METAGENOMIC ANALYSIS AND BIOHYDROGEN PRODUCTION FROM PINE NEEDLES USING DARK FERMENTATION

Dissertation submitted in partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

By

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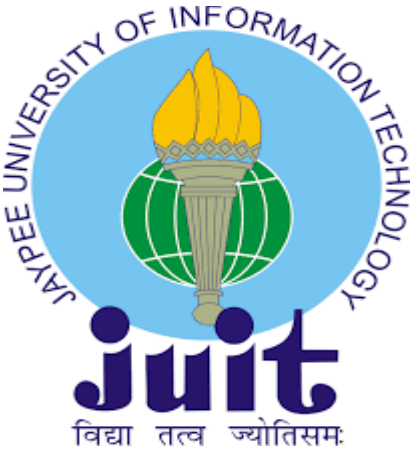

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MAY, 2024

DECLARATION

I hereby declare that the work presented in this report entitled "**Metagenomic Analysis and Biohydrogen Production from Pine Needles Using Dark Fermentation**" in complete fulfillment of the requirements for the award of the degree of **Master of Science in Biotechnology** submitted to the **Department of Biotechnology & Bioinformatics**, Jaypee University of Information Technology, Waknaghat is an authentic record of my own work carried out over a period from August 2023 to May 2024 under the supervision of **Dr. Sudhir Kumar,** Prof. & Head, and **Dr. Garlapati Vijay Kumar**, Associate Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, Himachal Pradesh. I also authenticate that I have carried out the above mentioned project work under the proficiency stream.

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

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This is to certify that the above statement made by the candidate is true to the best of my knowledge.

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CERTIFICATE

This is to certify that the work reported in the M.Sc. Biotechnology thesis entitled **"METAGENOMIC ANALYSIS AND BIOHYDROGEN PRODUCTION FROM PINE NEEDLES USING DARK FERMENTATION"**, submitted by Ms ISHA AGARWAL (225111018) at the Jaypee University of Information Technology, Waknaghat, India is a bonafide record of her original work carried out from August 2023 to May 2024 under our supervision. This work has not been submitted elsewhere for any other degree or diploma.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABBREVIATION	FULL FORM
°C	Degree Celsius
ADP	Adenosine Diphosphate
AFEX	Ammonia Fibre Explosion
AGL	Avantika Gas Limited
ATM	Atmospheric pressure
ATP	Adenosine Triphosphate
BHP	Biological Production of Hydrogen
BLAST	Basic Local Alignment Search Tool
Chl	Chlorophyll
CMC	Carboxymethyl Cellulose
CMISR	Continuous Mixed Immobilised Sludge Reactor
CNG	Compressed Natural Gas
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred Tank Reactor
Cu	Copper
DF	Dark Fermentation
DNA	Deoxyribonucleic acid
dNTPs	Dinucleoside Triphosphates
EC	Electrical Conductivity
EFB	Empty Fruit Bunch
Fd	Ferredoxin
Fe	Iron
GHG	Green House Gases
H_2	Hydrogen
HCl	Hydrochloric Acid
HPR	Hydrogen Production Rates
HRT	Hydraulic Retention Time
HY	Hydrogen Yield
IEA	International Energy Agency
K	Kelvin
LAB	Lactic Acid Bacteria
LB	Lignocellulosic Biomass
LCB	Lignocellulosic Biomass
MEC	Microbial Electrolysis Cells
MFCs	Microbial Fuel Cells
Mg	Magnesium
MHz	Mega Hertz
mL	Millilitres

Mn	Manganese
MPa	Mega Pascal
MTC	MICROBIAL TYPE CULTURE
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
NGHM	National Green Hydrogen Mission
Ni	Nickel
NTPC	National Thermal Power Corporation
NZE	Net Zero Emissions
OD	Optical Density
OLR	Organic Loading Rate
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
PPMV	Parts Per Million By Volume
PS	Photosystem
Psi	Pound force per square inch
rDNA	Recombinant DeoxyribonucleicAcid
RT	Room Temperature
SMR	Steam Methane Reforming
TCD	Thermal Conductivity Detector
TDS	Total Dissolved Solids
TVS	Total Volatile Solids
VS	Volatile Solids
WGS	Water Gas Shift

