 mL H_2 g^{-1} total sugar) was also achieved in the medium containing 59 g L^{-1} substrate. The yield obtained is 1/3 of the theoretical yield based on mol hydrogen mol^{-1} glucose.

Depending on the increase in the substrate concentration, the amount of lactic acid produced increased and there was an accumulation of lactic acid. Since it is known that the net hydrogen yield of lactic acid production is zero, it does not contribute to the production volume or yield. The accumulation of lactic acid in the environment at increasing substrate concentrations is related to the metabolism that the organism develops through its adaptation to high sugar.

The media containing 59 g L^{-1} substrate selected as the optimum substrate

concentration were prepared for fermentation by applying microwave pretreatment and the effect of the amount of organism on biohydrogen production was evaluated. The highest CHF was found to be 438 mL in the medium containing the highest concentration of organisms (2.5 g L^{-1}). However, no significant difference was observed between the bottles in terms of hydrogen production volumes. In next studies, higher organism concentrations can be studied to reveal this difference clearly. Almost 2/3 of the sugar concentration was depleted in the first 93 hours, even in the lowest organism-containing environment. This shows that the organism can easily adapt to the environment.

Based on CHF, yield and SHPR, the most appropriate organism concentration was chosen as 1 g L^{-1}. However, in the final stage, methane production was applied to all experimental bottles using the residual biomass of biohydrogen production. Methane volume was limited to only 25 mL. As a conclusion, high concentration of VFA in the medium might have an inhibitory effect on methane formation. In further studies, higher efficiency can be obtained when hydrogen and methane production processes are carried out simultaneously or in a continuous system. Methane can also be produced in a separate reactor by continuously removing VFA from the system. Separation of the reactors can help to overcome inhibitory effects of VFAs to methane bacteria.

5.2 Future Studies

Biohydrogen and biomethane production from mycotoxin containing fruits is a promising solution to find feedstock for these energy generation processes. Further studies need to performed to enhance biohydrogen and biomethane production. The following steps can be applied in future studies:

1. Different pretreatment methods can be applied to mycotoxic substances
2. Fed batch operation can be applied to enhance hydrogen and methane production
3. Sequential hydrogen and methane production can be achieved by using different reactors and seperating VFAs from hyrogen production media.

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APPENDICES

APPENDIX-1: Raw data and hydrogen production data from fruits with or without mycotoxins (DFM-, DFM+, RM-, RM+, DF-, D+-, R-, R+, G, F)

Table A.1.1 Raw data of DFM- medium

Dry fig with mycotoxin and without pretreatment (DFM-)						
Time (day)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.20	9.22	0	0	0	0
3	4.27	1.335	0.949	0.3238	0	0
4	4.75	0.834	3.464	0.443	0	0
5	5.6	0.644	5.247	0.55	0	0
6	5.65	0.586	6.06	0.56	0	0
7	6.5	0.57	6.6624	0.593	0	0
8	6.56	0.313	7.336	0.826	0	0
9	6.4	0.3285	7.448	0.893	0	0
10	6.2	0.2735	7.426	0.721	0	0
11	6.55	0.3175	7.563	0.719	0	0
12	6.48	0.295	7.441	0.717	0	0

Table A.1.2 Hydrogen production data of DFM- medium

Dry fig with mycotoxin and without pretreatment (DFM-)									
Time (day)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	300	300	0	0	0	0	0	0	0
3	295	305	0.07387	22.53035	315	0.07387	23.26905	45.79	1.8
4	285	315	0.03785	0	60	0.03785	2.271	48.07	1.89
5	279	321	0	0	0	0	0	48.07	1.89
6	272	328	0.03325	10.906	0	0.03325	0	58.97	2.32
7	266	334	0.06739	11.60226	0	0.06739	0	70.57	2.77
8	260	340	0.036	0	0	0.036	0	70.57	2.77
9	252	348	0.06615	10.7802	0	0.06615	0	81.35	3.2
10	246	354	0.06	0	0	0.06	0	81.35	3.2
11	241	359	0.05881	0	0	0.05881	0	81.35	3.2
12	236	364	0.04221	0	0	0.04221	0	81.35	3.2

Table A.1.3 Raw data of DFM+ medium

Dry fig with mycotoxin and with pretreatment (DFM+)						
Time (day)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.2	10.74	0	0	0	0
3	4.2	1.3	0.897	0.408	0	0
4	4.9	0.99	3.4109	0.477	0	0
5	5.7	0.67	5.525	0.524	0	0
6	5.85	0.5485	5.77	0.639	0	0
7	6.3	0.4715	6.497	0.735	0	0
8	6.4	0.3475	7.06	0.944	0	0
9	6.5	0.342	7.011	0.939	0	0
10	6.52	0.3	7.226	0.932	0	0
11	6.5	0.3115	7.1	0.925	0	0
12	6.5	0.268	7.197	0.946	0	0

