WHOLE GENOME SEQUENCING OF A NOVEL THERMOPHILIC ISOLATE UTILIZING THE OXFORD NANOPORE TECHNOLOGIES AND ADVANCING THE BIOTECHNOLOGICAL HYDROGEN PRODUCTION CAPACITY OF THE ISOLATE

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ABSTRACT

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Master's Program in Bioengineering

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Hydrogen is an excellent energy carrier and clean fuel with zero carbon emission. The production of hydrogen by biological source

s is of great importance in terms of sustainability. Thermophilic bacteria are frequently used in biohydrogen studies because they produce biohydrogen with a high yield. Anaerobic thermophiles, a type of thermophilic bacteria, can survive in an anoxic and hot environment and produce hydrogen from carbon monoxide using a special pathway, collectively called as water-

gas shift reaction (WGSR). Hot springs are one of the habitats of thermophilic anaerobic bacteria, and İzmir has several hot springs due to its geothermal location. For this purpose, the biohydrogen production potentials of mixed cultures obtained from 5 different hot springs in İzmir were determined, and the best hydrogen production was observed in the samples taken from Doğanbey, Seferihisar. The pure culture was obtained from mixed cultures by serial dilution and strake plate method. SEM and ESEM were used for morphological characterization whereas molecular characterization studies were carried out by whole genome sequencing via the Oxford Nanopore MinION. To optimize the growth conditions of the pure culture, the Box-Behnken design was made, and the hydrogen production efficiency was increased up to 30%. Bioreactor experimets were done with the best hydrogen producing strain and highest hydrogen production obtained at the end of 24^{th} h.

Keywords: Biohydrogen, water-gas shift reaction, thermophilic anaerobic microorganism, The Oxford Nanopore MinION, whole-genome sequencing

ÖZET

YENİ KEŞFEDİLMİŞ TERMOFİLİK İZOLATIN BİYOTEKNOLOJİK HİDROJEN ÜRETİM KAPASİTESİNİN ARAŞTIRILMASI VE OXFORD NANOPORE TEKNOLOJİLERİNİ TAM GENOM SEKANSLANMASI

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Hidrojen enerji verimi yüksek ve çevre dostu bir yakıttır. Hidrojenin biyolojik yollarla üretilmesi sürdürülebilirlik açısından oldukça büyük önem taşır. Termofilik bakteriler yüksek verimde biyohidrojen üretmeleri sebebiyle biyohidrojen çalışmalarında sıkça kullanılmaktadır. Termofilik bakterilerin bir türü olan anaerobic termofiller oksijensiz ve sıcak orta hayatta kalabilmekte ve su-gaz dönüşümü olarak da bilinen özel bir yolağı kullanarak karbonmonoksit gazından hidrojen üretebilmektedirler. Termal su

kaynakları, termofilik anaerobik bakterilerin habitatlarından biri olup, İzmir ili jeotermal konumu sebebiyle pek çok termal su kaynağına sahiptir. Bu amaçla, İzmir ilinde bulunan 5 farklı sıcak su kaynağından elde edilen karışık kültürlerin biyohidrojen üretim potansiyelleri belirlenmiş, en iyi hidrojen üretimi Doğanbey, Seferihisar kaplıcasından alınan örneklerde gözlenmiştir. Elde edilen izolatlardan seri seyreltme ve dökme plaka yöntemi ile saf kültür elde edilmiştir. Morfolojik karakterizasyon amacıyla ESEM ve SEM görüntüleme yapılmış olup, moleküler karakterizasyon çalışmaları için Oxford Nanopore MinION cihazı ile tüm genom sekanslama yapılmıştır. Elde edilen saf kültürün büyüme koşullarını optimize etmek amacıyla Box-Behnken dizaynı yapılmış ve hidrojen üretim verimi %30 oranında artırılmıştır. En iyi hidrojen üreten saf kültür ile biyoreaktör denemeleri kurulmuş ve en yüksek verim 24 saat sonunda elde edilmiştir.

Anahtar Kelimeler: Biyohidrojen, su-gaz dönüşümü, termofilik anaerobic mikroorganizma, The Oxford Nanopore MinION, tüm genom sekanslama

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LIST OF ABBREVIATIONS

16S rRNA: 16S Ribosomal Ribonucleic Acid

BBD: Box-Behnken Design

DNA: Deoxyribonucleic acid

ESEM: Environmental Scanning Electron Microscopy

GC: Gas Chromatography

HPLC: High Pressure Liquid Chromatography

PCR: Polymerase Chain Reaction

PNS: Purple non-sulphur

PS: Photosystems

SEM: Scanning Electron Microscopy

WGS: Whole-Genome Sequencing

WGSR: Water-Gas Shift Reaction

CHAPTER 1: INTRODUCTION

1.1. Hydrogen Energy

Hydrogen is a non-toxic gas without color, odor and is the lightest element. Hydrogen is an ideal energy carrier with the highest energy value on basis of mass (upper 140.9 MJ/kg, lower 120.7 MJ/kg) compared to other fuels (Bossel and Eliasson, 2002). An important difference between hydrogen from other fuels is that it can be produced from water with the help of solar or wind energy and only water is released when burned. However, to be used as energy, it must be separated from the compounds in nature (Das and Veziroğlu, 2001). The use of hydrogen, which is renewable energy, has become increasingly important since the 1990s because fossil fuel reserves decrease day by day and the combustion of fossil fuels causes environmental pollutions and global warming due to byproducts (Kim et al., 2006; Liu and Shen, 2004). In addition, it has been explained that hydrogen is a clean energy source and forms only H_2O when burned and does not form atmospheric polluting by-products (Mizuno et al. 2000).

Figure 1. Advantages of hydrogen (Source: Mizuno et al., 2000)

1.2. Hydrogen Production Techniques

Hydrogen is an artificial fuel that is produced from variety of raw materials including fossil fuels, water and biomass (Figure 2). The energy required in electrochemical and thermochemical hydrogen production processes is high and has harmful effects on the environment. However, the energy requirement of biological hydrogen production is low because they are operated at ambient pressure and temperature. Hydrogen production by the biological method is done by photosynthetic method, fermentative method, and hybrid systems. Although many researchers have focused on photosynthetic processes, the light conversion effect and production rate of this process are low. On the other hand, the high production rates of fermentative processes and no need for light have increased the interest in this method (Kim et al., 2006).

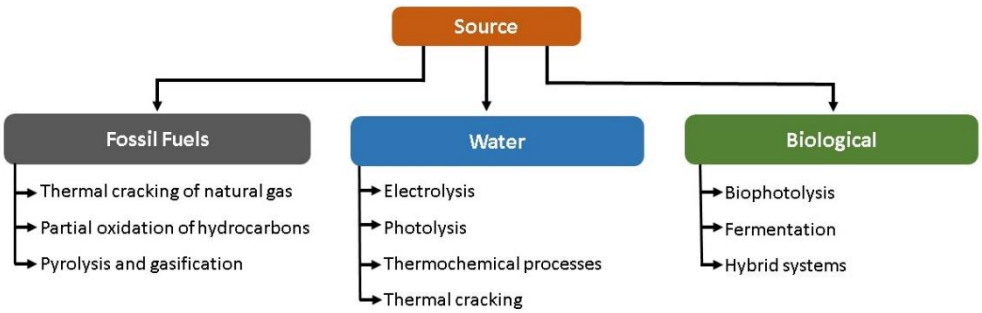

Figure 2. Hydrogen production routes from a variety of sources. (Source: Aziz et al., 2021)

1.3. Biological Hydrogen Production Methods

Biohydrogen is defined as hydrogen that is produced from renewable sources (solar, water, organic waste) biologically (Demirbas, 2009). However, utilization of biomass and waste materials as a substrate will be reduced the cost of the production. Biohydrogen is mainly produced by bacteria and algae. Hydrogen production yields from bacteria such as *Clostridium* spp. (Ortigueira et al., 2015; Shanmugam et al., 2018), *Enterobacter* spp. (Santiago et al., 2019) and *Bacillus* spp. (Mthethwa et al., 2019; Turhal et al., 2019) has higher compared to other algae. The biohydrogen production routes are divided into two major groups as; biophotolysis, photofermentation and dark fermentation (Sivaramakrishnan et al., 2021)(Figure 3).

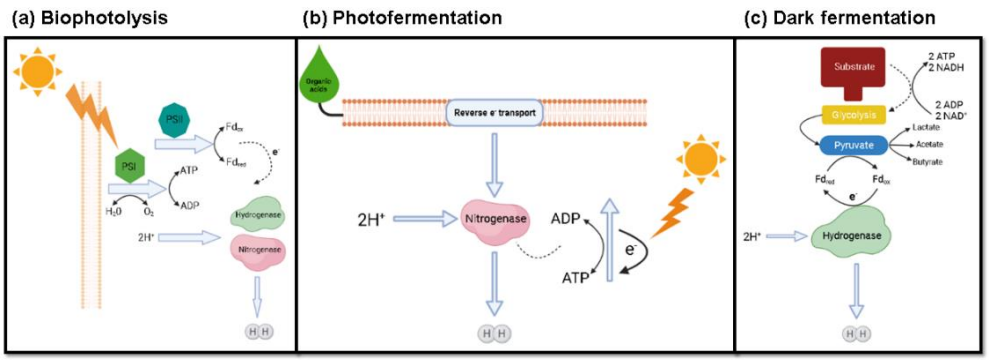

Figure 3. Schematic representation of biohydrogen production routes, created in BioRender. (Source: Sivaramakrishnan et al., 2021)

1.3.1. Biophotolysis

Biophotolysis provides hydrogen and oxygen production from water without the need for any organic compounds and is sustainable. The equation of biophotolysis is as follows(Azwar et al., 2014):

$$2H_2O + \text{solar energy} \rightarrow 2H_2 + O_2$$

This reaction takes place under anoxic conditions by cyanobacteria and microalgae. Photosystems 1(PSI) and 2 (PSII) play a role in absorbing solar energy and breaking down water into oxygen and hydrogen and hydrogen is used as an electron donor (Gadhamshetty and Sukumaran, 2011)(Figure 4).

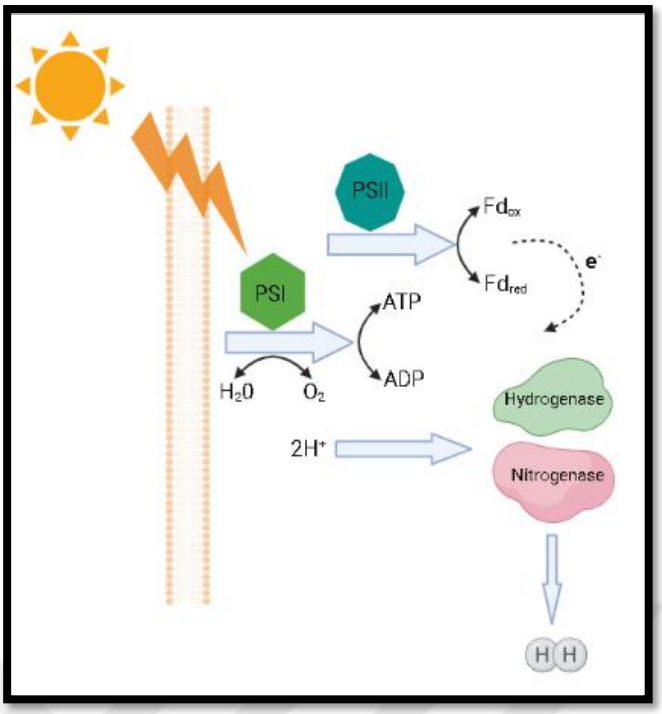

Figure 4. Schematic of biophotolysis reaction, created in BioRender (Source: Sivaramakrishnan et al., 2021)

Hydrogen production from different kinds of cyanobacteria such as Anabaena variabilis(Berberoğlu et al., 2008; Fedorov et al., 2001), Anabaena cylindrica (Miyamoto and Hallenbeck, 1979), Nostoc muscorum (Spiller et al., 1978) and algae such as Chlamydomonas spp.(Fouchard et al., 2005; Laurinavichene et al., 2006; Ohta et al., 1987; Tamburic et al., 2011) Platymonas subcordiformis (Guan et al., 2004), Chlorella sorokiniana Ce (Chader et al., 2009) has been reported in the literature. Biophotolysis is an attractive process as it only needs water and sunlight, whereas the separation of oxygen and hydrogen in the final product is costly(Gadhamshetty and Sukumaran, 2011). Also, the biohydrogen production rates are quite low and are not feasible for real-time applications(Levin et al., 2004)

1.3.2. Photofermentation

Photofermentation is described as conversion of organic substances into hydrogen with solar energy by nitrogenase enzyme under anaerobic conditions (Figure 5).

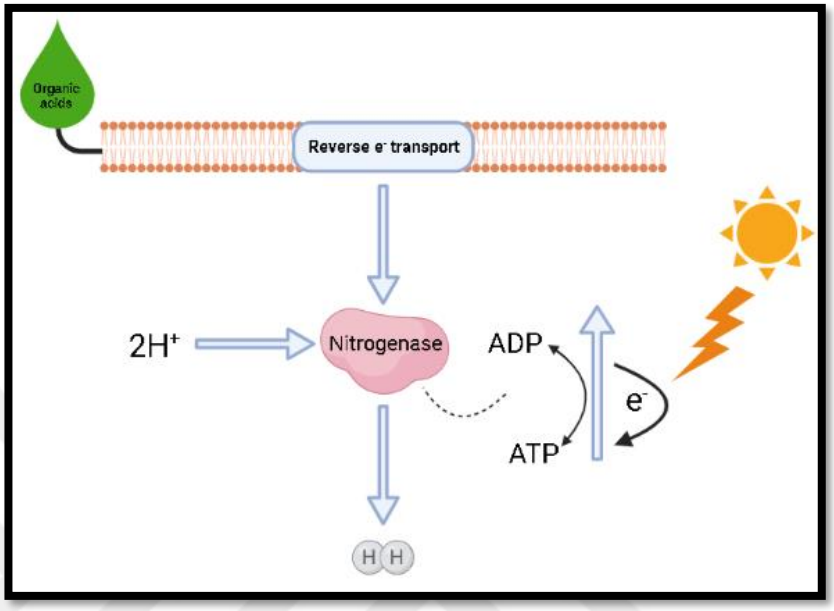

Figure 5. Schematic of photofermentation reaction, created in BioRender.(Source: Sivaramakrishnan et al., 2021)

The equation of photofermentation is as follows (Eroğlu et al., 2006):

$$\text{Organic acids} + 6H_2O + \text{solar energy} \rightarrow 12H_2 + 6CO_2$$

Biohydrogen production via photofermentation was performed through photosynthetic and purple non-suphur (PNS) bacteria(Azwar et al., 2014). Gram negative PNS bacteria such as *Rhodobacter* spp. (Ma et al., 2012; Uyar et al., 2009; Zhu et al., 2007) and *Rhodopseudomonas* spp. (Liu et al., 2009; Ren et al., 2009; Tian et al., 2010) are widely used for photofermentation. Pros of photofermentation can be listed as no carbon emission and theoretically high yield. The disadvantages are dependency of nitrogenase enzyme and dependency of environmental factors such as light intensity. Low light intensity dominantly affects the saturation of photosynthesis that causes the lower hydrogen production yields (Gadhamshetty and Sukumaran, 2011; Sivaramakrishnan et al., 2021).