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The present research uses an extremophilic consortium to produce hydrogen from lignocellulosic-enriched pine needles through dark fermentation (DF). DF constitutes, as aforementioned, sustainable techniques that use metabolism-coupled oxidation to produce hydrogen. It has recently emerged as a practical approach to decreasing GHG emissions while confronting climate change challenges. For the research, the sample was gathered from the Tattapani hot spring, 655 meters absl on the Satluj River's bank in the Mandi region of Himachal Pradesh, India. Metagenomics research revealed that Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria constituted the most prevalent bacterium phyla in thermal waters. Several operational variables, such as pH, temperature, and substrate concentration, were altered to increase hydrogen yield while retaining anaerobic conditions. Qualitative and quantitative tests were done for biohydrogen estimation, and favourable results were obtained. Sequencing was performed to identify the hydrogen-producing bacteria; *Anoxybacillus kestanbolensis* strain K1 was found to produce hydrogen in the sample.

Keywords: Extremophiles, Metagenomics, Pine Needles, Biohydrogen, Dark Fermentation

CHAPTER 1
INTRODUCTION

1.1 General

The escalating energy needs of industrialized nations have resulted in greater dependence on finite fossil fuel reserves. This reliance strains the dwindling fossil fuel supply and contributes to a concerning rise in global pollution levels. The heightened emission of greenhouse gases from burning fossil fuels has intensified the risks associated with global warming. Each year, approximately 6 gigatons of CO_2 flow into the environment through fossil fuel combustion.

The current concentration of CO_2 is approaching 350 parts per million by volume (ppmv), which can intensify the greenhouse effect and raise global temperatures. In recent decades, human activities have released organic carbon into the atmosphere, similar to carbon stored millions of years ago. Motor vehicles primarily contribute to global carbon monoxide (CO) and CO_2 emissions, accounting for over 70% and 19%, respectively. Based on current rates of consumption, reserves of oil (168.6 billion metric tonnes), natural gas (177.4 trillion cubic meters), and coal (847.5 billion metric tonnes) may be depleted within the next 133 years. Petroleum-based products first emerged as a dominant energy source approximately 150 years ago, when kerosene began to replace whale oil for lighting purposes. Concerns regarding climate change and the finite nature of global oil reserves have shifted attention toward developing renewable and sustainable power sources to address the expanding power requirements. Although, power assets serves a significant part in the current energy scenario rapid expansion of different, sustainable, environmentally friendly, and ecologically acceptable fuels is essential to meet the rising energy demands [1].

Renewable sources such as biohydrogen play a crucial role in addressing various global issues like protection of renewable power, ecological preservation, foreign exchange funds, economic and social challenges, particularly in rural areas. Biohydrogen emerges as an environmentally friendly substitute gas for engines that run on gasoline. The environmental advantages of biohydrogen are expected to fuel substantial growth in the vehicle fuel market in the coming years. Due to multiple factors, biohydrogen technology is relevant to

developed and developing nations. Traditional industrial methods hydrogen production methods involve steam reforming of gasoline, combustion of coal, and electrolysis of water using energy supplied by fuels made from carbon [2].

Industrial operations consume substantial energy and emit pollutants like CO_2 and GHGs. Apart from thermo-chemical methods like gasification, hydrogen generation can also be achieved through biological means. Two primary approaches for utilizing organisms in hydrogen production have been proposed. The first method involves directly synthesizing "photo-biohydrogen," wherein bacteria use sunlight to separate oxygen from water. Another approach involves leveraging biotechnologies centred on microorganisms capable of producing hydrogen. These biological methods offer promising avenues for generating clean and sustainable sources of hydrogen [2].

Biorenewable sources offer a viable substitute for traditional petroleum-based fuels. Many bioengineering approaches to deriving fuels from biochemical processes involve managing the organic by-products generated through photosynthesis. A significant portion of photosynthate is allocated towards producing timber, food, and animal feed. Biomass materials can be harnessed to create energy through various methods, including involving burning, oxidation, and reactions of biochemistry like C_2H_5OH, H_2, CH_4, and C_3H_8O production. Another alternative is to modify the photosynthetic process to enable hydrogen production. Additionally, cultivating energy crops and implementing the preceding processes presents another avenue for utilizing crops as energy sources [2].

Biomass holds promise for expediting the integration of hydrogen into the energy mix of the future. Nevertheless, the process of extracting hydrogen from biomass poses considerable challenges. There have yet to be any comprehensive technical trials to date. Biomass contains relatively low hydrogen content, approximately 6%, compared to methane's 25%, and its energy content is diminished due to O_2, which accounts for about 40% of its composition [2].

