OPTIMIZATION OF BIOHYDROGEN PRODUCTION YIELDS OF HOT SPRING MICROBIAL COMMUNITIES AND THEIR EVALUATION UTILIZING METAGENOMICS WITH NANOPORE SEQUENCING

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Master's Thesis

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ABSTRACT

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Akaçin, İlayda

Master's Program in Bioengineering

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Hydrogen gas and its utilization as an energy carrier through fermentative processes is an alternative green route for obtaining renewable and environmentally friendly energy sources and decreasing the dependency on fossil fuels. Hot spring microbial communities containing carboxydotrophic hydrogenogenic microorganisms are potential valuable contributors to the industrial applications as they have been observed to be adaptable to changing environmental circumstances, requiring less rigorous sterilizing standards, initiating a CO cycle in ecological processes as well as

in biotechnological processes. Carboxydotrophic hydrogenogenic microorganisms isolated from hot springs, utilize carbon monoxide (CO) as an electron and carbon source through carbon monoxide dihydrogenase and hydrogenase enzymes that through water-gas shift reaction, converting CO into molecular hydrogen (H_2). Their investigation and screening through high-throughput sequencing tools in their natural environments are vital for understanding the microbial dynamics in extreme terrestrial areas. In this study, 16S rRNA sequencing was performed using Oxford Nanopore Technologies MinION sequencer with DNA samples collected from 5 hot springs located in İzmir region and hot spring isolates were investigated by cultivation under 100% CO and 1 g/L yeast extract. H_2 production was monitored using gas chromatography, liquid by-products were monitored through high pressure liquid chromatography. H_2 production yields were optimized by Box Behnken design, highest H_2 yield reached was 0.40 mmol H_2/mmol CO with Doğanbey hot spring mixed culture.

Keywords: biohydrogen, hot spring microbial consortia, water-gas shift reaction, third generation sequencing, nanopore

ÖZET

KAPLICA MİKROBİYAL KOMÜNİTELERİNİN BİYOHİDROJEN ÜRETİM VERİMLERİNİN OPTİMİZASYONU VE NANOPORE SEKANSLAMASI KULLANILARAK METAGENOMİK PROFİLLERİNİN ÇIKARILMASI

Akaçin, İlayda

Biyomühendislik Yüksek Lisans Programı

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Kasım, 2022

Hidrojen gazının fermentatif süreçlerle biyoyakıt olarak üretilmesi, yenilenebilir ve çevre dostu enerji kaynakları kullanmak ve fosil yakıtlara olan bağımlılığı azaltmak için alternatif bir yeşil yol olarak görülmektedir. Kaplıcalardan izole edilen karboksidotrofik hidrojenojenik mikroorganizmalar, karbon monoksit dihidrojenaz ve hidrojenaz enzimleri aracılığıyla bir elektron ve karbon kaynağı olarak karbon monoksit gazını (CO) kullanarak moleküler hidrojene (H_2) dönüştüren su-gaz

değişim reaksiyonunu gerçekleştirmektedirler. Karboksidotrofik hidrojenojenik mikroorganizmaları içeren kaplıca mikrobiyal komüniteleri, değişen çevresel koşullara uyarlanabildikleri, sterilizasyon standartlarının daha az hassas olduğu proseslerde rol alabilmeleri, hem ekolojik hem biyoteknolojik süreçlerde CO çevrimi yapabildikleri gözlemlendiğinden endüstriyel uygulamalara değerli bir katkı sağlayabilecekleri düşünülmektedir. Doğal ortamlarında bu komünitelerin yeni nesil sekanslama teknolojileri aracılığıyla araştırmaları ekstrem doğal alanların mikrobiyal dinamiklerini anlamak için hayati önem taşımaktadır. İzmir bölgesinde bulunan 5 kaplıcadan alınan DNA örnekleri ile Oxford Nanopore Technologies MiniION sekanslama cihazı kullanılarak kaplıcaların 16S rRNA dizilemesi yapılmış ve kaplıca izolatları %100 CO ve 1 g/L maya ekstraktı altında kültive edilerek hidrojen üretim kapasiteleri araştırılmış, Gaz Kromatografisi ile H_2 üretimi izlenmiştir. H_2 üretim verimleri Box Behnken tasarımı ile optimize edilmiş olup, ulaşılan en yüksek H_2 verimi Doğanbey kaplıcası karışık kültürü kullanılarak 0.40 mmol H_2/mmol CO olarak gözlemlenmiştir.

Anahtar Kelimeler: biyohidrojen, kaplıca mikrobiyal komünite, su-gaz değişim reaksiyonu, üçüncü nesil dizileme, nanopore sekanslama

Dedicated to my wonderful family and beloved friends…

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LIST OF ABBREVIATIONS

$\Delta G0'$: standard free energy change

BBD: Box-Behnken design

bp: base pair

CH_4: methane

cm: centimeter

CO: carbon monoxide

CO_2: carbon dioxide

CODH: carbon monoxide dehydrogenase

DNA: deoxyribonucleic acid

ECH: energy-conserving hydrogenase

ELB: enzymatic lysis buffer

Fd: ferredoxin

GC: gas chromatography

Gt: gigatons

H_2: hydrogen

HPLC: high-performance liquid chromatography

M: molar

min: minute

mL: milliliter

mM: millimolar

mV: millivolt

NCBI: National Center for Biotechnology Information

NFW: nuclease-free water

NGS: next generation sequencing

NO_2: nitrous dioxide

ONT: oxford nanopore technologies

PBS: phosphate buffered saline

PCR: polymerase chain reaction

pg: picogram

rpm: revolutions per minute

rRNA: ribosomal ribonucleic acid

sec: second

SEM: scanning electron microscope

TGS: third generation sequencing

T_m: melting temperature

tsv: text separated values

UP: ultra-pure water

VFA volatile fatty acids

WGSR: water-gas shift reaction

µg: microgram

µL: microliter

CHAPTER 1: INTRODUCTION

1.1.Hydrogen

Industrialization of the modern world has led to a multitude of developments and technological advances for human civilization, applied in everyday life processes as transportation, residential, heating, industrial, commercial production and so forth. Since fossil fuels (coal, petroleum, natural gas) are dominantly used for these applications, excessive carbon-based fossil fuel consumption has caused extensive amounts of CO_2 emissions, following with accumulation of greenhouse gasses to the atmosphere, imprinting polluting traces on the environment (Muradov and Veziroglu, 2008). Increased degree of industrialization has resulted a major rise in the global atmospheric density of greenhouse gases, mainly carbon dioxide (CO_2), following with methane (CH_4) and nitrous oxide (NO_2), triggered the events of global warming and climate change (Yoro and Daramola, 2020). In addition to the massive demand for fossil fuels and their catastrophic environmental impact, carbon-based fossil fuels are also predicted to be depleted in the foreseeable future (Londoño-Pulgarin et al., 2021). In the 1900s reported CO_2 emissions levels were only 1.8 Gt and the most recently, in 2021 CO_2 emissions have reported to rise to a record level of 36.5 Gt (IEA, 2021).

As stated in many environmental reports and strategies developed against climate change, for instance The European Green Deal, reducing the use of fossil fuels and switching to renewable, clean and environmentally friendly energy sources is crucial for the reduction of CO_2 emissions. For this cause, decarbonization is a critical goal for a sustainable future (European Commission, 2020). Naturally carbon-free energy sources including geothermal, wind, solar, nuclear, biomass have become prominent for decarbonization strategies as their usage do not contribute to the rise of CO_2 levels in the atmosphere (Muradov and Veziroglu, 2008). On the other hand, inconsistent nature of these resources demands the utilization of effective storage mechanisms. Renewable technologies thus require to be stored as in form of chemical or electrical energy (Acar and Dincer, 2013).

Interest in hydrogen is increasing day by day due to its favorable properties as an energy carrier. Both electricity and hydrogen need to be produced from renewable energy sources for the confirmation of sustainable progress and the resolution of economic and environmental problems (Acar and Dincer, 2013).

Hydrogen is the molecule of two consisting gaseous hydrogen atoms, H_2, the first element in the periodic table, the most common substance in the universe, and a rich source of energy. Under normal conditions, hydrogen is a colorless, odorless and non-toxic gas that is harmless to the environment (Adolf et al., 2017). Hydrogen has three main utilization functions: as a fuel, an energy carrier and a raw material for chemicals and alternative fuels (such as ethanol). Hydrogen serves as a valuable energy carrier as it can be generated without emitting any greenhouse gases with renewable processes, is abundant in nature, has a high energy conversion efficiency and a higher calorific value than traditional fossil fuels (Dinçer et al., 2021). Hydrogen may also be stored in many ways (for example, with gas, liquid, or metal hydrides) as long as the appropriate regulations are ensued. Environmentally friendly hydrogen energy is fulfilled in terms of manufacture, storage, transit, and ultimate usage if hydrogen gas is obtained from renewable sources (Figure 1) (Dincer, 2012).

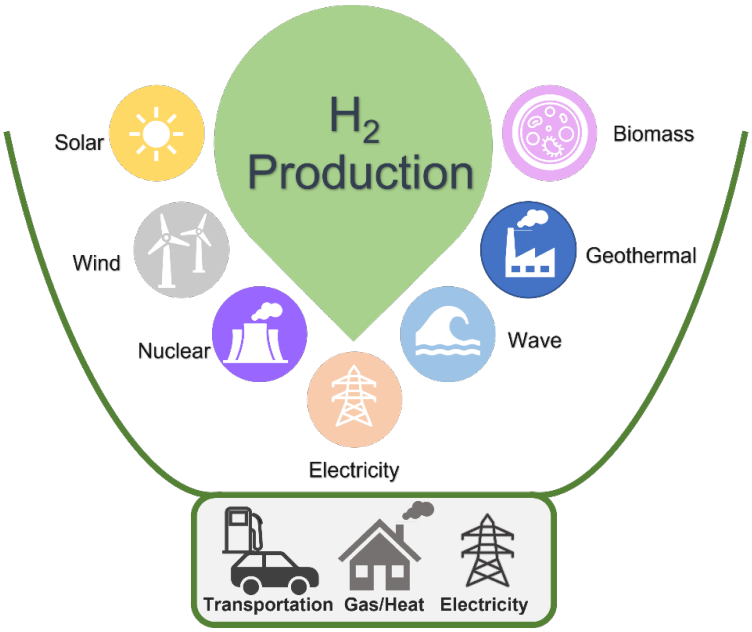


Figure 1. Hydrogen production from renewable energy sources and main application areas for hydrogen energy (Source: Dinçer et al., 2021).

1.2.Hydrogen Production Methods

Methodologies of hydrogen production fall into 6 different main classes: chemical, electrochemical, thermochemical, photochemical, radiochemical, and biochemical methods (Figure 2). Hydrogen production techniques can be classified under 3 main categories categorized by the type of source used:

Green hydrogen is the production of H_2 using only renewable resources. As the most common method, H_2 is produced by electrolysis of water using electricity obtained from renewable energies, as the electricity used derived from 100% renewable resources, H_2 is produced without CO_2, regardless of the electrolysis technology chosen.

Gray hydrogen is obtained from fossil fuels and is the most extensively used method today. Natural gas is converted to H_2 and CO_2 (by steam reforming) during natural gas production by heating. CO_2 generated during production is generally not consumed or converted.

Blue hydrogen is a type of gray hydrogen, however CO_2 produced by carbon capture and storage technology is accumulated and stored underground. CO_2 produced during H_2 production thus not released to the atmosphere and H_2 production can be made in a climate-neutral fashion (Dinçer et al., 2021).

In hydrogen production techniques, achieving sustainable, clean, economical, and dependable energy systems is critical. More than 90% of worldwide hydrogen production is reliant on fossil fuels (Bičáková and Straka, 2012). The ultimate objective of decarbonization is to reduce this proportion and eliminate or minimize fossil fuel-related greenhouse gas emissions (Parra et al., 2019). Therefore, instead of fossil fuels, hydrogen should be produced by utilizing renewable energy sources for a sustainable future.

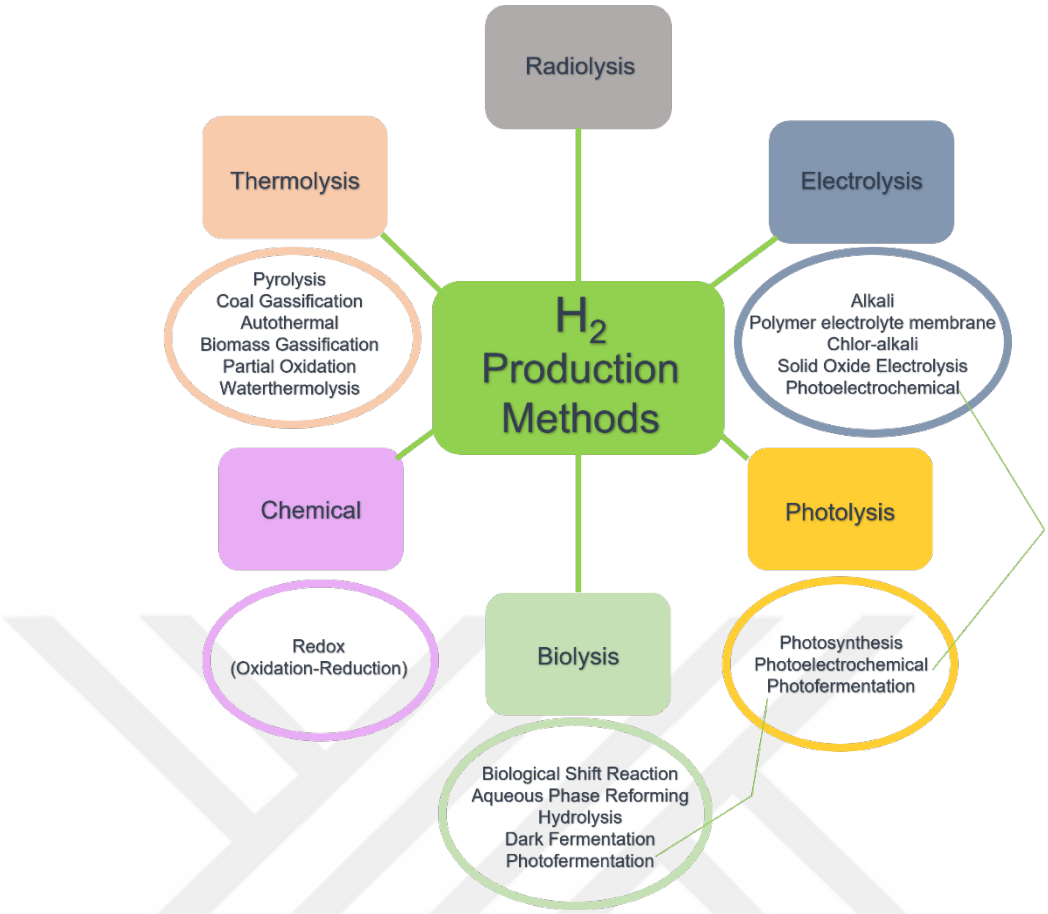


Figure 2. Hydrogen production methods and their classifications (Source: Oruc and Dincer, 2021)

1.2.1. Biological Hydrogen Production

Biohydrogen production is an important alternative to existing hydrogen energy technologies due to its renewable nature. Microbial hydrogen production is a habitual reaction occurred in many microorganisms taking place in oxygen-free environments.

Biohydrogen production can be carried out by four fundamental mechanisms: photo fermentation, direct biophotolysis, in-direct biophotolysis, dark fermentation (Hallenbeck and Benemann, 2002). Each of these mechanisms having their advantages and disadvantages and use different microorganisms, substrates and enzymes (Table 1).

Table 1. Biohydrogen production mechanisms, microorganisms used, reactions, catalyst enzymes, advantages and disadvantages (Source: Dinçer et al., 2021).

Mechanism	**Organism**	**Enzyme**	**Advantages**	**Disadvantages**
Photo fermentation	Purple non sulfur bacteria	Nitrogenase	H_2 is produced by solar energy and organic acids/wastes.	Bioreactor design challenges, low hydrogen production rates
Direct Biophotolysis	Green algae Cyanobacteria	[FeFe] Hydrogenase	Produced from water and sunlight	O_2 sensitivity Reactor design challenges Low yields of H_2
Indirect Biophotolysis	Cyanobacteria	[NiFe] Hydrogenase Nitrogenase	Heterocyst formation separates H_2 production from O_2 production	Reactor design challenges
Dark Fermentation	Anaerobic bacteria	Hydrogenase	High H_2 production rate Waste products and mixed cultures can be used Inexpensive Less maintenance	Low yields due to incomplete decomposition of organic matter

Photo fermentation is a method of producing H_2 using photons and biological sources. In low-nitrogen environments, photo-fermentative bacteria can produce hydrogen using nitrogenase enzyme and by utilizing organic acids, water, and light.

Direct biophotolysis method include regeneration of solar energy directly into hydrogen via photosynthetic processes in algae that involves separation of water molecules into hydrogen and oxygen. Hydrogenase enzyme acts as a catalyst in the conversion process of produced hydrogen ions into hydrogen gas (Chandrasekhar et al., 2015) (Kumar et al., 2019b).

Indirect biophotolysis refers to the mechanisms that produce hydrogen in two or three phases. In the first step, carbohydrates and oxygen are produced from water, CO_2 and solar energy. Cyanobacteria are employed in the second stage to produce hydrogen from carbohydrates, water and solar energy. Nitrogenase and hydrogenase enzymes found in these microorganisms can be used to produce H_2 (Acar et al., 2016).

Dark fermentation and production of H_2 occur in optimal conditions of temperature, pH, organic substrates and presence of anaerobic bacteria with hydrogenase enzyme (Kumar et al., 2019a). Organic acids, CO_2 and hydrogen can be produced when carbohydrates and water are utilized as substrates in fermentative processes (Acar et al., 2016).

Biohydrogen production by dark fermentative processes have gained significant attention as these processes have higher reported yields and production rates, lower energy requirements and various substrates and microorganisms can be applied (Saravanan et al., 2021).

1.2.2. Biological Water-Gas Shift Reaction for Hydrogen Production

In order to be fermentative biological hydrogen production to occur, microorganisms require carbon sources (organic and/or inorganic) to be used as a substrate and hydrogen production form carbohydrate-rich inputs is reported to be much higher than protein or fat-rich sources (Kim et al., 2013; Meher Kotay and Das, 2008).

Utilization of organic compounds by dark fermentation to produce biohydrogen is commonly applied in biotechnological processes, however use of inorganic carbon sources has not been studied extensively. Some anaerobic bacteria discovered to be successfully utilizing carbon monoxide (CO) as an inorganic carbon source, and this discovery has sparked an interest because as a result of this biological syngas conversion/fermentation, many added-value products such as biofuels (H_2 gas, ethanol, methane) and volatile fatty acids (acetic acid, propionic acid, butyric acid etc.) is produced (Henstra et al., 2007a).

Syngas or synthesis gas is a combination gas mostly made up of CO (30 to 60%), H_2 (25 to 30%), and CO_2 (5 to 15%) often produced from fossil fuels and conversion of resources such as biomass, sludges, coal, petroleum by thermochemical processes such as pyrolysis or gasification in high temperatures (Abubackar et al., 2016; Sipma et al., 2006; Sun et al., 2019).

CO-rich syngas is released from gasification and seen as a valuable reactant for bioprocesses. Although having toxicity effects, CO is a new and potential source to produce chemicals or fuels through a biological process called as water-gas shift reaction (WGSR) as utilization of CO is discovered to be achieved by a specific group of microorganisms called as carboxydotrophic microorganisms (Akhlaghi and Najafpour-Darzi, 2020; Phillips et al., 2017a).

The carbon present in CO can used as either carbon or energy source, or both. Consumption of H_2 as an energy source in the presence of CO and/or CO_2, can follow either the reductive acetyl-CoA or the Wood-Ljungdahl pathway to yield different chemicals or biofuels (Abubackar et al., 2019). Reductive acetyl-CoA pathway is evolved in strictly anaerobic conditions and this biochemical conversion pathway present in some bacteria and archaea was revealed by Wood and Ljungdahl (Ljungdahl and Wood, 1969; Munasinghe and Khanal, 2011). WGSR is defined in Eq. (1):

$$CO + H_2O \rightarrow CO_2 + H_2 \qquad \Delta G_0 = -20{,}1\ kJ\ mol^{-1} \quad (1)$$

In CO utilization, in addition to hydrogenogen microorganisms, acetogens can produce ethanol by using CO and H_2 as electron source and CO_2 as carbon source in the metabolic path traces that go through carbonyl western branch in the Wood-Ljungdahl pathway and consequently isopropanol, butyrate, butanol and polyhydroxy-butanol can be produced (Sun et al., 2019). Valuable industrial products such as acetate, formate, propionate, butyric acid and ethanol are reported to be produced by CO fermentation (Esquivel-elizondo et al., 2017; Henstra et al., 2007a; Köpke et al., 2011).

WGSR is a favorable production method of biohydrogen because the reaction occurs under ambient conditions with low energy requirements. Energy is captured by transfer of electrons form CO to H_2O and two types of enzymatic reactions take place in metabolisms of carboxydotrophic hydrogenogenic microorganisms.

Utilization of CO for the production of added-value products such as ethanol, acetate, butanol is proven to be more favorable from a standing point in thermodynamics when taking into consideration of the Gibbs free energies (ΔG°) compared to utilization of both CO and H_2 (Gunes, 2021). Syngas fermentation with utilization of only CO is spontaneous with negative values of ΔG° and production of ethanol from CO (-212.9 kJ/mol) is lower than ethanol production from CO_2 and H_2 (-79.1 kJ/mol) at thermophilic conditions (55°C) (Gunes, 2021; Phillips et al., 2017; Shen et al., 2018).

Hydrogenogenic carboxydotrophy metabolism yielding hydrogen production from CO is performed by enzyme complex of Ni-CODH/ECH and this enzymatic reaction takes place in anaerobic conditions as Ni-CODH enzyme catalyzes the reversible conversion of CO to CO_2 is sensitive to O_2 (Can et al., 2014; Fukuyama et al., 2020).

Two major enzymes are involved in the biological WGSR mechanism: [NiFe]-CO dehydrogenase (CODH), that converts CO to CO_2, and [NiFe]-hydrogenase, that generates H_2. CO is oxidized by CODH, releasing electrons that are required to reduce two protons to biohydrogen, which is catalyzed by the hydrogenase. Firstly, CO is converted to CO_2 through oxidation using carbon monoxide dehydrogenase (CODH) enzyme. Secondly, protons are reduced to H_2 with the help of first step, the oxidation step, and CODH-dependent hydrogenase enzyme (Figure 3) (Alfano and Cavazza, 2018; Munasinghe and Khanal, 2010; Sarangi and Nanda, 2020).