1.3.3. Dark Fermentation

Dark fermentation is a light-independent methods that carried out by variety of microorganisms under anaerobic conditions (Sarangi and Nanda, 2020). Facultative and obligate anaerobes can produce hydrogen by dark fermentation. The equation of dark fermentation is as follows (Kraemer and Bagley, 2008):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$$

Here, the substrate used as a primary energy (electron) source and degraded by glycolysis. Pyruvate is produced as a result of glycolysis reaction and this reaction resulted as production of volatile fatty acids such as butyrate, lactate and acetate (Elbeshbishy et al., 2017; Guwy et al., 2011). Hydrogenase enzyme catalyzes the hydrogen production, so that the hydrogen production is limited by enzymatic activity of hydrogenase enzyme (Figure 6).

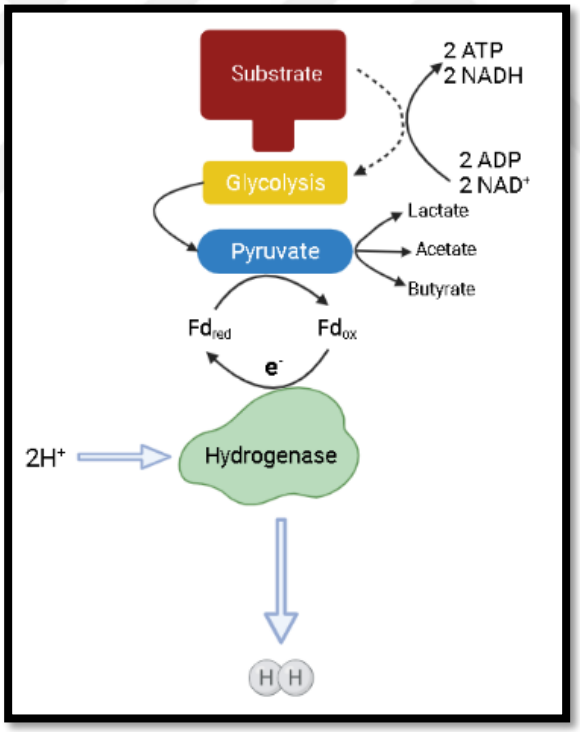

Figure 6. Schematic representation of dark fermentation, created in BioRender. (Source: Sivaramakrishnan et al., 2021)

Hydrogenase enzyme activity is pH dependent. The catalytic activity of the enzyme is higher at pH levels of 5.5 to 8.0 (Fang et al., 2002; Van Ginkel et al., 2005; Venkata Mohan et al., 2007). The main advantage of dark fermentation is hydrogen

production from butyrate utilization (Sharma, 2019). Although the H_2 formation rate is high in the dark fermentation method, which is a fermentative process, the hydrogen yield (mol H_2/mol substrate) is lower than other methods(Das and Veziroğlu, 2001).

Clostridium spp. (Cappelletti et al., 2011; Hu et al., 2014; Kumar et al., 2015; Lay et al., 2010; Lin et al., 2007; Qi et al., 2018), *Bacillus* spp. and *Enterobacter* spp. are widely used for production of biohydrogen via dark fermentation. Thermophilic microorganisms can survive at high temperatures and high temperatures stimulate the production of hydrogen from acetate pathway(Sivaramakrishnan et al., 2021). Hence, are promising and widely used for dark fermentation due to their high hydrogen production yields (Jayasinghearachchi et al., 2012; Liu et al., 2008; Maguire et al., 2021; De Vrije et al., 2010)

1.3.4. Water-Gas Shift Reaction (WGSR)

Biological water-gas shift reaction (WGSR) is a unique pathway and is described as production of CO_2 and H_2 from CO and water under anaerobic conditions (Figure 7).

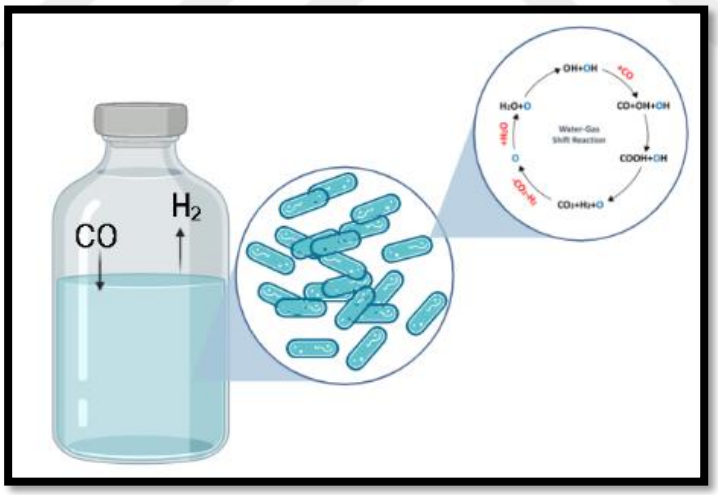

Figure 7. Schematic representation of water-gas shift reaction, created in BioRender. (Source: Newsome, 1980)

It is an exothermic reaction, and the oxygen is transferred to CO while CO_2 produced. With the help of carbon monoxide dehydrogenases and hydrogenases, carboxydotrophic hydrogenogenic microorganisms may convert CO (used as an electron and carbon source) to molecular H_2 (Akhlaghi and Najafpour-Darzi, 2020).

Carbon monoxide dehydrogenases catalyze the reversible reaction, which produces 1 mol CO_2 and 1 mol H_2 from CO and water (Diender et al., 2015). The equation of water gas shift reaction is as follows (Pezacka and Wood, 1984). Gibbs free energy is used to determine if a reaction will occur spontaneously. If the Gibbs free energy is negative for the given process, then the reaction will occur spontaneously. In the given scenario and it means that, the reaction takes place randomly at normal conditions.

$$CO + H_2O \rightarrow CO_2 + H_2 \quad \Delta G^o = -20.1 \text{ kJ/mol}$$

Mesophilic and thermophilic bacteria play significant role in production of hydrogen from water-gas shift reaction. Mesophilic bacteria such as *Rhodopseudomonas gelatinosa Rhodospirillum rubrum*, *Rubriviv axgelatinosus* are able to CO to H_2 under mesophilic temperatures (30-34 ^oC). The genus of *Carboxydothermus* spp. (Novikov et al., 2011; Slepova et al., 2009; Svetlichny et al., 1991; Yoneda et al., 2012), *Moorella spp.*(Alves et al., 2013), *Thermoanaerobacter* spp. (Balk et al., 2009), and *Thermosinus* spp.(Sokolova et al., 2004) are class of thermophilic bacteria an uptake CO to produce hydrogen under thermophilic conditions (55-73 ^oC).

Hot springs are rich in thermophilic anaerobic bacteria due to their high temperatures and low oxygen solubility environment. Hence, these bacteria are usually isolated from hot springs, as they have a high tendency to produce hydrogen by water-gas shift reaction (Kochetkova et al., 2011).

Table 1. Comparison of biological hydrogen production routes.(Source: Aziz et al., 2021)

Biological Route	Microorganisms	Substrate	Products	Reaction
Biophotolysis	• Algae • Cynobacteria	• Light • H_2O • CO_2	• H_2 • O_2 • Biomass	$H_2O \rightarrow H_2 + (1/2)\, O_2$
Photofermentation	• Photosynthetic bacteria	• Light • Organic wastes	• H_2 • CO_2 • Biomass • Organic acids	Organic acids$+6H_2O$+solar energy$\rightarrow$ $12H_2+ 6CO_2$
Dark fermentation	• Fermentative bacteria	• Organic wastes	• H_2 • CO_2 • Biomass • Organic acids	$C_6H_{12}O_6+2H_2O \rightarrow 2CH_3COOH+4H_2+2CO_2$
Water-gas shift reaction	• Thermophilic bacteria • Photosynthetic PNS bacteria	• CO • H_2O	• H_2 • CO_2	$CO+ H_2O \rightarrow CO_2+ H_2$

1.4. Hydrogen Production from Thermophilic Anaerobic Bacteria

Anaerobic term refers to an oxygen free environment, whereas thermophile term refers to microorganism that can survive under high temperatures. Anaerobic thermophilic bacteria are subclass of bacteria that can survive under anaerobic and high temperature conditions. Anaerobic thermophiles are divided into three groups according to temperature tolerance as thermophiles (50 to 64^{o}C) , extreme thermophiles (65 to 80 ^{o}C) and hyperthermophiles (above 80^{o}C) (Canganella and Wiegel, 2014).

The investigation of thermophilic anaerobes became popular because of their potential applications. They are able to produce temperature tolerant enzymes (Vieille and Zeikus, 2001), valuable organic compounds (Hosseini Koupaie et al., 2021) and energy from organic or inorganic substrates (Harnvoravongchai et al., 2020). Bioethanol, biomethane and biohydrogen can be produced as a biofuel by genera of *Clostridium, Carboxydocella, Thermincola, Thermosinus, Thermoanaero, Carboxydothermus, Carboxydobrachium, Anaerobaculum, Methanotorris, Methanococcus, Methanothermococcus* and *Methanotermobacter* (Srivastava et al., 2020).

Hot springs are known to acquire natural habitats of many thermophilic anaerobes due to features of high temperature and low oxygen solubility. Diverse anaerobic thermophilic bacteria isolated from variety of local hot springs in Turkey. *Geobacillus, Anoxybacillus* , *Bacillus* spp., were isolated from local hot springs in Turkey and identified with rep-PCR profiling and 16S rRNA sequencing (Adiguzel et al., 2009). The bacteria which is closely related to *Thermoananerobacter thermohydrosulfuricus* and *Thermoanerobacter siderophilus* that are able to utilize CO were isolated from hot spring in Ayaş, Turkey and identified with 16S rRNA sequencing and DNA-DNA hybridization methods (Balk et al., 2009).

1.5. Molecular Characterization Techniques of Bacterial Isolates

Microbiome experiments begin with removing host DNA and then classifying a set of sequencing reads, with each read assigned to a taxonomic category, followed by computing the relative abundance of different species in the sample. Molecular characterization of bacterial isolated consist of cloning and sequence analysis steps. Gene cloning is generally done via polymerase chain reaction (PCR) based methods and is based on the amplification of desired genetic material (RNA or DNA) with specific primers. Amplified genetic materials will be sequenced to determine genetic structure. There are several methods for DNA detection (Figure 8). PCR is often used method because it is efficient and rapid technology and there is no need for initial treatments. The final products of PCR should be detected by DNA detection method and these methods should be rapid and sensitive. DNA probe hybridization is a common technique that include sequence specific identification of ssDNA. Real-time PCR is another technique that targets the DNA based on amplification and used to detect particular DNA (Hatch et al., 2014). 16S rRNA regions are highly conserved regions and important genetic materials for sequence based bacterial analysis (Shougang Wang et al., 2022). These regions were amplified with specific primers via PCR and then sequenced to determine taxonomical classification of bacteria at strain even species level (Johnson et al., 2019). Whole genome sequencing (WGS) is one common approach to identify bacterial isolates and includes sequencing of all nucleic acid fragments in genome of the desired sample (Brown et al., 2017b). This method mainly used in environmental metagenomics studies.

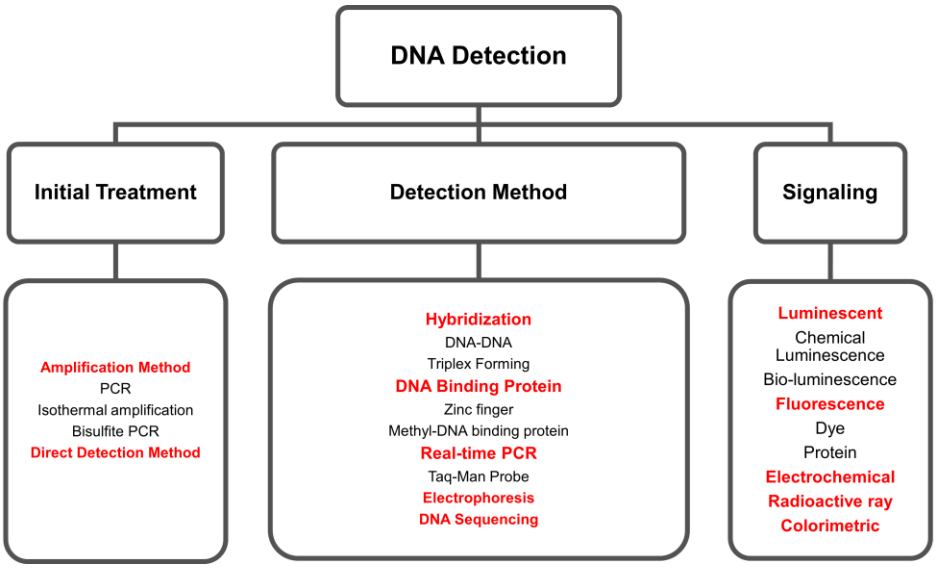

Figure 8. DNA detection strategies (Source: Koichi Abe and Kuzunori Ikebukuro, 2015)

DNA sequencing is another technology that offers sequencing of specific genes or even the whole genome of desired sample. The DNA sequencing are developing day by day. Sanger sequencing is the oldest method that is based on chain termination and has been used for three decades and classified as first generation sequencing (Sanger et al., 1977). Although it is a well-established method, demerits are that it is time-consuming and costly (Fujiyoshi et al., 2020). Since the preliminary report of sequencing in 2005 and continuing today, next-generation sequencing (NGS) technologies have fundamentally altered metagenomics research. They now bring valuable advantages in terms of speed, cost, quality, and precision in the ongoing search for the genetic material of microorganisms (Gupta, 2008)(Figure 9).

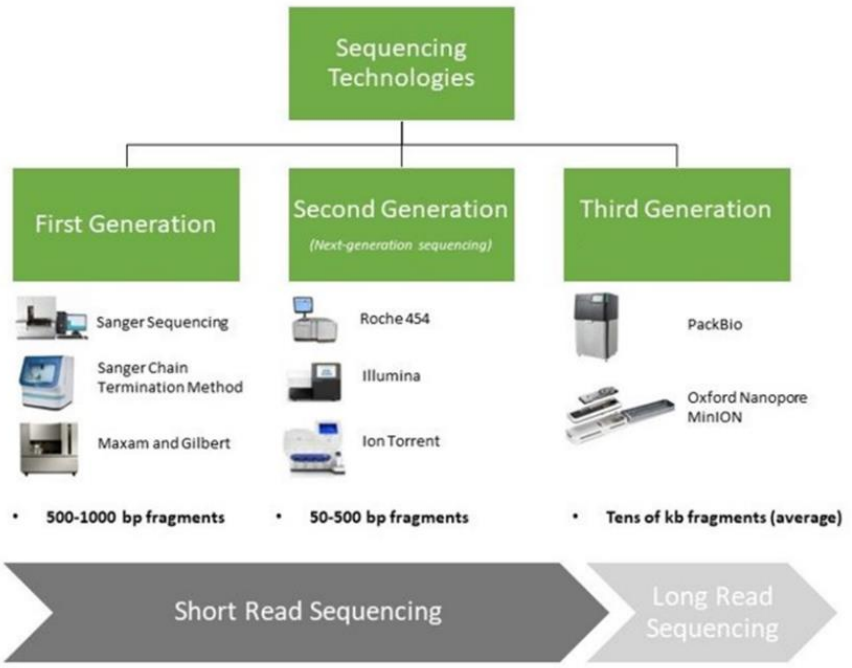

Figure 9. Classification of sequencing technologies. (Source: Cao et al., 2017)

The MinION by ONT is a TGS technology and is based on electrical current monitoring. The device consists of nanopores and when the bases passed through the nanopore, an electric signal will generate and monitored real-time. Each base (A, T, G,C) generates different electrical signals (Figure 10). Electrical current changes are recorded into a signal to be decrypted into specific DNA/RNA sequences (Mikheyev and Tin, 2014).