Table A.1.4 Hydrogen production data of DFM+ medium

Dry fig with mycotoxin and with pretreatment (DFM+)									
Time (hour)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	300	300	0	0	0	0	0	0	0
3	295	305	0.03724	11.3582	458	0.03724	17.05592	28.41	1.11
4	285	315	0.07747	13.044	35	0.07747	2.71145	44.17	1.73
5	279	321	0	0	0	0	0	44.17	1.73
6	272	328	0.03291	10.7944	0	0.03291	0	54.96	2.16
7	265	335	0.08293	16.987	0	0.08293	0	71.95	2.83
8	259	341	0.03783	0	0	0.03783	0	71.95	2.83
9	253	347	0.06146	8.426	0	0.06146	0	80.37	3.16
10	248	352	0.06804	2.623	0	0.06804	0	83	3.26
11	243	357	0.0662	0	0	0.0662	0	83	3.26
12	238	362	0.06302	0	0	0.06302	0	83	3.26

Table A.1.5 Raw data of RM- medium

Raisin with mycotoxin and without pretreatment (RM-)						
Time (day)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.2	12.42	0	0	0	0
3	4.31	1.037	0.35	0	0	0
4	4.8	0.749	2.245	0.372	0	0
5	5.15	0.621	5.423	2.144	0	0
6	5.9	0.513	6.736	0.4	0	0
7	6	0.493	7.475	0.4815	0	0
8	6.5	0.369	8.486	0.887	0	0
9	6.5	0.345	8.459	0.699	0	0
10	6.5	0.329	8.445	0.614	0	0
11	6.43	0.3375	8.362	0.6315	0	0
12	6.4	0.301	8.428	0.668	0	0

Table A.1.6 Hydrogen production data of RM- medium

Raisin with mycotoxin and without pretreatment (RM-)									
Time (hour)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	300	300	0	0	0	0	0	0	
3	295	305	0.05418	16.5249	305	0.05418	16.5249	33.05	1.30
4	283	317	0.0494	0	92	0.0494	4.5448	37.59	1.48
5	276	324	0	0	0	0	0	37.59	1.48
6	268	332	0	0	0	0	0	37.59	1.48
7	262	338	0.03417	11.54946	0	0.03417	0	49.14	1.93
8	255	345	0.03401	0.18399	0	0.03401	0	49.33	1.94
9	249	351	0.05144	6.32199	0	0.05144	0	55.65	2.19
10	244	356	0.04967	0	0	0.04967	0	55.65	2.19
11	239	361	0.04214	0	0	0.04214	0	55.65	2.19
12	234	366	0.04084	2.6629	0	0.04084	0	55.65	2.19

Table A.1.7 Raw data of RM+ medium

Raisin with mycotoxin and with pretreatment (RM+)						
Time (day)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.2	11.78	0	0	0	0
3	4.4	1.055	0.412	0	0	0
4	4.85	0.66	2.883	0.456	0	0
5	5.8	0.489	5.22	0.651	0	0
6	6	0.4875	7.061	0.4875	0	0
7	6.5	0.452	7.74	0.532	0	0
8	6.4	0.369	8.35	0.851	0	0
9	6.5	0.341	8.377	0.681	0	0
10	6.4	0.342	8.549	0.641	0	0
11	6.33	0.3265	8.387	0.642	0	0
12	6.6	0.229	8.347	0.671	0	0

Table A.1.8 Hydrogen production data of RM+ medium

Raisin with mycotoxin and with pretreatment (RM+)									
Time (hour)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	300	300	0	0	0	0	0	0	0
3	295	305	0.03796	11.57841	595	0.03796	22.58739	34.17	1.34
4	286	314	0.04719	3.23925	75	0.04719	3.53925	40.94	1.61
5	279	321	0	0	0	0	0	40.94	1.61
6	272	328	0.03292	10.79776	0	0.03292	0	51.74	2.04
7	265	335	0.03854	2.11314	0	0.03854	0	53.86	2.12
8	258	342	0.03356	0	0	0.03356	0	53.86	2.39
9	251	349	0.05243	6.82055	0	0.05243	0	60.67	2.39
10	246	354	0.05033	0	0	0.05033	0	60.67	2.39
11	240	360	0.04892	0	0	0.04892	0	60.67	2.39
12	235	365	0.04855	0	0	0.04855	0	60.67	2.39