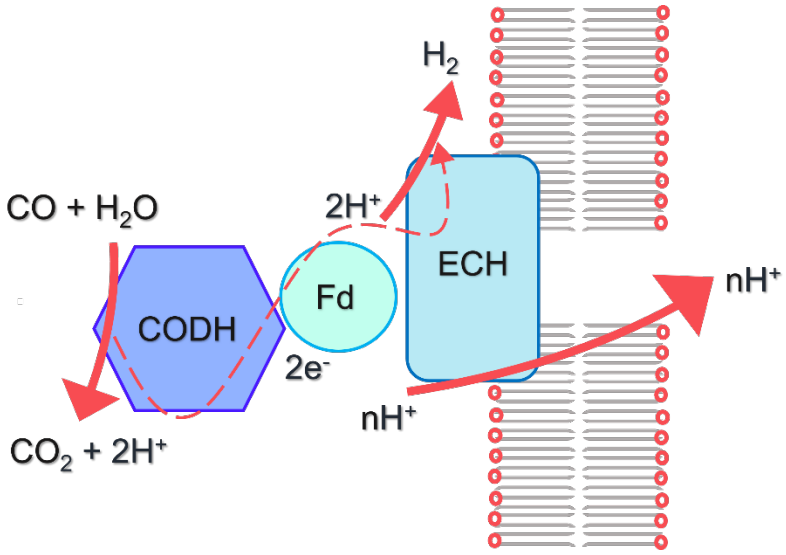


Figure 3. Water-Gas Shift Reaction and its schematic representation in carboxydotrophic hydrogenogenic microorganisms (CODH: CO dehydrogenase; Fd: ferredoxin (CooF); ECH: energy conserving hydrogenase (Source: Alfano and Cavazza, 2018).

As carbon monoxide is a toxic gas, harmful to the environment and released from gasification of fossil fuels. In many industrial activities conversion of this toxic waste gas with biotechnological processes to valuable products such as biofuels is a major action in terms of sustainable environmental development and have been studied by their potential biotechnological applications (Alfano and Cavazza, 2018; Diender et al., 2015a). Therefore, this thesis aims to do research on carboxydotrophic microorganisms and their biotechnological processes to be developed for industrial applications.

1.2.2. Carbon Monoxide Converting Microorganisms for Biohydrogen Production

As main components of syngas is CO, CO-utilizing carboxydotrophic microorganisms play an essential role in syngas (or CO) fermentation. Carboxydotrophic organisms are distinguished by their capacity to grow on CO and using it as a sole carbon and energy source. CO-utilizing carboxydotrophic microorganisms can be categorized into 4 main groups: methanogenic archaea, hydrogenogenic bacteria, sulfate-reducing bacteria and acetogenic bacteria (Henstra et al., 2007b).

Psychrophiles, mesophiles, thermophiles, and hyperthermophiles are four groups of microorganisms categorized based on their optimal growth temperature. Carboxydotrophic species that thrive in syngas/CO-rich gas mixtures are both mesophilic (37 to 40 °C) and thermophilic (55 to 80 °C). Carboxydotrophic microorganisms were mostly known as being mesophilic, but the numbers of novel thermophilic microorganisms discovered that can grow on CO have increased and furthermore these carboxydotrophic thermophiles are predominantly classified as carboxydotrophic hydrogenogenic microorganisms (Fukuyama et al., 2020).

Hydrogenogenic microorganisms can produce hydrogen using various substrates and carbon sources. Hence, carboxydotrophic hydrogenogens are able to oxidize CO and utilize H_2 by chemolithoautotrophic growth and through the conversion of CO and H_2O to H_2 and CO_2 in anaerobic conditions (Sipma et al., 2006; Sokolova et al., 2009). Anaerobic hydrogenogenic bacteria and purple non sulfur photosynthetic bacteria in the absence of a light source and under basic growth circumstances, are involved in the biological waste-gas shift process (Sarangi and Nanda, 2020). Thermophilic anaerobic bacteria are significantly important for the biohydrogen production processes performing Water-gas shift reaction as thermophilic carboxydotrophic hydrogenogens reported to be more abundant than mesophilic species, high temperatures can contribute to the increasing levels of gas diffusion.

Thereby both rapid supply of CO gas to the microorganism and elimination of H_2 accumulation is achieved, consequently promoting the hydrogenogenic CO metabolism, making thermophilic microorganisms superior biocatalysts for CO-dependent biohydrogen production than their mesophilic counterparts (Alfano and Cavazza, 2018; Fukuyama et al., 2020).

Among the thermophilic bacterium, the Firmicutes phylum compose of more than half of the hydrogenogenic and CO oxidizing species where optimal growth temperatures of these microorganisms range between 55-73 °C and most of them are strictly anaerobic (Fukuyama et al., 2020).

Even though the vast of known hydrogenogenic microorganisms are belong in the bacteria domain, some hyperthermophilic archaea have been reported to grow in hydrogenogenic CO oxidation conditions, such as Thermococcus onnurineus, a typical sulfur-reducing hyperthermophilic archaeon that has been demonstrated to use WGSR and employ the necessary metabolic pathway in its genome to use CO and to yield CO_2 and H_2 (Kochetkova et al., 2011; Lee et al., 2008; Sokolova et al., 2009).

Carboxydothermus hydrogenoformans was discovered and identified as the first carboxydotrophic hydrogenogen bacterium living in strictly anaerobic and thermophilic conditions, capable of oxidizing CO and producing H_2 (Svetlichny et al., 1991). This hydrogenogenic organism, its genome and metabolic pathways were extensively studied, as *C. hydrogenoformans* being an extremely thermophilic microorganism living in high temperatures and using CO as a sole carbon source, converting H_2O to H_2 in its metabolism and could be a model organism for biotechnological processes for enhancing biohydrogen production yields (Svetlichny et al., 1991; Svetlitchnyi et al., 2001; Wu et al., 2005).

Thermophilic, hydrogenogenic CO-utilizing microorganisms have been reported to be discovered and isolated from many different geographical habitats such as hot springs (Balk et al., 2009; Novikov et al., 2011; Parshina et al., 2005a; Slepova et al., 2006; Sokolova et al., 2009), marine sediments (Inoue et al., 2019; Yoneda et al., 2013), deep sea hydrothermal mats (Kim et al., 2001; Kozhevnikova et al., 2016; Sokolova, Jeanthon, et al., 2004), and soil (Mohr et al., 2018).

Investigations on natural habitats for the discovery of these microorganisms capable of grow on CO is still ongoing and 37 different isolates were reported to discovered in 2020 (Fukuyama et al., 2020). Frequency of CO oxidizing microorganisms in the environment and their dynamics as microbial communities are an appealing topic of investigation for understanding how a CO oxidizing and H_2 producing microbial consortia is shaped in nature.

1.3. Microbial Communities and Mixed Cultures for Biohydrogen Production

Microbial communities and their utilization in dark fermentative biohydrogen production is a crucial part of the fermentation processes as the biocatalytic actions of consortia reported to achieve efficient biohydrogen yields. When compared with a pure culture, in spite of a competition between strains, mixed cultures offer some advantages such as being economically viable, easy to obtain and naturally diverse in terms of containing various microorganisms able to coexist (Levin et al., 2004; Parshina et al., 2005a; Sarangi and Nanda, 2020). In the matter of CO conversion to H_2 investigations, pure or co-cultures are dominantly studied compared to mixed cultures. However, investigations on mixed cultures could potentially play an important role in the discovery of new CO-oxidizing species and their diversity in microbial communities (Kochetkova et al., 2011).

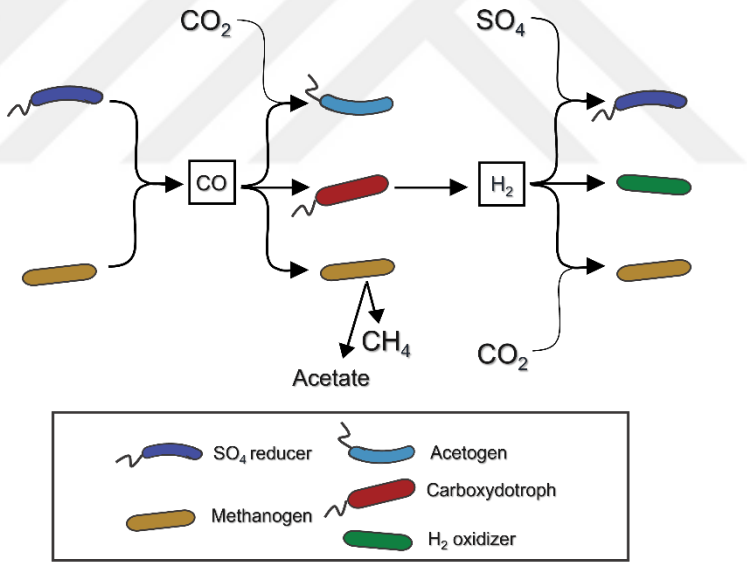


Figure 4. Schematic representation of carboxydotrophic microbial communities (Source: Techtmann et al., 2009)

In Figure 4, mechanism of carboxydotrophy and metabolic trade between different molecules in a mixed consortia consisting of a variety of microorganisms is schematized.

Sulfate reducers and methanogens can be used to produce CO, which can then be used to promote acetogenesis, methanogenesis, or carboxydotrophy hydrogenogenic activities. CO utilizing species such as acetogens can synthesize acetate by occurrence of CO dehydrogenase in acetogens. Acetate, methane and H_2 are all produced as a result of this process, all playing key roles in thermophilic ecosystems (Techtmann et al., 2009).

Wood/Ljungdahl pathway involves the synthesis of acetate with two different branches: methyl and carbonyl. Both branches reduce CO_2 into acetate and biomass through acetyl-CoA pathway. Fixation of CO_2 to the methyl and carbonyl level is achieved by reducing equivalents derived from various substrates such as organic substrates (ethanol and glucose) and inorganic (CO and H_2). Methanogens and sulfate-reducers also employ the acetyl-CoA pathway, thus acetyl-CoA pathway and involved microorganisms in this pathway are highly valuable in carbon cycling. Microbial consortia can use the CO_2 released by WGSR mechanism of microorganisms, H_2 production by WGSR also consequently results in the production of CO_2 which then be cycled in the microbial consortium's mechanism (Drake et al., 1997; Schiel-Bengelsdorf and Dürre, 2012).

Acetate, CO2 and H_2 can be consumed by methanogenic archaea to CH_4 and presence of these methanogens can lower the hydrogen production yields in a microbial consortium. This can be prevented by blocking the methanogenesis with pre-treatments such as heat, chemical and aeration-based strategies (Pachapur et al., 2019). CO-oxidizing species reported to be protecting other community members from toxic effects of CO, even if the CO concentration in the headspace reaches 100% (Techtmann et al., 2009). Once carboxydotrophs paired with sulfate reducing microorganisms, a metabolic occurrence called as cross-feeding is observed. Carboxydotrophic microorganisms oxidize CO and eliminate the toxicity effect against CO-sensitive organisms and produce H_2. Hydrogen can be used by other H_2 oxidizers as a substrate and can be removed by sulfate reduction by SO_4 reducer microorganisms, consequently decreasing the inhibition caused by end-product formation (Parshina et al., 2005a; Techtmann et al., 2009).

Microbial consortia in their natural habitats considered to be reflecting the most authentic reference between microbial dynamics, symbiosis and relations in microbial communities containing carboxydotrophic hydrogenogenic microorganisms still needed to be enlightened. For this purpose, the investigations on microbial consortia must be carried out using microbiological and molecular tools.

1.3.1. Hot Spring Microbial Community Dynamics on Biohydrogen Production

Hot springs, where geothermally heated water is released from cracks in the Earth's crust, have been found to be home to a diverse range of microorganisms known as thermophiles, which can survive and even flourish in extreme temperatures. Studies conducted at Yellowstone National Park hot springs (Brock, 1967) pioneered hot spring microbial community investigations (Omae et al., 2019).

Gasification processes occurring at high temperatures utilizes syngas and consequently CO gas found naturally at hot environments, such as hot springs. Investigation of hot springs for reaching further knowledge regarding the microbiology of CO-conversion can be obtained through the genomic investigations of these habitats. Very limited amount of information on ecology of carboxydotroph hydrogenogens are reported to this date, as these microorganisms are low in abundance in thermal waters compared to other microbial community members (Table 2). Sensitive identification studies using genomic tools such as metagenomic analysis are needed to better understand the ecological function, distribution and diversity of thermophilic hydrogenogenic carboxydotrophs in their natural habitats, hot springs.

Table 2. Thermophilic carboxydotrophic hydrogenogenic organisms isolated from hot springs (Source: Alfano and Cavazza, 2018; Fukuyama et al., 2020)

Organism	**Domain**	**Temperature °C**	**Reference**
Carboxydothermus hydrogenoformans	Bacteria/Firmicutes	70	(Svetlichny et al., 1991)
Thermincola carboxydiphila 2204	Bacteria/Firmicutes	55	(Sokolova et al., 2005)
Thermincola ferriacetica	Bacteria/Firmicutes	60	(Zavarzina et al., 2007)
Thermosinus carboxydivorans Nor1	Bacteria/Firmicutes	60	(Sokolova, González, et al., 2004)
Carboxydocella sporoproducens DSM 16521	Bacteria/Firmicutes	60	(Slepova et al., 2006)
Thermosinus carboxydivorans R1	Bacteria/Firmicutes	73	(Sokolova et al., 2007)
Caldanaerobacter subterraneus subsp. pacificus DSM 12653	Bacteria/Firmicutes	70	(Sokolova et al., 2001)
Carboxydothermus islandicus SET	Bacteria/Firmicutes	65	(Novikov et al., 2011)
Carboxydothermus pertinax Ug1	Bacteria/Firmicutes	65	(Yoneda et al., 2012)
Carboxydothermus siderophilus 1315	Bacteria/Firmicutes	65	(Slepova et al., 2009)
Dictyoglomus carboxydivorans	Bacteria/Dictyoglomi	80	(Sokolova et al., 2009)
Thermofilum carboxyditrophus 1505	Archaea/Crenarchaeota	90	(Sokolova et al., 2009)

1.3.2. 16S rRNA Gene Sequencing for Microbial Community Analysis

Metagenomics is the examination of detection of all genes present in an organism and their structures, functions and expressions. Metagenomics' main goal is to investigate microorganisms and their functions, distribution and diversity in the course of their community by sequencing, or arraying, genetic fragments obtained from the complete microbiome at the same time (Handelsmanl et al., 1998; Horgan and Kenny, 2011; Latorre-Pérez et al., 2021)

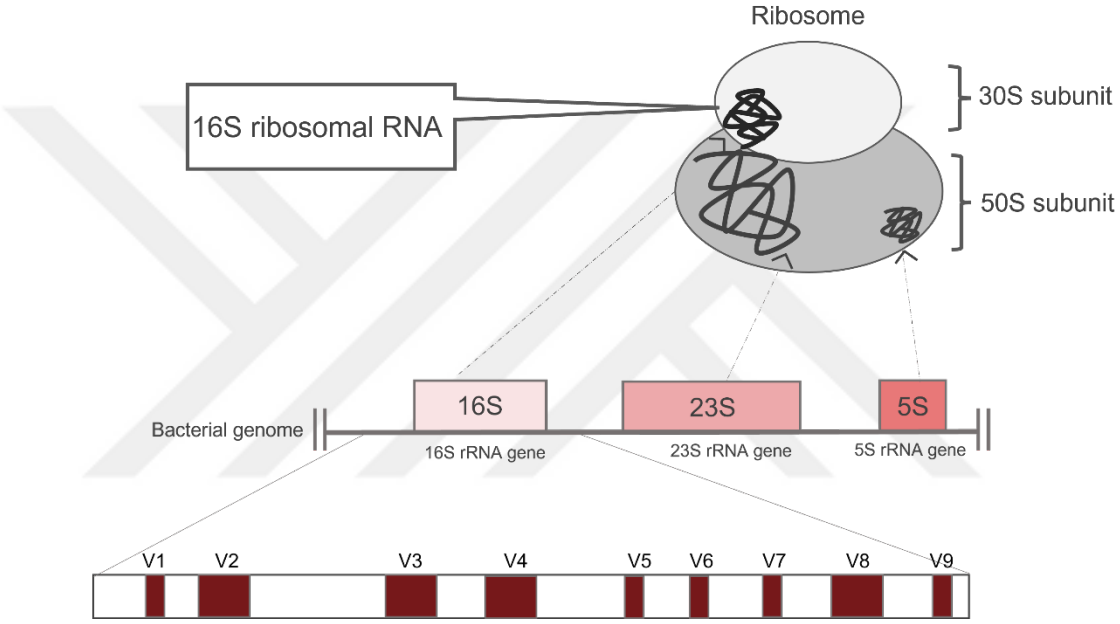


Figure 5. Schematic representation of 16S rRNA gene and ribosomal complex. White boxes indicate conserved regions and burgundy boxes indicate hypervariable regions (Source: Fukuda et al., 2016)

Ribosome, playing an important role in protein synthesis as a cell component, is composed of proteins and RNA subunits (30S ribosomal subunit,50S ribosomal subunit). Each subunit containing ribosomal RNAs that encoding genes; 16s rRNA in 30S subunit, 23S and 5S rRNA in 50S ribosomal subunit. The 16S rRNA gene (~1600 nucleotides) generates a 30S ribosomal RNA molecule, which is found in all prokaryotic cells and contain nine hypervariable regions (V1–V9) that show significant sequence variability among bacteria (Figure 5).

Meanwhile the 16S gene is separated by constant regions called as "housekeeping genes" that are reported to remain mostly conserved over the time of the evolutionary process of bacteria and archaea. Conserved 16s rRNA genes enable identification and classification of bacteria and archaea in complex biological samples, such as an environmental sample. (Fukuda et al., 2016; Olsen et al., 1986; Woese et al., 1990).

Phylogenetic characterization of a microbial community based on 16S rRNA amplification and comparison with known 16S rRNA sequences from organisms firstly introduced 16s rRNA sequencing (Schmidt et al., 1991). Later on, this approach is used in a study by(Barns et al., 1994) on hot spring microbial communities, the first reported metagenomic analysis of an extreme temperature environment based on 16S rRNA gene was conducted in Yellowstone National Park, USA (Panda et al., 2018). With the help of newly developed long-read sequencing platforms, 16S region can be covered by all V1-V9 hypervariable regions.

1.4. DNA Sequencing Platforms

Sequencing technologies have radically revolutionized metagenomics research since their discovery in 1997, delivering crucial benefits including as resolution, accuracy, and speed. Sanger sequencing based on chain termination method was developed as a first DNA sequencing technique in 1977 (Sanger et al., 1977), dominantly used for clinical diagnosis purposes. Due time-consuming nature and high costs of this technique, DNA sequencing technology was not very widespread (De Cario et al., 2020; Zheng et al., 2019).

In 2005, with the introduction of next-generation sequencing (NGS) technologies to the market that are able to sequence genomic material with lower costs and faster workflows, the genomic sampling of uncultured microorganisms had been made possible and our understanding of microbial diversity was improved significantly. The constant development of sequencing platforms enabled DNA sequencing to be more available with reduced costs and greater resolutions over the time and opened up a new understanding for the discovery and monitorization of novel species from environmental samples (Panda et al., 2018).

In 2005 the first NGS platform, Roche's (Basel, Switzerland) 454 pyrosequencing, was launched followed up with development of Illumina (San Diego, USA) and Ion Torrent™ (Thermo Fisher, USA). These technologies are classified as second-generation technologies. In 2012, a high throughput and long read platform was introduced by Pacific Biosciences (PacBio®, Menlo Park, CA, USA), being the first third-generation sequencing platform, following the launch of Oxford Nanopore Technologies MinION sequencer (Oxford Nanopore Technologies, London, UK) in 2014 (White et al., 2016).

Table 3. Classification of sequencing technologies (Source: PacBio, 2021).

Sequencing Technologies		
Short-read sequencing		Long-read sequencing
First Generation (500-1000 bp fragments)	Second Generation (5-500 bp fragments)	Third Generation (10-30 kb fragments)
Sanger Sequencing	Roche 454	Pacific Biosciences
Maxam and Gilbert	Illumina	**Oxford Nanopore Technologies**
	Ion Torrent	

Sequencing technologies are mainly classified for their sequencing length in base pairs (bp) (Table 3). Long-read sequencing platforms can sequence more than 1500 base pairs and are able to cover the full-length 16S region. In contrast, short-read sequencing platforms can only sequence 500 bp or less (~300 bp), only partially covering the 16S rRNA gene. Short read platforms issued low resolution of species level identification and misclassifications as their 300 bp readings generally targets the partial variable areas, the V3-V4 region of 16S rRNA gene, which is reported to show similarity between microorganisms, establishing the long read platforms superior in terms of taxonomical resolution and identification of prokaryotes (Jeong et al., 2021).

Each DNA sequencing platforms having their advantages and disadvantages, their strengths can be implemented to their suited application areas. Microbial community analysis using 16S rRNA gene require long-read sequencing platforms that can cover the complete 16S region for better taxonomical classification and functional profiling of uncultured organisms.

1.4.1 Nanopore Sequencing

Oxford Nanopore MinION® technology (ONT), is a sequencing method performed using the deviations in the electric current that emerges specific to each nucleotide as a single DNA sequence passes through a protein nanopore. MinION® sequencer, the product using the nanopore sequencing developed in 2014, containing up to 2,048 nanopores, is the smallest sequence analysis device and a molecular tool available today that can be applied even in field applications, with only size of a USB flash, weighs less than 450 g with 10x3x2 cm dimensions (Figure 6) (Bayley, 2015).

Figure 6. The Oxford Nanopore Technologies MinION sequencer (Source: Oxford Nanopore Technologies, 2022)

In Oxford Nanopore MinION® technology (ONT), contrasting many other technologies, sequencing depends on direct detection and sequencing of a single-stranded DNA (ssDNA) molecule with distinctive electrical current changes of bases, rather than nucleotide incorporation (Buck et al., 2017; Weirather et al., 2017).

DNA sequencing with nanopore sequencing distinguishes from other NGS and TGS platforms as it enables on-field applications with rapid library preparation and real-time acquisition of genomic data alongside being cost efficient (Charalampous et al., 2019).

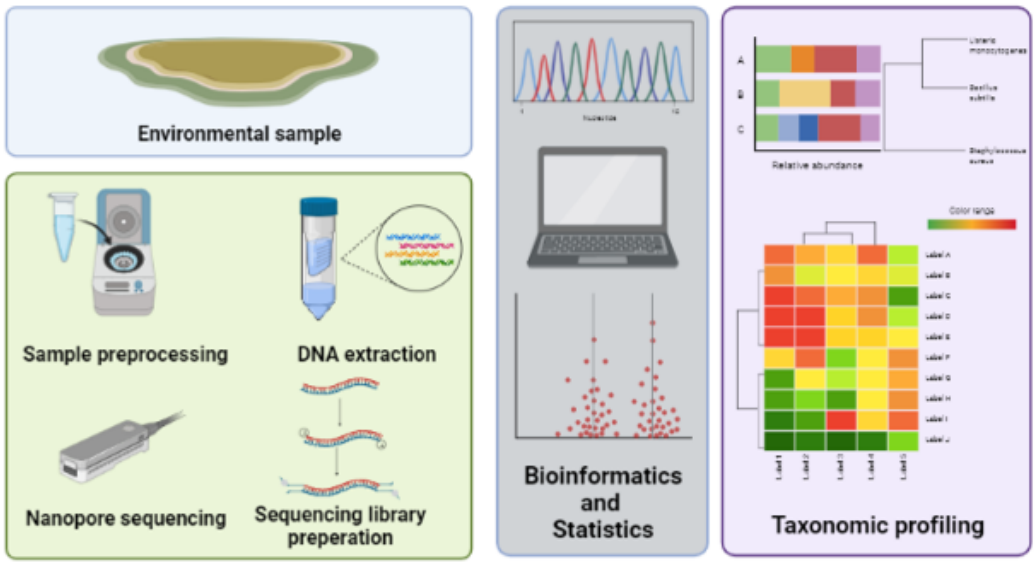


Figure 7. Workflow for nanopore sequencing of environmental samples. Created with BioRender (Source: Latorre-Pérez et al., 2021)

As schematized in Figure 7, MinION workflow demonstrated to be suitable to the essence of environmental microbial community analysis as being portable with simplified workflows and can be used on-site applications with a single computer requiring no heavy laboratory equipment (Ciuffreda et al., 2021; Oliva et al., 2020). Nanopore sequencing have been reported in the literature to be employed in environmental microbial community studies (Reddington et al., 2020; Samson et al., 2019; Urban et al., 2021) and considered a powerful tool for screening, taxonomically identifying microbial community members.