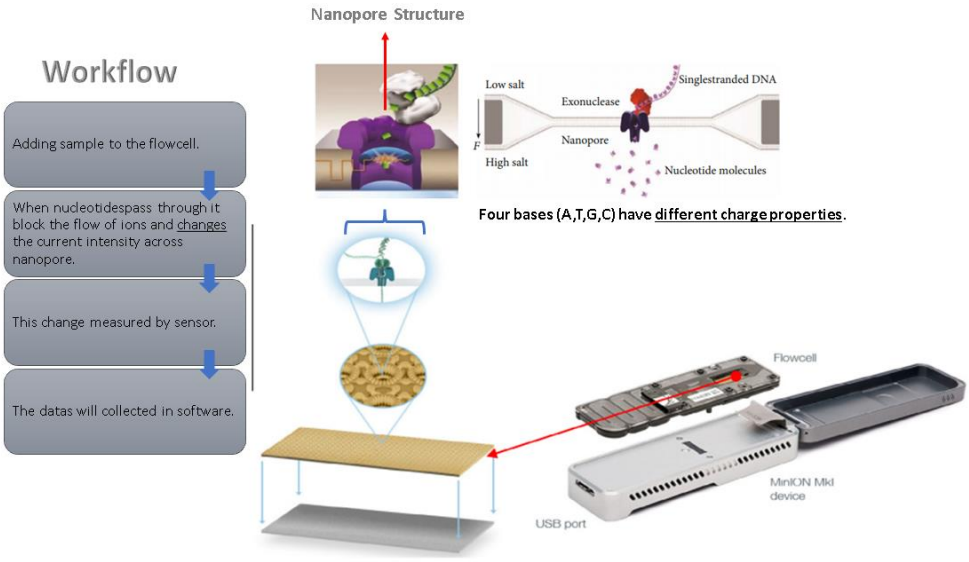

Figure 10. The MinION by ONT device and general workflow (Source: Chen et al., 2020)

1.6. The Aim of the Study

The hot springs are important source for thermophilic bacteria. The aim of this study is identification of the microorganisms that lives in hot springs located in in İzmir, Turkey in terms of biohydrogen production and then the whole genome sequencing of hydrogen-producing species with the MinION by ONT. Therefore, hot springs were selected according to temperatures and water samples were collected. Collected samples were cultured in enrichment medium and with 100% CO. Hydrogen and carbon dioxide production and carbon monoxide consumption were monitored regularly. The most efficient hydrogen producing mix culture was determined and pure culture was obtained by dilution plating method. The morphological characterization of pure isolates was examined by gram staining, ESEM and SEM imaging. The whole genome sequencing of the pure isolate was performed with the MinION by ONT. It is aimed to successfully complete whole genome sequencing studies and to find a new carboxidotrophic hydrogenogic species that produces hydrogen with high yields. In this way, hydrogen production potentials of local hot spring in İzmir were evaluated.

CHAPTER 2: METHODOLOGY

2.1. Sampling from Hot Springs and Cultivation Conditions

2.1.1. Sampling Sites

Five hot springs (Doğanbey, Nebiler, Bademli, Yıldızburnu and Bergama) are distrubuted in İzmir, Turkey and were selected according to temperature. The water samples from five locations were collected at aseptic conditions into sterile flasks in the summer season of 2021. Samples were taken aseptically to avoid contamination and air gaps. pH, temperature, and oxidation-reduction potential (ORP) were measured *in situ* by a portable multiparameter (Milwaukee, USA). Samples were stored in heat-proof bags and brought to the laboratory as quickly as possible. Table 2 contains the global positioning system (GPS) locations and properties of the hot springs that were sampled.

Table 2. Geographical location, pH, ORP, and temperature of the sampled hot springs.

Sampling Site	GPS Location	Environmental Temperature(ºC)	Incubation Temperature(ºC)	pH	ORP(mV)
Doğanbey	38°07'32.8"N 26°54'35.8"E	64.7	65	6.72	22.8
Nebiler	39°09'32.0"N 26°54'12.1"E	56.2	50	6.86	
Bademli	38°59'50.2"N 26°48'00.9"E	43	50	6.75	43.8
Yıldızburnu	38°18'59.9"N 26°20'55.4"E	38-43	45	7.2-7.3	2
Bergama	39°14'14.3"N 27°18'23.4"E	56.2	65	7.88	-54.8

2.2. Cultivation Conditions and Experimental Set-Up

Figure 11 represents the general workflow of experimental design.

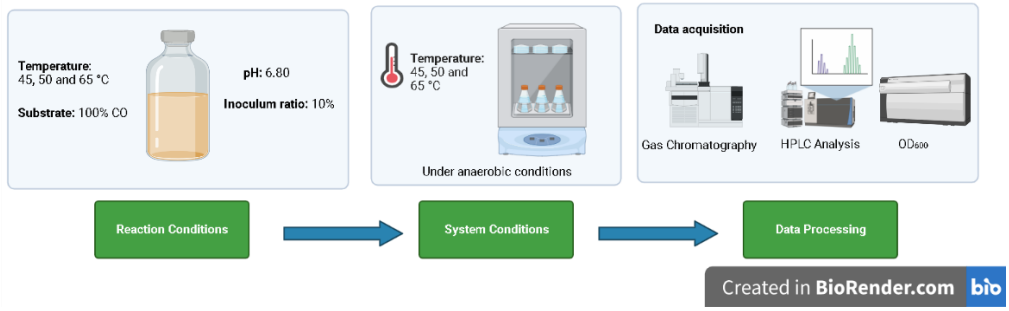

Figure 11. Schematic representation of workflow of experimental design.

Anaerobic medium contains macro and microelements, buffers, vitamins, and resazurin to detect oxygen in the culture. The anaerobic medium used in this study for one liter contains 1.0 g NH_4Cl; 0.33 g $MgCl_2.6H_2O$; 0.1 g $CaCl_2.6H_2O$; 0.33 g KCl; 0.5 g KH_2PO_4; 0.5 g $NaHCO_3$; 1.0 g $Na_2S.9H_2O$; 0.001 g resazurin; 1.0 ml trace element solution (per liter distilled water; 1.5 g nitrilotriacetic acid; 3.0 g $MgSO_4$; 0.5 g $MnSO_4$; 1.0 g NaCl; 0.1 g $FeSO_4$; 0.1 g $CaCl_2$; 0.1 g $CoCl_2$; 0.1 g $ZnSO_4$; 0.01 g $CuSO_4$; 0.01 g $AlK(SO_4)_2$; 0.01 g H_3BO_3; 0.01 g Na_2MoO_4) and 1 ml vitamin solution (1 liter distilled for water; 2.0 mg biotin; 2.0 mg folic acid; 2.0 mg pyridoxine hydrochloride; 10.0 mg riboflavin; 5.0 mg thiamine; 5.0 mg nicotinic acid; 5.0 mg pantothenic acid; 0 1 mg vitamin B12; 5.0 mg p-aminobenzoic acid; 5.0 mg thioctic acid). The medium is enriched with 1.0 g/l yeast extract to stimulate microbial growth. The pH was adjusted to 6.80 using 1.0 M HCl and 1.0 M NaOH. The 100 ml serum bottles containing 54 ml of culture medium were prepared and crimp-sealed with sterile butyl rubber stoppers. Before the inoculation, the medium was flushed with 100 % nitrogen gas to provide anoxic conditions and until the color of the resazurin is losing. Afterward, the medium was sterilized by autoclaving at 121°C for 15 min.

2.3. Isolation and Selection of Hydrogen Producing Bacteria

Figure 12 represents the isolation workflow of pure culture from mix cultures.

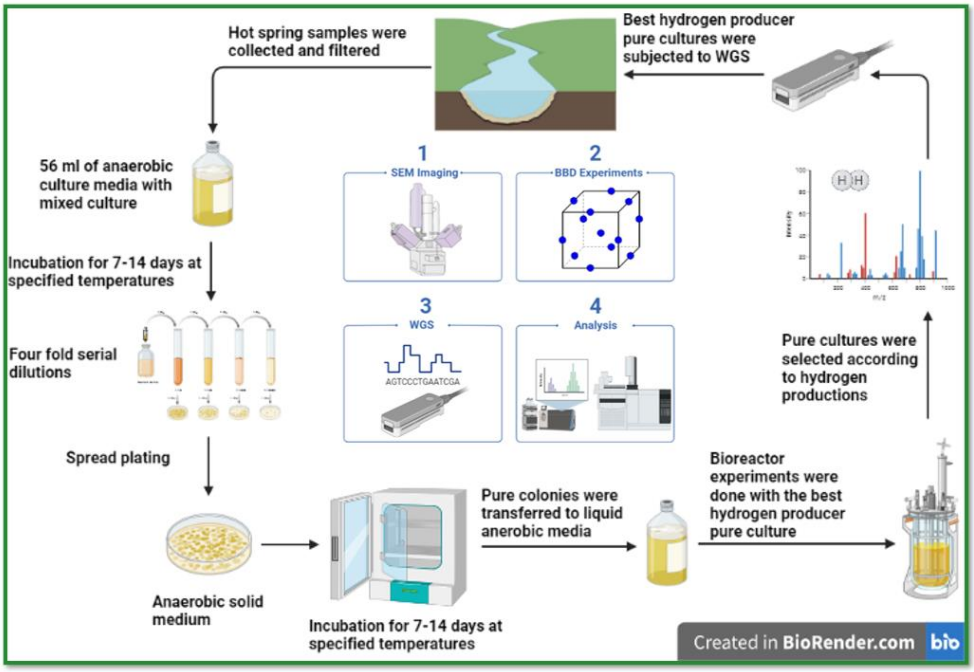

Figure 12. Isolation workflow of pure culture from mix culture.

2.3.1. Inoculation

Collected water samples were filtered with a 0.22 µm cellulose nitrate membrane filter (Hypore, Malaysia). The biomass was harvested, and bottles were inoculated with 10 % (v/v) under sterile conditions (Figure 13). After inoculation, the bottles were flushed with 100 % carbon monoxide gas for 30 s. Incubation temperatures were chosen according to environmental temperatures (45, 50, and 65 ^{o}C) (Table 2). Cultures were incubated for 7-20 days at specified temperatures in incubators. The experiments were carried out in duplicates. The mix cultures were monitored regularly for biohydrogen production and studies continued with the culture that showed the highest biohydrogen production.

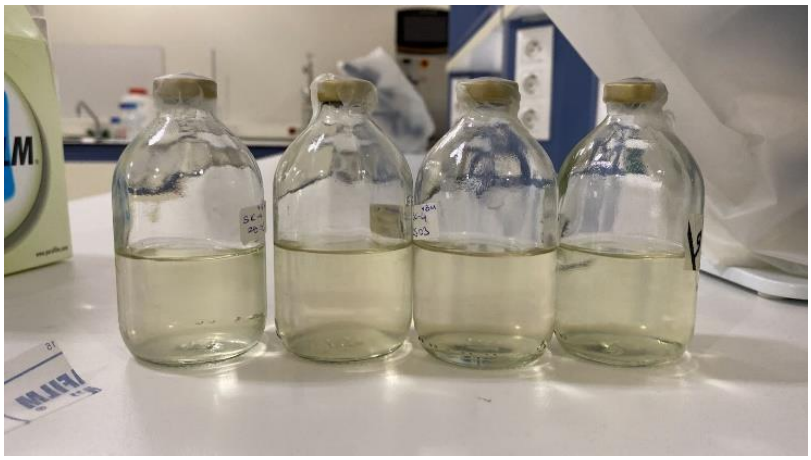

Figure 13. Culture bottles.

2.3.2. Isolation of Pure Culture from Mixed Culture

Isolating pure cultures was performed using liquid enrichment and streak plate methods. The solid medium was obtained by adding 2 % (v/v) agar to an anaerobic liquid medium. The hot spring cultures were sub-cultured on the solid medium and single colonies were isolated regularly to obtain a pure culture. After obtaining the same general appearance on the colonies on the solid medium, single colonies were transferred into an enriched liquid medium. An enriched liquid medium was obtained by addition of 1 g/l yeast extract to the anaerobic medium.

2.4. Growth Profiling of Pure Culture

2.4.1. Volatile Suspended Solid Analysis

Volatile suspended solids (VSS) were calculated to determine the biomass in the medium. The pure culture grown for 4 days in an anaerobic liquid medium was used as a stock culture. 1000 ml of sterile anaerobic liquid medium was inoculated at 15% (v/v) from stock culture and incubated at 60 ^{o}C. For VSS, cellulose nitrate membrane filters were dried in an oven at 110 ^{o}C for 1 h and cooled in a desiccator for 30 min and tarred (W1). At regular intervals of 12 hours, a 10 ml sample was aliquoted and filtered through 0.22 µm cellulose nitrate membrane filter and then dried in an oven at 110 ^{o}C for 1h. The filter was then cooled in a desiccator for 30 min and weighed (W2). VSS was conducted for each sampling and calculated from equation 1.

$$\boldsymbol{mg\ total\ solids/\ l} = [(W2 - W1) * 1000)] \div sample\ volume(ml) \quad (1)$$

2.4.2. Spectroscopic Measurements

The pure culture was cultivated at 60 ^{o}C in the sterile enriched anaerobic medium. At regular intervals of 12 h, a 1.5 ml sample was aliquoted and optical density at 600 nm was recorded by UV-Visible spectrophotometer (Lambda 750(Perkin Elmer, USA)). An uninoculated, sterile anaerobic medium was used as a blank. This procedure was conducted for each sampling.

2.4.3. pH Measurement

The pH of the mediums was monitored regularly to estimate microbial growth. The pH was affected by the accumulation of acids and any other by-products in the medium. Acidic pH levels inhibit hydrogen production so that organic acid production will increase(Chong et al., 2009). 5.0 ml of sample was taken from the culture aseptically by 5.0 ml syringe and measured by a portable multiparameter meter (Milwaukee, USA).

2.4.4. Dilution Plating and Viable Counts

The dilution plating technique is commonly used in to evaluate the number of bacteria in a sample, and it involves inoculating the culture on an agar medium after serial dilutions at a certain rate and counting viable bacteria (Jett et al., 1997). The pure culture was inoculated at 15% (v/v) and incubated at 60 ^{C} for 14 days. At regular intervals of 12 h, a 1.0 ml sample was taken from the medium, and serial dilution was performed. Serial dilutions were made with 0.9 % (w/v) sterile peptone water. 1.0 ml sample was taken with a 1.0 ml sterile syringe from the medium and transferred to the microbiology tube containing 9.0 ml of 0.9 % sterile peptone water for the first dilution tube (10^{-1}), then homogenized by pipetting. 1.0 ml suspension was transferred to the second tube (10^{-2}) and briefly vortexed. These steps were repeated until the sample is diluted 10^{-4}. 1.0 ml of sample was taken from each of the 4-fold diluted samples and inoculated on agar plates. (Figure 14). Trials were carried out to obtain colonies with a countable range (30-300). The inoculated Petri dishes were incubated in an oven at 60 °C and waited for 7 days. Bacteria amount was calculated as CFU per square centimeter of agar. The data were given in $\log_{10}$ CFU/ml (Figure 18). Growth rate constant and generation time were calculated as a function of the number of viable

cells and where t_2 is the end time of the log phase and t_1 is the start time of the log phase (Eq. 2 and Eq. 3).