Table A.1.9 Raw data of DF- medium

Dry fig without mycotoxin and without pretreatment (DF-)						
Time (day)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.2	11.74	0	0	0	0
3	4.1	3.8	1.119	0.832	0.242	0
4	5.7	0.91	1.72	1.35	0.197	0
5	6.28	0.503	1.66	1.68	0	0
6	6.83	0.45	2.365	1.704	0	0
7	6.4	0.49	2.85	1.72	0	0
10	6.4	0.453	5.986	1.61	0	0
11	6.5	0.3815	5.96	1.604	0	0
12	6.5	0.435	6.08	1.61	0	0

Table A.1.10 Hydrogen production data of DF- medium

Dry fig without mycotoxin and without pretreatment (DF-)									
Time (hour)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	300	300	0	0	0	0	0	0	0
3	295	305	0.03163	9.64715	495	0.03163	15.65685	25.30	1.00
4	288	312	0.304	85.20085	248	0.304	75.392	185.90	7.31
5	282	318	0.0333	0	78	0.0333	2.5974	188.49	7.42
6	275	325	0.2689	76.8031	0	0.2689	0	265.30	10.44
7	270	330	0.2552	0	0	0.2552	0	265.30	10.44
10	265	335	0.2111	0	0	0.2111	0	265.30	10.44
11	258	342	0.185	0	0	0.185	0	265.30	10.44
12	253	347	0.169	0	0	0.169	0	265.30	10.44

Table A.1.11 Raw data of DF+ medium

Dry fig without mycotoxin and with pretreatment (DF+)						
Time (day)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.2	12	0	0	0	0
3	4.12	2.95	0.733	0.511	0.55	0
4	6.11	0.8	1.79	1.25	0.475	0
5	6.3	0.473	1.8	1.52	0	0
6	6.8	0.38	2.196	1.508	0	0
7	6.5	0.439	2.48	1.44	0	0
10	6.4	0.4	3.16	1.51	0	0
11	6.3	0.3525	3.515	1.485	0	0
12	6.42	0.397	4.32	1.407	0	0

Table A.1.12 Hydrogen production data of DF+ medium

Dry fig without mycotoxin and with pretreatment (DF+)									
Time (hour)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	300	300	0	0	0	0	0	0	0
3	295	305	0.03289	10.03145	540	0.03289	17.7606	27.79	1.09
4	289	311	0.273	74.87155	195	0.273	53.235	155.90	6.13
5	283	317	0.034	0	55	0.034	1.87	157.77	6.21
6	277	323	0.1778	46.6514	0	0.1778	0	204.42	8.04
7	272	328	0.2305	18.1746	0	0.2305	0	222.59	8.76
10	267	333	0.195	0	0	0.195	0	222.59	8.76
11	262	338	0.1732	0	0	0.1732	0	222.59	8.76
12	256	344	0.163	0	0	0.163	0	222.59	8.76

Table A.1.13 Raw data of R- medium

Raisin without mycotoxin and without pretreatment (R-)						
Time (day)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.2	14.56	0	0	0	0
3	4.12	3.72	0.617	0.538	0.485	0
4	6.14	0.67	1.54	0.946	0.505	0
5	6.47	0.465	1.23	1.18	0	0
6	6.5	0.381	2.366	1.142	0	0
7	6.2	0.385	2.82	1.06	0	0
10	6.4	0.354	5.92	1	0	0
11	6.6	0.324	6.15	1	0	0
12	6.42	0.353	6.49	0.965	0	0

Table A.1.14 Hydrogen production data of R- medium

Raisin without mycotoxin and without pretreatment (R-)									
Time (hour)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	300	300	0	0	0	0	0	0	0
3	295	305	0.03353	10.22665	505	0.03353	16.93265	27.16	1.07
4	287	313	0.1943	50.58925	230	0.1943	44.689	122.44	4.82
5	281	319	0.0331	0	48	0.0331	1.5888	124.03	4.88
6	276	324	0.1902	51.0659	28	0.1902	5.3256	180.42	7.10
7	271	329	0.1693	0	0	0.1693	0	180.42	7.10
10	265	335	0.1412	0	0	0.1412	0	180.42	7.10
11	259	341	0.129	0	0	0.129	0	180.42	7.10
12	254	346	0.114	0	0	0.114	0	180.42	7.10

Table A.1.15 Raw data of R+ medium

Raisin without mycotoxin and with pretreatment (R+)						
Time (day)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.2	13.92	0	0	0	0
3	4.13	1.76	0.724	0.521	0.465	0
4	6.23	0.59	0.925	0.9	0.414	0
5	6.56	0.405	1.55	1.06	0	0
6	6.5	0.334	2.19	1.081	0	0
7	6.55	0.385	2.4	1	0	0
10	6.4	0.377	3.8	1	0	0
11	6.55	0.301	4.97	0.9	0	0
12	6.55	0.3305	6.27	0.95	0	0