Nanopore sequencing has become an appealing and ever-growing tool for real-time in-field genomic characterization of complex environmental samples, improving the detection of unknown organisms, owing it to the development of rapid protocols and analysis pipelines for metagenomic profiling (Akaçin et al., 2022).

1.5. Statistical Experimental Design

For many years, statistical experimental procedures have been widely employed for optimization strategies such as experiment screening or studying optimal operational parameters on production. Optimization of operating conditions and parameters (one-factor-at-a-time) has been widely utilized to determine the influence of a single element and to describe how individual factors interact. Using response surface approach, different research has been undertaken to determine the interaction between distinct individual components (RSM). One of the most often used design for fitting response surfaces is Box Behnken Response Surface Methodology or Box Behnken Design (BBD) (Ferreira et al., 2007).

BBD statistical design require 3 layers of each component (high, medium and low) and is a rotatable or nearly rotational, useful technique for three-level complete factorial designs. BBD is an efficient tool for 3-level full factorial designs as response calculations can be mode at intermediate levels that have not been experimentally studied and represents the interactive effects of different operational parameters on the response by developing a quadratic model and performing statistical significancy tests to check the model's adequacy with ANOVA (Patel et al., 2016; Sekoai, 2016).

BBD can be an effective tool for the optimization of biological processes where various operational parameters directly effects the microorganism growth and production yields such as substrate content, pH, temperature. Thus, implementation of statistical design to the biotechnological studies is vital for improving production yields.

1.6. The aim of study

In this thesis, it is aimed to perform metagenomic microbial community analysis of hot springs located in Izmir region with 16S rRNA sequencing using a third-generation sequencing technology, Oxford Nanopore Technologies' MinION device.

Hot springs' microbial community diversity and their capacity of hosting hydrogen producer thermophiles able to perform CO fermentation was studied and established. Isolates from hot springs were tested for growth in anaerobic and thermophilic medium with a 100% CO feeding followed by sequencing DNA.

CO gas was used as a carbon and electron source by putative carboxydotrophic hydrogenogenic microorganisms isolated from hot springs to produce H_2 utilizing WGSR with carbon monoxide dihydrogenase and hydrogenase enzymes.

Following the batch productions and subculturing in anaerobic and thermophilic conditions, two different Box-Behnken statistical experimental designs with 3 independent variables in 3 levels were implemented to increase the production efficiency of mixed culture thermophilic hydrogenogenic microorganisms.

CHAPTER 2: METHODS

2.1. Sampling and Cultivation of the Hot Spring Isolates

2.1.1. Sampling Procedure

5 different hot springs were selected as sampling points: Doğanbey, Çeşme, Dikili Bademli, Dikili Nebiler and Bergama hot springs. Hot springs were visited from the dates of 25/05/2021 to 23/08/2021 for sampling. Thermal water samples were collected with 2 bottles of 500 mL and 2 bottles of 250 mL from selected points using sealed and autoclavable plastic bottles and transferred to the laboratory in a heat-insulated container. pH, temperature and ORP measurements were made from each collection point (Table 16) with a portable pH meter (MW 105 Max, Milwaukee, USA). The samples were placed in thermal bags to maintain their temperature and taken to the laboratory for experiments.

The collected hot spring water samples were used in two different workflows:

a) DNA isolation was performed with the hot spring water samples (5 L of volume) without any enrichment and stored at -20°C for 16S rRNA analysis (See section 2.3.2.1. DNA Isolation Using IndiSpin Pathogen Kit)

b) the collected samples were centrifuged, and the collected pellet was transferred to the anaerobic and thermophilic enrichment medium with 10% inoculation (See section 2.1.1. Cultivation Procedure). After the serial cultivation and analysis of the cultures, DNA isolation was performed and stored at -20°C for 16S rRNA analysis (See section 2.3.2.1. DNA Isolation Using GeneJET Genomic DNA Purification Kit).

2.1.2. Cultivation of Hot Spring Isolates

Enrichment basal culture medium was prepared and used to propagate anaerobic carboxydotrophic cultures. Trace elements to be used in the environment have been determined to create a highly selective environment and increase the efficiency of CO oxidization (Meyer and Schlegel, 1983). Accordingly, the contents of macronutrients, micronutrients and vitamins for a 1 L of growth medium was prepared as specified in Table 4 (Sokolova et al., 2002).

pH of the nutrient medium was fixed to 6.8 with 0.5 M HCl and 54 mL of prepared basal medium was transferred to glass bottles with a total volume of 100 mL, and the bottles were closed with stoppers and metal caps. O_2 in the headspace of the closed bottles was removed with N_2 gas. Then, sterilization of medium containing cultivation bottles was carried out in autoclave at 121°C and 15 minutes.

Table 4. Medium and contents for the enrichment and isolation of thermophilic, anaerobic and carboxydotrophic microorganisms per 1 L of growth medium

Macronutrients	**Micronutrients**	**Vitamins**
1.0 g NH_4Cl	1.5 g nitrilotriacetic acid	2.0 mg biotin
0.33 g $MgCl_{2.6}H_2O$	3.0 g $MgSO_4$	2.0 mg folic acid
0.1 g $CaCl_2.{}_6H_2O$	0.5 g $MnSO_4$	2.0 mg pyrotoxin hydrochloride
0.33 g KCl	1.0 g NaCl	10.0 mg riboflavin
0.5 g KH_2PO_4	0.1 g $FeSO_4$	5.0 mg thiamine
0.001 g resazurin	0.1 g $CaCl_2$	5.0 mg nicotinic acid
0.5 g $NaHCO_3$	0.1 g $CoCl_2$	5.0 mg pantetoic acid
1.0 g $Na_2S.{}_9H_2O$	0.1 g $ZnSO_4$	0.1 mg of vitamin B12
1 g yeast extract	0.01 g $CuSO_4$	5.0 mg of p-aminobenzoic acid
	0.01 g $AlK(SO_4)_2$	5.0 mg of thioctic acid
	0.01 g H_3BO_3	
	0.01 g Na_2MoO_4	

After the samples were brought to the laboratory, they were taken into sterile 50 mL falcon tubes and centrifuged at 1398 x *g* for 10 minutes (Hanil Science Industrial, South Korea) and pellets were obtained. First, 0.1 mL of 10x vitamin solution was added to 100 mL airtight and sealed vials containing 54 mL of sterile anaerobic thermophilic medium (Table 4), and 6 mL (10%) collected pellet was inoculated. Inoculated bottles were labeled with the name of the sampling point, the incubation temperature and date. Bottles were fed with a 100% CO gas for 30 seconds. After the bottles were inoculated, they were placed in an incubator with a temperature appropriate to their isolation temperature (45, 55, 60 or 65 °C).

2.2. Characterization of the Hydrogen Producing Cultures

2.2.1. Subculturing

Subculturing of cultures was conducted by inoculating 6 mL of culture into freshly prepared medium with 100% CO feeding for 30 seconds. GC analysis was carried out with the headspace gas of cultivated bottles for H_2 production and CO consumption along with spectrophotometric analysis at OD_{600nm} for verification of microbial growth and HPLC analysis for by-product formation daily.

2.2.2. Scanning Electron Microscopy (SEM) Imaging

Along 5 different hot springs, best hydrogen producing isolates Çeşme, Doğanbey and Bergama mixed cultures were screened using Scanning Electron Microscopy (SEM) (FEI Quanta 250 FEG Philips, Netherlands) imaging for the detailed examination of their size and shape.

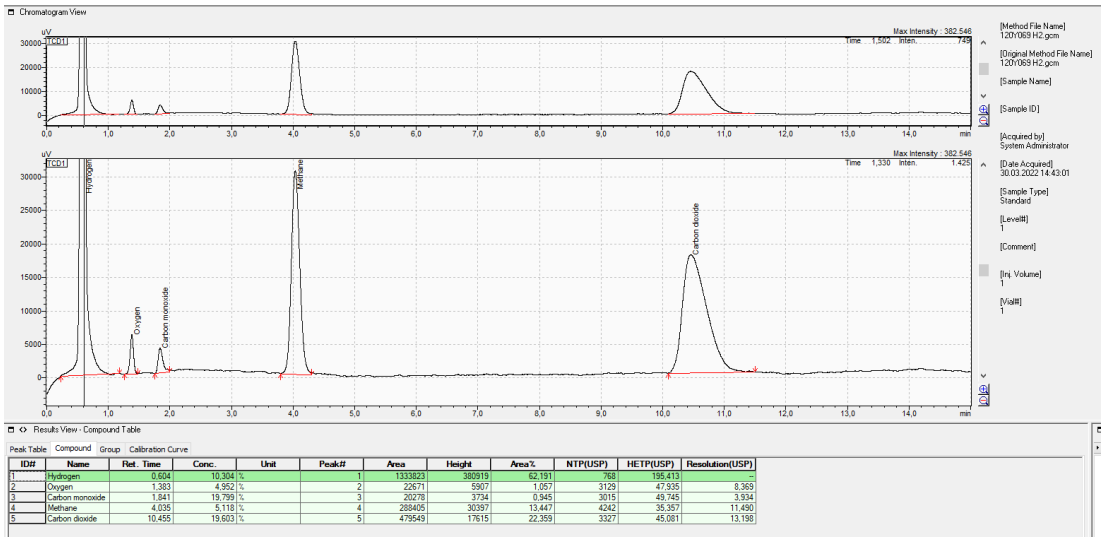
15 mL of liquid culture were transferred to sterile falcon tubes and then centrifuged 1789 x *g* for 10 minutes, supernatant was discarded. The pellet was suspended in a phosphate buffered saline (PBS) solution (4,43 g NaCl, 0,546 g of Na_2HPO_4 and 0.138 g of NaH_2PO_4 in 500 mL of UP water) with a pH of 7.5 and centrifuged (1789 x *g* for 10 min). Supernatant was discarded and the pellet re-suspended in 2.5% (v/v) formaldehyde solution for 1 h for cell fixation.

Following the formaldehyde fixation, the falcon tubes were centrifuged again (1789 x *g* for 10 min) and supernatant was discarded. The pellet was re-suspended with PBS and centrifuged again in (1789 g for 10 minutes) for the final pellet collection. Obtained pellet was fixed by dropping final pellet on to glass slides and the matte side of a piece of aluminum foil and air dried in an incubator with a temperature of 37°C. For SEM imaging, samples were prepared with a coating of gold particles and examined.

2.3. Analytical Methods

2.3.1. Analysis of Gaseous Products Using Gas Chromatography

Headspace gas samples of cultivated bottles were analyzed daily by a gas chromatography (Shimadzu, Nexis GC-2030, Japan) with a thermal conductivity detector (TCD) equipped with a Wide Bore Injector (WBI) and a Restek ShinCarbon ST 100/120 packed column using a 100 mL glass syringe (Sanitex, Turkey). Nitrogen was the carrier gas. The oven, injector and detector temperatures were 60, 150 and 150 °C, respectively. GC was calibrated accordingly using a calibration gas mixture containing hydrogen (5%), oxygen (5%), carbon monoxide (10%), methane (5%), carbon dioxide (20%) and nitrogen (40%) (Figure 8).



ID#	Name	Ret. Time	Conc.	Unit	Peak#	Area	Height	Area%	NTP(USP)	HETP(USP)	Resolution(USP)
1	Hydrogen	0,604	10,304	%	1	1333823	380919	62,191	768	195,413	--
2	Oxygen	1,383	4,952	%	2	22671	5907	1,057	3129	47,935	8,369
3	Carbon monoxide	1,841	19,799	%	3	20278	3734	0,945	3015	49,745	3,934
4	Methane	4,035	5,118	%	4	288405	30397	13,447	4242	35,357	11,490
5	Carbon dioxide	10,455	19,603	%	5	479549	17615	22,359	3327	45,081	13,198

Figure 8. Calibration peaks for hydrogen, oxygen, carbon monoxide, methane and carbon dioxide gasses in gas chromatography

Cultivated bottles were analyzed by GC firstly for 0^{th} hour analysis for calculating CO percentage in the headspace of the bottle, and GC analysis was carried out daily for CO consumption and H_2 and CO_2 production, verifying the water-gas shift reaction.

2.3.2. Analysis of Microbial Growth Using UV-Visible Spectrophotometer

Monitoring the growth of microbial population was analyzed with a UV-Vis spectrophotometer Lambda 750 (Perkin Elmer, USA) and optical density at 600 nm measurement. Culture samples were taken into quartz cuvettes and growth medium was used as a blank sample.

2.3.3. Analysis of Volatile Fatty Acid and Ethanol Production Using High Performance Liquid Chromatography

Volatile fatty acids (VFA) including acetic acid, butyric acid, formic acid, propionic acid, and ethanol and their concentrations were determined by High Pressure Liquid Chromatography (HPLC) (Thermo Fisher Scientific, USA). Analysis was conducted with a refractive index detector (RID), an autosampler, and a HyperREZ XP 8 µm, 300 x 7.7 mm column (Thermo Fisher Scientific, USA). Ultra-pure water was used as mobile phase at a flow rate of 0.6 mL min^{-1}, column temperature set at 85 °C. Processing of analytical data carried out using the Chromeleon™ 7 Chromatography Data System software (Intelligent Tech, Inc., USA).

The samples collected from the medium on the days of 0,3,7 and 10 were transferred to 1.5 mL eppendorfs to precipitate the pellet inside and centrifuged at 9358 x *g* for 5 minutes and the supernatant was taken. The collected supernatant was filtered with a 0.22 µm syringe tip PTFE HP syringe filter (GVS Filter Technology, USA) and placed in the HPLC device for analysis.

2.4. Molecular Methods

2.4.1. 16s rRNA Archaea Primer Design

Archaea primers were specifically designed for PCR amplification of 16S rRNA regions of thermophilic archaea that are likely to be isolated from the Izmir province. Thermophilic and anaerobic archaea species reported in hot spring microbial community studies were screened and selected from literature investigations. Archaea sequences were downloaded from NCBI database in FASTA format (Table 5).

Table 5. Archaeal species that are likely to be found in thermal waters in and around İzmir, living at a temperature of 50-65 ˚C, around pH 6.00-7.00

Organism	**Accession Code**	**Reference**
Thermococcus barophilus	NC_014804	(Omae et al., 2019)
Thermococcus guaymasensis DSM11113	NZ_CP007140	
Thermococcus onnurineus NA1	NC_011529	
Thermococcus paralvinellae	NZ_CP006965	
Thermofilum adornatum	NC_022093	
Methanothermobacter thermautotrophicus	NC_000916	
Methanomethylovorans thermophila strain L2FAW	NR_043089	(Chan et al., 2015)
Methanomassiliicoccus luminyensis	HQ896499	
Methanococcus aeolicus	(NC_009635)	
Methanocaldococcus jannaschii	NC_000909	(Sokolova et al., 2009)
Archaeoglobus fulgidus	NZ_CP006577	
Thermosphaera aggregans	NC_014160	(Canganella and Wiegel, 2014)
Methanocaldococcus vulcanius	NC_013407	
Methanocella conradii	NC_017034	
Methanothermobacter marburgensis	NC_014408	(Wagner and Wiegel, 2018)
Methanosarcina thermophila	NZ_CP009501	

In order to detect potential species, archaea species and their entire genomes were combined on the same text file. *Thermococcus celer*, a thermophilic archaeal species isolated from a hot spring and its 16s rRNA partial sequence was selected as an exemplary (Barns et al., 1994). Archaeal sequence (NCBI Reference Sequence: NR_113295.1) and the FASTA file was downloaded via NCBI.

For the alignment the 16S rRNA sequence (query) and the merged archaeal sequences (subject) via BLAST+, a Python code was written to give only the accession codes of the merged archaeal sequences. The software printed the access codes of the archaea and codes were also given to BLAST+, to load archaea sequences from its own database.

The command 'blastn -query ".\Methanocaldococcus 16S.txt" -subject Merged_archaea.fasta -outfmt 7 > output.tsv' entered to the BLAST+ software Tabular with comment lines has been selected as a formatting option to save the results as output.tsv format in an Excel file. The start and end of the sequences in common to the 16s sequence and the archaea sequence in the tsv file were determined as the query start (q.start) and end points (q.end).

A Python called SeqExtractor was coded, to detect the archaea name and sequences of from the 'merged_archaea.fasta' file which initially given in the Python code to detect the 8th (query start) and 9th (query end) rows in the query table in tsv file given by the BLAST software. Query start and end points from the archaea sequences were extracted and printed as alignments. Archaea accession codes and alignment sequences were recorded in a file named '16ssequences.fasta'. These sequences were aligned with the EUGENE Multiple Sequence Alignment software. Some sequences were found to be inverted (sequences starting with T…), otherwise the sequences were very similar and aligned.

16S sequences were also aligned with the ClustalX Multiple Sequence Alignment software (Figure 11). Reverse complementary sequences of reversed sequences were obtained and reverse complementary sequences were loaded into the ClustalX software together with the sequences and realigned (Figure 11). Forward and reverse archaeal primers were determined according to the obtained alignment results (Table 19).

Table 6. Universal 16S rRNA Bacterial primers

Universal Bacteria Primers	
Tailed-27F: 5'TTTCTGTTGGTGCTGATATTGCA GAGTTTGATCMTGGCTCAG 3'	**Tailed-1492R:** 5'ACTTGCCTGTCGCTCTATCTTC CGGTTACCTTGTTACGACTT 3'

Universal 16S rRNA bacterial sequences were sourced by the company Nucleus Genetik Urunleri A.S. and 16S primers were manufactured and supplied by the company Sentebiolab Biyoteknoloji (Table 6). In order to obtain the same T_m (melting temperature) value of the primers to perform PCR under the same conditions as the primers to be used in bacteria, the T_m value of the specific bacterial primers was first found on the website (NEB T_m Calculator, 2022) and the Archaea primer sequences were shortened accordingly (Table 19).

2.4.2. DNA Isolation

DNA Isolation of thermal waters collected from hot springs was performed using the IndiSpin Pathogen Kit (Indical Bioscience GmbH, Germany), a DNA isolation kit suitable to be used for environmental samples. 5 L of thermal water samples collected from each region were isolated for DNA and stored for future analysis. IndiSpin DNA isolation kit was used for thermal water samples collected from hot springs. Isolation steps were performed according to the manufacturer's instructions:

First, the solutions in the kit were prepared: Isopropanol was added to Buffer ABC, 40 mL of 99.5% ethanol to Buffer AW2, 25 mL of 99.5% ethanol to Buffer AW1 and homogenized. 20 µl of Proteinase K to break down proteins and Buffer VXL (30%-50% guanidine hydrochloride, 1%–10% t-Octylphenoxypolyethoxyethanol (Trixon-100)) used to lyse cells were added onto 200 µl filtered pellet. Pulse vortexing was performed and the sample was kept at room temperature for 15 minutes of contact time.

The droplets on the surface were removed by centrifugation at 1497 x *g* for 3 seconds. 350 µL of Buffer ACB was added to ensure optimal binding of the nucleic acids in the medium to the silica membrane. Lysates, which were vortexed by flickering, were transferred to collection tubes with a spin column, which allowed the DNA to attach but did not allow the adhesion of factors such as unwanted nucleic acids and salts that could cause negative effects in the PCR process. Eppendorfs were centrifuged at 5989 x *g* for 1 min to remove unwanted fractions.

The supernatant was poured and 600 µL of Buffer AW1 was added onto the collection tube to denature the proteins in the column and allowed them to separate from the column, and centrifuged at 5989 x *g* for 1 min. The supernatant was discarded and 600 µL of Buffer AW2 was added to wash out the salts present in the column and centrifuged at 5989 x *g* for 1 min. The supernatant was discarded, and the column was centrifuged at 18341 x *g* for 2 min to remove residual liquids and to better isolate the DNA. 100 µL of Buffer AVE was added and centrifuged at 18341 x *g* for 1 min to separate and collect the DNA bound to the column. The obtained DNA samples were labeled and stored at -20 °C.

DNA Isolation of cultivated samples were performed using GeneJET Genomic DNA Isolation Kit (ThermoFisher Scientific, US) which is a convenient DNA extraction kit for cultivated samples. Hydrogen production observed from the mixed cultures established from the samples taken from the hot springs in each region were determined and GC analyzes were carried out regularly. DNA isolation was performed from hydrogen-producing cultures.

In the DNA isolation kit procedure, it was stated that up to $2x10^9$ cells should be obtained for isolation (OD_{600} of 1.0 = 8 x 10^8 cells/mL). OD600 values were calculated using a calculator designed according to microorganisms based on *E. coli* culture (Agilent OD Bacterial Calculator, 2022). As seen in Table 7, cell amounts were sufficient for isolation and DNA isolations were performed accordingly.

For the DNA isolation of gram-negative bacteria, cultures in 2 mL volume obtained were centrifuged at 5000 x *g* for 10 minutes. 180 µL of Digestion Solution, that is included in the kit and known to lyse cells, was added to the pellet and the pellet was resuspended. In order to break down the proteins, 20 µL of Proteinase K solution was added and homogenized by vortexing. The prepared samples were incubated in a preheated 56 °C stirrer for 30 minutes until the cells were completely lysed.

Table 7. OD_{600nm} measurements of cultures for DNA isolation

Isolated Hot Spring	**Sample Name**	**Average OD_{600nm}**	**Bacterial cells/mL**
Doganbey	K-Syngas-65	2.2211±0.002	1.78 x 10^9
	K-YE+G-65-Şekerli(7)	1,0871±0.004	8.7 x 10^8
	K-65-(1108)	0.7114±0.01	5.69 x 10^8
	K-YE+G-65	2.0246±0.002	1.62 x 10^9
	K-syngas-65-Şekerli	1.1002±0.003	8.8 x 10^8
Cesme	Ç1-50-Şekerli(5)	2.4924±0.01	1.99 x 10^9
	Ç1-50-Şekerli(4)	2.3052±0.005	1.84 x 10^9
	Ç1-50(5)	1.7578±0.007	1.41 x 10^9
Bergama	B1-YE-65	1.2388±0.009	9.91 x 10^8
	B1-YE-Şekerli-65	1.6885±0.002	1.35 x 10^9

The samples were vortexed by adding 20 µL of RNase A solution, known to catalyze the degradation of RNA and incubated at room temperature for 10 minutes. 200 µL of lysis solution was added to the samples and vortexed for 15 seconds to mix homogeneously. 400 µL of the previously prepared 50% ethanol solution was added to the samples, pipetted and homogenized.