μ (specific growth rate) =2.303(log((CFU)/ml)2- log((CFU)/ml))1 / (t2-t1) (2)

g (generation time) = log(2.303)/μ (3)

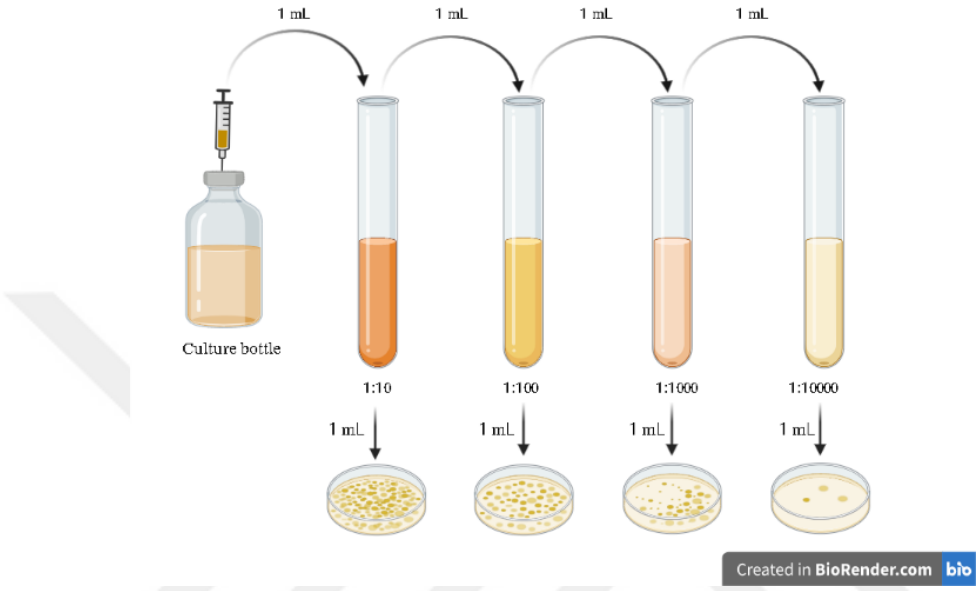

Figure 14. Serial dilution technique, created in Biorender.

2.5. Optimization of Culture Conditions by Box- Behnken Experimental Design

Box–Behnken design ensures modeling of the response surface (Box and Wilson, 1992) and it can be used for biohydrogen production processes. Two different designs were made. The first design is for the optimization of the process parameters, and the second design includes the variation of metal concentrations added to the medium to increase the hydrogen production (Table 3 and Table 4). These three factors and levels were selected according to literature and result from previous studies. Design-Expert Software (Design Expert v13, USA) was used for Box Behnken Design and a set of 17 experiments at 3 levels with 3 independent parameters were performed and comparatively analyzed the effects of parameters on biohydrogen yields. The values of each experiment that results in the maximum value of response (biohydrogen yield) will be selected as optimal conditions.

Table 3. Factors and levels selected for the Box-Behnken Design 1 (Design Expert v13, USA).

Factors		Levels		
		1 (Low)	2 (Middle)	3 (High)
1	Temperature (ºC)	55	60	65
2	pH	5.0	7.0	9.0
3	CO feed (ml)	5	10	15

Table 4. Factors and levels selected for the Box-Behnken Design 2 (Design Expert v13, USA).

Factors		Levels		
		1 (Low)	2 (Middle)	3 (High)
1	Fe (mg/l)	50	150	250
2	Zn (mg/l)	25	87.5	150
3	Ni (mg/l)	10	55	100

2.6. Morphological Characterization of the Pure Culture

2.6.1. Gram Staining

Morphological characterization of hydrogen-producing pure cultures was done through microscopic observations. The colony morphology of pure culture was observed under the light microscope (Optika, Italy) concerning size and shape by gram staining. Gram staining was done according to the manufacturer's instructions (Rokim, Turkey). First, the slides were immersed in 70 % (w/w) ethanol and air-dried. For liquid culture, 1-2 drops of the sample were dripped on slides and heat fixed. For solid culture, single colonies on agar were taken with a needle and heat fixed. The primary stain which is crystal violet was dripped onto the slide and waited for 1 min then gently rinsed with water. The gram’s iodine solution was dripped on and waited for 1 min then rinsed with alcohol. The secondary stain, which is safranin was dripped on the slide and stayed for 1 min then rinsed with water. The slides were then air-dried and observed with a light microscope.

2.6.2. ESEM and SEM Imaging

The size, shape, and surface features of pure culture were screened in more detail by Scanning Electron Microscopy (SEM) (FEI Quanta 250 FEG (Philips, Netherlands) and Environmental Scanning Electron Microscopy (ESEM) (FEI Quanta 250 FEG (Philips, Netherlands). Firstly, liquid cultures were centrifuged (1789 *x g* for 10 min). Next, the pellet was suspended in phosphate-buffered saline (PBS, 4.4325 g NaCl, 0.5465 g Na_2HPO4, and 0.138 g NaH_2PO_4 in 500 ml DI water; pH 7.5) (Sigma Aldrich, USA) solution and centrifuged again. After that, the pellet was resuspended in 2.5 % (v/v) formaldehyde for 1 h and washed again. The samples were dehydrated through a graded ethanol series for 10 min for each (25 %, 50 %, 70%, 95 %, 100 % (v/v)). Finally, the final suspension was fixed and air-dried until it gets solidified. Samples were coated with gold particles for 90, before SEM imaging.

For ESEM analysis, the final suspension was mixed with 2% (w/v) sodium alginate at a ratio of 1:1 and vortexed until it gets homogenized. This mixture was added dropwise through a syringe into 100 ml 2 % (w/v) $CaCl_2$. The beads were allowed to harden for 1 h with continuous stirring on a magnetic stirrer and examined with an ESEM. ESEM imaging is generally used for biological samples due to the ease of sample preparation. Figure 15 shows the encapsulated bacteria prior to ESEM imaging.

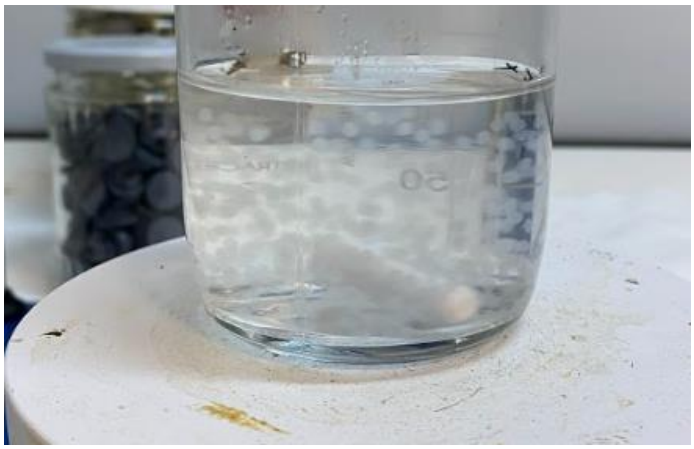

Figure 15. Hydrogel encapsulated bacteria for ESEM imaging.

2.7. Analytical Methods

2.7.1. Gas Chromatography (GC) Analysis

The selection of the best biohydrogen-producing mixed culture among 5 different hot spring isolates were evaluated considering the amount of hydrogen and consumption of carbon monoxide. The pure culture was obtained from these mixed cultures, and the best hydrogen-producing strain was determined by hydrogen production yields (Table 5). Therefore, hydrogen production and carbon monoxide consumption of cultures were regularly monitored by gas chromatography (Figure 16).

Headspace gas was collected periodically and directly from bottles with a 1000 µl gas-tight syringe (Hamilton, Nevada) and was analyzed by gas chromatography (Shimadzu, Nexis GC-2030, Japan) equipped with a Thermal Conductivity Detector (TCD) with a Wide Bore Injector (WBI) and a Restek ShinCarbon ST 100/120 packed column. The temperature of the injector, column, and detector were 150, 60, and 150 ^{o}C, respectively. Nitrogen was used as a carrier gas.

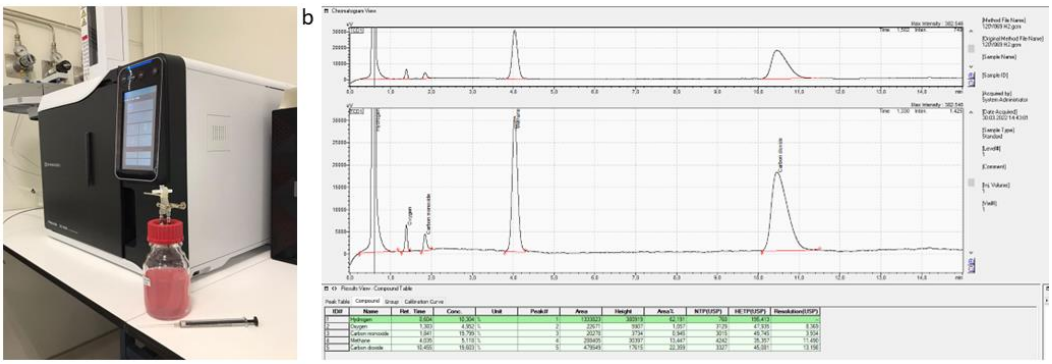

Figure 16. (a) Shimadzu, Nexis GC-2030 GC and (b) chromatogram

2.7.2. High-Pressure Liquid Chromatography (HPLC) Analysis

2.7.2.1. Processing Method Development

The processing methods are a key element of HPLC analysis. The processing method includes analytes and their levels. Standard solutions (1000 ppm) of acetic acid, citric acid, butyric acid, formic acid, propionic acid, and ethanol were separately diluted with ultra-pure water to 3.0, 1.5, 0.75, 0.325, 0.1625 ppm concentrations. Standards were prepared with serial dilutions and were then injected into the HPLC

device. After fitting the chromatograms, a processing method suitable for the analysis of VFAs was attained.

2.7.2.2. VFA Analysis

Volatile fatty acids (VFA) and alcohols are usually produced along with hydrogen production and are crucial to understanding the production pathways of bacteria (Kumar et al., 2019) The analyzed VFA included acetic acid, citric acid, butyric acid, formic acid, and propionic acid, whereas alcohols included ethanol. 1.0 ml of sample was taken from culture and was centrifuged 9358 *x g* for 5 min, the supernatant was taken to a sterile 1.5 ml syringe (Beybi, Turkey) and filtered through a 0.22 µm PTFE HP syringe filter(GVS Filter Technology, USA). Filtered samples were transferred to 2.0 ml HPLC vials. The concentration of VFA and alcohols in the medium were analyzed by a High-Pressure Liquid Chromatography (HPLC) (Thermo Fisher Scientific, USA) equipped with a refractive index detector (RID) and autosampler. Chromatographic separations were performed at 85 ^{o}C HyperREZ XP, 8 µm, 300 x 7.7 mm column. A mobile phase was ultra-pure water and degassed by ultrasonicator for 15 min before use. The flow rate of the mobile phase was 0.6 ml min^{-1} and the sampling volume is 10.0 µl. Analytical data were processed by the Chromeleon™ software.

2.8. Molecular Characterization

Molecular characterization studies conducted in 4 steps: DNA extraction, DNA barcoding, DNA sequencing and finally the bioinformatic analysis. The workflow of the molecular characterization studies is given below (Figure 17).

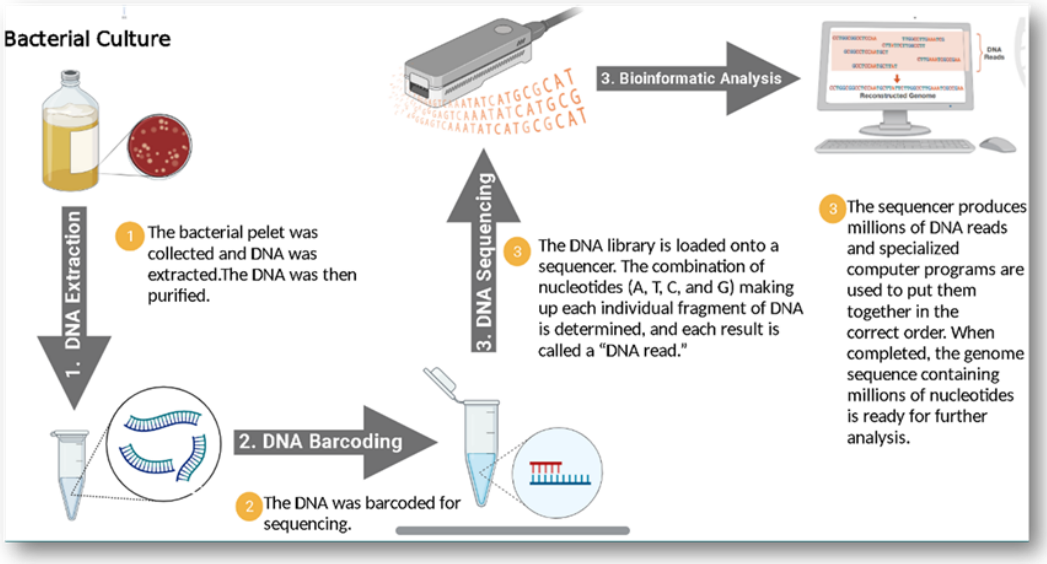

Figure 17. Flow chart of the molecular characterization

2.8.1. Genomic DNA (gDNA) Isolation

The cultures with the hydrogen production were determined by considering the hydrogen values obtained by gas chromatography. gDNA isolation was carried out in Doğanbey culture, where hydrogen production was observed. gDNA isolation was performed according to the manufacturer's instructions with Thermo Fisher GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA). Since the bacteria were found to be gram-positive because of gram staining, the gram-positive bacteria gDNA isolation procedure specified in the kit was applied. First, stock solutions of 1 M Tris-HCl (2.42 g Tris-HCl in 20 ml water; pH: 8.0) and 0.5 M EDTA (1.46 g EDTA in 10 ml water) were prepared. 5.0 ml of 1 M Tris-HCl, 1.0 ml of 0.5 M EDTA, and 3.0 ml of Triton X-100 were dissolved in 250 ml water and homogenized to obtain gram-positive bacteria enzymatic lysis buffer (ELB). 180 µl of ELB per sample and 20 mg/ml of lysozyme were prepared (Table 5). ELB is an enzyme-containing buffer that degrades the outer cell wall of gram-positive bacteria. Liquid cultures were transferred to the sterile 50.0 ml falcons and centrifuged at 5000 *x g* for 10 min. The pellet was suspended in 2.0 ml of supernatant in a 2.0 ml microcentrifuge tube and optical density (OD) was measured at 600 nm wavelength with a UV-Vis spectrophotometer. Measurements were done in three replicates.

Table 5. Enzymatic lysis buffer components and amounts were prepared according to the number of samples.

Sample Number (+1)	**Total ELB Volume (µl)**	**Lysozyme (mg)**
(18+1) = 19	3420	68.4

Samples were centrifuged 10 min at 5000 *x g* and the supernatant was discarded. 180 µl enzymatic lysis buffer was added and incubated at 37 ºC for 30 min. 200 µl lysis solution and 20 µl Proteinase K were added and mixed by pipetting.