Table A.1.16 Hydrogen production data of R+ medium

Raisin without mycotoxin and with pretreatment (R+)									
Time (hour)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	300	300	0	0	0	0	0	0	0
3	295	305	0.03718	11.3399	130	0.03718	4.8334	16.17	0.64
4	289	311	0.157	37.4871	93	0.157	14.601	68.26	2.69
5	283	317	0.0329	0	15	0.0329	0.4935	68.75	2.70
6	278	322	0.1768	46.5003	25	0.1768	4.42	119.68	4.71
7	273	327	0.1546	0	0	0.1546	0	119.68	4.71
10	268	332	0.1187	0	0	0.1187	0	119.68	4.71
11	262	338	0.112	0	0	0.112	0	119.68	4.71
12	257	343	0.105	0	0	0.105	0	119.68	4.71

Table A.1.17 Hydrogen production data of control medium (glucose)

Glucose (G)									
Time (day)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	300	300	0	0	0	0	0	0	
3	295	305	0.1008	30.744	80	0.1008	8.064	38.81	1.53
4	287	313	0.07767	0	223	0.07767	17.32041	56.13	2.21
5	279	321	0.1016	8.30289	220	0.1016	22.352	86.78	3.41
6	270	330	0.0339	0	160	0.0339	5.424	92.21	3.63
7	263	337	0.03869	1.85153	0	0.03869	0	94.06	3.70
8	255	345	0.218	62.17147	0	0.218	0	156.23	6.15
9	248	352	0.1961	0	0	0.1961	0	156.23	6.15
10	242	358	0.1895	0	0	0.1895	0	156.23	6.15
11	237	363	0.1812	0	0	0.1812	0	156.23	6.15
12	232	368	0.1731	0	0	0.1731	0	156.23	6.15

Table A.1.18 Hydrogen production data of control medium (fructose)

Fructose (F)									
Time (day)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	300	300	0	0	0	0	0	0	0
3	295	305	0.0436	13.298	65	0.0436	2.834	16.13	0.63
4	286	314	0.0335	0	55	0.0335	1.8425	17.97	0.71
5	279	321	0.1205	28.1615	230	0.1205	27.715	73.85	2.91
6	272	328	0.03306	0	285	0.03306	9.4221	83.27	3.28
7	265	335	0.2595	76.08882	105	0.2595	27.2475	18.61	7.34
8	259	341	0.0331	0	0	0.0331	0	18.61	7.34
9	249	351	0.2167	64.7746	0	0.2167	0	251.38	9.89
10	244	356	0.2054	0	0	0.2054	0	251.38	9.89
11	238	362	0.19127	0	0	0.19127	0	251.38	9.89
12	233	367	0.1817	0	0	0.1817	0	251.38	9.89

APPENDIX-2: Raw data and hydrogen production data from different substrate concentrations experiments (11.8 g L^{-1}, 29.5 g L^{-1}, 59 g L^{-1}, 88.5 g L^{-1}, 118 g L^{-1})

Table A.2.1 Raw data of substrate concentration of 11.8 g L^{-1}

Initial substrate concentration of 11.8 g L^{-1}						
Time (h)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.2	11.65	0	0	0	0
21	4.78	2.53	1.6	0.765	6.3	0.508
45	6.2	1.88	1.88	1.36	5.76	0.386
69	6.75	0.8	4.4	2.46	2.98	0.259
93	6.8	0.66	5.18	2.94	1.68	0.329
141	6.85	0.8	5.66	3.198	0.635	0.376
168	7.1	0.44	5.5	3.187	0	0.38
190	7	0.58	6.01	3.4	0	0.4
214	6.8	0.52	6	3.28	0	0.439
237	6.8	0.527	6.06	3.26	0	0.416
262	6.77	0.61	6.22	3.32	0	0.429
333	6.77	0.49	6.19	3.3	0	0.429
356	6.8	0.31	6.29	3.2	0	0.422

Table A.2.2 Hydrogen production data of substrate concentration of 11.8 g L^{-1}

Initial substrate concentration of 11.8 g L^{-1}									
Time (h)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	100	210	0	0	0	0	0	0	0
21	96	214	0.1753	37.5142	100	0.1753	17.53	55.0	2.0
45	92	218	0.242	15.2418	50	0.242	12.1	82.4	3.1
69	90	220	0.198	0	22	0.198	4.356	86.7	3.2
93	88	222	0.155	0	7	0.155	1.085	87.8	3.3
141	86	224	0.126	0	0	0.126	0	87.8	3.3