1.2 Biohydrogen

Biohydrogen is an eco-friendly and naturally occurring organic substance from plants, animals, and microorganisms. The word is derived from the Greek words "bio," indicating living, "hydro," signifying water, and "genes," which means "created of" or "growing." Typically produced by bacteria, biohydrogen serves as a biofuel sourced from recycled organic matter. Industrial methods for biohydrogen production, such as thermophilic fermentation, dark fermentation, photofermentation, and gas purification, offer promising

solutions. These processes may involve anaerobic digestion and the conversion of methane from biogas into hydrogen via steam reforming [2].

Biohydrogen, generated by microalgae and bacteria, currently lacks practical production methods. Nonetheless, several promising models suggest future growth possibilities for the process. Biohydrogen encompasses hydrogen production from various biomass materials through chemical, thermochemical, biological, biochemical, and bio-photolytic pathways [2].

As an extremely plentiful substance in the cosmos, hydrogen poses a pressing challenge to produce from renewable sources. However, biohydrogen derived from renewables emerges as a promising solution for sustainable energy. Compared to petroleum and coal, hydrogen is a source of clean energy that relies minimally on hydrocarbons. This potential alternative to traditional fuels is not only clean and environmentally friendly but emits water rather than greenhouse gases upon combustion [2].

1.3 DF

DF, a biological decay method, is an up-and-coming method for managing organic wastes and is crucial in sustainable bioenergy production. According to a 2018 World Bank study, global waste production is anticipated to reach 3.5 billion tonnes in the year 2050, comprising biodegradable residues via farming areas constituting over fifty per cent of it. Managing such substantial squandered quantities sustainably is imperative, and dark fermentation emerges as a solution offering dual advantages management and renewable energy extraction. While methane is the predominant bioenergy produced from organic wastes, there is a growing demand for hydrogen generation as part of the hydrogen economy, serving as an alternative to fossil fuel-derived hydrogen. Dark fermentation presents notable advantages over other biological processes due to its low light dependency (in contrast to photosynthetic methods), capacity for high biohydrogen production rates, environmental friendliness, versatile substrate utilization, and lower energy intensity. Moreover, using biodegradable squanders as substrates for DF H_2 generation holds potential economic competitiveness, given the abundance, renewability, affordability, and high biodegradability of organic wastes. Several sustainable biological wastes, such as sake lees, cassava, sago palm glucose, rice stalks, vegetable-based food waste, date pods, sugarcane cultivation molasses, maize stover, alligator weeds palm kernel liquid and straw from wheat, have been studied as possible sources for DF –based H_2 production.

The utilization of including natural and blended cultures as inoculants in DF reactors has been explored. Opting for a mixed culture system proves more advantageous than a pure culture system due to its diverse microbial populations adept at efficiently degrading different substrates. The absence of stringent aseptic conditions streamlines handling procedures and minimizes operational expenses. Various microbial groups within mixed cultures, including H_2makers, non-biohydrogen manufacturers, and H_2 users like methane-producing and homoacetogens, contribute to complexity of the biochemical environment. Despite extensive research efforts, the biological processes underlying dark fermentation remain elusive. Biohydrogen production involves a unique microbial community and intricate trophic relationships [3].

The entire reaction was described as follows:

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 4H_2 + 2CO_2 + 2CH_3COOH \quad G^0 = -206kJ \qquad (1.1)$$

Equation (1.1) demonstrates that 1 mole of glucose can yield up to 4 moles of hydrogen. However, microorganisms utilize glucose for their growth and maintenance, producing volatile fatty acids (VFAs). As a result, the actual yield of H_2 production is lower than the theoretical yield [4].

1.4 Pine Needles

The Chir-pine tree is the most extensively distributed throughout Himachal Pradesh (*Pinus roxburghii*). Pine trees are typically located at high altitudes, and the shape of their leaves makes it possible for them to develop quickly by preventing snow and rain from staying on top of them. In Himachal Pradesh, forests encompass almost 67% of the state's total land area, providing a habitat for a staggering variety of flora and fauna. Among all the region1, 25,885 acres of land were covered with Pine trees. The primary characteristics of pine needles, namely their high flammability and acidic nature, are the essential characteristics required in biomass to manufacture biofuel.