Samples were then incubated at 56 ºC for 50 min a shaking incubator. 20 µl RNase A was added to the sample and homogenized then incubated at room temperature for 10 min. 400 µl 50 % (v/v) ethanol solution was added to the sample and homogenized. Samples were transferred to GeneJET Genomic DNA Purification Column and centrifuged 1 min at 6000 *x g*. 500 µl Wash Buffer I added to the top of the column and centrifuged 1 min at 8000 *x g*. 500 µl Wash Buffer II was added to the top of the column and centrifuged at max speed (27000 *x g*) for 3 min, then the column was transferred to a new 1.5 ml microcentrifuge tube. To collect the gDNA attached to the column, 200 µl of elution buffer was added to the midpoint of the column and incubated at room temperature for 2 min, then centrifuged 1 min at 8000 *x g*. The purified gDNA was stored at -20 ºC.

2.8.2. Fluorometric Quantification of gDNA

The isolated gDNA samples were quantified with a Qubit Fluorometer (Thermo Fisher Scientific, ABD). Qubit is used to determine the amount of DNA in the sample according to the absorbance value obtained as a result of the binding of fluorescent dyes at certain wavelengths to the target molecule (Thermo Fisher, 2021). Isolated DNA samples were measured with a Qubit device using Quant-iT™ 1X dsDNA HS Assay Kit (Thermo Fisher, USA) according to the manufacturer's instructions. A working solution was prepared with 1 µl of Qubit Reagent and 199 µl of Qubit Buffer for each sample. Firstly, standards were prepared. 190 µl of the working solution was taken into 0.5 ml thin-walled tubes and 10 µl of standard 1 and 2 were added,

respectively. 2 µl of DNA sample and 198 µl of working solution were added to 0.5 ml thin-walled tubes. All tubes were vortexed for 3 s and incubated at room temperature for 2 min. Tubes were then measured with Qubit 3.0. Fluorometer.

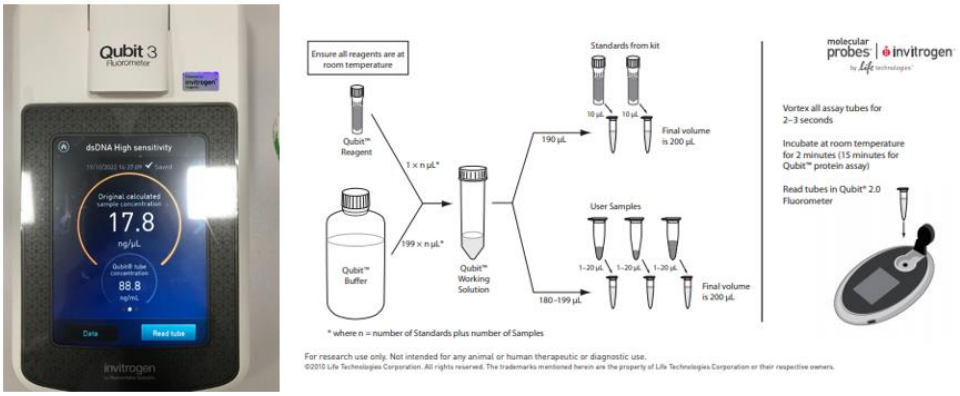

Figure 18. Qubit 3.0 fluorometer and sample preparation method

2.9. Bioreactor Experiments

Microsparger design was made to maximize gas diffusion, and experiments were carried out with a glass tank reactor and microsparger (Figure 19).

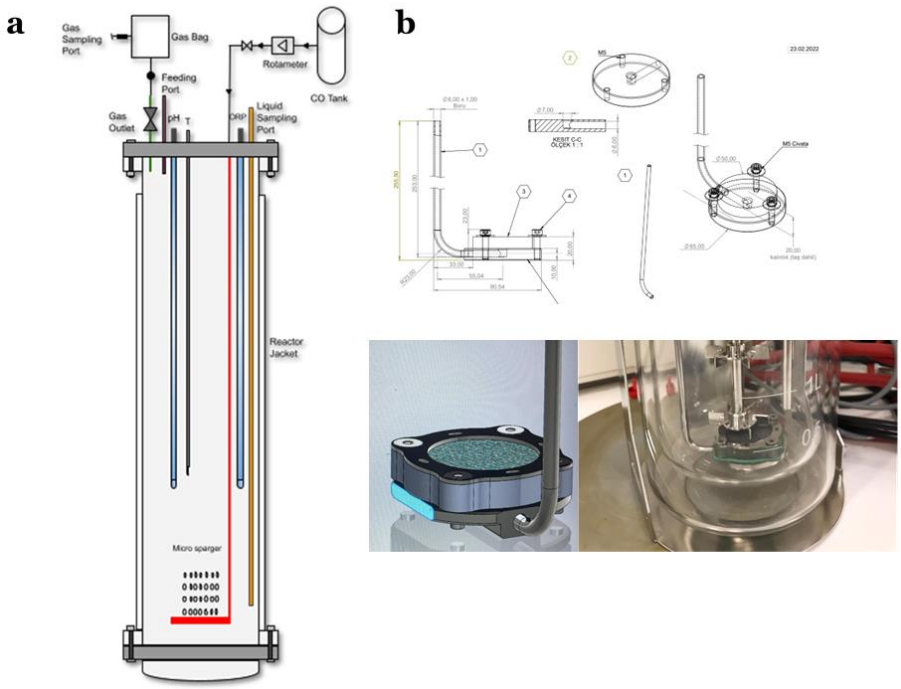

Figure 19. (a)Bioreactor and (b)micro sparger design

2.9.1. Feedstock and inoculum preparation

Pure culture derived from the Doğanbey hot spring was maintained under anaerobic conditions in 1 L serum bottles containing 800 ml of anaerobic medium containing 1 g/l of yeast extract (Figure 19). Rezasurin was added as a redox indicator. The medium (excluding the vitamin solution and reducing agent) was autoclaved at 121 °C for 20 min and cooled to room temperature. Then nitrogen gas was used to purge the medium for 5 min to remove dissolved oxygen. The vitamin solution and reducing agent were then added to the medium using a 0.22 µm sterile filter under aseptic and anaerobic conditions. The medium pH was adjusted to 6.8 prior to autoclave. Culture media were then purged with 100 %CO at the time of inoculation. A total of 200 ml seed culture was inoculated into the bioreactor.

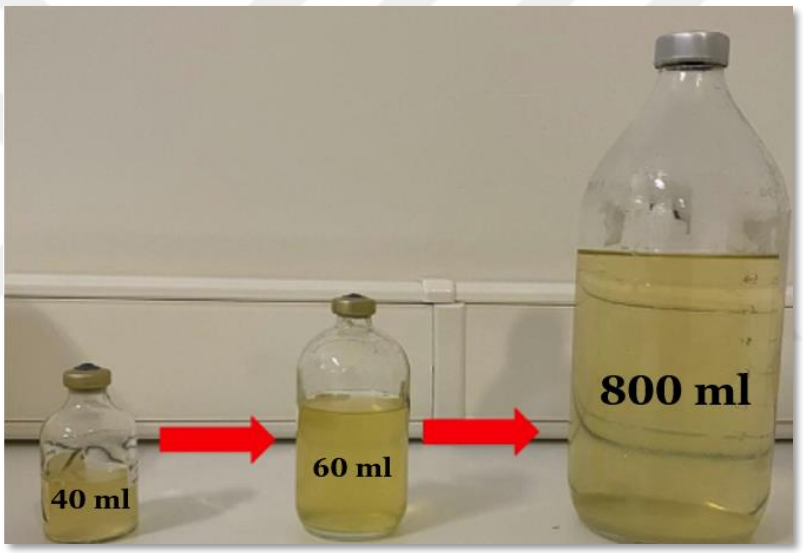

Figure 20. Scale-up experiments and feedstock

2.9.2. Experimental design and procedure

The bioreactor experiments were conducted in 2 L glass tank bioreactor (Biostat B, Sartorius, Germany) with working volume 1.5 L. The composition of the initial medium was the same as that used in the subculture. The micro sparger was used to feed carbon monoxide and nitrogen into the bioreactor with very fine bubbles and consists of a sparger pipe and a porous frit made of stainless steel (Figure 21). The micro sparger reduces bubble size and increase both gas transfer and k_{La} to reduce gas consumption and improve upstream reactor yields. In all experiments, headspace of the bioreactor was firstly purged with nitrogen gas for 5 min to create an anaerobic environment. The bioreactor was fed with 100% CO and pH adjusted to 6.8. The

continuous stirring with a speed of 30 rpm was conducted. The reactor was operated in batch mode for 15 days.

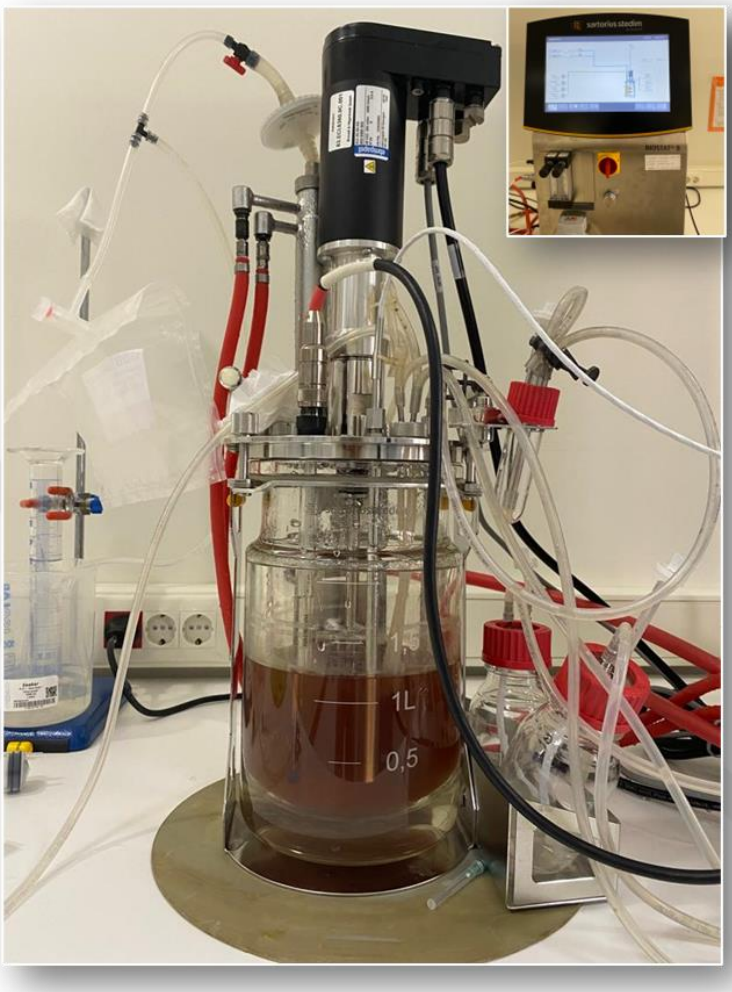

Figure 21. Bioreactor tank, sampling port and experimental design

2.9.3. Analyses OF Bioreactor Sampling

The exhaust gas compositions (CO, H_2, and CO_2) were analyzed by a real-time with gas chromatography (See Section 2.6.1.). The ethanol and volatile fatty acid (VFA) concentrations were determined by HPLC device (See Section 2.6.2).

2.10. Whole-Genome Sequencing of Pure Cultures

The whole genome sequencing of the pure cultures was conducted by The Oxford Nanopore MinION and workflow is given below.

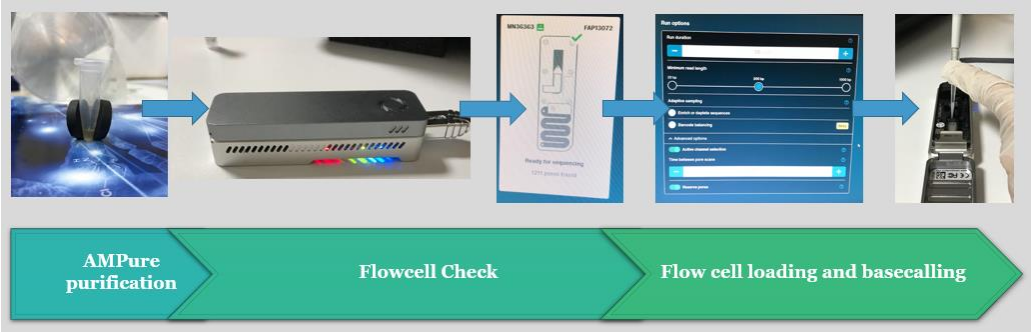

Figure 22. The Whole-Genome Sequencing workflow.

2.10.1. Library Preparation and Barcoding

Whole-genome sequencing (WGS) is a method for identifying entire genomes of organisms and is also known as full genome sequencing (Brown et al., 2017a). The whole genomes of the best biohydrogen producer strains have been sequenced using the Oxford Nanopore (ONT) MinION device and are Doğanbey and Yıldızburnu cultures. The standard protocol of the manufacturer was used and involves the library preparation, priming, and loading of the SpotON flow cell, sequencing, and analysis. Samples were prepared for sequencing according to SQK-RBK004(ONT, England). The rapid barcoding sequencing kit protocol. All kit components were used at room temperature and spun down briefly before use. 400 ng 7.5 µl of previously isolated genomic DNA (gDNA) was transferred into DNA LoBind tubes. 2.5 µl of fragmentation mix (RB01-12) was used for each sample. The mixture was then incubated at 30 ºC for 1 min and 80 ºC for 1 min. The tubes were transferred on ice. All barcoded samples were polled and AMPure XP beads purification (Beckman Coulter Inc., USA) was performed. To this end, an equal volume of AMPure XP beads was added to the mixture and incubated at room temperature for 5 min. The sample was spun down and kept on a magnet until beads settled down, and the supernatant was poured out. Beads were washed with 200 µl %70 (v/v) freshly prepared ethanol without disturbing the pellet. Ethanol was then removed and air-dried. Beads were resuspended in 10 µl of 10 mM Tris-HCl pH 8.0 with 50 mM NaCl buffer at room

temperature for 2 min. 10 µl of eluate transferred into a new tube. After the purification step, 1 µl of RAP was added to 10 µl barcoded DNA and incubated at room temperature for 5 min.

2.10.2. Flow Cell Priming and Loading

EXP-FLP002 kit was used for the flow cell priming step. The kit components are Flush Tether (FLT), Flush Buffer (FB), Sequencing Buffer (SQB) and Loading Beads (LB). 30 µl of FLT and mixed with FB and used as flow cell priming solution. MinION Mk1B lid and priming port were opened. 800 µl of priming solution loaded into priming port and waited for 5 min. 200 µl of priming mix was loaded into the priming port to complete flow cell priming. The library was prepared by addition of 34 µl of SQB, 25.5 µl of LB, 4.5 µl of nuclease-free water, and 11 µl of DNA library. The final volume was 75 µl. The prepared library was mixed and 75 µl of the sample was loaded into the flow cell via the SpotON sample port (Figure 23).

Figure 23. Flow cell loading.

2.10.3. Data Acquisition and Base-calling

The Nanopore device control, data acquisition, and real-time data analysis were carried out by MinKNOW software. The device was plugged into the computer and MinKNOW was started. First, the available pores were detected. After the samples

were loaded into the flow cell the sequencing was started and the data stream was observed for 24 h.

2.10.4. Flow Cell Wash

EXP-WSH004 kit is used for flow cell wash and contains Wash Mix (WMX) and Wash Diluent (DIL). The washing step was done according to the manufacturer's instructions. 2 µl of WMX and 398 µl of DIL were mixed and used as washing solution and loaded into the flow cell via the priming port and waited for 60 min. The flow cell was stored at +4 ^{o}C.