The samples were transferred to the collection column included in the kit and centrifuged at 6000 x *g* for 1 minute. The supernatant in the collection tube was poured and the samples were transferred to the new collection tube. 500 µL of 'Wash Buffer I' was added to the column and centrifuged at 8000 x *g* for 1 minute. The supernatant was poured, 500 µL of wash buffer II was added to the column and centrifuged for 3 minutes at maximum speed (27.040 x *g*). The collection tube containing the supernatant was discarded and the column was transferred to sterile 1,5 mL eppendorf.

In order to collect the DNA attached to the column, 200 µL of Elution Buffer was added to the midpoint of the column and incubated for 2 minutes at room temperature. It was then centrifuged at 8000 x *g* for 1 minute. The obtained DNAs were labeled and brought to -20 °C.

For the DNA isolation of gram-positive bacteria, differently from the gram negative isolation procedure, firstly enzymatic lysis buffer (ELB) was prepared. For this purpose, stock solutions were prepared. For 1 M Tris-HCl stock solution, 2.42 g Tris-HCl was weighed with a precision balance (Sartorius Stedim Biotech, Germany) and dissolved in 20 mL ultra-pure (UP) water and adjusted to pH 8.0. For 0.5 M EDTA stock solution, 1.46 g of EDTA was weighed and added into 10 mL of water, homogenized by stirring on a magnetic stirrer for one day. Prepared stocks were homogenized by adding 5 mL of 1 M Tris-HCl, 1 mL of 0,5 M EDTA and 3 mL of Triton X-100, and UP water to a final volume of 250 mL, respectively. Lysis buffer was freshly prepared by adding lysis buffer and lysozyme used within 24 hours to eliminate loss of activity of the enzyme (Table 8). The cultures in 2 mL volume obtained as specified in the preliminary preparation section were centrifuged at 5000 x *g* for 10 minutes and their supernatants were poured. 180 µL of lysis buffer was added to the pellet and incubated at 37 °C for 30 minutes. After incubation, 200 µL lysis solution and 20 µL of Proteinase K were added and homogenized by vortexing. Rest of the procedure were applied accordingly to the DNA isolation of gram-positive bacteria procedure.

Table 8. Enzymatic lysis buffer components and amounts prepared according to the number of samples

Number of Samples (+1)	Total volume of ELB (µL)	Lysozyme (mg)
(18+1) = 19	3420	68.4

Qubit 3 fluorometer (Thermo Fisher Scientific, USA) was used for determining the DNA quantities of isolated samples. A total of 15 samples taken from 5 regions and isolated from DNA were brought to room temperature by removing them from -20°C. Quant-iT™ 1X dsDNA HS Assay Kit (Thermo Fisher) was used for sample preparation.

Qubit protocol included a working solution, that was prepared by taking 17 µL of Qubit Reagent and 3383 µL of Qubit Buffer for 15 samples, considering the margin of error. 1 µL for each sample from Qubit Reagent in the kit and 199 µL for each sample from Qubit Buffer.

First, the standards were prepared. For this purpose, 190 µL of the working solution was taken and 10 µL of the standard 1 and 2 included in the kit were added to it, and 0.5 mL thin-walled eppendorfs were prepared with a final volume of 200 µL.

2 µL of DNA samples were taken, 198 µL of working solution was added, and 0.5 mL thin-walled eppendorfs were prepared with a final volume of 200 µL. Prepared standards and samples were vortexed for 2-3 seconds to ensure homogeneous mixing. Samples were incubated for 2 minutes at room temperature. Firstly, standard 1 and standard 2 were read by selecting the dsDNA option on the device. The prepared samples were placed in the device in order, readings were made and the measurements were recorded (Table 20).

2.4.4. 16S rRNA Polymerase Chain Reaction (PCR)

For the amplification of 16S rRNA region of the DNA samples an PCR trial using 16S rRNA archaeal and bacterial primers were set up. Custom designed archaeal and universal bacterial primers were homogenized by adding 2 µL of nuclease-free water (NFW) to 10 µM primer in the amount specified in the user manual and homogenized by taking 20 µL of the prepared archaeal and bacterial primers from the forward and reverse primers, and 160 µL of NFW was added on it, a total of 200 µL of primer mix was used.

For PCR (Blue-Ray Biotech, China) optimization, trials were performed in the range of 65-55°C and with 5 different DNA samples. In the PCR optimization experiment, in order to prevent DNA degradation, the primers were vortexed by adding 100 mM Tris-EDTA (TE) buffer, pH 8.0, according to the amount written in the guide (20 µL). Primers were heated at 57 °C for 30 min.

In order to determine the lengths between primers, the distance between primers and elongation time were determined by entering the base sequences at the ends of the primers using the Primer3plus site. The distance between the two primers was found to be 1600bp and the elongation time was calculated as follows:

1000 bp → 1 min ; 600 bp → 40 sec
Total elongation time was calculated as 1 min 40 sec.

PCR mix was prepared according to the Oxford Nanopore Kit procedure (Table 9). 5 µL of Doganbey spring sample containing 2500 ng/mL was taken and 495 µL of NFW was added to it. Thus, initially 100 pg of DNA was obtained. The following amounts were homogenized and PCR was performed:

Table 9. PCR mix

Volume	Reagent
5 µL	DNA stock
25 µL	PCR master mix (Sigma Aldrich, USA)
2 µL	Primer mix
18 µL	NFW

PCR products were run on a 2% agarose gel. For this purpose, 50 mL of 1x Tris base, acetate, and 1 g of agarose in EDTA(TAE) buffer were added and heated in the microwave oven for 5 minutes until homogenization was achieved without reaching to the boiling point. 1% agarose gel cooled down to room temperature, 3 µL of GelRed (Biotium, Australia) fluorescent nucleic acid dye, which is used to stain dsDNA, ssDNA or RNA in agarose gels or polyacrylamide gels, was added.

The resulting gel mixture was poured into the gel electrophoresis tray, taking care not to form air bubbles, and was allowed to harden. After the gel dried, the comb was removed and the gel containing chamber was rotated, and the current was adjusted to flow through the (-) side to the (+) side. 1x TAE buffer was added in an amount to cover the surface and cover the gel. 5 µL of Biomatik 1 kb DNA ladder plus (Biomatik, Canada) was loaded into the first well in order to ensure to determine the kb value of the bands in case of primers not performing.

5 µL of each of the PCR products was added to the drops of Loading Dye Solution (Sigma Aldrich, USA) on parafilm in order to increase the density of the DNA and allow it to precipitate into the wells during electrophoresis and appear in the gel by staining. This process was done at the end to prevent the evaporation of DNA, Loading Dye Solution containing two biomarker dyes: Bromophenol blue (advancing at 150 bp) and Xylose (proceeding at 750 bp) was added to the PCR samples. In this way, two band images were obtained: fast and slow moving. PCR products were mixed with Loading Dye Solution by pipetting and loaded into the wells and samples were run on agarose gel.

A separate PCR setup was prepared for the verification of archaeal primers. For this purpose, DNA samples that were expected to contain archaeal DNA with a high probability were selected, such as direct thermal water isolation (IndiSpin) samples, and the template DNAs were calculated according to the Qubit results, and the PCR experiment was repeated with archaea and bacterial primers at 52°C. As a result of PCR, samples were run on agarose gel with the same procedure.

Qubit protocol was applied to 47 DNA samples, excluding the direct water isolation samples for which DNA measurements were made previously. As a result of the Qubit results, the amount of DNA for each sample was recorded and the amount of DNA to be loaded for PCR was calculated for each sample.

PCR was performed with a total of 79 samples by adding a bacterial primer mixture to all of the samples isolated from the liquid cultures (57 samples) and additionally adding archaeal primers to the samples isolated directly from thermal water (22 samples). 47 samples of the PCR experiment were prepared with 500 µL of template DNA, 2 µL of primer mix, 18 µL of NFW and 25 µL of master mix, together with the required NFW Amount (mL) for PCR. Cultures that are not isolated from agar petri dishes were prepared separately to contain both bacterial and archaeal primers.

For the purification of PCR products, samples were transferred to eight strips and 50 µL of AmPure (Beckman Coulter, USA) (1:1 volume) was added to each. The samples were homogenized by directing up and down for 5 minutes. The samples were spin down and the AmPure beads were precipitated as brown-colored pellets, and the supernatant was observed as colorless. The magnet was contacted with the base of the strip containing the samples, and the beads holding the magnetic DNA were attached to the magnet and kept at the bottom, and the supernatant was discarded with a pipette. 200 µL of freshly prepared 70% ethanol was added per sample and then fresh ethanol was added by pipetting ethanol. The samples were spinned down and left to dry for 30 seconds with the lid open. Since 24 µL of sample will be added for the barcoding step, 50 µL of NFW water was added to the beads. The samples were kept at 4°C until the remaining steps of the experiment were conducted.

2.5. 16S rRNA Sequencing with Oxford Nanopore Technologies MinION Sequencer

MinION flow cell check was performed by connecting MinION device to the computer and placing it t inside the device (Figure 8). Flow Cell Check was initiated via the software and by heating at 37°C. 1366 active pores were detected.

2.5.1. PCR Amplicon Barcoding

24 µL of DNA purified with AmPure was withdrawn and transferred to new strips. Barcodes numbered 1-96 in the kit were added to the pure stock DNA in the strips as 1 µL in order. 25 µL of NEB Long AMP Primer Mix (Taq Polymerase) was added to each sample. PCR was performed under the conditions specified in the protocol (Table 10).

Table 10. PCR conditions specified in the ONT 16 Barcoding Kit

Cycle Step	Temperature (°C)	Time (second)	Number of Cycles
Initial denaturation	95	60	1
Denaturation	95	20	25
Annealing	55	30	25
Extension	65	120	25
Final extension	65	300	1
Hold	4	∞	-

Qubit samples and standards were prepared according to the Qubit protocol. 5 µL of DNA samples obtained after PCR amplicon barcoding were transferred to Qubit tubes and the amount of DNA was measured. Qubit test was repeated again by taking 1 µL of DNA for exact amount measurements on Qubit from samples with high amount of DNA (>20.000 ng/mL). The amounts calculated for pooling according to the DNA amount results were taken from the amounts in the table and transferred to 1,5 mL eppendorf (Table 11).

Due to some DNA amount values were being too high, instead of using a concentration of 1 µg, it was adjusted to 3 µg (865 µL in volume) and pooled and the DNA was divided into three (260 µL). PCR products and their DNA amounts were quantified with Qubit and required volumes for pooling the barcoded DNA samples for reaching total amount of 3000 ng of DNA was calculated accordingly (Table 11).

Table 11. Volume of each barcode for pooling based on Qubit results for 79 samples (* Samples with PCR archaea primer, DW: Direct water isolation samples)

Barcode	Isolation Source	Qubit result (ng/mL)	Volume (uL)	Total Target Amount(ng)
1	Doğanbey	880	43	3000
2	Çeşme	2392	16	3000
3	Bergama	9920	4	3000
4	Çeşme	48400	1	3000
5	Bergama	7160	5	3000
6	Çeşme	1248	30	3000
7	Bergama	872	44	3000
8	Çeşme	1552	24	3000
9	Bergama	932	41	3000
10	Bergama	3724	10	3000
11	Doğanbey	9960	4	3000
12	Doğanbey	22800	2	3000
13	Doğanbey	44100	1	3000
14	Doğanbey	9400	4	3000
15	Çeşme	2012	19	3000
16	Bergama	2924	13	3000
17	Bergama	24000	2	3000
18	Doğanbey	21200	2	3000
19	Doğanbey	19920	2	3000
20	Doğanbey	13960	3	3000
21	Çeşme	22400	2	3000
22	Bergama	1956	19	3000
23	Çeşme	3104	12	3000
24	Bergama	12440	3	3000
25	Çeşme	6840	6	3000

Table 11 (continued). Volume of each barcode for pooling based on Qubit results for 79 samples (* Samples with PCR archaea primer, DW: Direct water isolation samples)

Barcode	Isolation Source	Qubit result (ng/mL)	Volume (uL)	Total Target Amount(ng)
26	Bergama	14360	3	3000
27	Çeşme	23600	2	3000
28	Bergama	44300	1	3000
29	Bergama	51000	1	3000
30	Doğanbey	44400	1	3000
31	Doğanbey	20000	2	3000
32	Doğanbey	22000	2	3000
33	Bergama	3896	10	3000
34	Çeşme	1432	27	3000
35	Doğanbey	21600	2	3000
36	Bergama	22400	2	3000
37	Bergama	15000	3	3000
38	Doğanbey	45900	1	3000
39	Bergama	16080	2	3000
40	Doğanbey	21200	2	3000
41	Çeşme	20400	2	3000
42	Doğanbey	23200	2	3000
43	Doğanbey	21600	2	3000
44	Dikili Bademli	22400	2	3000
45	Dikili Bademli	4560	8	3000
46	Doğanbey (DW)	2724	14	3000
47	Doğanbey (DW)	808	47	3000
48	Doğanbey (DW)	48200	1	3000
49	Doğanbey (DW)	43400	1	3000
50	Doğanbey (DW)	1088	35	3000
51	Doğanbey (DW)	20400	2	3000
52	Çeşme (DW)	23600	2	3000
53	Çeşme (DW)	40100	1	3000
54	Dikili Bademli (DW)	12520	3	3000
55	Dikili Bademli (DW)	1036	37	3000
56	Bergama (DW)	24000	2	3000
57	Nebiler (DW)	3492	11	3000
58	Bergama*	13720	3	3000

Table 11 (continued). Volume of each barcode for pooling based on Qubit results for 79 samples (* Samples with PCR archaea primer, DW: Direct water isolation samples)

Barcode	Isolation Source	Qubit result (ng/mL)	Volume (uL)	Total Target Amount(ng)
59	Bergama*	19160	2	3000
60	Doğanbey*	41800	1	3000
61	Bergama*	23600	2	3000
62	Doğanbey*	47100	1	3000
63	Çeşme*	23200	2	3000
64	Doğanbey*	6320	6	3000
65	Doğanbey*	2164	18	3000
66	Dikili Bademli*	2364	16	3000
67	Dikili Bademli*	20800	2	3000
68	Doğanbey (DW)*	2224	17	3000
69	Doğanbey (DW)*	796	48	3000
70	Doğanbey (DW)*	55000	1	3000
71	Doğanbey (DW)*	41700	1	3000
72	Doğanbey (DW)*	1284	30	3000
73	Doğanbey (DW)*	1804	21	3000
74	Çeşme (DW)*	780	49	3000
75	Çeşme (DW)*	11160	3	3000
76	Dikili Bademli (DW)*	21600	2	3000
77	Dikili Bademli (DW)*	996	38	3000
78	Bergama (DW)*	1216	31	3000
79	Nebiler (DW)*	1044	36	3000

DNA Purification of Barcoded PCR Products

Pooled DNA sample was taken into a separate tube with 280 µL of pooled DNA and 280 µL of AmPure beads were added (1:1 volume). The sample was mixed by gently up and down for 5 minutes, the beads were contacted with DNA, and the supernatant was discarded by spinning down for 5 minutes. 625 µL of freshly prepared 70% ethanol was added onto DNA and AmPure and withdrawn with a pipette. This process was repeated 2 times.

The sample was spinned-down, and the tube cap was kept open for 30 seconds for allow it to dry. 50 µL of NFW was added to the bead in eppendorf. After waiting for 2 minutes, the pellet was removed and the beads and NFW contacted. By spinning down, the supernatant containing the pure pooled DNA was taken and transferred to a separate eppendorf.

End Repairing and DNA Purification

Reagents specified in Table 12 were added to the DNA containing 47 µL of pure adapter, for the total volume to be completed to 60 µL. Pure pooled DNA with added reagents was incubated 5 minutes in 20 °C and 5 more minutes in 65°C in thermal cycler.

Table 12. End repair reagents

Volume (µL)	Reagent
1	DNA CS
3.5	Neb FFPE Buffer
3.5	Neb Ultra II Buffer
2	DNA FFPE Repair Mix
3	Ultra II Enzyme Mix

DNA purification protocol was repeated by adding 1:1 volume of AmPure beads to 60 µL DNA sample.

Adaptor Ligation and Clean Up Protocol

25 µL of thawed and spinned-down LNB buffer, 10 µL of NEBNext Quick Ligase and 5 µL of Adapter Mix (AMX) were added to 60 µL of pure DNA sample, and the total volume reached to 100 µL. The tube was vortexed with flickering for 30 seconds, spinned-down for 20 seconds, and incubated for 10 minutes at room temperature. For 100 µL DNA sample, 40 µL AmPure was added, the tube was rotated up and down for 5 minutes to mix the beads, and the supernatant was discarded by spinning down. 250 µL of Short Fragment Buffer (SFB) was added to the sample, the pellet was lifted again, spin down and the supernatant was discarded. This process was repeated 2 times.

The sample was dried for 30 min. 15 µL of Elution Buffer was added to the sample, and the pelleted sample was incubated for 10 minutes at room temperature on the magnet until it became colorless. Qubit measurement was performed by taking 1 µL sample from the tube and the amount of DNA was measured as 30000 ng/mL. 1 µL of Rapid Adaptor (RAP) solution was added onto the pure DNA, mixed slowly and spin down. It was incubated for 5 min at room temperature. Sample to be loaded into MinION in a total volume of 75 µL was prepared according to the volumes specified in Table 13.

Table 13. Reagents for DNA library loading to flow cell

Volume (µL)	Reagent
10	Elution Buffer (EB)
2	DNA library
37.5	Sequencing Buffer (SQB)
25.5	Loading Beads (LB)

2.6. MinION Sequencing and Metagenomic Analysis

Priming solution was prepared by adding 30 µL of Flush Tehter (FLT) directly onto the Flush Buffer (FB) tube. Flow cell was placed inside the MinION device and connected to the MinION computer and software. A 1000 µL automatic pipette was adjusted to the volume of 200 µL and placed in the port in the flow cell, and slowly turned to 220-230 µL. In this way, the storage buffer in the flow cell was withdrawn with the elimination of formation of air bubbles.

Prepared 800 µL priming solution was added from the priming port and left for incubation for 5 minutes. 200 µL of priming solution was added again from the priming port, this time with the SpotON cover of the flow cell open (Figure 9).

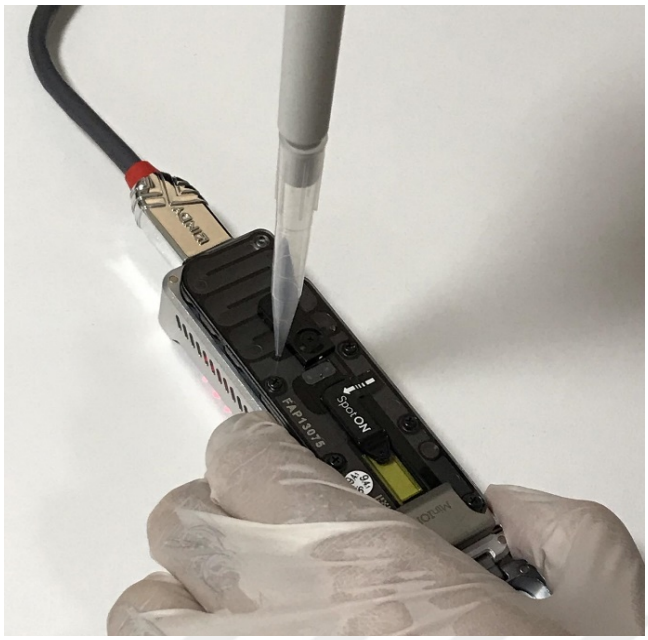


Figure 9. MinION flow cell priming

By initiating sequencing, DNA sequencing data flow from the device through its pores was observed for 24 hours (Figure 10).

Figure 10. MinION 16S rRNA sequencing data flow

Completion of sequencing and flow cell clean up

After providing sufficient data flow at the end of 24 hours, sequencing was stopped but base calling was continued. Wash Buffer (B) in the wash kit was thawed at room temperature, and Wash Buffer (A) was kept frozen as it contains DNase I. 20 µL Wash Buffer (A) and 380 µL Wash Buffer (B) were combined in one eppendorf. Waste liquid was withdrawn from the waste port of the flow cell with the help of a micropipette. The automatic pipette was brought to 200 µL and the air bubbles were removed from the priming port. 400 µL Wash Buffer was loaded into the priming port, the priming port cover was closed and left for incubation for 30 minutes. Flow Cell was stored in refrigerator at 4°C later to be used for future studies.

16S rRNA metagenomic sequencing analysis results were obtained with workflow using EPI2ME 16S-BLAST ® (Oxford Nanopore Technologies, UK) and Kraken 2 (Wood et al., 2019), analysis of metagenomics classification results and Sankey visualization were obtained with Pavian tool (https://fbreitwieser.shinyapps.io/pavian/).

2.7. Optimization of Biohydrogen Production Yields Using Box Behnken Design

2.7.1. Optimization of Biohydrogen Production Yields Using Box Behnken Design with Operational Parameters

Optimization experiments were carried out with the statistical experiment design method of Box-Behnken Design methodology. In order to determine the effect of the independent variables in the hydrogen production process, the CO volume (mL), temperature and pH, experiments were carried out at 3 levels as shown in Table 14 and 15.

Table 14. Factors and levels selected for the Box-Behnken Design

Factors	**Levels**		
	1 (Low)	**2 (Middle)**	**3 (High)**
Temperature (°C)	55	60	65
pH	5.0	7.0	9.0
CO feed (mL)	5	10	15

H_2 production and by-product formation, which are the dependent variables during the optimization process, entered as a response to the Design Expert (StatEase, v13, USA) software. After the analysis, the relationship between the factors and the response was compared whether they were statistically significant as a result of the ANOVA evaluation and the fit with the quadratic model was tested.

Table 15. Factors and levels selected for the Box-Behnken Design (Design Expert v13, USA).

Run	Temperature (°C)	pH	CO volume (mL)
1	60	7	10
2	65	7	5
3	65	5	10
4	60	9	5
5	55	7	5
6	55	7	15
7	60	7	10
8	60	7	10
9	60	7	10
10	60	5	5
11	55	9	10
12	65	9	10
13	60	5	15
14	65	7	15
15	60	7	10
16	60	9	15
17	55	5	10

Anaerobic bottles containing 50 mL basal medium, and 6 mL of inoculated microbial culture were fed with CO gas by ranging volume (5,10 and 15 mL) with an airtight syringe. During these experiments, Gas Chromatography (GC) with a TCD (thermal conductivity detector) detector was used to determine the concentrations of H_2, CO and CO_2 and by-product (VFA and ethanol) analysis was conducted with HPLC.

2.7.1. Optimization of Biohydrogen Production Yields Using Box Behnken Design with Bivalent Metal Concentration of Growth Medium

In order to determine the effect of the concentrations of the micronutrients which are the bivalent metals present in the growth medium for the biological hydrogen production process optimization experiments were carried out with the statistical experiment design method of Box-Behnken Design methodology.