They are surprisingly complex in their composition. They contain various organic and inorganic compounds that contribute to their diverse functions. They are lignocellulose biomasses. Here are some key components:

1.4.1 Organic Compounds:

- **Cellulose:** The most abundant component, making up around 45-51% of the dry

weight. It provides structure and strength to the needle.

- **Hemicelluloses:** These polysaccharides account for about 20-25% of the dry weight and contribute to cell wall structure and water absorption.
- **Lignin:** This complex molecule makes up 30-35% of the dry weight and provides rigidity and resistance to decomposition.
- **Essential oils:** These aromatic compounds, typically made up of terpenes and monoterpenes, are responsible for the characteristic pine scent. They also have antimicrobial and insecticidal properties.
- **Flavonoids:** These antioxidants protect the needles from free radical damage and contribute to their anti-inflammatory properties.
- **Carotenoids:** These pigments give some pine needles their yellow or orange colour and are also antioxidants.
- **Waxes:** These fatty substances coat the needles, protecting them from water loss and harsh weather conditions.

1.4.2 Inorganic Compounds:

- **Minerals:** Pine needles are rich in minerals like potassium, calcium, magnesium, phosphorus, and iron. These are vital for plant growth and can be beneficial when used for compost or fertilizer.
- **Water:** Fresh pine needles contain about 50-70% water, which helps maintain their flexibility and prevents them from drying out.

Lignocellulose materials—such as pine needles are the most abundant biomass energy source worldwide. Pine wastes are a biomass resource that is both economical and environmentally friendly. Less than 50% of a tree is typically employed to produce the final goods; the remaining portion is left as unused resources, normally burned or dumped in landfills [5][6].

1.5 Tattapani Hot Spring

The Tattapani hot spring is situated at a height of 655 meters absl, on the Satluj River's bank, in the Mandi district of Himachal Pradesh, having geographical coordinates 31°14’ 56” N, 77° 5’ 10’’E, India (Fig.1.1). The water was collected from this hot spring and sampled. From a geographical perspective, the site of the hot springs is very peculiar because the river water temperature was measured at around 5°C–6°C. In contrast, the hot water temperature in the

reservoir was 65°C. For microbial diversity research, hot water samples were taken from the hot springs that sprung up from various locations along the river's banks [7].

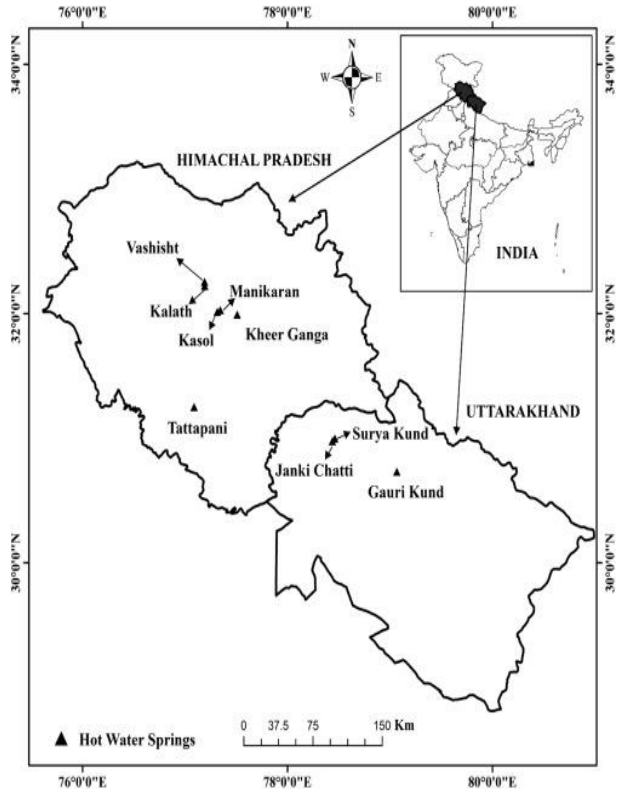

Fig. 1. 1 Map of Himachal Pradesh showing Tattapani

CHAPTER 2

REVIEW OF LITERATURE

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Biohydrogen is regarded as potentially the power source of the future due to its distinctive characteristics in environmentally friendly power generation, managing waste, and its substantial levels of energy. Several researchers and producers are now expressing an interest in the financial implications of generation. This section discusses production technologies, types of feedstocks used, microbiological, biochemical, molecular biology, and other viewpoints on the biological production of hydrogen (BHP). the pH level, substrate type, ambient temperature, stirring speed, retention of hydraulic force duration, and hydrogen relative pressure are all important components of dark fermentation.