Table A.2.2 continues

Time (h)	V_{liquid} (mL)	V_{gas} (mL)	CH_2/100	$VH_{2(head\ space)}$ (mL)	$V_{total\ gas}$ (mL)	CH_2/100	$V_{total\ gas}$*CH_2 (mL)	Vcumulative H_2 (mL)	Cumulative H_2 (mmol)
168	84	226	0.104	0	0	0.104	0	87.8	3.3
190	82	228	0.0903	0	0	0.0903	0	87.8	3.3
214	80	230	0.0642	0	0	0.0642	0	87.8	3.3
237	78	232	0.0762	0	0	0.0762	0	87.8	3.3
262	76	234	0.0677	0	0	0.0677	0	87.8	3.3
333	74	236	0.058	0	0	0.058	0	87.8	3.3
356	72	238	0.0567	0	0	0.0567	0	87.8	3.3

Table A.2.3 Raw data of substrate concentration of 29.5 g L^{-1}

Initial substrate concentration of 29.5 g L^{-1}						
Time (h)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.2	22.3	0	0	0	0
21	4.66	8.44	1.84	0.539	7.85	0.777
45	4.75	4.85	1.764	0	16.14	0.707
69	5.98	2.6	2.05	0	16.26	0.622
93	6.6	1.32	3.174	2.14	14.16	0.153
141	6.55	1.4	5.48	3.16	10	0.358
168	6.8	0.814	6.03	6.878	6,5	0.381
190	6.8	0.929	6.49	6.72	3.15	0.382
214	6.8	0.86	6.74	6.05	0	0.401
237	6.7	0.84	6.82	7.12	0	0.446
262	6.7	0.81	7.25	7.41	0	0.614
333	6.7	0.83	7.53	7.5	0	0.617
356	6.8	0.59	7.5	7.7	0	0.607

Table A.2.4 Hydrogen production data of substrate concentration of 29.5 g L^{-1}

Initial substrate concentration of 29.5 g L^{-1}									
Time (h)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	100	210	0	0	0	0	0	0	0
21	95	215	0.18	38.7	105	0.18	18.9	57.6	2.1
45	92	218	0.169	0	8	0.169	1.352	59.0	2.2
69	89	221	0.246	17.524	33	0.246	8.118	84.6	3.1
93	87	223	0.29	10.304	45	0.29	13.05	107.9	4.0
141	85	225	0.1953	0	21	0.1953	4.1013	112.0	4.2
168	83	227	0.152	0	17	0.152	2.584	114.6	4.3
190	81	229	0.128	0	0	0.128	0	114.6	4.3
214	79	231	0.111	0	12	0.111	1.332	116.0	4.3
237	77	233	0.09	0	4	0.09	0.36	116.3	4.3
262	75	235	0.09	0.18	0	0.09	0	116.5	4.3
333	73	237	0.0768	0	0	0.0768	0	116.5	4.3
356	71	239	0.0755	0	0	0.0755	0	116.5	4.3

Table A.2.5 Raw data of substrate concentration of 59 g L^{-1}

Initial substrate concentration of 59 g L^{-1}						
Time (h)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.2	47,4	0	0	0	0
21	4.57	27.1	1.76	0	8.27	1.06
45	4.7	23	1.65	0	16.84	1.05
69	5	12.45	1.61	0	27.09	0.976
93	5.1	8.46	1.75	0	36.57	1
141	5.2	6.76	1.28	0	38.93	0.806
168	6	2.93	2.4	0	39.84	0.517
190	6.65	2.62	2.48	2.86	37.68	0
214	6.5	2.53	3.67	4.44	34.78	0.136
237	6.6	2.35	4.33	6.4	28.95	0.18
262	6.7	1.94	4.64	8.09	23.02	0.207
333	6.7	1.72	6.2	9.27	16.17	0.226
356	6.9	0.8	6.45	9.38	13.17	0

Table A.2.6 Hydrogen production data of substrate concentration of 59 g L^{-1}

Initial substrate concentration of 59 g L^{-1}									
Time (h)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	100	210	0	0	0	0	0	0	0
21	96	214	0.154	32.956	130	0.154	20.02	53.0	2.0
45	93	217	0.142	0	5	0.142	0.71	53.7	2.0
69	89	221	0.137	0	3	0.137	0.411	54.1	2.0
93	86	224	0.14	1.083	0	0.14	0	55.2	2.1
141	83	227	0.102	0	0	0.102	0	55.2	2.1
168	80	230	0.266	38.026	58	0.266	15.428	108.6	4.0
190	77	233	0.304	9.652	75	0.304	22.8	141.1	5.2
214	75	235	0.253	0	25	0.253	6.325	147.4	5.5
237	73	237	0.224	0	17	0.224	3.808	151.2	5.6
262	71	239	0.18	0	18	0.18	3.24	154.5	5.7
333	69	241	0.147	0	35	0.147	5.145	159.6	5.9
356	67	243	0.144	0	10	0.144	1.44	161.0	6.0