Iron (Fe), zinc (Zn) and nickel (Ni) were selected as independent variables and experiments were tested in batch optimization experiments carried out in 17 different 100 mL sterile glass serum bottle reactors as shown in Table 16 and 17. Doğanbey mixed culture was selected as model culture for BBD optimization, as it showed consisting growth and H_2 production (Table 19 and Table 20).

Table 16. Factors and levels selected for the Box-Behnken Design

Factors	**Levels**		
	1 (Low)	**2 (Middle)**	**3 (High)**
Fe^{+2} (mg/L)	50	150	250
Zn^{+2} (mg/L)	25	87.5	150
Ni^{+2} (mg/L)	10	55	100

Anaerobic bottles containing 50 mL basal medium, and 6 mL of inoculated microbial culture were fed with 10 mL CO gas with an airtight syringe. During these experiments, Gas Chromatography (GC) with a TCD (thermal conductivity detector) detector was used to determine the concentrations of H_2, CO and CO_2 and by-product (VFA and ethanol) analysis was conducted with HPLC.

H_2 yield (mmol H_2 / mmol CO), which is the dependent variable during the optimization process, entered as a response to the Design Expert (StatEase, v13, USA) software and the relationship between the factors and the response was compared whether they were statistically significant as a result of the ANOVA evaluation and the fit with the model was tested.

Table 17. Factors and levels selected for the Box-Behnken Design (Design Expert v13, USA).

Run	Fe^{+2} (mg/L)	Zn^{+2} (mg/L)	Ni^{+2} (mg/L)
1	250	87.5	100
2	250	25	55
3	50	25	55
4	150	150	10
5	150	25	10
6	50	150	55
7	250	25	10
8	150	150	100
9	150	87.5	55
10	150	87.5	55
11	150	150	100
12	50	87.5	10
13	250	150	55
14	50	87.5	100
15	150	87.5	55
16	150	87.5	55
17	150	87.5	55

2.8. Biohydrogen Production with Anaerobic Thermophilic Mixed Culture Using a Continuous Stirred Tank Reactor (CTSR)

Bioreactor trials were established with Biostat® B (Sartorius, Germany) bioreactor and with the addition of microsparger designed for microbubble production (Figure 11).

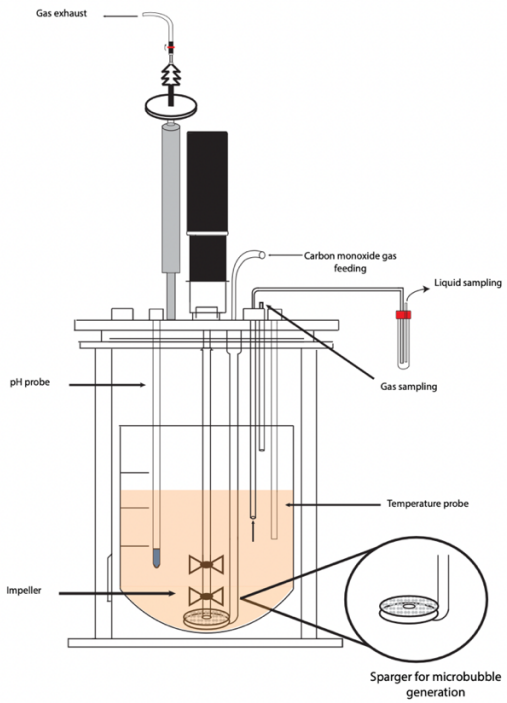


Figure 11. Designed bioreactor set-up for biohydrogen production with CO-fermentation

A bioreactor volume of ⅔ was determined as the working volume, and 1300 mL of anaerobic thermophilic growth medium with a pH of 7.00 (Table 4) was prepared and transferred to the bioreactor tank (Figure 12). All connections of the bioreactor tank were closed with the help of clips and prepared for sterilization. Before the autoclave sterilization, the medium was nitrogenized for 1 minute, and the oxygen was removed for an anaerobic environment. For pH stabilization, a 1 M NaOH solution was prepared and connected to the feed port of the reactor tank. Sterilization was completed by autoclaving the reactor at 121 °C for 15 minutes.

After the sterilization, the connections between bioreactor and sterilized tank were made and the system was installed. The water circulator was turned on and set to 10 °C, after the circulator reached the desired temperature, the pump was turned on and the bioreactor jacket surrounding the reactor tank was filled with water After the jacket in the reactor tank was filled with water, the temperature was set to working temperature of 60 °C (Figure 12).

In order to distribute the temperature homogeneously throughout the tank, the impeller was turned on and set to 30 rpm. Under aseptic conditions, 200 mL of mixed culture (inoculation rate of 15%) was inoculated to the bioreactor with a peristaltic pump. Following the inoculation, CO gas was fed to the reactor tank for 1 minute. GC analysis was performed by sampling the headspace gas.

Bioreactor was sampled daily 10 mL sample was taken from the sampling port with the help of a sterile syringe and used for taxonomical profiling of mixed Doğanbey culture and analyzing the change in microbial consortia in terms of abundance of different microorganisms with time. H_2 production and CO consumption was monitored by the sampling of the headspace gas produced in the bioreactor with the gas chromatography.

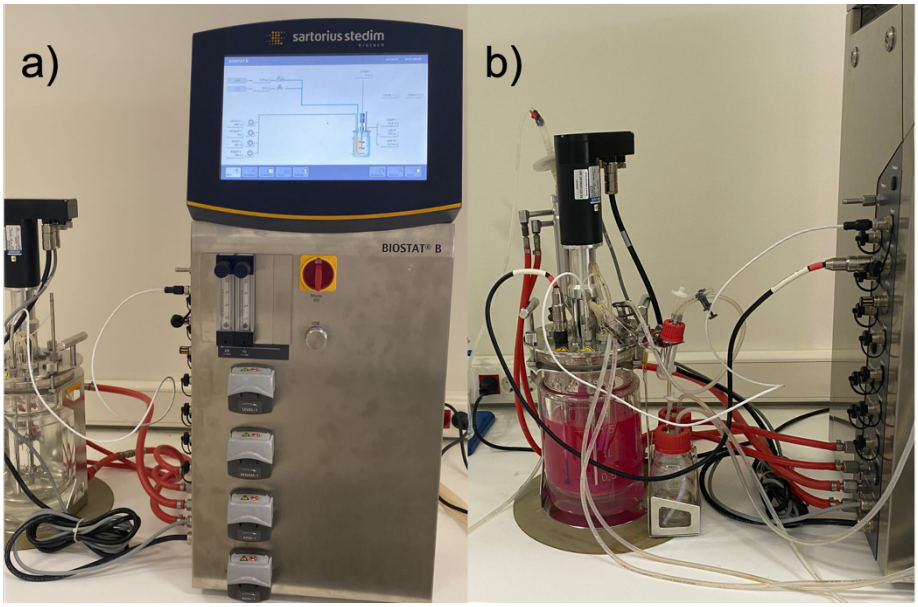


Figure 12. a) Bioreactor set-up before and b) bioreactor set-up after medium sterilization

CHAPTER 3: RESULTS AND DISCUSSION

3.1. Results of Sampling Procedure and Cultivation of the Hot Spring Isolates

In Table 18, temperature, pH and ORP measurements of 5 different hot springs were specified.

Doğanbey hot spring was the most suitable in terms of a thermophilic and anaerobic environment, with the temperature values above 60°C and an ORP value of 43.8 mV. Bergama and Dikili Nebiler hot springs were also observed in thermophilic temperatures (>50°C), however Çeşme and Dikili Bademli hot springs were colder (~40°C). pH values of all 5 hot springs were around 6.88-7.88, which is suitable for carboxydotrophic hydrogenogenic growth (Fukuyama et al., 2020).

Table 18. Temperature, pH and ORP measurements from 5 different hot springs

Hot Spring	Collection Location	Temperature (°C)	pH	ORP (mV)
Doğanbey	Upstream	77.3	6.42	43.8
	Midstream	52.0	7.09	-13.2
	Downstream	49.2	7.43	-23.1
Çeşme	Yıldızburnu	38-43	7.2	-
Dikili Nebiler	1	53.8	7.04	-
	2	56.2	6.86	-
Dikili Bademli	Sea	43.2	6.75	-
	Pool	34.5	6.85	-
Bergama		56.2	7.88	-54.8

Cultivation of isolates from 5 different hot springs were performed accordingly (See section 2.1.2. Cultivation of Hot Spring Isolates) and regularly analyzed for microbial growth, H_2 production and by-product formation, ethanol and VFAs (Table 18). Growth medium was supplemented with 1 g/L of yeast extract, cultivation and enrichment of the isolates were significantly improved by yeast extract addition to the medium. As carboxydotrophic hydrogenogens are reported in the literature to be using a combination of CO and yeast extract for substrates, electron acceptors/donors to produce H_2 (Kochetkova et al., 2011; Parshina et al., 2005b; Sokolova et al., 2005; Yoneda et al., 2012; Zavarzina et al., 2007).

Supplementation of organic substrates such as rumen fluid or yeast extract reported to significantly enhancing microorganisms' growth on CO. This strategy shortens the adaptation time to the toxic effects of CO, increases the capacity of microorganisms to handle more higher concentrations of CO (up to 100%) and boosts the growth mechanisms by cutting down on the lag phase time period (Chang et al., 2007; Kerby and Zeikus, 1983; Sharak Genthner and Bryant, 1987).

3.2. Characterization of the Hydrogen Producing Cultures

3.2.1. Subculturing

Considering the results of the GC analysis performed with headspace gas samples of the cultures established in the previous experiments, cultures showing a regular hydrogen production trend were determined and these cultures were transferred to fresh nutrient medium. Culture bottles containing isolates from Çeşme (Yıldızburnu), Bergama, Dikili (Bademli) and Doğanbey thermal springs were routinely subcultured on supplemented fresh nutrient media and feeding with CO gas every two weeks.

Regular GC analysis was followed hydrogen production of the subcultures, the presence and production of hydrogen were detected in all of them (Figure 13 and Table 19). Methane production was observed in some samples, this could indicate that methanogenic species are present in mixed microbial consortia. Some methanogenic bacteria and archaea are reported to be able to oxidize CO as an energy and carbon source (Brady et al., 2015). Also, some methanogens are reported to consume the hydrogen produced in the environment (Yoshinaga et al., 2015).

Table 19. 12th day analysis results of gaseous products of subcultured isolates from 4 different hot springs on 26 /07/21.

Sample Name	Isolation Source	Incubation Temperature (°C)	H_2%	CH_4%	Date
Ç1-YE+G	Çeşme Yıldızburnu	55	0.420	5.806	26/07/2021
Ç2-YE+G	Çeşme Yıldızburnu	55	1.443	0	26/07/2021
B2-YE+G	Dikili Bademli	45	0.050	3.623	26/07/2021
B2	Dikili Bademli	45	1.293	0	26/07/2021
B2-YE+G	Dikili Bademli	45	0.128	0	26/07/2021
K-YE+G-1	Doğanbey	65	1.389	0	26/07/2021
K-YE+G-2	Doğanbey	65	0.846	0	26/07/2021
B1-MIX	Bergama	65	1.084	0.625	26/07/2021
K-SYNGAS	Doğanbey	65	0.432	0	26/07/2021
B1-YE	Bergama	65	0.372	0	26/07/2021
B2-YE	Bergama	65	0.367	0.203	26/07/2021
B2-YE	Bergama	55	0.129	0	26/07/2021
B1-YE	Bergama	55	0.744	0	26/07/2021
Control	Bergama	65	0	0	26/07/2021

All of the cultures detected and routinely followed according to the analysis results of gaseous products, which are followed regularly, showed hydrogen production capacity. H_2 production is observed in all cultures 2 days after being transferred to the fresh medium, and the cultures continue to consume carbon monoxide until the 13th day (Figure 13). Among 5 different hot springs: Doğanbey, Bergama and Çeşme were observed in most consistent H_2 production. However, samples from Dikili hot springs (Nebiler and Bademli) indicated poor H_2 production capacity with little to no H_2 observed.

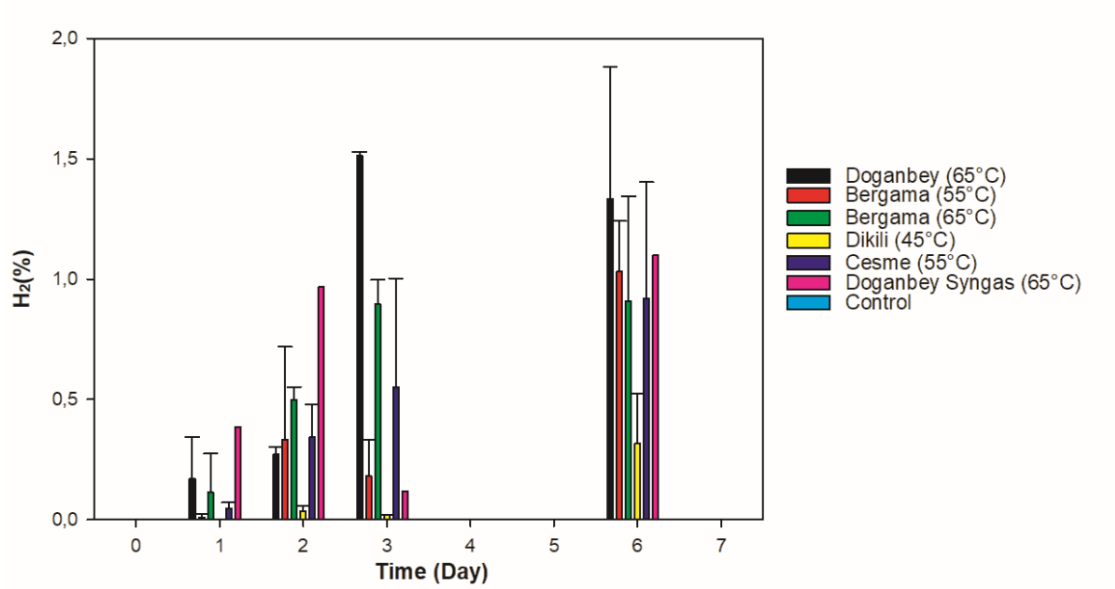


Figure 13. 1-week hydrogen production of subcultured isolates from 4 different hot springs: Doğanbey, Bergama, Dikili and Çeşme

3.2.1. Scanning Electron Microscopy (SEM) Imaging

SEM imaging of 3 different H_2-producing hot spring microbial communities showed that these communities are consisting of bacilli and cocci shaped bacteria with various sizes and morphological features.

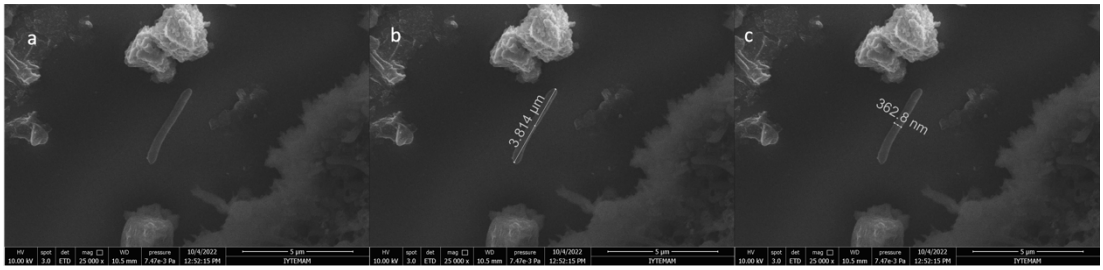


Figure 14. SEM images and dimensions of a single bacilli-shaped bacterium isolated from Doğanbey hot spring

A single bacilli-shaped bacteria with the length of 3.814 µm and width of 362.8 nm was observed (Figure 14). This single thermophilic and anaerobic microorganism showed significant morphological similarity to the genera of Thermoanaerobacter, Carboxydocella and Bacillus associated with CO conversion to hydrogen and organic acids (Balk et al., 2009; Slepova et al., 2006).

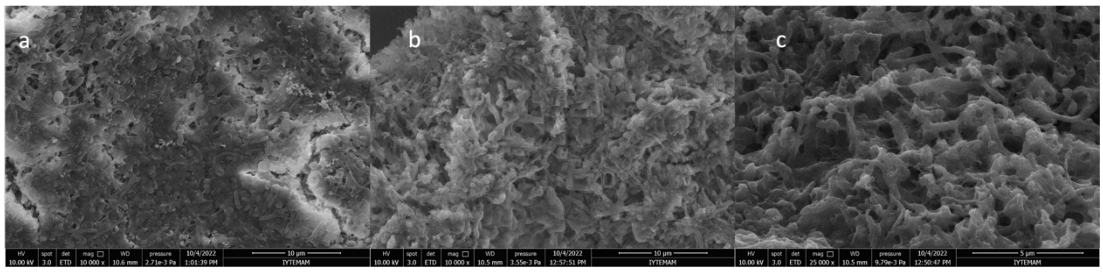


Figure 15. SEM images of bacilli-shaped bacterium isolated from Doğanbey hot spring

Doğanbey hot spring consisted of bacilli-shaped bacteria, intertwined as a matrix form throughout the sample (Figure 14 and 15). A dense bacterial population was observed.

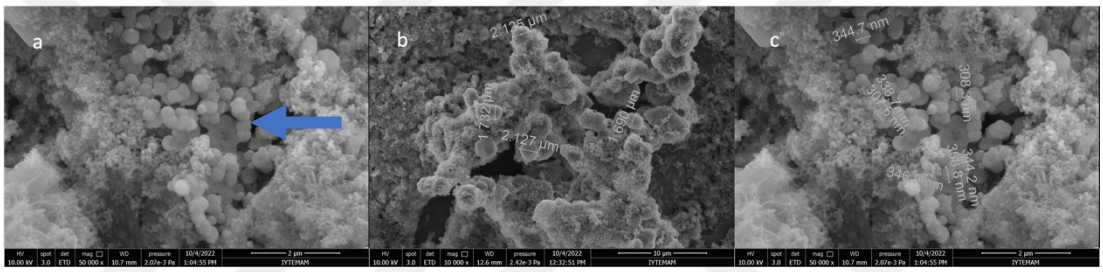


Figure 16. SEM images and dimensions of cocci-shaped bacterium isolated from Çeşme hot spring

Çeşme samples exhibit mainly cocci-shaped bacterium with various sizes from 1.60 to 2.127 µm in length and 307.6 to 620.1 nm in width (Figure 16 and 17).

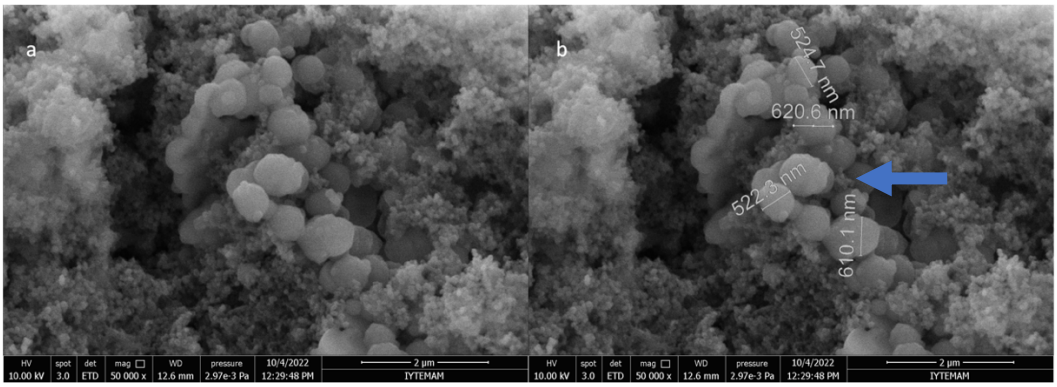


Figure 17. (a) SEM images and (b) dimensions of cocci-shaped bacterium isolated from Çeşme hot spring

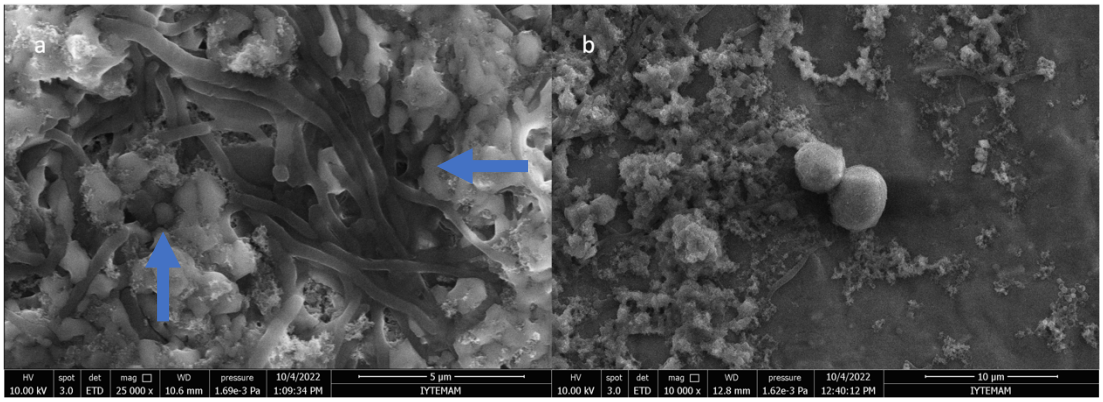


Figure 18. SEM images of (a) bacilli and (b) cocci-shaped bacterium isolated from Bergama hot spring

In Bergama samples, both bacilli and cocci-shaped bacterium were observed together (shown with blue arrow), being very abundant in both morphological features. Bacilli-shaped bacterium of 1.850 µm in length and cocci-shaped bacterium 344.3 to 455.3 nm in width were identified (Figure 18 and 19).

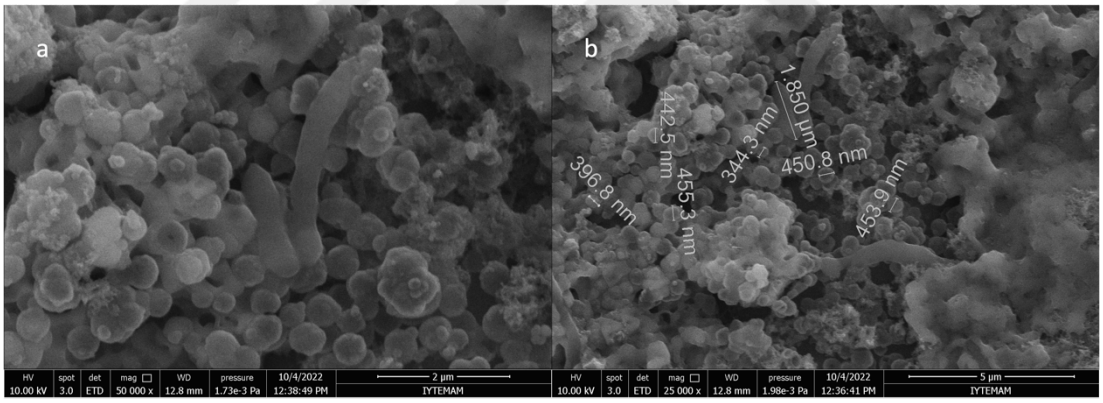


Figure 19. (a) SEM images and (b) dimensions of bacilli and cocci-shaped bacterium isolated from Bergama hot spring

3.3. Analysis Results for Hot Spring Microbial Communities

Hot spring microbial communities and their cultivations was analyzed daily with GC analysis of gaseous products, UV-Vis spectrophotometry analysis for OD_{600} measurements and HPLC analysis for VFA and ethanol (by-product) production.