2.1 BIOHYDROGEN PRODUCTION TECHNOLOGIES

Biological techniques for producing biohydrogen have been categorised into two types: microbiological conversion and thermochemical synthesis. Biological necessitates an abundance of certain H_2-producing enzymes to produce hydrogen. These methods primarily include degradation, biophotolysis, and bioelectrochemical pathways. Conversely, thermochemical synthesis processes require elevated temperatures to generate biohydrogen [8].

2.1.1 Fermentation

Fermentation is a chemical reaction in which microbes nitrogenases, hydrogenases, and enzymes transform organic compounds into fuel. The procedure includes the oxidative breakdown of naturally occurring substances, and the result is governed by the catalyst, organic substrate, and other parameters used. Decomposition may take place under both anaerobic and aerobic circumstances, with anaerobic digestion being more advantageous because it creates hydrogen, acidic substances and alcoholic compounds that are suitable for use in industry. The light demands of the microorganisms involved characterise two major fermentation processes: DF and photofermentation. DF decomposes biomass into biological material into hydrogen, alcohol, and acids in the absence of radiation and under oxidative circumstances. Conversely, photofermentation involves photovoltaic microorganisms to transform biological compounds to CO_2 and H_2 in the accessibility of daylight and anaerobic

circumstances. Few investigations blended these two fermentation techniques to increase the output of H_2generation [9].

2.1.1.1 DF

DF is a process in which bacteria produce biohydrogen in an anaerobic environment without light. Nevertheless, this procedure is not commonly viewed as beneficial due to its low hydrogen production and the generation of several byproducts. Equations 2.1 and 2.2 illustrate the major processes that produce biohydrogen in dark fermentation.

$$2H^+ + 2e^- = H_2 \quad (2.1)$$

$$C_6H_{12}O_6 + 6H_2O = 6CO_2 + 12H_2 \quad (2.2)$$

$$C_6H_{12}O_6 + 2H_2O = 2CH_3COOH + 2CO_2 + 4H_2O \text{ (Acetic Acid Pathway)} \quad (2.3)$$

$$C_6H_{12}O_6 = CH_3COOH + CH_3CH_2COOH + CO_2 + H_2 \text{ (Propionic Acid Pathway)} \quad (2.4)$$

$$C_6H_{12}O_6 + 6H_2O = CH_3CH_2CH_2COOH + CO_2 + 2H_2 \text{ (Butyric Acid Pathway)} \quad (2.5)$$

Equation (2.1) shows how hydrogen is generated by reducing protons with electrons released during the decomposition of a carbon source. This process relies heavily on enzymes like [Ni-Fe]-hydrogenase and [Fe-Fe]-hydrogenase. Despite the conceptual expectation of an output of 12 mol/mol $C_6H_{12}O_6$based on Equation (2.2), practical constraints limit this yield to 4 mol/mol. However, thermophilic fermentation has the potential to increase hydrogen yield. The comparatively modest H_2generation throughout DF is frequently caused by the development of several byproducts, such as CH_3COOH, CH_3CH_2COOH, and $C_4H_8O_2$ along with H_2. Dark fermentation uses a variety of substrates, including farm residues, sewage effluent, food waste, and effluent, each with its chemical composition, to generate hydrogen. Glucose or sugar-rich feeds were demonstrated to generate greater amounts of H_2 compared to protein or lipid-rich substrates, with an immediate connection throughout H_2production and feed amount. Cultivating the inoculum is another important step in increasing biohydrogen production. This pre-treatment is critical because it suppresses the activity of hydrogen-consuming bacteria known as hydrogenotrophic methanogens, which contribute to the reduced yield of biohydrogen. Pre-treatment is essential for boosting bio-hydrogen generation since it promotes the growth of hydrogen-producing bacteria while reducing the prevalence of hydrogen-consuming bacteria.

Dark fermentation releases bio-hydrogen by combining diverse organic waste products and microbes. These microorganisms are categorised to three types depending on their temp necessities: thermophiles ranging from (40-65°C), mesophiles (25-45°C), and psychrophiles (0-25°C). *Clostridium* and *Enterobacter* were two of the best regularly employed mesophilic bacteria for bio-hydrogen generation, while *Thermoanaerobium* is usually referred to as a thermophilic microbe. They can also be categorised according to their energy expenditure in an atmosphere full of O_2.