2.10.5. Bioinformatic Analysis

The Nanopore device control, data acquisition, and real-time data analysis were carried out by MinKNOW software. The computational analysis of the sequencing data is critical for the accurate and complete characterization of the microbial community. To this end, Guppy software was used for base-calling whereas Velvet Assembler was used for De novo assembly. Velvet output files are then passed to Kraken Tool for classification and Pavian for visualization. Pavian provides Sankey visualization of samples and read count comparisons between samples (Figure 24).

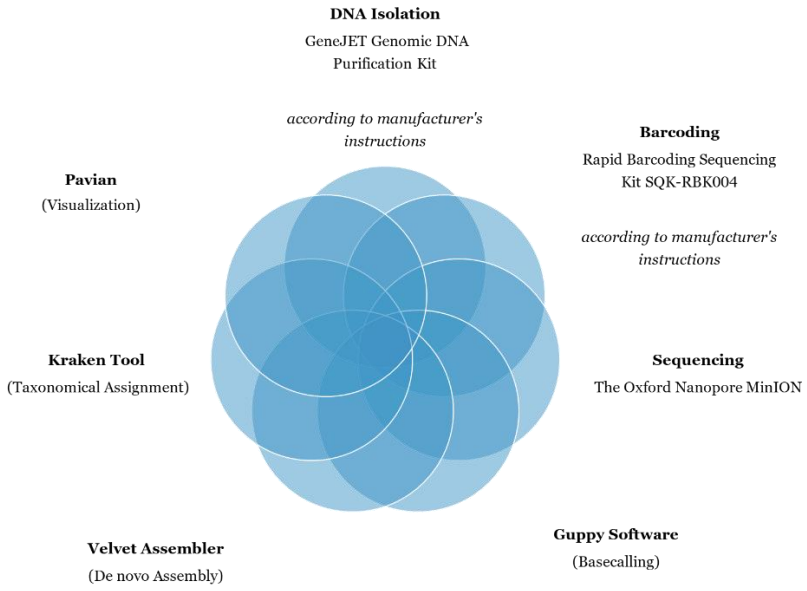

Figure 24. Protocol workflow

CHAPTER 3: RESULTS AND DISCUSSION

3.1. Sampling Sites and Cultivation Conditions

The temperature of the sampling sites is in the mesophilic to thermophilic range(38-64.7^{0}C). Collected samples were incubated at temperatures close to the environmental temperatures of hot springs (Table 6). The growth medium was supplemented with 1 g/l yeast extract to stimulate microbial growth. Microbial growth and hydrogen production in the samples taken from lower environmental temperatures were observed to be considerably lower than in the samples taken from higher temperatures. While the best hydrogen production was observed in Bergama, Doğanbey and Yıldızburnu samples; relatively lower hydrogen production was observed in Bademli and Nebiler samples (Figure 25) (Table 6).

Table 6. Biohydrogen production yields of five different hot spring mix cultures.

Hot Spring	**Incubation Temperature (°)**	**H_2 (%)**	**CO (%)**	**Yield (ΔH_2/ ΔCO)**
Doğanbey	65	1.868	10.297	0.13
Yıdızburnu	55	1.262	20.859	0.12
Bademli	45	0.857	26.146	0.09
Nebiler	55	0.649	26.142	0.06
Bergama	55	2.099	12.032	0.18

Figure 25. Doğanbey (a) and Bergama (b) hot springs.

3.2. Isolation and Selection of Hydrogen Producing Pure Culture

According to gas chromatography results, Bergama, Yıldızburnu and Doğanbey cultures produced hydrogen from carbon monoxide. A total of seven pure cultures were isolated from liquid mix cultures based on colony morphology on agar. Colony morphologies of bacteria are bacilli to cocci shape. Gram staining observations showed that mostly micro-shaped bacilli and gram-negative bacteria were found in the pure cultures. The isolates of Bergama are bacilli and cocci shape, and both are gram-negative; the isolates of Yıldızburnu are bacilli shape and gram-negative; the isolates of Doğanbey are bacilli shape and gram-positive (Table 7).

Table 7. Colony morphology and gram stain of pure culture.

Hot Spring	**Colony Number**	**Colony Morphology**	**Gram-Stain**
Bergama	1	Bacilli	Gram-negative
	2	Cocci	Gram-negative
	3	Cocci	Gram-negative
Yıldızburnu	1	Bacilli	Gram-negative
	2	Bacilli	Gram-negative
Doğanbey	1	Bacilli	Gram-positive
	2	Bacilli	Gram-positive

All isolates were primarily transferred into an enriched liquid medium and screened for hydrogen production. Among the seven isolates, six of them produced

hydrogen (Table 8). The environmental conditions of Yıldızburnu were not suitable for thermophilic hydrogenogenic carboxydotrophs. Nevertheless, the first colony of Yıldızburnu was yielded as 0.33 mmol H_2/mmol CO. The environmental conditions of Bergama and Doğanbey hot springs were close to thermophilic conditions and were likely to contain thermophilic hydrogenogenic carboxydotrophs. The third colony of Bergama was yielded as 0.31 mmol H_2/mmol and the highestyield was observed in the second colony of Doğanbey at 0.43 mmol H_2/mmol CO. Both colonies are gram-positive and bacilli shape. The second colony of Doğanbey was transferred into agar to liquid medium regularly and hence was selected for further characterization studies.

Table 8. Biohydrogen yields of pure cultures.

Hot Spring	**Colony Number**	**Yield (mmol H2/ mmol CO)**
Bergama	1	-
	2	0.12
	3	0.31
Yıldızburnu	1	0.33
	2	0.07
Doğanbey	1	0.12
	2	0.43

3.3. Growth Profiling of Hydrogen Producing Pure Culture

Growth profiling experiment of the Doğanbey pure culture conducted with VSS, optical density and viable counting. Figure 26 represents the changes in VSS concerning time. The VSS value increased in the first 12 h followed by a decreasing trend. An increasing trend could not be observed in the VSS values, but it should increase with the increase in biomass. There may be many reasons for this. First, there may have been sampling errors even though the sampling volume was the same. Secondly, deviations may have occurred in drying and desiccator times. Thirdly, there may be a situation caused by the filters. Since the measured amount is at the milligram level, it is a very error-prone measurement. The medium contains undissolved ingredients. It may be that the medium ingredients that are held in the filter as

suspended solids in the media are consumed by microorganisms depending on time. This may cause a decrease in VSS values.

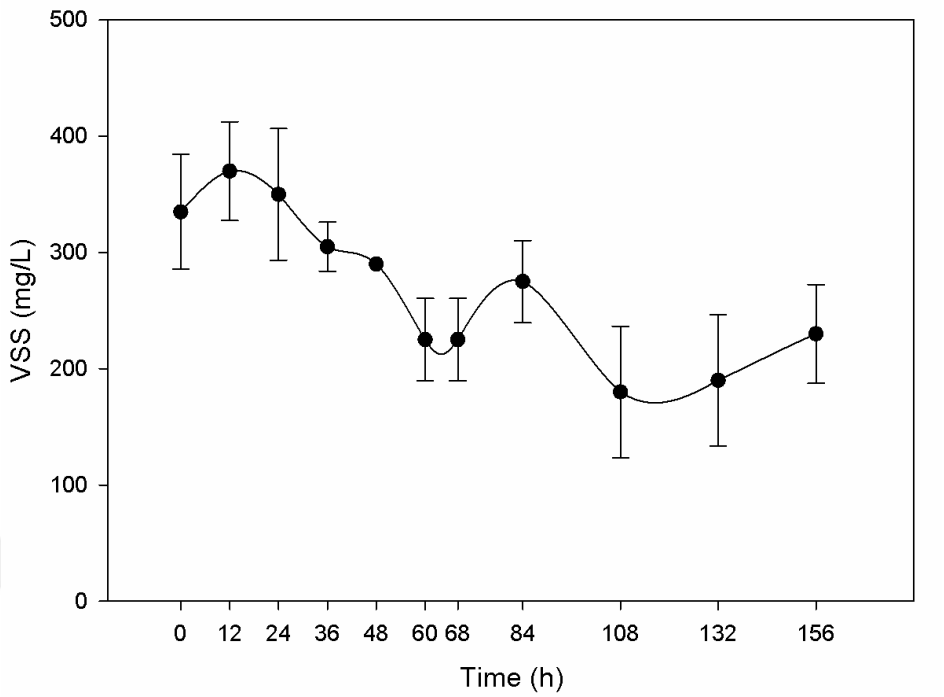

Figure 26. VSS values of the Doğanbey pure cultures.

Figure 27(a) represents the growth curve characteristics of Doğanbey pure culture. This curve was obtained by logarithmic transformation of optical density values. The first phase of the growth curve is the lag phase and is the time it takes for microorganisms to adapt to the environment and no change in optical density is observed in this phase. It can be said that there is no lag phase in the growth curve. The reason for this may be that the stock culture used is in the logarithmic phase so quickly adapted, the transferred medium and the stock culture medium ingredients and cultivation conditions are the same. For these reasons, microorganisms can be said to have passed into the logarithmic phase without the lag phase. The second phase of the growth curve is the logarithmic phase. Here, the growth of microorganisms is highest and a continuous increase in optical density is observed. Microorganisms are in the logarithmic phase between 0-36 hours in the growth curve and the doubling time is 36 h. The third phase of growth is the stationary phase, and the optical density is constant, followed by the fourth phase, the death phase, and the optical density values decrease. 36-84 hours is the stationary phase, after 84 hours it is the death phase. Growth rate constant and generation time are calculated from Equation 2 and Equation 3 and where

t_2 is 36 h and t_1 is 12 h. Generation time of the pure culture is 1.892 h, and the growth rate constant is 0. 159 h^{-1}.

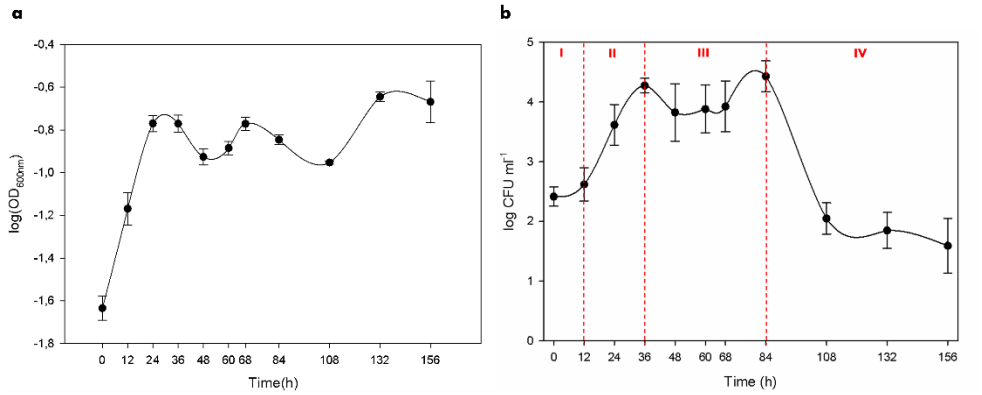

Figure 27. log OD_{600nm} (a) and log CFU versus time(b) curve of Doğanbey pure culture. (Lag phase(I); log phase (II); stationary phase(III) and death phase(IV).

Figure 27(b) represents the colony-forming unit (CFU) per milliliter versus the time curve of Doğanbey pure culture. The curve was obtained by logarithmic transformations of viable counts during the culture period. There is no significant increase in values between 0-12 hours. However, it is seen that there is a logarithmic increase in the 12–48-hour range. Values are stationary in the 48–84-hour range and can be called the stationary phase. After 84 hours, it can be said that there is a decrease in the values and there is a death phase. If we evaluate it together with the OD_{600nm} curve, the viable count and optical density values confirm each other. It can be said from here that Doğanbey pure culture is in the lag phase between 0-12 hours; is in the log phase between 12-48 hours; is in the stationary phase between 48-84 hours and finally in the death phase between 84-156 hours.

3.4. Box-Behnken Experimental Design (BBD)

3.4.1. Box-Behnken Design I

Table 9 represents the trial sets made with the Design-Expert program. All trials were conducted at the same time and regular intervals of 24 h, all samples were monitored by gas chromatography for hydrogen production.

Biohydrogen yields of the 4, 12, 13, and 16th runs were quite high compared to others. It is seen that the common parameters are 60 ^{o}C temperature and pH 9. Almost all the sets have different CO amounts. The results are given in Table 9.

Table 9. Box-Behnken three-factor experimental design and biohydrogen yields from Doğanbey pure culture.

Run	**Temperature (^{o}C)**	**pH**	**CO amount (ml)**	**Yield (mmol H_2/mmol CO)**
1	60	7	10	0.18
2	65	7	5	0.17
3	65	5	10	0.07
4	60	9	5	0.44
5	55	7	5	0.20
6	55	7	15	0.30
7	60	7	10	0.31
8	60	7	10	0.20
9	60	7	10	0.23
10	60	5	5	0.33
11	55	9	10	0.38
12	60	9	10	0.40
13	60	5	15	0.41
14	65	7	15	0.29
15	60	7	10	0.36
16	60	9	15	0.41
17	55	5	10	0.07

p-values lower than 0.05 indicates that the model terms are significant(Neill and Johnson, 1984). As reported in Table 10, the p-value of the model is 0.1942 and model is not significant for biohydrogen yield as a response. Non-significant lack of fit means

that the variables has significantly affect the response and model fits the experimental data (Neill and Johnson, 1984). The lack of fit F-value of the model is 2.24 and not significant. p-value of the pH is 0.0263 and lower than 0.05. pH is a significant model term. It can be said from this that the pH has great impact on biohydrogen production yield. Biohydrogen production yields are higher at pH 9. p-values greater than 0.1 indicate the model terms are not significant(Neill and Johnson, 1984). Hence, temperature (p:0.9424) and CO addition (p: 0.3460) has no significant effect on biohydrogen production yield (Table 10). Maximum biohydrogen yield of 0.44 mmol H2/mmol CO with 60 ^{o}C, pH 9-, and 5-ml CO addition.

Table 10. ANOVA results of Quadratic model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.1572	9	0.0175	1.96	0.1942	not significant
A-Temperature	0.0001	1	0.0001	0.0056	0.9424	
B-pH	0.0703	1	0.0703	7.88	0.0263	
C-CO addition	0.0091	1	0.0091	1.02	0.3460	
AB	0.0001	1	0.0001	0.0112	0.9187	
AC	0.0001	1	0.0001	0.0112	0.9187	
BC	0.0030	1	0.0030	0.3388	0.5788	
A^2	0.0354	1	0.0354	3.97	0.0866	
B^2	0.0182	1	0.0182	2.04	0.1964	
C^2	0.0242	1	0.0242	2.71	0.1440	
Residual	0.0625	7	0.0089			
Lack of Fit	0.0392	3	0.0131	2.24	0.2259	not significant
Pure Error	0.0233	4	0.0058			
Cor Total	0.2197	16				

Standard deviation of the model is 0.0945 and R^2 is 0.7155. Adjusted R2 value is 0.3498 and it means that is model not good as to predict the effects of new variables on response (Table 11).

Table 11. Fit statistics of model.