H_2 production yields of samples were calculated by GC analysis results from headspace gas in means of formed H_2 and consumed CO as Eq. (2):

$$yield = \Delta H2\ (mmol\ )/\Delta CO\ (mmol) \quad (2)$$

H_2 productions of the cultivations are substantially influenced by sample collecting sites and their environmental conditions. Suitable thermophilic conditions were found in Doğanbey and Bergama hot springs (Table 18). As a result, the cultures of Doğanbey (0.13 mmol H_2/mmol CO) and Bergama (0.18 mmol H_2/mmol CO) hot springs demonstrated higher hydrogen yields compared to moderately thermophilic conditions among the rest of the hot springs (Table 20).

Microbial growth of cultivated samples was analyzed by OD_{600} measurements with UV-Vis spectrometry. As seen in Table 20, Dikili Nebiler and Bademli hot springs showed poor growth. Isolates cultivated in an anaerobic and thermophilic medium could not be sufficient enough for reproducing the microorganisms collected from their natural habitats, along with these hot springs did not fully show thermophilic and anoxic conditions in the first place (Table 18). Consequently, designed growth conditions were not suitable for these hot spring isolates to grow. Doğanbey, Çeşme and Bergama cultivations displayed moderate and consistent microbial growth in the medium.

Table 20. Maximum H_2% production yields, OD600 values, VFA and ethanol production analysis of 5 different cultivated hot spring isolates.

Hot Spring	Yield (ΔH_2 (mmol) / ΔCO (mmol))	OD_{600}	Ethanol (mM)	Propionic Acid (mM)	Formic Acid (mM)
Doğanbey	0.13	0.235	0.034	0.218	0.024
Çeşme	0.12	0.179	0.038	0.015	0.042
Dikili Bademli	0.09	0.095	-	-	-
Dikili Nebiler	0.06	0.097	-	-	-
Bergama	0.18	0.226	0.05	-	-

Produced VFA's and ethanol in this study, as a result of CO fermentation of hot spring isolates were investigated and summarized in Table 20. Although presence of ethanol, acetic acid, butyric acid, propionic acid and formic acid were detected, they are in very small amounts compared to other reported VFA productions. 0.218 mM (10.012 mg/L) and 0.042 (1.948 mg/L) of formic acid were produced by Doğanbey and Çeşme samples, respectively. Çeşme samples showed highest ethanol and propionic acid production with 0.038 mM (1.774 mg/L) and 0.015 mM (1.13 mg/L) respectively. According to the literature, (Henstra et al., 2007a) maximum formic acid production of 6.8 mM. (Esquivel-Elizondo et al., 2017)0.84 mM of formic acid, 1.1 mM of ethanol and 7.79 mM of propionic acid produced as a result of CO fermentation. Esquivel-Elizondo et al., 2017 investigated the structure and functions of a mixed culture containing anaerobic CO-consuming microbial community, and conducted a HPLC analysis where by-products of acetate, formate, propionate, butyrate and ethanol were observed. Archaeoglobus fulgidus, an obligate anaerobic and hyperthermophile archaeon that can use CO gas as a substrate, was used for CO fermentation.

It was determined as a result of organic acid analysis that this organism formed acetate and formate in the presence of CO and sulphate. In addition to acetate and formate, propionate and butyrate with little quantities (<0.5 mM) were also analyzed by HPLC (Henstra et al., 2007a). Another study where CO gas was used as a substrate for the cultivation of *C. hydrogenoformans*, products of propionic acid and butyric acid organic acids formed as a result of CO fermentation (Henstra and Stams, 2004).

3.4. Molecular Methods

3.4.1. 16S rRNA Archaea Primer Design

PCR trial conducted with custom designed 16S rRNA primers for archaea showed the successful amplification of 16S region of the DNA samples isolated from hot springs (See section 3.4.4. 16S rRNA Polymerase Chain Reaction (PCR)).

Table 21. Thermophilic bacterial and archaeal 16S rRNA primers

Primers	**Forward**	**Reverse**
Bacteria Primers (Universal)	Tailed-27For: 5' TTTCTGTTGGTGCTGATATTGC AGAGTTTGATCMTGGCTCAG 3'	Tailed-1492Rev: 5' ACTTGCCTGTCGCTCTATCTTCC GGTTACCTTGTTACGACTT 3'
Archaea Primers (Custom designed)	For27: ACTTGCCTGTCGCTCTATCTTC TACGGCTACCTTGTTACGAC	Rev621: TTTCTGTTGGTGCTGATATTGCCT GAAACTTAAAGGAATTGGC Rev1413: TTTCTGTTGGTGCTGATATTGCA CKGCTCAGTAACACGTG

Literature investigations and selection for 16S rRNA genes from reported thermophilic archaeal species that are likely to be found in İzmir hot springs (Table 5) were obtained by aligning using ClustalX software (Figure 20) and successfully applied to the primer design (Table 21). Presence of archaeal species in hot springs were able to be detected with custom designed primers (See section 3.6. MinION Sequencing and Metagenomic Analysis).

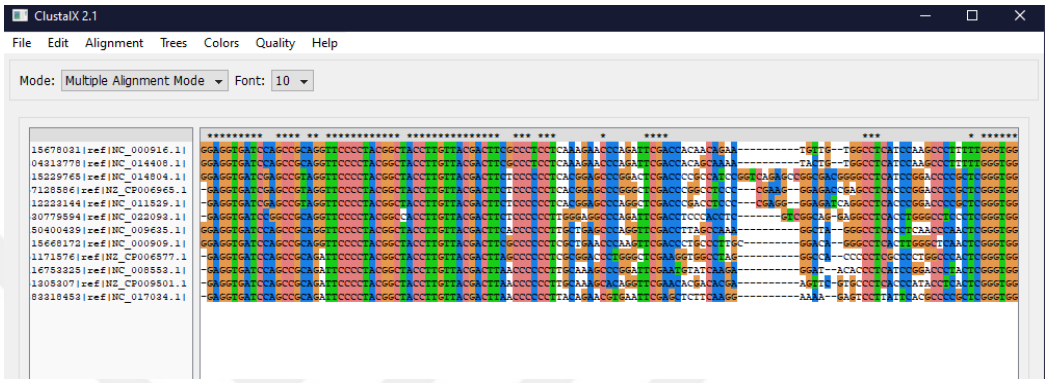


Figure 20. Alignment results on ClustalX with the archaea species that are likely to live in the hot springs in İzmir with literature research.

Quantification of DNA with Qubit 3 fluorometer results given in Table 20 shows that DNA quantities were out of range in the upstream samples taken from Doğanbey (K1 and K2), the Bademli pool sample (D-B2) , Nebiler (N1 and N2) and Bergama (P1). Water outlet points were not fully determined, so it was not possible to do the sampling directly from the source in Bademli pool sample.

Sample problem applied for Nebiler sample, no direct samples could be taken from the source, the hot water was flowing from the fountain and therefore the bacterial uptake was minimal. Bergama sample, on the other hand, sufficient amount of DNA could not be obtained due to the error due to sampling, since the amount obtained in the control was 904 ng/mL.

Table 22. DNA Quantities from 5 different hot spring from Qubit readings.

Isolation Source - Location	Sample Name	DNA Quantity (ng/mL)
Doganbey - Upstream	K1	Out of range
	K2	Out of range
Doganbey - Midstream	O1	999
	O2	383
Doganbey - Downstream	B1	2320
	B2	1460
Cesme	C1	943
	C2	863
Dikili (Bademli) - Sea	D-B1	517
Dikili (Bademli) - Pool	D-B2	Out of range
Dikili (Nebiler)	N1	Out of range
	N2	Out of range
Bergama	B	193
	P1	Out of range
	P2	904

In Doğanbey samples, on the other hand, DNA amounts were observed at the desired level in the head and middle part, but out of range in the upstream region. Another reason for the DNA amounts to be out of range or lower than desired is thought to be the volume taken from the samples. Considering the kit procedure, it was seen that it can vary between 1-20 µL depending on the amount of DNA in the sample.

Since the isolated samples were not pre-enriched and were only water samples collected from the region, the microorganism density and thus the DNA amount might be low. For this reason, it was concluded that the amount of sample (2 µL) taken may not have been sufficient to determine the DNA amounts.

Table 23. Second trial for Doğanbey upstream samples and the DNA quantities obtained with Qubit.

Number	Sample Name	Isolation Source	DNA Quantity (ng/mL)
1	K1	Doğanbey Upstream	Out of range
2	K2	Doğanbey Upstream	28,4

For this reason, on 23/08/2021, the region was visited again and the sample was collected and DNA isolation was performed again. DNA amounts were determined with the Qubit device as stated above. Unlike the first experiment, measurements were performed with 5 µL of DNA (Table 23). Sufficient bacterial density cannot be obtained in the Doğanbey upstream samples without pre-enrichment in the first trial (Table 22), but 28.4 ng/mL DNA was obtained in the second trial (Table 23).

3.4.4. 16S rRNA Polymerase Chain Reaction (PCR)

The bacterial band was most prominent at 56.8°C (indicated by the blue arrow as seen in Figure 21). However, an archaeal band could not be observed. Reasoning for this could be due to the high temperature selections determined for PCR, and it was decided to set up another PCR trial set at a lower temperature for archaea primers.

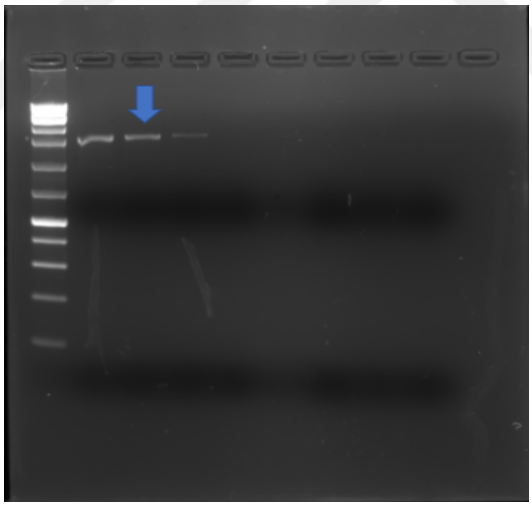


Figure 21. Agarose gel image obtained as a result of PCR experiment.

PCR trial with custom designed 16S primers for thermophilic archaea revealed that a band was observed in agarose gel image (Figure 22), meaning that custom designed archaeal primers are able to amplify the 16S rRNA region of the archaeal species present in DNA samples and suitable to be used for hot spring DNA samples obtained from İzmir hot springs.

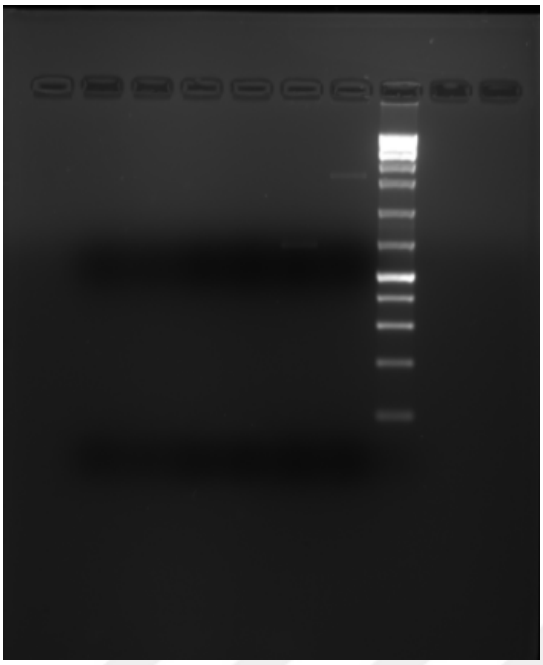


Figure 22. Gel image of PCR experiment trial with archaea primers.

Table 24 shows the amount of DNA to be loaded for PCR was calculated for each sample according to the Eq.(3):

$$Amount\ required\ for\ PCR\ (\mu L)\ = Total\ amount\ of\ PCR\ mix\ prepared\ (50\ \mu L) \times \frac{amount\ of\ DNA\ required\ for\ PCR\ (500\ \mu L)}{amount\ of\ DNA\ in\ the\ sample\ (ng/ml)} \quad (3)$$

Addition of NFW according to the DNA amount of each sample was calculated according to the Eq.(4):

$$NFW\ \ to\ be\ added\ (\mu L) = 500 - \ Amount\ required\ for\ PCR(\mu l) \quad (4)$$

Table 24. Calculation results for Qubit DNA amount of DNA samples and NFW amount to be added for PCR

Isolation Technique	**Sample Name**	**PCR Sample Number**	**DNA Amount (ng/mL)**	**Amount Required for PCR (µL)**	**NFW to be added (µL)**
Gram Positive Isolation	6	19	656	15.24	484.76
	K	20	107	93.46	406.54
	5-ş	21	357	28.01	471.99
	2-ş	22	972	10.29	489.71
	4-ş	23	-	-	-
	3-b	24	1108	9.03	490.97
	4	25	604	16.56	483.44
	2	26	1412	7.08	492.92
	Ç	27	106	94.34	405.66
	3-a	28	664	15.06	484.94
	3-b-ş	29	472	21.19	478.81
	6-ş	30	448	22.32	477.68
	7	31	572	17.48	482.52
	7-ş	32	416	24.04	475.96
	3a-ş	33	1260	7.94	492.06
	5	34	972	10.29	489.71
	8	35	768	13.02	486.98
	B1-YE	36	1132	8.83	491.17
Direct Water Isolation	B2-YE	37	696	14.37	485.63
	K-YE+G-2	38	277	36.1	463.9
	B1-MIX	39	334	29.94	470.06
	K-SYNGAS	40	1148	8.71	491.29
	Ç2-YE+G	41	1136	8.8	491.2
	K-SYNGAS	42	464	21.55	478.45
	K-YE+G-1	43	298	33.56	466.44
	B2-45	44	820	12.2	487.8
	B2-YE+G-45	45	948	10.55	489.45
	Doğanbey Upstream	47	28.4	352.11	147.89

Table 25 (continued). Calculation results for Qubit DNA amount of DNA samples and NFW amount to be added for PCR

Isolation Technique	Sample Name	PCR Sample Number	DNA Amount (ng/mL)	Amount Required for PCR (µL)	NFW to be added (µL)
Gram Negative Isolation	6	1	572	17.48	482.52
	5-ş	2	328	30.49	469.51
	2-ş	3	1380	7.25	492.75
	4-ş	4	512	19.53	480.47
	3-b	5	556	17.99	482.01
	4	6	114	87.72	412.28
	2	7	768	13.02	486.98
	Ç	8	81.6	122.55	377.45
	3-a	9	274	36.5	463.5
	3-b-ş	10	227	44.05	455.95
	6-ş	11	716	13.97	486.03
	7	12	392	25.51	474.49
	7-ş	13	301	33.22	466.78
	K	14	120	83.33	416.67
	5	15	206	48.54	451.46
	8	16	800	12.5	487.5
	3-b-ş	17	824	12.14	487.86
	K7	18	20.4	490.2	9.8

3.5. MinION 16S rRNA Sequencing and Metagenomic Analysis

Classification according to the 16S rRNA metagenomic data showed that more than 97% of microbial communities in İzmir hot springs are consisting of bacterial species. Bacteria/archaea ratio were increased in Doğanbey, Çeşme and Bergama samples by cultivation under anaerobic thermophilic and CO-supplemented conditions except for the Dikili Bademli samples, however this might be caused by a sequencing error (Table 26).

Table 26. Classification summary of 16S rRNA metagenomic analysis of 5 different hot springs in İzmir

Isolation Source	Isolation Technique	Number of raw reads	Classified reads	Unclassified reads	Microbial reads	Bacterial reads
All Samples	Direct Water + Cultured Isolation	6.391.436	99.4%	0.641%	99.3%	97.8%
Doğanbey	Direct Water Isolation	877.400	99.3%	0.699%	99.2%	89.8%
	Cultured Isolation	1.902.906	99.8%	0.196%	99.8%	99.7%
Çeşme	Direct Water Isolation	248.028	99.9%	0.0867%	99.9%	96.3%
	Cultured Isolation	291.211	98.2%	1.82%	98.2%	98.1%
Dikili Bademli	Direct Water Isolation	50.438	99.8%	0.151%	99.8%	99.4%
	Cultured Isolation	258.004	98.2%	1.75%	98.2%	98.2%
Dikili Nebiler	Direct Water Isolation	1.148	12%	88%	12%	11.9%
Bergama	Direct Water Isolation	125.354	99.5%	0.534%	99.5%	99.2%
	Cultured Isolation	999.771	99.6%	0.436%	99.6%	99.4%

Cultivation of the isolates successfully enriched the microbial growth, as number of raw reads significantly increased compared to direct water isolation reads. In natural water resources, microbial communities are lower in abundance compared to culture conditions as harsh environmental conditions such as absence of nutrients, fluctuation of water content, temperature and pH by ecological disruption and substrates deeply effects the microbial life (Curtis and Sloan, 2004; Hays et al., 2015)

Metagenomic Analysis of Doğanbey Hot Spring

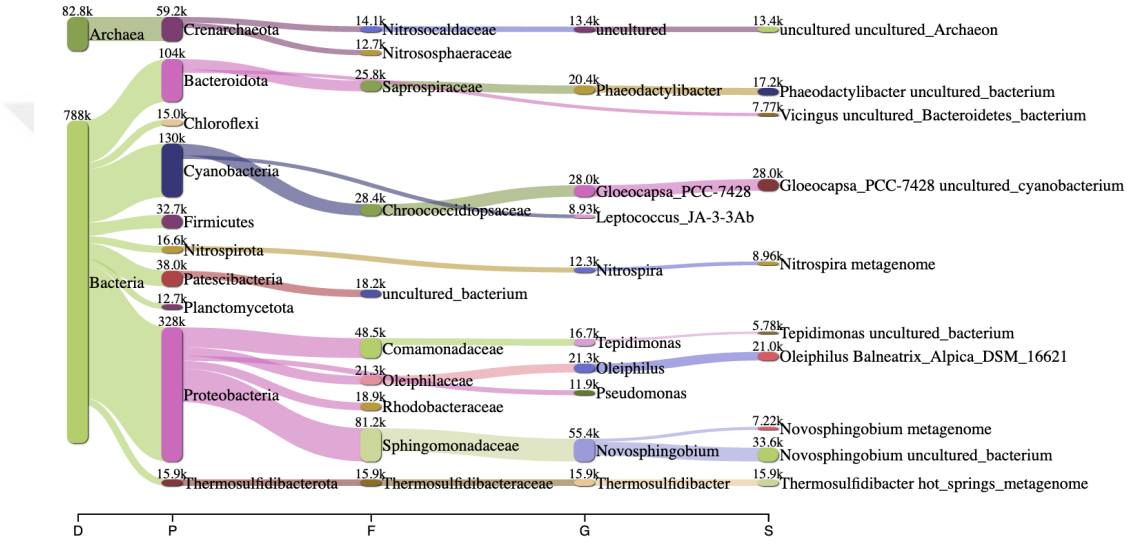


Figure 23. 16S rRNA Taxonomical Analysis of Doğanbey Hot Spring

Based on the species obtained as a result of 16S rRNA metagenomic sequencing for Doğanbey hot spring and given in Figure 23, it was seen that Doğanbey hot spring hosts dominantly *Novospingobium* species belong in *Proteobacteria* genus (*Novosphingobium arabidopsis, Novosphingobium sediminis* and *Novosphingobium aromaticivorans*) which are microorganisms that frequently encountered in thermal water resources, (Walker et al., 2010).

Sulfate producer *Thermodesulfovibrio yellowstonii* and hydrogen oxidizer *Thermodesulfovibrio hydrogeniphilus* species were detected (Haouari et al., 2008; Henry et al., 1994).

Among the 5 hot spring water communities, the hot spring, where the presence of archaea population is most evident where more than 80k reads were obtained, was Doğanbey thermal spring. Archaea population in the hot spring was determined as a result of 16S analysis and three archaeal species were dominantly seen (*Nitrosopumilus ureiphilus, Nitrosopumilus maritimus, Nitrososphaera viennensis*).

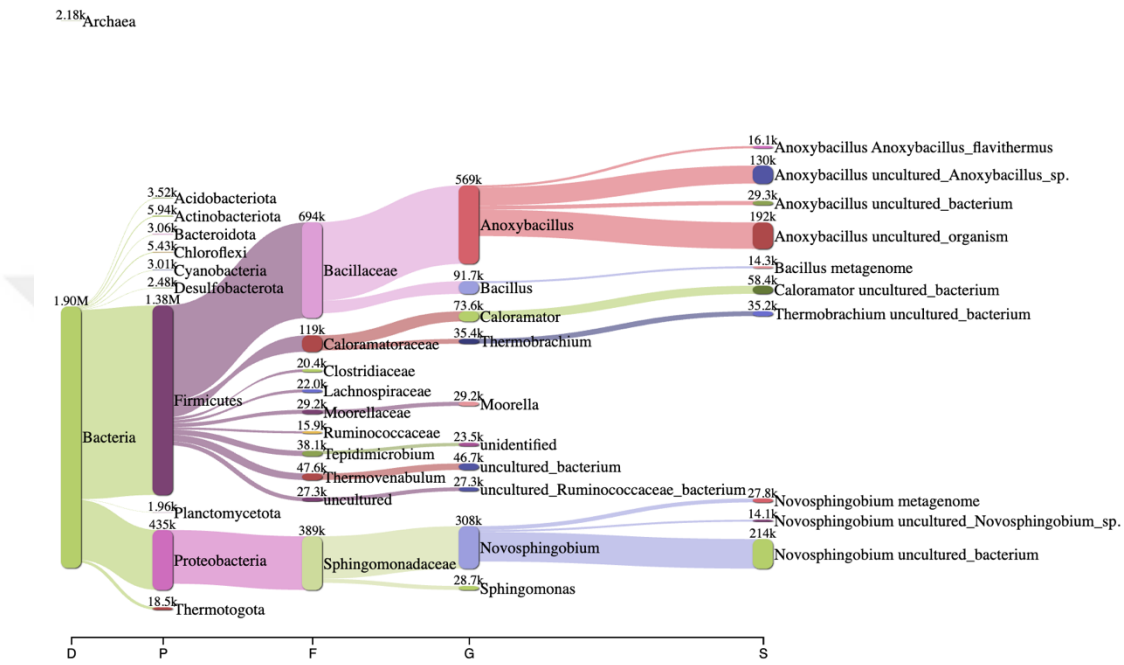


Figure 24. 16S rRNA Taxonomical Analysis of Isolates from Doğanbey Hot Spring

As seen in Figure 24, species that show hydrogen production in the community become dominant after the samples are taken into anaerobic and thermophilic culture medium and after feeding with 100% CO gas. Bacterial species belong in Firmicutes genus *Anoxybacillus* and *Caloramator* have been dominantly observed which have been reported to produce H_2 (Hniman et al., 2011). *Moorella stamsii* species which is a thermophilic carboxydotrophic hydrogenogen was also detected in the culture (Alves et al., 2013) and *Tepidimicrobium ferriphilum* species were also seen in the sample that reported to grow on syngas (Liu et al., 2020).