Facultative microorganisms such as *Enterobacteria cloacae*, *Enterobacteraerogens*, and *Enterobacter asburiae* can conduct both aerobic and anaerobic fermentation processes. On the other hand, obligate anaerobic bacteria like *C.paraputrificum*, *R.albus*, and *C.beijerinckii* can only grow in an atmosphere devoid of O_2. Gram-positive bacteria, such as Enterobacter and Clostridium, are frequently utilisedforenormousH_2 generation due to their rapid multiplication rate and ability to form endospores. Several microbes includes LAB such as *K.pneumoniae*, and *Cellulomonas*, as well as thermophilic archaea like *Thermotoga neapolitana* and *Caldicellulosiruptor saccharolyticus*, have shown promising outcomes in bio-hydrogen production output through DF processes [10].

2.1.1.1.1 Microbiology and Biochemical Pathways of Dark Fermentation

A carbohydrate-rich feedstock is anaerobically digested through dark fermentation processes by H_2-generating microbes, such as facultative and obligate anaerobes. H_2 is generated during disposal of surplus electrons by the hydrogenase enzyme. In anaerobic conditions, protons (H^+) act as electron acceptors, eliminating the e^- produced through the combustion of biological substrates and creating H_2. On the other hand, aerobic respiration reduces oxygen and produces water as a result.

During the absence of light degradation of glucose as an instance medium, hydrogen-producing microbes first metabolise $C_6H_{12}O_6$ to pyruvate via glycolytic pathways, generating (ATP) from (ADP) and a decreased form of (NADH).

The slow breakdown of intricate carbohydrates by hybrid anaerobic bacteria can produce an array of metabolites and residues, which are regulated by operational conditions including type of substrate, loading rate, potential of hydrogen, and other ecological factors. These factors also enhance the structure of the population of microbes in bioreactors. Acetate and butyrate are among the most prevalent outcomes of dark fermentation. Facultative anaerobes commonly undergo the following metabolic processes during DF:

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2.6)$$

$$C_6H_{12}O_6 \longrightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2.7)$$

While the process of metabolism favours CH_3COOH production, the predicted output of hydrogen is 4 mol for each mole of $C_6H_{12}O_6$, resulting in about 545 mL H_2 per gram of hexose at 25°C, as given in Equation (2.6). In contrast, imagine the ultimate result is butyric acid. In such an instance, the hydrogen yield drops to 2 mol per mole of glucose, resulting in around 272 mL H_2 per gram of hexose at 25°C, as stated in Equation 2.7. However, the real H_2 yield is generally lesser than the theoretical value because a portion of the substances is used for biomass formation and there is a chance of substrate breakdown via alternate reactions that fail to generate hydrogen.

$$C_6H_{12}O_6 + 2H_2O \longrightarrow CH_3CH_2OH + CH_3COOH + 2CO_2 + 2H_2 \quad (2.8)$$

Clostridium butyricum, an extensively researched clostridia species, is recognised as generating predominantly butyric acid during the process of fermentation, with residues of acetate and hydrogen. A different approach fermentation process utilises *Clostridium articum*, which generates propionate, however, this pathway uses hydrogen alternatively. Furthermore, chemical reactions that produce solely ethanol and lactic acid do not release hydrogen, as indicated in Eqs (2.10) and (2.11).

$$C_6H_{12}O_6 + 2H_2 \longrightarrow 2CH_3CH_2COOH + 2H_2O \quad (2.9)$$

$$C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2 \quad (2.10)$$

$$C_6H_{12}O_6 \longrightarrow CH_3CHOHCOOH + 2CO_2 \quad (2.11)$$

It was observed that an association between B/A ratios and hydrogen emissions during dark glucose fermentation in Continuous Stirred Tank Reactor (CSTR) systems. An AB/A ratio greater than 2.6 showed effective hydrogen generation by anaerobic bacteria. In mixed culture dark fermentation, a B/A ratio of 3:2 generally yields 2.5 mol H_2 /mole of fermented hexose, as shown in Equation (2.12).

$$4C_6H_{12}O_6 + 2H_2O \longrightarrow 3CH_3CH_2CH_2COOH + 2CH_3COOH + 8CO_2 + 10H_2 \quad (2.12)$$

Contrary to this, Ghimire et al. in 2015 observed in their investigation that using lignocellulosic substrates to the B/A the proportion could fail to precisely represent the

biohydrogen yield, especially in batch experiments where homoacetogenic activity dominates. Therefore, elevated acetate volumes might not constantly signify a higher hydrogen yield. Certain homoacetogens like *Clostridium aceticum* can reduce hydrogen generation by transforming hydrogen as well as carbon dioxide to acetate, or by directly converting hexose to acetate. Nevertheless, analyzing biodegradable compounds may present insights into fermentation pathways and, consequently, the efficiency of hydrogen production.