Std. Dev.	0.0945	**R^2**	0.7155
Mean	0.2794	**Adjusted R^2**	0.3498
C.V. %	33.82	**Predicted R^2**	-2.0189
		Adeq Precision	5.0884

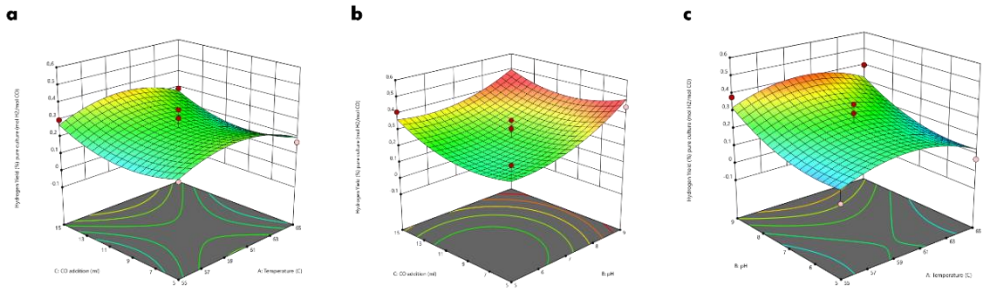

Figure 28. Effects of different parameters on the hydrogen yield for pure culture. (a) temperature (y-axis) and pH (x-axis) (b) CO addition (y-axis) and temperature(x-axis) (c) CO addition (y-axis) and pH(x-axis).

Validation experiments were carried out for validation of Box-Behnken results. Five different runs were selected for validation experiments and trials one with three replicates. The maximum hydrogen production yield obtained at 4th run as 0.44 mmol H_2/mmol CO in BBD experiments. 16th and 13th run yielded as 0.41 mmol H_2/mmol CO. Results confirmed the repeatability and show that the maximum yield of 0.46 mmol H_2/mmol CO for the 4th run and the second high yield was achieved on the 16th run (Table 12).

Table 12. Validation results for biohydrogen production yield of Doğanbey pure culture.

Run ID	Yield (mmol H_2/mmol CO)
4	0.46
7	0.32
12	0.39
16	0.44
17	0.06

3.4.2. Box-Behnken Design II

Table 13 represents the trial sets and the results made with the Design-Expert program. All trials were conducted at the same time and regular intervals of 24 h, all samples were monitored by gas chromatography for hydrogen production. Biohydrogen yields of the 2, 14, and 17th runs were quite high compared to others.

Table 13. Box-Behnken three-factor experimental design and biohydrogen yields from Doğanbey pure culture.

Std	A:Fe	B:Zn	C:Ni	Hydrogen Yield
Unit	mg/L	mg/L	mg/L	mmol H_2/ mmol CO
1	50	25	55	0.22
2	250	25	55	0.4
3	50	150	55	0.22
4	250	150	55	0.04
5	50	87.5	10	0.09
6	250	87.5	10	0.12
7	50	87.5	100	0.28
8	250	87.5	100	0.08
9	150	25	10	0.23
10	150	150	10	0.08
11	150	25	100	0.23
12	150	150	100	0.15
13	150	87.5	55	0.21
14	150	87.5	55	0.21
15	150	87.5	55	0.13
16	150	87.5	55	0.35
17	150	87.5	55	0.21

p-values lower than 0.05 indicates that the model terms are significant(Neill and Johnson, 1984). As reported in Table 10, the p-value of the model is 0.0472 and model is significant for biohydrogen yield as a response. Non-significant lack of fit means that the variables has significantly affect the response and model fits the experimental data (Neill and Johnson, 1984). The lack of fit F-value of the model is 0.6847 and not significant. p-value of the Zn is 0.0151 and lower than 0.05. Zn addition is a significant model term. It can be said from this that the Zinc has great impact on biohydrogen production yield. Biohydrogen production yields are higher at 87.5 Zn concentration. p-values greater than 0.1 indicate the model terms are not significant(Neill and Johnson, 1984). Hence, Fe (p:0.4186) and Ni (p: 0.3005) has no significant effect on biohydrogen production yield (Table 10). Maximum biohydrogen yield of 0.4 mmol H_2/mmol CO with 250 mg/L Fe, 25 mg/L Zn and 55 mg/L Ni concentrations.

Table 14. ANOVA results of Quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.1000	6	0.0167	3.28	0.0472	significant
A-Fe	0.0036	1	0.0036	0.7118	0.4186	
B-Zn	0.0435	1	0.0435	8.57	0.0151	
C-Ni	0.0060	1	0.0060	1.19	0.3005	
AB	0.0324	1	0.0324	6.38	0.0300	
AC	0.0132	1	0.0132	2.61	0.1375	
BC	0.0012	1	0.0012	0.2414	0.6338	
Residual	0.0508	10	0.0051			
Lack of Fit	0.0255	6	0.0042	0.6717	0.6847	not significant
Pure Error	0.0253	4	0.0063			
Cor Total	0.1508	16				

Standard deviation of the model is 0.0742 and R^2 is 0.6634. Adjusted R^2 value is 0.4614 and it means that is model not good as to predict the effects of new variables on response (Table 15).

Table 15. Fit statistics of model.

Std. Dev.	0.0712	**R^2**	0.6634
Mean	0.1912	**Adjusted R^2**	0.4614
C.V. %	37.26	**Predicted R^2**	0.0691
		Adeq Precision	7.1641

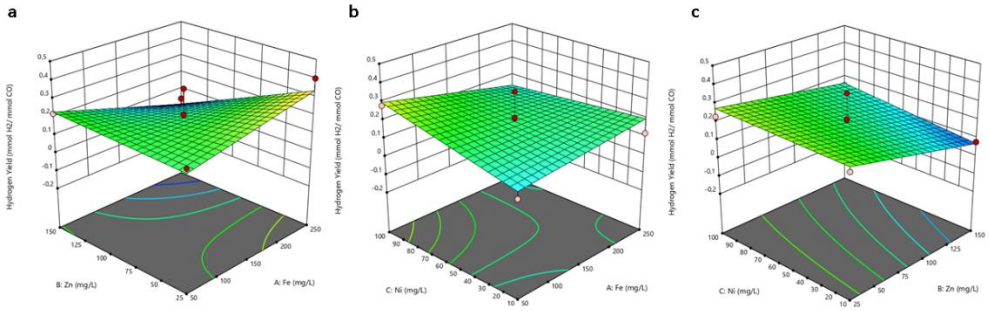

Figure 29. Effects of different parameters on the hydrogen yield for pure culture. (a) Fe (y-axis) and Zn (x-axis) (b) Fe (y-axis) and Ni (x-axis) (c) Zn (y-axis) and Ni(x-axis)

3.5. Morphological Characterization of Hydrogen Producing Pure Culture

3.5.1. Gram Staining

Gram staining results show that the Doğanbey pure culture is bacilli shape and gram-positive. According to agar examinations, the surface of the colony is rough, and opacity is transparent with convex elevation. Mainly, *Clostridium* and *Bacillus* species are gram-positive and thermophilic hydrogenogenic carboxydotrophs (Ueno et al., 2001; Wu et al., 2005). The results confirmed that the Doğanbey pure culture is a potential thermophilic hydrogenogenic carboxydotroph (Figure 31).

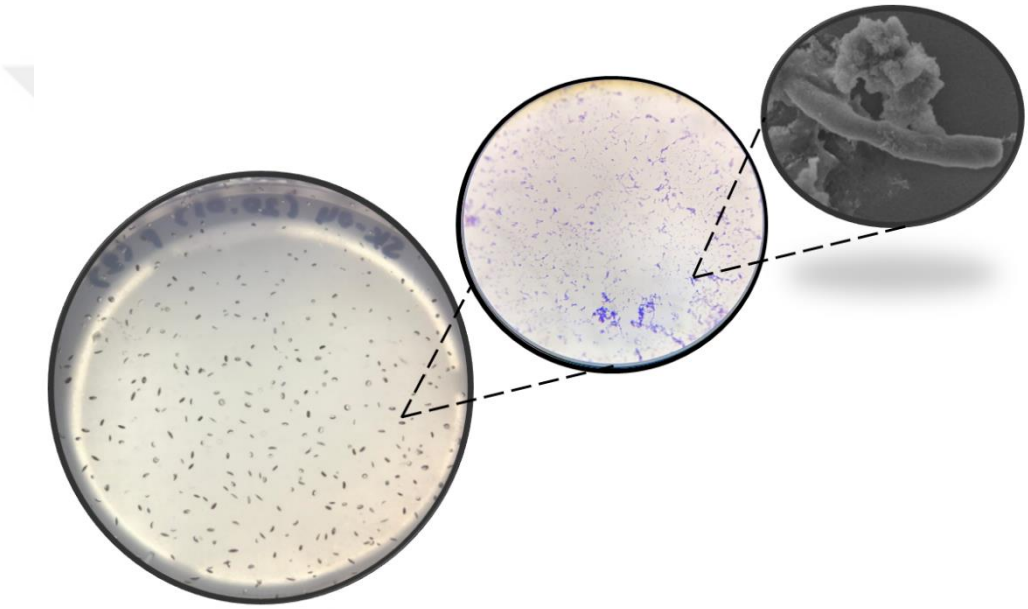

Figure 30. Colony morphology, gram-staining, and SEM result of the Doğanbey pure culture.

3.5.2. SEM and ESEM Imaging

The samples were imaged at different magnifications. The SEM images of the Doğanbey pure culture confirmed that the bacteria present in this culture are bacilli shape and the size of the bacteria is around 1.893 to 5.671 μm in length and 468.5 to 562.1 nm in width. The bacilli are arranged in chains in streptobacilli shape. The shape of a few bacteria was deformed due to the dehydration step. Several bacteria are observed under the surface due to salt residues that are from PBS solution (Figure 32).

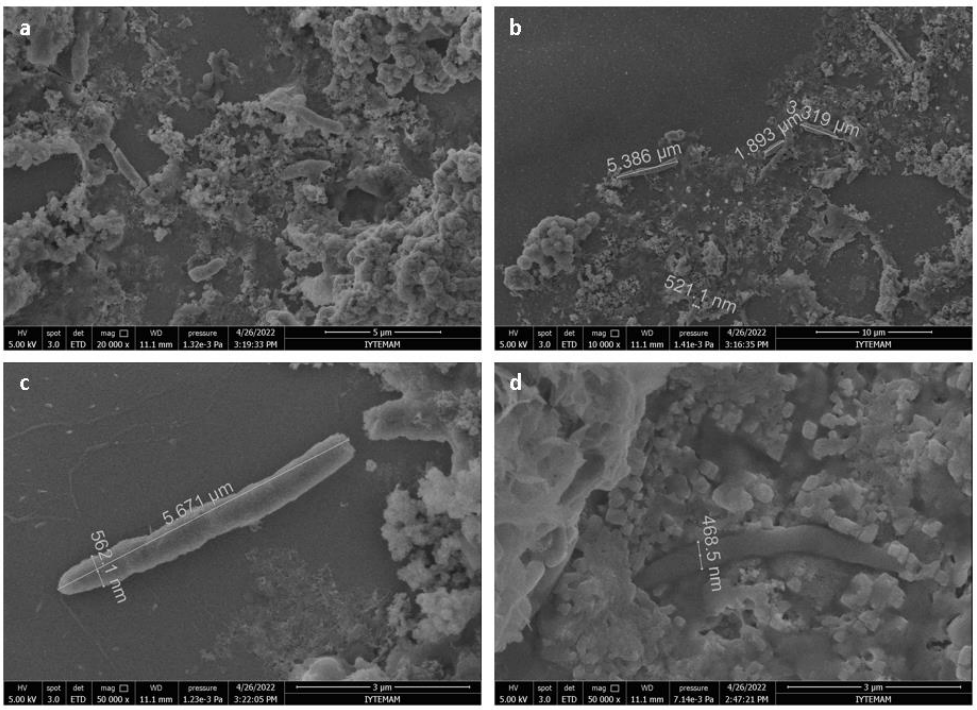

Figure 31. SEM images of the Doğanbey pure cultures at different magnifications.

ESEM imaging shows that the bacteria is bacilli shape with a 5.021 µm length. Since liquid samples are used in ESEM imaging, the clarity of the samples is lower compared to SEM (Figure 33).

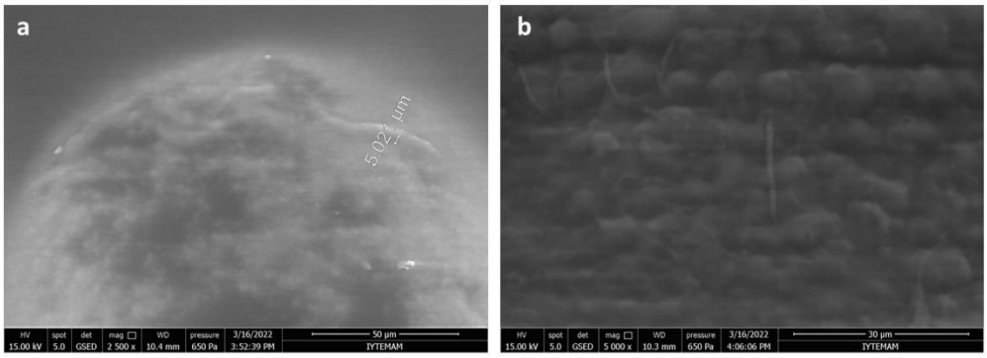

Figure 32. ESEM images of the Doğanbey pure cultures at different magnifications.

Based on gram staining, microscopic examination, and VFA analysis, the present microorganisms may belong to the genera *Thermoanaerobacter*, *Carboxydocella*, and *Bacillus*, which are associated with carbon monoxide conversion to hydrogen and organic acids. The morphological features (size and form) obtained through SEM imaging, as well as the growth characteristics obtained from growth profiling, led to the conclusion that hydrogen-producing and CO-using bacteria could probably be members of the *Carboxydocella* genus (Slepova et al., 2006).

3.6. Analytical Methods

Figure 33 shows the cumulative hydrogen and carbon dioxide (CO_2) production and carbon monoxide (CO) consumption during the culture period of the Doğanbey pure culture. The trials were conducted in two replicates and standard deviations were calculated. The amount of carbon monoxide decreases over time, and hydrogen and carbon dioxide amounts increases. The initial CO amount is 22.326 % and the final amount is 13.632%; 8.694 % of CO was consumed by bacteria. The final amount of CO_2 is 6.4875% and the final hydrogen amount is 7.69 % after 156 h, respectively. CO is the main carbon source in this study and the amount was decreased over time. Also, CO_2 production was observed. Here can be said that the WGSR takes place in the Doğanbey pure cultures and hydrogen was produced as a result of the WGSR pathway (Younesi et al., 2008)(Figure 34). The partial list in the literature includes several photosynthetic bacteria but it needs a carbon source other than CO to grow (Jung et al., 2002; Zhu et al., 2001). The Doğanbey pure culture uptake CO, produced hydrogen, and doesn't need any other carbon source. Here can be said that the Doğanbey pure culture is not photosynthetic bacteria. The yield is calculated as a function of hydrogen production and CO consumption (Mohr et al., 2019). The overall yield at the end of the 156 h is 0.56 mmol H_2/ mmol CO (Table 16). The partial list in the literature includes higher biohydrogen yields. Mohr et al., 2019 reported yields of up to 0.808 mmol H_2/mmol CO. The hydrogen yields of thermophilic microorganisms utilizing glucose as a substrate conversion were higher than CO as a substrate and reported to be 2.64 mmol H_2/mmol glucose (Sivaramakrishnan et al., 2021)

Table 16. Hydrogen, carbon monoxide, carbon dioxide amounts and yields of the Doğanbey pure culture.