Table A.2.7 Raw data of substrate concentration of 88.5 g L^{-1}

Initial substrate concentration of 88.5 g L^{-1}						
Time (h)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.2	79.8	0	0	0	0
21	4.58	42.7	2.22	0	8.7	1.32
45	4.7	33.9	1.45	0	18	1.05
69	5	31.7	1.44	0	9.27	1.19
93	5.25	24.16	1.53	0	33.2	1.24
141	5.1	20.96	1.46	0	38.39	0.853
168	5.3	11.78	1.097	0	24.24	0
190	5.5	6.135	1.26	0.47	59.58	0.747
214	6.3	5.815	2,74	0	59.34	0.183
237	6.6	4.87	3.02	0.902	57.41	0
262	6.5	3.85	3.52	2.09	58.62	0
333	6,7	3.67	3.51	3.35	51.36	0
356	6,8	1.95	3.72	5.76	50.31	0

Table A.2.8 Hydrogen production data of substrate concentration of 88.5 g L^{-1}

Initial substrate concentration of 88.5 g L^{-1}									
Time (h)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	100	210	0	0	0	0	0	0	0
21	97	213	0.1255	26.7315	103	0.1255	12.9265	39.66	1.47
45	91	219	0.122	0	11	0.122	1.342	41.00	1.52
69	88	222	0.1138	0	11	0.1138	1.2518	42.25	1.57
93	85	225	0.108	0	0	0.108	0	42.25	1.57
141	82	228	0.106	0	0	0.106	0	42.25	1.57
168	77	233	0.096	0	0	0.096	0	42.25	1.57
190	74	236	0.088	0	0	0.088	0	42.25	1.57
214	70	240	0.16	17.632	30	0.16	4.8	64.68	2.40
237	67	243	0.183	6.069	18	0.183	3.294	74.05	2.75
262	65	245	0.217	8.696	67	0.217	14.539	97.28	3.62
333	63	247	0.211	0	34	0.211	7.174	104.46	3.88
356	61	249	0.2	0	8	0.2	1.6	106.06	3.94

Table A.2.9 Raw data of substrate concentration of 118 g L^{-1}

Initial substrate concentration of 118 g L^{-1}						
Time (h)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.2	85.1	0	0	0	0
21	4.61	52.3	0	0	7.55	0
45	4.65	49	1.88	0	16.49	0
69	4.8	46	0	0	17.64	0
93	5.15	38.43	2.18	0	28	0
141	5.1	40.8	2.3	0	34.74	0
168	5.5	30.33	2.28	0	41.27	0
190	5.5	25.86	1.39	0	46.87	0.775
214	5.5	14.8	1.26	0	58.3	0.687
237	5.5	19.1	1.26	0	65.79	0.667
262	5.3	11.36	1.12	0	79.42	0.7
333	5.9	10	1.17	0	83.58	0.479
356	6.4	8.56	3	0	81.46	0.145
380	6.63	7	4.18	0	76.51	0

Table A.2.10 Hydrogen production data of substrate concentration of 118 g L^{-1}

Initial substrate concentration of 118 g L^{-1}									
Time (h)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	100	210	0	0	0	0	0	0,00	0,00
21	97	213	0.1057	22.5141	135	0.1057	142695	36.78	1.37
45	94	216	0.0546	0	9	0.0546	0.4914	37.28	1.39
69	91	219	0.122	14.9244	21	0.122	2.562	54.76	2.04
93	88	222	0.114	0	0	0.114	0	54.76	2.04
141	85	225	0.113	0.117	0	0.113	0	54.88	2.04
168	82	228	0.102	0	0	0.102	0	54.88	2.04
190	79	231	0.099	0	0	0.099	0	54.88	2.04
214	75	235	0.101	0.866	0	0.101	0	55.74	2.07
237	72	238	0.0956	0	0	0.0956	0	55.74	2.07
262	69	241	0.098	0.8652	0	0.098	0	56.61	2.10
333	66	244	0.1	0.782	0	0.1	0	57.39	2.13
356	63	247	0.156	14.132	0	0.156	0	71.52	2.66

APPENDIX-3: Raw data and hydrogen production data from different organisms concentrations experiments (0.5 g L^{-1}, 1 g L^{-1}, 1.5 g L^{-1}, 2 g L^{-1}, 2.5 g L^{-1})