Clostridia are the predominant hydrogen-producing microbes in mesophilic blended cultures with a pH of 5.5. Based on rDNA sequence analysis, scientists discovered that three *Clostridium* species (Clostridiaceae) accounted for 64.6% of all microorganisms, after19% by Enterobacteriaceae and 3.1% by *Streptococcus bovis* (Streptococcaceae). Additionally, new findings by others showed that subdominant species, despite their modest abundance, can have a major impact on hydrogen generation. While *E. coli* can increase the output of H_2 by controlling its metabolic routes towards acetate and butyrate generation, other species ecosystems, such as *Bacillus sp*. and *Lactobacillus sp.*, can reduce H_2 supply by promoting lactate formation [11]

2.1.1.2 Photofermentation

Gest and Kaman first recognised the use of photo-fermentation for generating biohydrogen using photosynthetic microbes in 1949. After that time, it has proven effective in creating high-purity hydrogen without producing oxygen. Photo-fermentation produces biohydrogen through nitrogenase-catalyzed processes as organic compounds breakdown in an environment of light energy, which is aided by photosynthetic organisms or anaerobic cultures of bacteria such as Rhodobium, Rhodobacter, Rhodospirillum, and Rhodopseudomonas. This technology is gaining popularity because to the benefits of using a types of raw materials and optimal substrate utilisation. Furthermore, it is known for its environmental friendliness and ability to generate hydrogen on a massive scale at moderate temperatures and pressures. The photo-fermentation reaction may be stated thereby in Eq. 2.13:

$$C_6H_{12}O_6 \rightarrow 6CO_2 + 12H_2 \quad \text{(Photosynthetic bacteria)} \quad (2.13)$$

Within a usual photo-fermentation organised a photoreactor of sufficient capacity is put in a chamber with a constant level of light to carry out hydrogen generation tests. In the beginning, photo-fermentation slurry containing various volumes of substrate, culture, and medium is added to the photoreactor. Throughout fermentation, the photoreactor's base pH is

adjusted to an optimal level. This correction is made including 5 M sodium hydroxide or 5 M hydrochloric acid. The photo-reactor is then purged either nitrogen or argon for 5 minutes to keep it devoid of oxygen. Earlier studies revealed that the temperature is typically maintained at 30°C. The hydrogen extraction procedure then begins with steady stirring for a few weeks, involving sampling at 12-hour intervals. A specialised bag is used for storing hydrogen, and its concentration is tested with a gas chromatograph.

Despite its potential, photo-fermentation for biohydrogen production presents several challenges. Photosynthetic bacteria are limited in efficiently harnessing solar energy, leading to low effectiveness of light transformation for biohydrogen generation. These microbes also require pristine and ideal environmental conditions to develop and produce hydrogen. Furthermore, nitrogenase enzymes demand substantial activation energy, resulting in considerable energy demand during the photo-fermentation activity. Furthermore, the cell shadowing effect limits the diffusion of light through the photoreactor, lowering light intensity and decreasing biohydrogen output. In large-scale applications, substantial land coverage is required to set up an effective anaerobic photobioreactor [12].

2.1.1.3 Biophotolysis

This mechanism is comparable to photosynthesis that happens in both algae and plants and instantly converts light into H_2. Biophotolysis, also known as freshwater-splitting photosynthesis, is a method by which hydrogen is produced utilising only water and UV rays, aided by oxygenic photosynthesis-producing microorganisms such as cyanobacteria and green microalgae. Green microalgae require FeFe-hydrogenase, but heterocystous cyanobacteria utilise nitrogenase. There are two basic types of biophotolysis: direct and indirect [13].