Cultivated Doğanbey mixed culture has become dominant in H_2-producer species after their cultivation under thermophilic and anaerobic laboratory conditions to promote hydrogenogenic growth. Abundance of H_2-producers were significant where more than 50% of the microorganisms belong in thermophilic and hydrogenogenic bacteria: Caloramator (28%), Moorella (14%), Anoxybacillus (9%) and Clostridium (2%).

By enriching the samples in growth medium, *Methanothermobacter thermautotrophicus* archaea, which is a hydrogen consumer methanogen was able to reach a significant population in the community (Yoshinaga et al., 2015). This archaeon could be consuming the hydrogen produced by hydrogenogens in the sample. Microbial consortia dynamics include the coexistence of microorganisms that produce and consume hydrogen (Wang and Yin, 2021). Higher yields of hydrogen production were observed in the cultures belong to Doğanbey hot spring compared to other hot spring samples (Table 19). Presence of thermophilic and anaerobic bacteria species in the microbial community constructed a suitable mixed culture for hydrogen production by converting CO gas.

Metagenomic Analysis of Çeşme Hot Spring

According to the results obtained from the Çeşme hot spring with direct isolation from water, it is seen that the *Novospingobium* bacteria species, which live in milder temperature conditions (30-45 °C), dominate the community (Figure 25) (Hyeon et al., 2017). This is a reasonable finding considering the temperature of hot spring was measured as 38-43°C (Table 18).

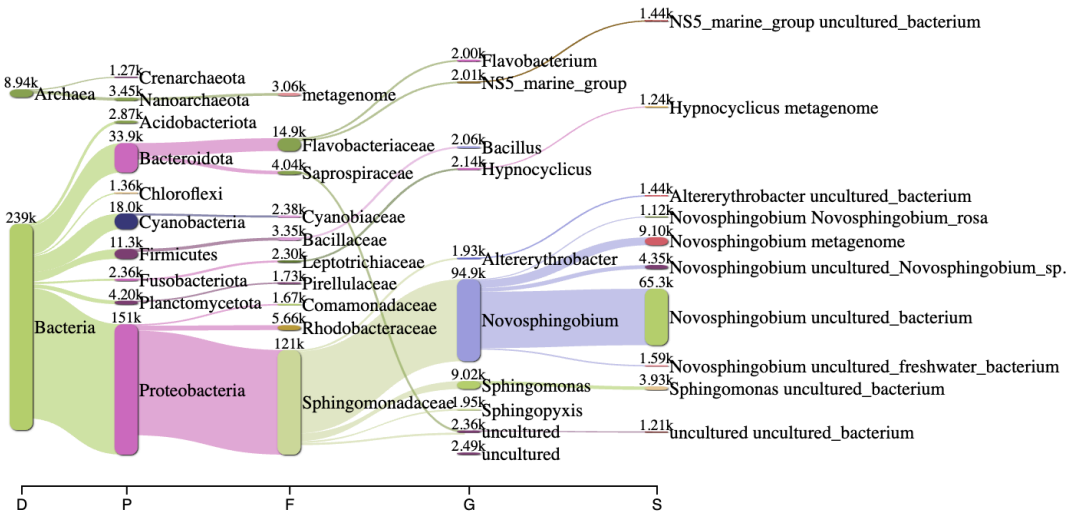


Figure 25.16S rRNA Taxonomical Analysis of Çeşme Hot Spring

By culturing water samples in growth medium under anaerobic and thermophilic conditions, the population of thermophilic bacteria such as *Anoxybacillus, Caloramator, Moorella* and *Thermosinus* in the community was increased (Figure 26). This rate of was quite low (<1%) in the samples directly isolated from Çeşme hot spring, however successful enrichment and reproduction of anaerobic, thermophilic and hydrogen producing species was achieved with the suitable cultivation conditions. Hydrogen production was observed in the cultures originated with Çeşme hot spring (Table 19).

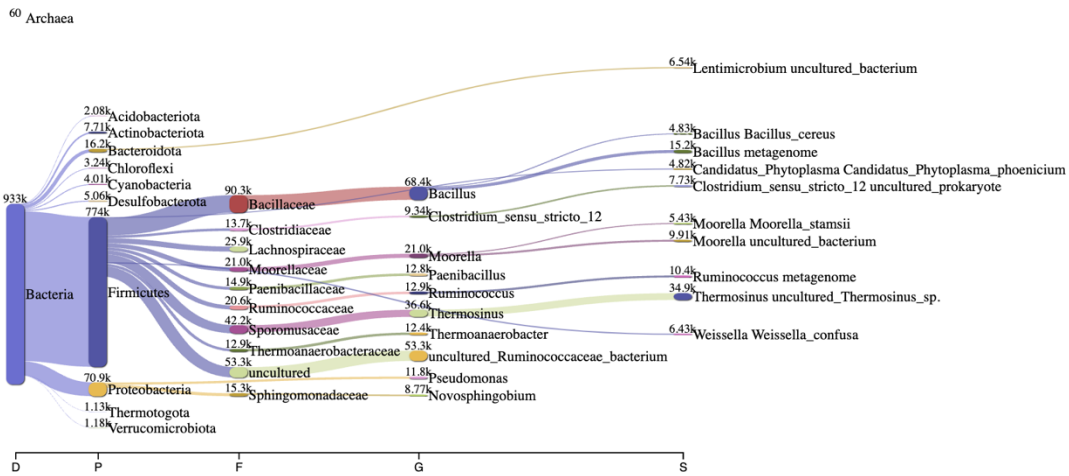


Figure 26. 16S rRNA Taxonomical Analysis of Isolates from Çeşme Hot Spring

Metagenomic Analysis of Dikili Bademli Hot Spring

Figure 27 demostrates that metagenomics of Dikili Bademli hot spring mainly consisting of (up to 60%) *Novosphingobium* species. This hot spring habituating to some thermophilic species (Firmicutes), but in very low abundance. According to Table 18, this hot spring's temperature was varying from 38-43 °C, meaning that only moderately thermophilic species such as *Novosphingobium* can be dominantly consist in this microbial consortium.

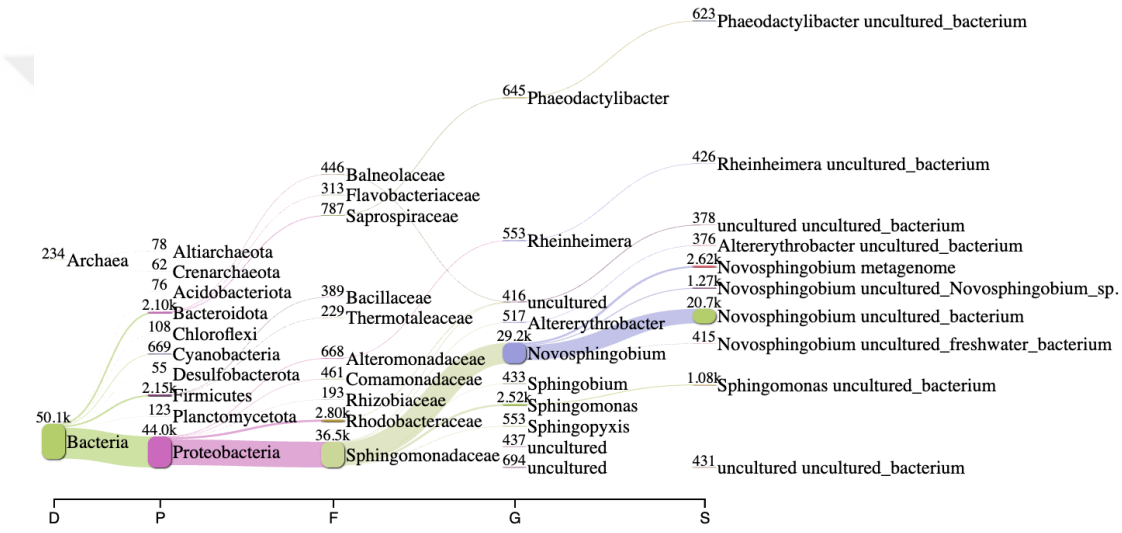


Figure 27. 16S rRNA Taxonomical Analysis of Dikili Bademli Hot Spring.

Dikili Bademli region samples were able to be reproduced in liquid culture. As shown in Figure 28, thermophilic and anaerobic bacterial species such as *Treponema, Thermosinus, Clostridium, Caloramator, Moorella, Symbiobacterium* could be reproduced in culture, although small amounts of hydrogen production were observed in these cultures (Table 19 and Table 20).

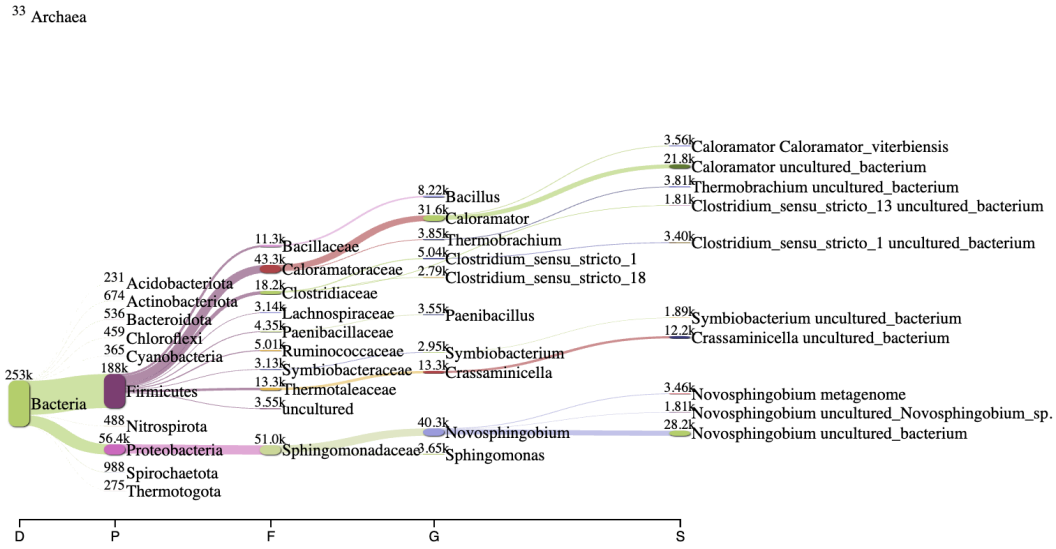


Figure 28. 16S rRNA Taxonomical Analysis of Isolates from Dikili Bademli Hot Spring.

Metagenomic Analysis of Dikili Nebiler Hot Spring

Unlike Dikili Bademli region, Dikili Nebiler hot spring isolates could not be continuously grown in the culture medium after it was isolated directly from the water, DNA isolation from the culture could not be performed. DNA isolation from direct water obtained from this region, 16S metagenomic analysis results were also obtained in a very low number of reads, and the bacterial population in the region was found to be unfavorable, dominant in Lactobacillus species (Figure 29). For the Dikili Nebiler region, most of the samples taken by isolation from direct water showed insufficient DNA amount even in the Qubit measurement after PCR. The reason for this is seen as the source water obtained from the region first passed through a bath in the facility and then given out as a continuous flow, and this flow preventing the formation of any bacterial population.

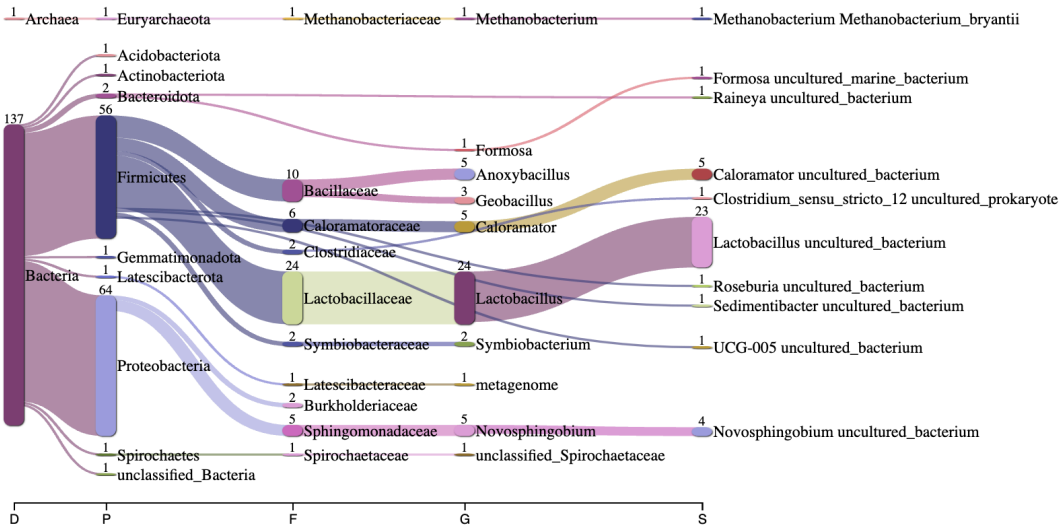


Figure 29. 16S rRNA Taxonomical Analysis of Dikili Nebiler Hot Spring.

Metagenomic Analysis of Bergama Hot Spring

Anaerobic and thermophilic bacteria *Hydrogenophaga* and *Alishewanella* genera, which were previously isolated from hot springs, were observed with the taxonomic classification obtained as a result of sequencing (Figure 30) made by sequencing directly from the Bergama hot spring (Ghilamicael et al., 2017; Lin et al., 2017).

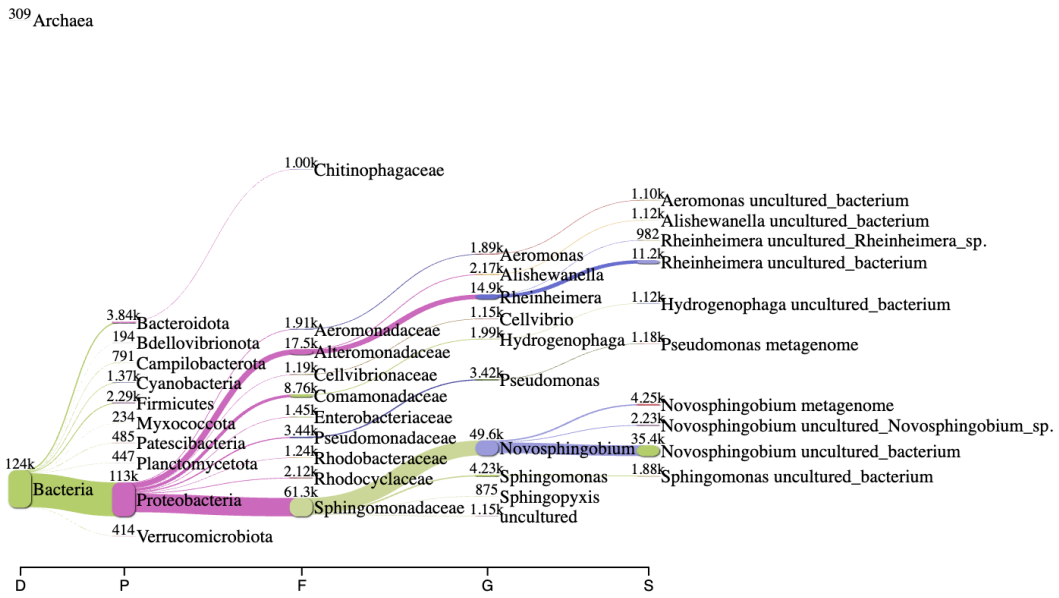


Figure 30. 16S rRNA Taxonomical Analysis of Isolates from Bergama Hot Spring.

As a result of growing water cultures taken from Bergama hot spring in culture medium, the population of thermophilic and anaerobic bacteria (Figure 31) in the community was increased (*Thermodesulfovibrio, Anoxybacillus, Geobacillus, Thermosediminibacter, Caloramator* and *Symbiobacterium*). Hydrogen production was observed as a result of GC analyzes in most of the cultures of the species isolated from Bergama hot spring (Table 19).

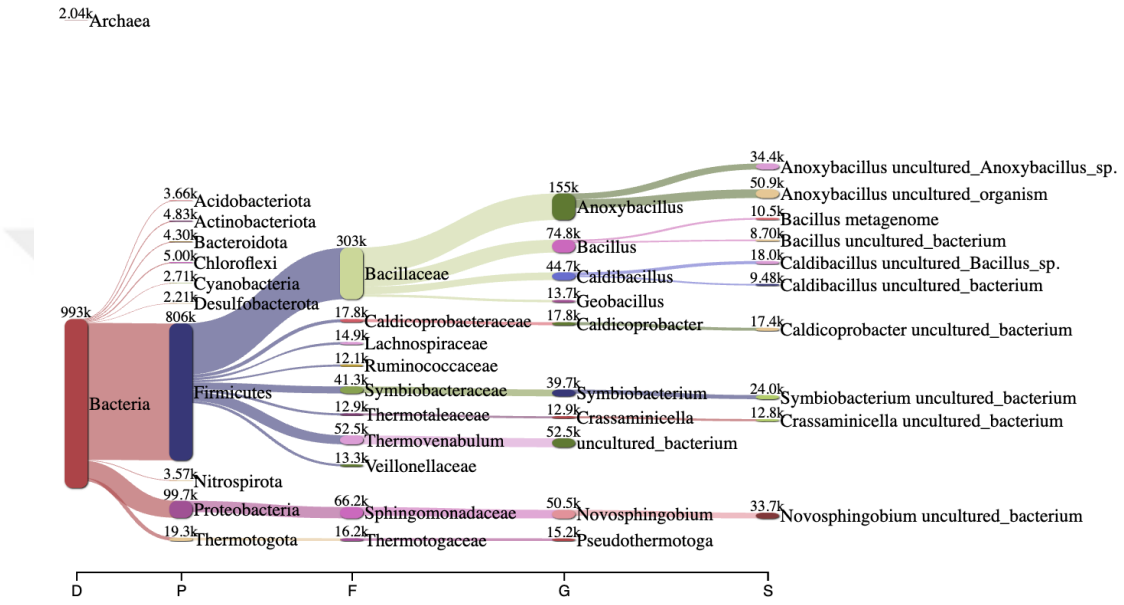


Figure 31. 16S rRNA Taxonomical Analysis of Cultivated Bergama Hot Spring Isolates

Metagenomic Analysis of 5 Hot Spring Thermal Water and Cultivates Isolates

According to taxonomic classification data obtained from all hot springs (Figure 32); In the samples belonging to 5 hot springs, bacteria (95%) with 6,376,218 readings and archaea with 85,786 readings (1%) were classified as taxonomic in the Superkingdom class. 4% of the readings could not be classified. *Novosphingobium* species capable of degrading gram-negative, aromatic compounds (*Novosphingobium sediminis, Novosphingobium aromaticivorans, Novosphingobium arabidopsis*) was found to be dominant (17%), but because this bacterial species is moderately thermophilic (37–45 °C) and aerobic, it is not considered as one of the bacterial species of interest in terms of H_2 production within the scope of the study (Xian et al., 2019).

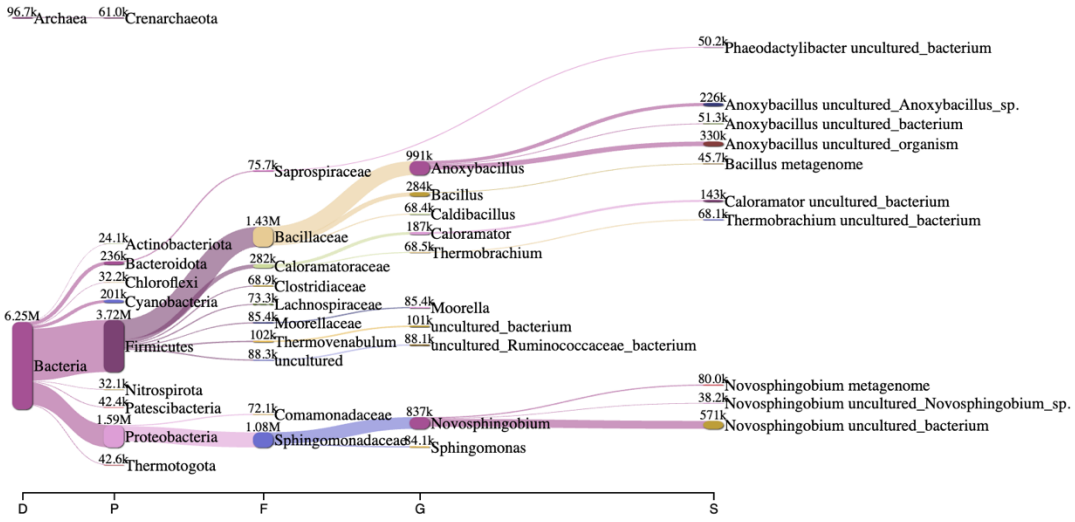


Figure 32. 16S rRNA Metagenomic analysis and taxonomic classification of hot spring microorganisms in İzmir region

Among the bacterial communities, *Moorella (Moorella humiferrea, Moorella thermoacetica, Moorella gylcerini)*, a gram-positive thermophilic and anaerobic bacterium, is seen as the dominant genus after *Novospingobium* species (15*%).* Thermophilic, anaerobic and gram positive *Anoxybacillus (Anoxybacillus kamchatkensis and Anoxybacillus flavithermus)* species and gram positive, facultative anaerobic and moderately thermophilic Caloramator species (*Caloramator australicus* and *Caloramator quimbayensis*) with 3% population density were determined. *Moorella, Anoxybacillus* and *Caloramator* genera, which emerge dominantly in the community, are species that have spore-forming properties and were previously isolated from hot springs (Nepomnyashchaya et al., 2012; Ogg and Patel, 2009; Panosyan et al., 2021).

Table 27. Carboxydotrophic hydrogenogenic microorganisms detected in İzmir hot springs

Genus	**Carboxydotrophic hydrogenogenic microorganism**	**%**	**Reference**
Methanosarcina	*Methanosarcina acetivorans*	0.000052	(Rother and Metcalf, 2004)
Thermincola	*Thermincola ferriacetica*	0.004672	(Zavarzina et al., 2007)
Moorella	*Moorella thermoacetica*	0.000935	(Diender et al., 2015)
	Moorella stamsii	0.254945	
Thermosinus	*Thermosinus_carboxydivorans_Nor1*	0.005706	(Sokolova, González, et al., 2004; Techtmann et al., 2012; Toshchakov et al., 2018)
	Thermosinus_carboxydivorans	0.000660	
Carboxydothermus	*Carboxydothermus_pertinax*	0.000052	(Yoneda et al., 2012)
Carboxydocella	*Carboxydocella_ferrireducens*	0.006472	(Slobodkin et al., 2006; Sokolova et al., 2002)
	Carboxydocella_thermautotrophica	0.001532	
	Carboxydocella_sp._930	0.000501	
	Carboxydocella_sp._961	0.000475	
	Carboxydocella_sp._1244	0.000079	
	Carboxydocella_sp._1503	0.000079	
Caldanaerobacter	*Caldanaerobacter Caldanaerobacter_subterraneus*	0.003196	(Bao et al., 2002; Fardeau et al., 2015, 2004; Kozina et al., 2010)
	Caldanaerobacter Thermoanaerobacter_sp._RH0804	0.000528	

Table 27 demonstrates the presence of carboxydotrophic hydrogenogenic organism findings in 5 different hot springs in İzmir. These microorganisms are very low in abundance in İzmir thermal waters (<0.02), as this was an expected finding from reported investigations that thermophilic, CO-oxidizing and H_2 producing microorganisms are rare in nature (<0.1%) and usually found in a dormant state, which is associated with little to zero growth (Brady et al., 2015; Fukuyama et al., 2020; Yoneda et al., 2015).