Time (h)	H_2 (%)	CO (%)	CO_2 (%)	Yield (ΔH_2 / ΔCO)
0	0	22.33±1.64	0	0
12	0.05±0.03	20.34±0.70	0.05±0.00	0
24	2.12±0.63	19.15±0.71	1.61±0.03	0.11
36	4.17±0.59	18.50±0.97	3.69±0.04	0.23
48	5.45±0.07	18.34±1.48	4.83±0.09	0.30
60	6.60±0.63	17.09±2.27	4.44±0.65	0.39
68	6.84±0.31	17.09±2.05	4.90±0.46	0.40
84	6.47±0.52	16.01±0.41	5.04±0.09	0.40
108	7.59±0.16	15.63±0.82	6.87±0.12	0.49
132	7.67±0.69	15.33±1.79	6.83±0.46	0.50
156	7.69±.072	13.63±0.79	6.49±0.37	0.56

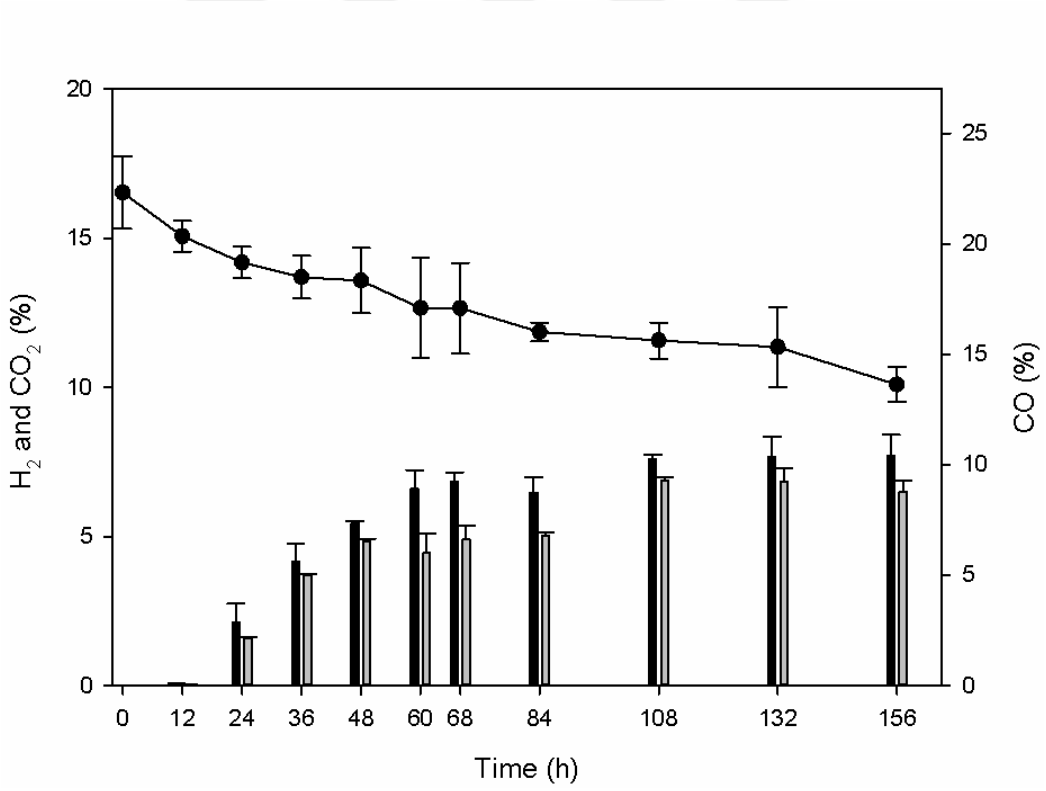

Figure 33. CO consumption, H_2 and CO_2 production of hydrogen-producing pure cultures during the cultivation with an initial gas atmosphere of 100 % CO (H_2(black), CO_2 (gray), CO (line))

VFA production was an important parameter for estimating the metabolic pathway of hydrogen production (Kumar et al., 2019). The initial concentration of formic acid was 0.3255 mg/l and the final concentration was 0.1773 mg/l. 0.1482 mg/l formic acid was metabolized by bacteria in the medium during the culture period. 0.1901 mg/l of acetic acid is produced and is the highest, meanwhile propionic acid and ethanol production were also observed at 0.0353 mg/l and 0.0427 mg/l respectively (Figure 35). The propionic acid and ethanol concentrations are quite low compared to formic and citric acid. The lower propionic acid concentrations indicate higher efficiency of biological hydrogen production (Chen et al., 2002; Han and Shin, 2004). Consequently, as shown in Figure 34, concentrations of propionic acid were slightly less compared to other VFAs. Formic acid can be degraded intracellularly in 2 ways. The first way is reversible CO_2 hydrogenation (HCOOH <-> CO_2 +H_2) and the second way is a reversible reaction, which is the conversion of formic acid to CO_2 and water(HCOOH<-> CO+H_2O) (Tedsree et al., 2011). Formic acid concentration decreases during the culture period. The Doğanbey pure culture may use the formic acid as a substrate for hydrogen production(Matsumoto and Nishimura, 2007). Also, the first reaction's Gibbs free energy predicts that it will take place ($\Delta G = -48.4$ kJ mol^{-1}).

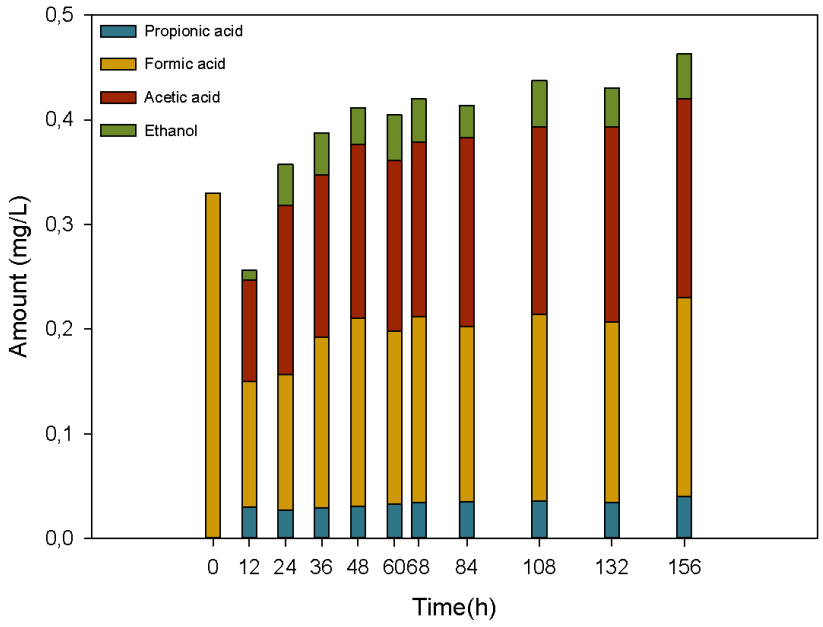

Figure 34. The amount of VFAs in the culture medium.

3.7. Bioreactor Experiments

Bioreactor experiments showed maximum hydrogen production at the end of 24 hours as 3,678%. There is an increase in the amount of oxygen in the reactor with time (Table 17).

Table 17. Headspace gas content of the bioreactor

Sample ID	**H_2%**	**O_2%**	**CO%**	**Hour**	**Day**
SK-4	0	6.551	35.150	0	0
SK-4	3.678	2.466	29.103	24	1
SK-4	2.076	7.407	23.173	48	2
SK-4	1.889	6.804	20.92	72	3
SK-4	3.403	3.105	18.164	96	4
SK-4	2.121	8.267	8.691	168	7

The hydrogen production yields of thermophilic microorganisms from organic substrates (glucose, sucrose, and cellulose) were reported comparatively high than from CO, ranging from 3.3 to 0.9 mol H_2 /mol CO (O-Thong et al.,2019). Koskinen et al., 2008 isolated and enriched an anaerobic and thermophilic culture, obtaining H_2 yields up to 3.2 mol H_2/mol glucose. Biohydrogen production with WGS pathway yields are relatively low compared to organic substrates and could be improved by scale-up and immobilization methodologies to increase biomass. Najafpour et al., 2004 reported a yield of 0.65 mmol H_2/mmol CO. Mohr et al., 2019 reported yields up to 0.808 mmol H_2/mmol CO, while an equimolar conversion of 1.08 H_2/CO was reported by the same group in a different study (Mohr et al.,2018). In this study, the maximum biohydrogen yield of bioreactor experiment is 0,5 mmol H_2/mmol CO which is above average in terms of yield (Table 18).

Table 18. Literature survey

Pathway	**Yield**	**Reference**
Fermentation	3.2 mmol H_2/mmol glucose	Koskinen et al.,2008
Fermentation	0.9-3.3 mmol H_2/mmol glucose	O-Thong et al.,2019
WGSR	0.65 mmol H_2/mmol CO	Najafpour et al.,2004
WGSR	0.808 mmol H_2/mmol CO	Mohr et al.,2019
WGSR	1.08 mmol H_2/mmol CO	Mohr et al.,2018
WGSR	**0.56 mmol H_2/mmol CO**	This research

3.8. Molecular Characterization of Hydrogen-Producing Pure Culture

gDNA isolation was performed for the whole genome sequencing (WGS) experiment, and the target gDNA amount is 54000 ng/ml according to the ONT Rapid Barcoding Sequencing kit protocol. gDNA quantification was carried out by Qubit fluorometer and as shown in Table 19 gDNA amounts of the Doğanbey pure culture isolates were considerably less than the target amount.

Table 19. gDNA amounts of the Doğanbey pure culture.

Sample ID	**gDNA Amount (ng/ml)**
1	448
2	572
3	416
4	107

To obtain more concentrated gDNA, gDNA was lyophilized and quantified with a Qubit fluorometer. Table 20 represents the final concentrations of gDNA after lyophilization. Although more concentrated gDNA is obtained by lyophilization, the concentrations are comparatively less than the target gDNA amount.

Table 20. gDNa amounts after lyophilization.

Sample ID	gDNA Amount (ng/ml)
1	6,500
2	14,380
3	10,280
4	10,700

A literature review was performed for manual gDNA isolation to obtain more concentrated gDNA from the Doğanbey pure culture. Vingataramin and Frost, 2015 develop a quick and easy gDNA extraction method. Table 21 represents the comparison of the final gDNA amounts from the kit and manual protocol. The manual extraction procedure yielded 4-fold higher compared to kit extraction. Although, the amount is still 71% lower than the target amount.

Table 21. gDNA amounts from kit versus manual protocol.

Sample ID	**gDNA Amount(ng/ml)**	
	Kit	**Manual**
1	2,460	-
2	3,630	-
3	3,470	-
4	-	15,500

Whole-genome sequencing was performed with the lyophilized gDNA of the Doğanbey pure culture (Table 22). Table 22 represents the cumulative reads using the MinION device. The whole genome of a gram-positive *Bacillus subtilis* is nearly $4.2x10^6$ base (Bolotin and Borchert, 1997). So, the cumulative reads are not enough to estimate the whole genome of the Doğanbey pure culture. Brown et al., 2017b reported that, genome preamplification with Φ29 polymerase increase the gDNA amount from 1 μg to 5 ng.

Table 22. Cumulative reads using the MinION device.

Sample ID	Cumulative Reads
1	433
2	34,444
3	75,762
4	81,004

DNA isolation was performed on the 2nd day of pure culture samples for WGS. The resulting DNA concentrations were measured with a Qubit (Table 23). The obtained DNA amounts were prepared with the Rapid Barcoding Sequencing (SQK-RBK004) kit. The starting amount of DNA is 400 ng/ 7.5 µl according to the kit instructions. For this purpose, 100 µl elution was made to obtain more concentrated DNA. Samples were prepared according to the manufacturer's instructions.

Table 23. gDNA amounts of the Doğanbey pure culture.

Sample ID	**gDNA Amount(ng/ml)**
1	8,080
2	4,880
3	7,330

Barcodes 11 and 12 were used and DNA were purified with AMPure before the barcoding. After the AMPure, the DNA amounts were increased up to 26 and 33 ng/µl. The flow cell was prepared for loading and the prepared samples were loaded into the flow cell.

3.9. Bioinformatic Analysis

The sequencing took about 20 hours and yielded 40 GB of data. Sequencing reads collected from the sample via MinKNOW software were passed to Guppy software for base-calling after that, Velvet Assembler was used for De novo assembly. Velvet output files are then passed to Kraken Tool for classification and Pavian for visualization. Pavian provides Sankey visualization of samples and read count

comparisons between samples. Sequencing results was analyzed at the time of sequencing and approximately 20% of the total obtained reads were determined to belong to the Leifsonia family.

CHAPTER 4: CONCLUSIONS

Biohydrogen is a clean fuel with zero carbon emission and powerful alternative to fossil fuels due to high energy content. There are specific metabolic pathways for biohydrogen production and is produced by various microorganisms. Anaerobic thermophiles are the most prominent bacteria living under extreme environment and are important in terms of biohydrogen production. These bacteria have a unique pathway called as water-gas shift reaction (WGSR) and converts CO and H_2O to CO_2 and hydrogen with the help of carbon monoxide dehydrogenases enzyme. It means that thermophilic anaerobes can produce hydrogen without any carbon source, but CO. CO is the main reason of air pollution whereas CO utilization can be the solution. The biohydrogen production ability of anaerobic thermophiles makes them attractive tools for biohydrogen studies. Scientists have been researching nature to find novel biohydrogen producer strains. In this aim, the local hot springs in İzmir, Turkey are investigated with this research.

The isolated microorganisms from five different hot spring produced hydrogen at low yields. So that, the mix cultures were subcultured to selective isolation of the best hydrogen producing species. The Doğanbey pure culture has the highest hydrogen yield as 0.43 mmol H_2/mmol CO. Thereafter, the characterization studies were performed to identify morphological characteristics. Gram staining results show that the isolate is gram-positive and bacilli shape. ESEM and SEM imaging confirmed that bacteria is bacilli shape, and the size of the bacteria is around 1.893 to 5.671 μm in length and 468.5 to 562.1 nm in width. The growth characteristics were determined by determining the biomass, optical density, and viable counting. Generation time of the pure culture is 1.892 h and the growth rate constant is 0. 159 h^{-1}.

Box-Behnken Design experiments were conducted to obtain high hydrogen production yields and to stimulate growth. It was seen that the pH greatly affects the biohydrogen production, the yield is highest at pH 9 compared to pH 7 and pH 5. The temperature and CO have no significant effect on biohydrogen yield ($p<0.05$) and maximum biohydrogen yield of 0.44 mmol H_2/mmol CO with 60 ^oC, pH 9, and 5 ml CO addition. Results are validated with another experimental setup with same conditions and confirmed that the highest biohydrogen yield was obtained under the

condition of 60 ^{o}C, pH 9-, and 5-ml CO addition. The biohydrogen yield after BBD experiments were 30% higher (Table 13).

Whole genome sequencing of the best hydrogen producing isolates were performed and sequencing results were analyzed at the time of sequencing and approximately 20% of the total obtained reads were determined to belong to the Leifsonia family.

Briefly, the identification and characterization of thermophilic anaerobic bacteria from local hot spring and biohydrogen production studied. The results show that the İzmir hot springs are hosting variety of hydrogen producer microorganisms. Molecular identification and characterization of the newly found bacteria that isolated from Doğanbey hot spring will have significant impact on literature.

CHAPTER 5: FUTURE SUGGESTIONS

This research has significant impact on both literature and real-life applications. The hydrogen production yield of the hot spring isolates is promising in terms of biotechnological applications. Hydrogen production via Doğanbey pure culture by biotechnological water-gas shift reaction, industrial syngas as a sole carbon source, can be significantly reduced carbon emissions. However, the purification of the produced hydrogen and then its storage using the required technology, for example, a hydrogen fuel cell, and its use as an energy source in various fields are the promising aspects of this study.

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