Table A.3.1 Raw data of organism concentration of 0.5 g L^{-1}

Organism concentration of 0.5 g L^{-1}						
Time (h)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.3	43.8	0	0	0	0
21	4.25	40.3	1.28	0	5.96	2.16
45	4.57	31	0.98	0	13.6	1.97
93	5.87	13.4	0.912	0	22.24	1.8
124	5.25	6	0	5.48	21.4	2.3
164	6.75	1.63	2.76	11.84	5.8	2.18
189	6.8	2	3.67	15	0	2
213	6.85	1.86	3.2	15.27	0	1.1
237	6.9	1.65	3.24	13.98	0	0.76
260	6.92	1.02	3.4	14.3	0	0.58
332	7	0.72	3.6	14	0	0.51

Table A.3.2 Hydrogen production data of organism concentration of 0.5 g L^{-1}

Organism concentration of 0.5 g L^{-1}									
Time (h)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	100	210	0	0	0	0	0	0	0
21	98	212	0.033	6.996	95	0.033	3.135	10.13	0.38
45	96	214	0.0337	0.2158	0	0.0337	0	10.35	0.38
93	94	216	0.091	12.4442	22	0.091	2.002	24.79	0.92
124	92	218	0.464	81.496	314	0.464	145.696	251.99	9.37
164	90	220	0.462	0.488	195	0.462	90.09	342.56	12.74
189	88	222	0.292	0	67	0.292	19.564	362.13	13.46
213	86	224	0.25	0	23	0.25	5.75	367.88	13.68
237	84	226	0.222	0	15	0.222	3.33	371.21	13.80
260	82	228	0.218	0	0	0.218	0	371.21	13.80
332	80	230	0.1	0	0	0.1	0	371.21	13.80

Table A.3.3 Raw data of organism concentration of 1 g L^{-1}

Organism concentration of 1 g L^{-1}						
Time (h)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.3	40.7	0	0	0	0
21	4.3	32.55	1.37	0	7.23	2.2
45	4.7	32.6	1	0	15.3	1.9
93	5.12	4.3	0	6.2	15.1	2.4
124	5.9	4.26	0	8.33	14.1	2.67
164	6.85	1.73	3.4	14.28	0	0
189	6.8	2	3.95	14.5	0	0
213	6.9	1.69	3.95	14.9	0	0.98
237	6.75	1.45	4.26	13.56	0	0.95
260	6.75	1.3	4.4	13.47	0	0.98
332	6.9	0.95	4.8	13.5	0	1

Table A.3.4 Hydrogen production data of organism concentration of 1 g L^{-1}

Organism concentration of 1 g L^{-1}									
Time (h)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	100	210	0	0	0	0	0	0	0
21	98	212	0.033	6.996	100	0.033	3.3	10.30	0.38
45	96	214	0.0336	0.1944	5	0.0336	0.168	10.66	0.40
93	94	216	0.472	94.7616	446	0.472	210.512	315.93	11.75
124	92	218	0.454	0	95	0.454	43.13	359.06	13.35
164	90	220	0.4	0	105	0.4	42	401.06	14.91
189	88	222	0.298	0	37	0.298	11.026	412.09	15.32
213	86	224	0.26	0	17	0.26	4.42	416.51	15.49
237	84	226	0.23	0	15	0.23	3.45	419.96	15.61
260	82	228	0.2	0	3	0.2	0,6	420.56	15.64
332	80	230	0.193	0	0	0.193	0	420.56	15.64

Table A.3.5 Raw data of organism concentration of 1.5 g L^{-1}

Organism concentration of 1.5 g L^{-1}						
Time (h)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.3	42.3	0	0	0	0
21	4.25	29.6	1.36	0	7.46	2.25
45	4.57	24.6	1.05	0	15.3	2.05
93	4.87	5.73	0	6.77	18	2.1
124	5.25	3.87	0	6.9	17.5	2.27
164	6.75	2.2	4	13.4	0	2
189	6.8	2.7	4.9	14.5	0	0
213	6.85	2.5	4.6	13.7	0	0.745
237	6.9	1.86	4.7	13.8	0	0.6
260	6.92	1.52	4.8	13.8	0	0.6
332	6.9	1.3	4.9	13.6	0	0.7