3.6. Optimization of Biohydrogen Production Yields Using Box Behnken Design

3.6.1. Optimization of Biohydrogen Production Yields Using Box Behnken Design with Operational Parameters

The maximum biohydrogen yield obtained for mixed culture was at 60°C, pH 7.00 and 10 mL CO addition (0.28 mmol H_2/ mmol CO) (Table 28). ANOVA results showed that quadratic model ($p<0.05$) is not significant (p-value: 0.0669). The R^2 value of this model was 0.8071, suggesting that the model was able to explain 80% of the variations in the data. Model can predict the response with more accuracy as R^2 value approach to 1 (Moodley and Gueguim-Kana, 2017). Thus, BBD model investigating 3 operational parameters cannot be sufficiently tested against their significance in H_2 production yield (Figure 33). However, higher H_2 yields were obtained compared to other trials (Table 19). Where maximum H_2 production yield was 0.13 mmol H_2 /CO mmol for Doğanbey culture, BBD optimization experiments using the same culture yielded up to 0.28 mmol H_2 /CO mmol.

Fermentative biohydrogen production was studied with BBD in the literature, dominantly using organic substrates and waste products for H_2 production (Grohmann et al., 2018; Hitit et al., 2017; Moodley and Gueguim-Kana, 2017; Rai et al., 2019; Sekoai, 2016; Singla et al., 2017; Varrone et al., 2012; Vi et al., 2017; Wang et al., 2021). Hitit et al., 2017 reported high yields of H_2 (6.4 mol H_2/mol glucose) by optimizing substrate concentration.

Salakkam et al., 2017 increased the cumulative hydrogen production by 60 % with BBD optimization with variables of substrate concentration, micronutrient concentration and pH. Moodley and Gueguim-Kana, 2017 reported a 45% increase in H_2 production comparatively to a non-optimized experimental set up.

Table 28. Hydrogen production yields with Box Behnken Optimization

Run	Fe^{+2} (mg/L)	Zn^{+2} (mg/L)	Ni^{+2} (mg/L)	Yield (mmol H_2/ mmol CO)
1	250	87.5	100	0.19
2	250	25	55	0.16
3	50	25	55	0.01
4	150	150	10	0.17
5	150	25	10	0.03
6	50	150	55	0.24
7	250	25	10	0.25
8	150	150	100	0.17
9	150	87.5	55	0.27
10	150	87.5	55	0.01
11	150	150	100	0.25
12	50	87.5	10	0.00
13	250	150	55	0.01
14	50	87.5	100	0.15
15	150	87.5	55	0.28
16	150	87.5	55	0.00
17	150	87.5	55	0.00

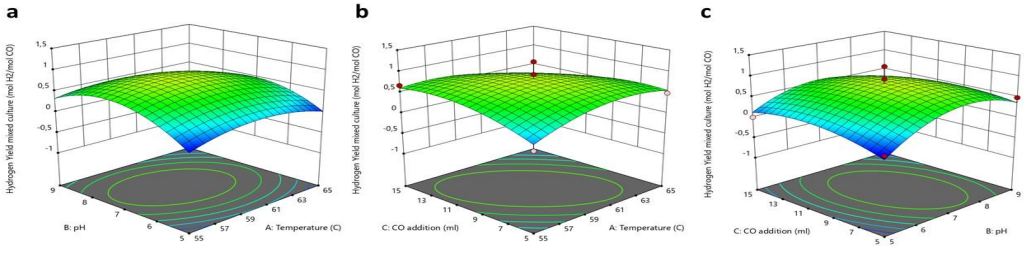


Figure 33. Effects of different parameters on the hydrogen yield for mixed culture (a) temperature (y-axis) and pH (x-axis) (b) CO addition (y-axis) and temperature (x-axis) (c) CO addition (y-axis) and pH (x-axis)

Validation trials were also conducted, selecting best and worst yielding runs, and to test the capacity of experimental set to reproduce (Table 29). Model validation did not give an increase in H_2 yield (0.26 mmol H_2 /CO mmol). However, the validation data demonstrated a compatible pattern with the original data.

Table 29. Hydrogen production yields of validation trials with Box Behnken Optimization

Run	Temperature (°C)	pH	CO volume (mL)	Yield (mmol H_2/ mmol CO)
2	65	7	5	0.18
3	65	5	10	0.04
7	60	7	10	0.11
12	65	9	10	-
15	60	7	10	0.26

Table 32 represents the comparison of hydrogen production yields with CO-utilizing microorganisms, with various concentrations of CO gas. Mohr et al., 2019 presented significantly improved yields, highest yield among in literature 0.808 mmol H_2 / CO mmol, with different concentrations of CO. Present study using a mixed culture isolated from a hot spring presents highest yield of 0.28 mmol H_2 /CO mmol.

Considering that mixed cultures include H_2 producer species along with H_2 oxidizers, it is expected to obtain lower yields of H_2, as produced H_2 could also be consequently consumed in the culture. Also, microorganisms isolated from their natural habitats need longer times for adaptation, especially higher concentrations of CO. As CO content in natural habitats are low (~0.0002%), microbial communities are not exposed to high concentrations of CO and their adaptation to CO and abilities to oxidize CO improves with time, serial transfers and subculturing (Lee et al., 2016; Pérez et al., 2008).

3.6.2. Optimization of Biohydrogen Production Yields Using Box Behnken Design with Bivalent Metal Concentration of Growth Medium

A three factor Box Behnken design was used to optimize multiple parameters based on the process parameters studied during the single parameter optimization. An analysis of the BBD revealed the effect of independent variables metal concentration on hydrogen yield.

The maximum biohydrogen yield obtained for mixed culture (0.40 mmol H_2/ mmol CO) was with a bivalent metal composition of 150 mg/L Fe^{+2}, 25 mg/L Zn^{+2}, 10 mg/L Ni^{+2} (Table 25). ANOVA results showed that quadratic model ($p<0.05$) significant (p-value: 0.0374) with a standard deviation of 0.0657. The R^2 value of this model was 0.8416, suggesting that the model was able to explain 84% of the variations in the data. BBD model investigating 3 different metal concentrations' effect on biological hydrogen production was sufficiently tested (Figure 33).

H_2 yields were optimized compared to other trials (Table 19 and Table 27). Where maximum H_2 production yield was 0.28 mmol H_2 / CO mmol for Doğanbey culture, BBD optimization experiments using the same culture yielded up to 0.40 mmol H_2 /CO mmol.

Srikanth and Mohan, 2012 investigated the effect of bivalent metal ions for the acetogenic biohydrogen production and demonstrated that Fe^{+2}, Zn^{+2} and Ni^{+2} and their concentrations in the growth medium. Growth medium used in this study (Table 4), included a Fe^{+2} and Zn^{+2} concentration of 100 mg/L and no presence of Ni^{+2}.

Table 30. Hydrogen production yields with Box Behnken Optimization

Run	Fe^{+2} (mg/L)	Zn^{+2} (mg/L)	Ni^{+2} (mg/L)	Yield (mmol H_2/ mmol CO)
1	250	87.5	100	0.02
2	250	25	55	0.1
3	50	25	55	0.06
4	150	150	10	0.2
5	150	25	10	0.4
6	50	150	55	0.22
7	250	25	10	0.04
8	150	150	100	0.1
9	150	87.5	55	0.06
10	150	87.5	55	0.09
11	150	150	100	0.1
12	50	87.5	10	0.29
13	250	150	55	0.02
14	50	87.5	100	0.17
15	150	87.5	55	0.05
16	150	87.5	55	0.01
17	150	87.5	55	0.02

Results of the BBD optimization suggested that addition of nickel to growth medium along with an increase in iron and a decrease in zinc concentration significantly improved biohydrogen production yields for a thermophilic and anaerobic mixed culture with CO-utilization (Table 30 and Figure 34).

Model equation for hydrogen yield was obtained as Eq. (5):

$$Hydrogen\ Yield = 0.504545 + 0.000044 * Fe - 0.002600 * Zn - 0.008941 * Ni - 9.60000e^{-6} * Fe * Zn + 5.55556e^{-6} * Fe * Ni + 0.000018 * Zn * Ni - 7.00000e^{-7}Fe^2 + 0.000016 * Zn^2 + 0.000046 * Ni^2 \quad (5)$$

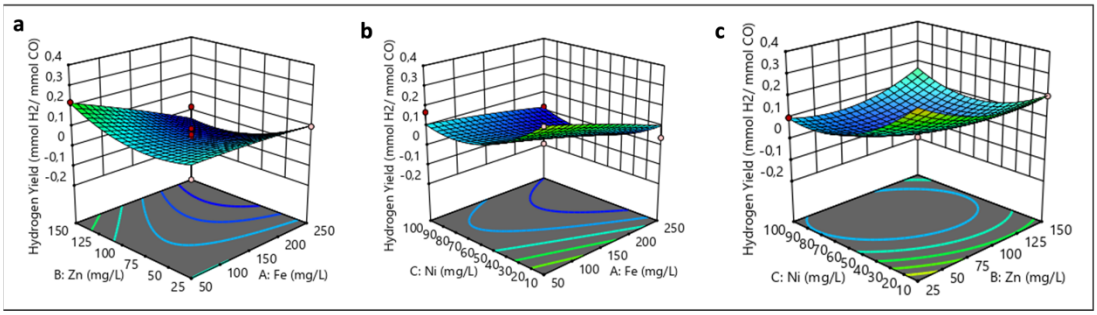


Figure 34. Effects of different parameters on the hydrogen yield for mixed culture (a) Fe^{+2} (mg/L) (y-axis) and Zn^{+2} (mg/L) (x-axis) (b) Fe^{+2} (mg/L) (y-axis) and Ni^{+2} (mg/L) (x-axis) (c) Zn^{+2} (mg/L) (y-axis) and Ni^{+2} (mg/L) (x-axis)

Where the model formula (Eq.5) is used for the optimization of hydrogen yields in Design Expert software, a sample run formulated as 50 mg/L Fe^{+2}, 25 mg/L Zn^{+2} and 10 mg/L Ni^{+2} calculated to be resulting in a maximum hydrogen yield of 0.360 mmol H_2/ mmol CO which is lower in yield compared to actual design (0.40 mmol H_2/ mmol CO) indicating that the actual Box-Behnken design of 17 runs produced maximum hydrogen yield.

3.7. Biohydrogen Production with Anaerobic Thermophilic Mixed Culture Using a Stirred Tank Reactor

After 4 days of batch production using Sartorius Biostat® B bioreactor, a significant decrease in pH was observed, initial pH of the fermentation broth was 7.00 and final pH was 5.72 (Figure 35). The pH decrease indicates the production of organic acids, particularly VFA's as these organic acids are by-products of CO-fermentation (Roy et al., 2016).

pH of the fermentation broth and accumulation of by-products (ethanol and VFA's) effects the carbon and electron transfer between substrate and cell. At acidic pH, intercellular diffusion is interfered and an energy shift from bacterial growth to transportation of fatty acid metabolites can occur, which causes low yields (Zhang et al., 2013). A decrease in H_2 yields from 0.25 to 0.04 mmol H2/ mmol CO could be related to the pH of fermentation broth inclining to an acidic phase after the 2nd day of the mixed culture bioreactor (Figure 36 and Table 31).

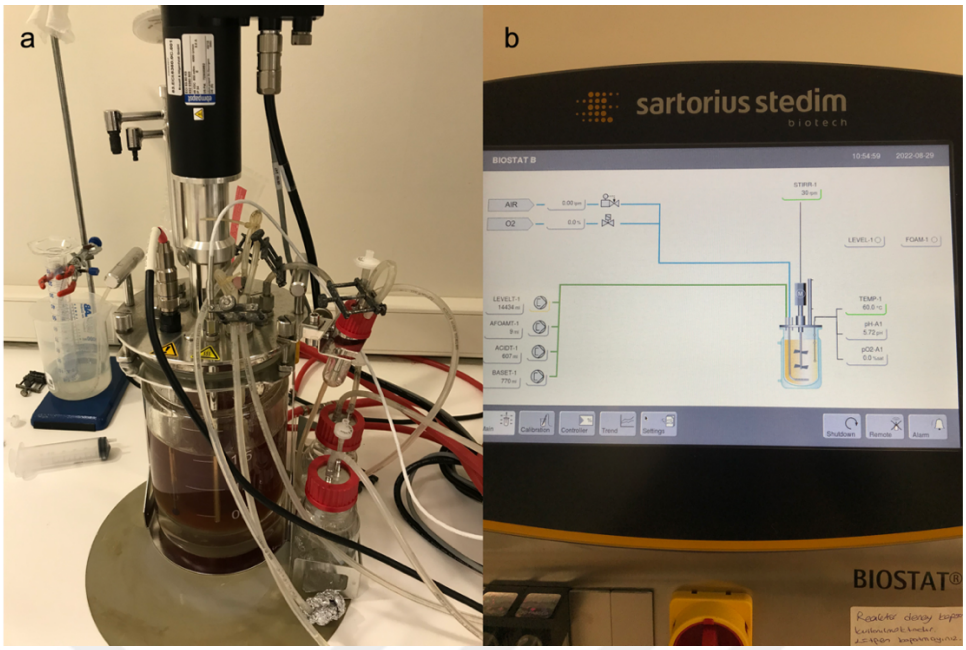


Figure 35. (a) 14-day bioreactor set-up for the biohydrogen production using Doğanbey mixed culture (b) bioreactor configurations of temperature and pH

CO-fermentation utilized in this study has reported lower yields compared to other syngas fermentation studies in the literature (Table 32). Uttermost, low H_2 yields was interpreted as the inability to provide a 100% anaerobic environment under bioreactor operating conditions and the presence of hydrogen-consuming and methane-producing species within the scope of mixed consortia directly isolated from hot spring. For H_2 low yields caused by methanogenesis, where methanogenic bacteria use H_2 as a side reaction when a mixed culture is used, inhibition of methanogenic bacteria can be achieved by addition of bromoethanosulfonic acid (Shen et al., 2018).

H_2 production using mixed cultures as an inoculum source with syngas fermentation could result in lower production yields (Table 32). Also, for eliminating disadvantage of using a mixed culture is by using pretreatment methods (physical, chemical or combined methods) to obtain higher H_2 yields by inhibiting H_2-consuming bacteria and conserve the population of H_2-producing bacteria in mixed cultures and seed sludges.

Table 31. Biohydrogen production yields of Doğanbey mixed culture with a CSTR bioreactor

Sample ID	ΔH_2	ΔCO	Day	Yield (mmol H_2/ mmol CO)
Reactor_Mix	0	0	0	0
Reactor_Mix	0.973	19.925	1	0.05
Reactor_Mix	1.638	6.684	2	0.25
Reactor_Mix	0.445	3.573	5	0.12
Reactor_Mix	0.174	2.706	7	0.06
Reactor_Mix	0.111	2.676	8	0.04

By employing pretreatment methods, mixed cultures and their advantageous properties such as being flexible to environmental distress (change in pH, temperature, aeration conditions) and synergetic relations in substrate consumption can be used in benefit for high yield H_2 production (Wong et al., 2014). Especially, heat pretreatment in seed sludge indicated higher H_2 yields and 3-fold higher hydrogenase enzyme gene expression in a study. Heat pretreatments reported to be eliminating non-spore forming hydrogen-consuming bacteria and stimulate the spore germination in *Clostridium* genus which is one of the most fundamental H_2-producers where Doğanbey mixed culture used in this study includes 2% of *Clostridium* species in the microbial consortia.

CO-fermentation and its effectiveness in terms of the substrate being attainable to microorganism is measured as achieving improved gas- liquid interfacial areas in fermentation process. Small-scale gas bubbles effectively enhance the microorganism' ease of access to CO gas, this approach can be achieved by increasing agitation speeds. However, higher energy input results in both augmented energy requirements and stress to cells (Bredwell et al., 1999; Bredwell and Worden, 1998; Mohammadi et al., 2011; Roy et al., 2016).

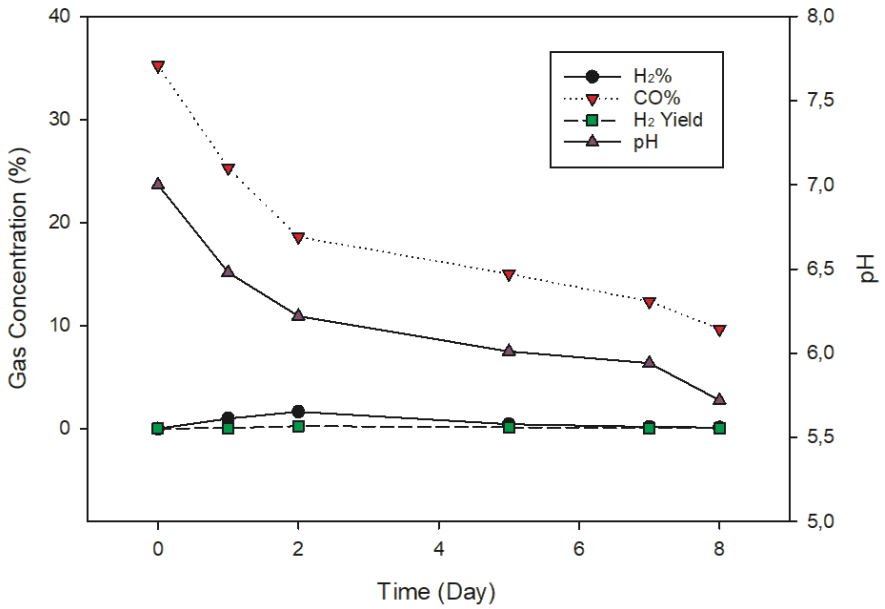


Figure 36. ΔH_2, ΔCO, pH and H_2 production yields results with mixed culture

Microbubble (diameter: 50-100 μm) strategies using disc sparger designs aimed for an alternative solution of gas and liquid mass transfer limitations in syngas fermentation by increasing gas solubility, improving the CO uptake and consequently the improved CO-utilization of microorganisms for hydrogen production (Bredwell and Worden, 1998). Disc spargers for the utilization of gas delivery as a bioreactor configuration reported in the literature being as a powerful tool for taking a step further in terms of industrialization of syngas fermentation to the grater scales with being economically viable as they increase CO solubility in aqueous phase (Munasinghe and Khanal, 2012; Yasin et al., 2019).

Agitation speed, gas flow rate and bubble size are all parameters that affect kLa value in conventional CSTR bioreactor operations. However, to increase kLa value configurations of these parameters demand either continuous input of gaseous substrate or increased power inputs that result in increased shear stress and costs (Keryanti et al., 2019).

Table 32. Comparison of biohydrogen production yields with syngas fermentation

Culture Type	Substrate (%CO)	Yield (mmol H_2 /CO mmol)	Reference
Pure culture	75%	0.808	(Mohr et al., 2019)
Pure culture	36%	0.748	(Mohr et al., 2019)
Pure culture	55%	0.65	(Najafpour et al., 2004)
Mixed microbial consortium	100%	0.40	In this study

Addition of immobilization methodologies such as attachment or entrapment of bacteria to a surface eliminates shear stress on cells and lowers the energy inputs significantly and mass transfer rates increase in gas-liquid interface by the transfer of gaseous substrate (such as syngas or pure CO) in bioreactor.

Emphasizing the immobilization technique by the use of bubble column or biofilm reactors such as Monolithic biofilm reactor (MBR), Trickle bed reactor (TBR) and hollow-fiber membrane biofilm reactor (HFMBR) on improving mass transfer rates as well as achieving greater process stability was investigated in the literature. MBR and HFMBR technologies reported to be enhancing volumetric mass transfer (kLa) value for CO as well as CO conversion yields (Gunes, 2021; Kreutzer et al., 2005; Shen et al., 2014a, Shen et al.,2014b; Yasin et al., 2019).

For improved yields of H_2 by using hot spring mixed cultures, a bioprocessing strategy based on pretreatment of microbial consortia to suppress H_2-consuming bacteria and preserve H_2-producing bacteria and implementing immobilization techniques on bioreactors could significantly enhance H_2 production yields by improving mass transfer rate in the gas-liquid interface and resulting in a more concentrated microbial mass.

It is widely recognized that biohydrogen production can be achieved through use of a variety of organic substrates in various literature investigations. However, there are three comparable studies in which water-gas shift reaction is demonstrated with inorganic CO as a sole carbon source, resulting in a unique method of obtaining biohydrogen. Comparative hydrogen yields of this study are summarized in Table 32, and further suggestions were made in the discussion section to improve the biohydrogen yields using inorganic CO as a carbon source and utilizing water-gas shift reaction with using hot spring mixed culture where higher biohydrogen yields to be obtained as a prominent study in the literature.

CHAPTER 4: CONCLUSIONS

As a conclusion, this study successfully applied microbiological methods of cultivating, enriching and growing hot spring isolates along with the implementation of molecular biology methods of taxonomic classification with third-generation sequencing of thermophilic and anaerobic isolates that have the biohydrogen production capacity. Isolates were successfully enriched in an anaerobic, thermophilic growth medium with a 1 g/L yeast extract supplementation and 100% CO gas inoculation. 5 different hot spring mixed cultures were monitored regularly for their CO conversion capacity to H_2 gas. Doğanbey hot spring selected the best for H_2 production with highest yields among 5 different hot springs, and its metagenomic analysis showed that hot spring consortium cultivations dominantly included *Anoxybacillus* and *Caloramator* species belong in Firmicutes genus, which have been reported to including most of the thermophilic carboxydotrophic hydrogenogenic bacterium. Compared to the H_2 production yields reported in the literature, the results obtained in this study were comparatively inadequate. In order to obtain higher hydrogen yields, as a future study for increasing H_2 production yields establishing an anaerobic bioreactor setup could be applied with bioreactor configurations to improve the process stability and gas solubility with increased mass transfer rates.

Hot spring isolates showed the capacity of converting CO-containing gases such as synthesis gas. Implementing synthesis gas for the conversion of clean fuels with the help of bioprocesses is a sustainable and recent strategy. As synthesis gas is released as a result of incomplete combustion reactions of industrial establishments and is one of the main causes of air pollution due to toxic carbon monoxide in its content, conversion of the components in the synthesis gas into a clean energy source is a very advantageous approach in terms of sustainable environment, natural resource management and health despite the accelerating climate change. Carboxydotrophic hydrogenogenic species in the microbial community isolated from the hot springs are promising findings in biotechnological applications. This novel study investigated the potential of Izmir hot springs' capacity of habituating thermophilic species that perform syngas fermentation for green hydrogen production.

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