Table A.3.6 Hydrogen production data of organism concentration of 1.5 g L^{-1}

Organism concentration of 1.5 g L^{-1}									
Time (h)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	100	210	0	0	0	0	0	0	0
21	98	212	0.0334	7.0808	70	0.0334	2.338	9.42	0.35
45	96	214	0.036	0.6232	9	0.036	0.324	10.37	0.39
93	94	216	0.466	92.952	320	0.466	149.12	252.44	9.39
124	92	218	0.415	0	48	0.415	19.92	272.36	10.13
164	90	220	0.341	0	110	0.341	37.51	309.87	11.52
189	88	222	0.27	0	49	0.27	13.23	323.10	12.01
213	86	224	0.228	0	30	0.228	6.84	329.94	12.27
237	84	226	0.198	0	19	0.198	3.762	333.70	12.41
260	82	228	0.188	0	3	0.188	0.564	334.26	12.43
332	80	230	0.169	0	11	0.169	1.859	336.12	12.50

Table A.3.7 Raw data of organism concentration of 2 g L^{-1}

Organism concentration of 2 g L^{-1}						
Time (h)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.3	47.7	0	0	0	0
21	4.4	44.4	1.44	0	7.96	2.1
45	4.7	23	1.18	0	15.93	2
93	4.75	3.75	0	5.65	22.65	2
124	5.7	4.7	0	6	22.1	2.28
164	6.9	2.7	4.1	15.72	0	0
189	6.7	2.4	5.14	13.3	0	1.6
213	6.7	2.15	5.37	14.66	0	0.52
237	6.7	1.8	5.74	14.56	0	0.558
260	6.7	1.32	5.96	14.9	0	0.6
332	6.75	1.2	6.3	15	0	0.66

Table A.3.8 Hydrogen production data of organism concentration of 2 g L^{-1}

Organism concentration of 2 g L^{-1}									
Time (h)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	100	210	0	0	0	0	0	0	0
21	98	212	0.0338	7.1656	86	0.0338	2.9068	10.07	0.37
45	96	214	0.0377	0.9022	5	0.0377	0.1885	11.16	0.42
93	94	216	0.43	84.8122	438	0.43	188.34	284.32	10.57
124	92	218	0.386	0	52	0.386	20.072	304.39	11.32
164	90	220	0.31	0	155	0.31	48.05	352.44	13.10
189	88	222	0.226	0	38	0.226	8.588	361.03	13.42
213	86	224	0.19	0	18	0.19	3.42	364.45	13.55
237	84	226	0.164	0	14	0.164	2.296	366.74	13.64
260	82	228	0.156	0	13	0.156	2.028	368.77	13.71
332	80	230	0.126	0	8	0.126	1.008	369.78	13.75

Table A.3.9 Raw data of organism concentration of 2.5 g L^{-1}

Organism concentration of 2.5 g L^{-1}						
Time (h)	**pH**	**Total Sugar Concentration (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Lactic Acid (g/L)**	**Propionic Acid (g/L)**
0	7.3	46.1	0	0	0	0
21	4.45	33.5	1.4	0	7.2	2.12
45	4.75	16.1	1.14	0	19.2	2
93	5.22	3.2	0	7.2	14.3	3.1
124	6.2	4.2	0	8.73	10.56	3.17
164	6.6	1.7	4.48	13.74	0	0
189	6.5	2.1	5.38	15	0	0
213	6.55	1.78	4.64	12.5	0	1.4
237	6.6	1.8	4.87	12.5	0	1.4
260	6.5	1.45	5.1	12.36	0	1.47
332	6.55	1	5.85	12.5	0	1.5

Table A.3.10 Hydrogen production data of organism concentration of 2.5 g L^{-1}

Organism concentration of 2.5 g L^{-1}									
Time (h)	**V_{liquid} (mL)**	**V_{gas} (mL)**	**CH_2/100**	**$VH_{2(head\ space)}$ (mL)**	**$V_{total\ gas}$ (mL)**	**CH_2/100**	**$V_{total\ gas}$*CH_2 (mL)**	**Vcumulative H_2 (mL)**	**Cumulative H_2 (mmol)**
0	100	210	0	0	0	0	0	0	0
21	98	212	0.0337	7.1444	85	0.0337	2.8645	10.01	0.37
45	96	214	0.04	1.4156	4	0.04	0.16	11.58	0.43
93	94	216	0.46	90.8	530	0.46	243.8	346.18	12.87
124	92	218	0.397	0	110	0.397	43.67	389.85	14.49
164	90	220	0.33	0	100	0.33	33	422.85	15.72
189	88	222	0.261	0	33	0.261	8.613	431.47	16.04
213	86	224	0.232	0	12	0.232	2.784	434.25	16.15
237	84	226	0.213	0	14	0.213	2.982	437.23	16.26
260	82	228	0.162	0	4	0.162	0.648	437.88	16.28
332	80	230	0.078	0	0	0.078	0	437.88	16.28