2.1.1.3.1 Direct biophotolysis

Green algae and cyanobacteria utilise sunlight at wavelengths ranging from 400 to 700 nm to produce power for photosynthesis. Whenever struck by direct sunlight, these microorganisms use enzymes such as nitrogenase or hydrogenase to generate hydrogen. Water divides utilising the energy of light at a wavelength of 680 nm, yielding protons, electrons, and oxygen, as illustrated in equation 1. The e^{-1}s created in Eq (2.14) are then transported through PS II and PS I until there are enough electrons to reduce ferredoxin (Fd). Eq (2.15)

demonstrates how the hydrogenase enzyme uses the reduced Fd to convert $NADP^+$ to NADPH. This reduction step is important for the production of hydrogen [8].

$$2H_2O + light(h\nu) = O_2 + 4H^+ + 4e^- \quad (2.14)$$

$$2H^+ + 2Fd\ (reduction) \leftrightarrow 2Fd\ (oxidation) + H_2 \quad (2.15)$$

2.1.1.3.2 Indirect Biophotolysis

Indirect biophotolysis is a two-step sun process that alters sunlight to chemical power in the type of carbohydrates. In the initial stage, solar energy generates oxygen and carbohydrates. Green algae produce both starch and glycogen. Reducing nitrogen (N_2) during Equation (2.16) promotes carbohydrate synthesis while limiting oxygen levels, which boosts hydrogen generation. The next phase entails turning carbohydrates into CO_2 and H_2 utilising sunlight in an anaerobic condition with less oxygen volumes, as shown in equations (2.17) and (2.18).

$$6CO_2 + 12H_2O + light(h\nu) = C_6H_{12}O_6 + 6CO_2 \quad (2.16)$$

Table 2.1 summarises major research efforts focused on the generation of biohydrogen from algal cells and archaea. Scientists investigated biohydrogen generation adopting four cyanobacterial types: *Desertifilum sp.* IPPAS B-1220, *Synechocystis sp*. PCC 6803, *Phormidium corium* B-26, and *Synechococcus sp.* Hydrogen generation was measured as moles of hydrogen per gram of chlorophyll (Chl) in an hour.

$$C_6H_{12}O_6 + 2H_2O = 4H_2 + 2CH_3COOH + 2CO_2 \quad (2.17)$$

$$CH_3COOH + H_2O\ light(h\nu) = 8H_2 + 4CO_2 \quad (2.18)$$

Table 2.1. Hydrogen production from different microalgae by biophotolysis [13]

Microalgae/cyanobacteria	Process condition	Light Intensity (W/m^2)	H_2 Production
Nostoc PCC 7120	The BG110 medium was used, accompanied by a combination of red and white light, while the atmosphere was changed to 100% Ar or a mixture of Ar/N_2 (20/80).	18.9	6.3 ml/L/h
***Clostridium reinhardtiicbn* 1–48**	The experiment utilized Tris-acetate-phosphate medium in an environment containing 5% CO_2, and it was conducted under dark anaerobic conditions.	426.7	40.3 ml/kg
8yngby ssp. (benzoate as a carbon source)	During the late exponential phase, the basal media was used with 600 mg/l of benzoate.	31.8	16.1μmol H_2/g Chl a/h
***Clostridium reinhardtii* (CC124)**	TAP medium devoid of sulphur.	64.0	1.5 ± 0.1ml/L/h
***Clostridium reinhardtii* CC-425 strain**	TAP medium containing sulphur.	121.7	0.9μmol/mg Chl/h

Among these bacteria, *Synechocystis sp.* PCC 6803 accumulated the most hydrogen, attaining 0.038 mole H_2/mg Chl/h after 2 dark hours, while *Desertifilum sp.* IPPAS B-1220 generated 0.228 mole H_2/mg Chl/h after 7 light hrs. It was observed that H_2 and O_2 generation in *C.reinhardtii* mutant strains using photosystem I light. During 18 hours of continuous PS I light exposure; they reported hydrogen yields of 222 dm^3/kg for carbon 1-48 (a chlorophyll-b-deficient mutant) and 176 dm^3/kg for VHLR-S4 (a high light tolerance mutant). The maximum hydrogen output of 368 dm^3/kg was obtained in carbon 1-48 when subjected to a 1.5-hour light and dark cycle [14].

2.1.4 Microbial Electrolysis Cells

During previous years, it had been rapid advancement in (MECs) as a novel method for H_2 production from various substrates. MECs are essentially modified microbial fuel cells (MFCs), a technique which has been under investigation for many years. By utilizing MECs as an electrically driven H_2 generation platform, a broad spectrum of organic substrates can be converted into H_2 under externally applied potential. This approach, also known as electro-fermentation or biocatalyzed electrolysis cells, involves a slight input of external voltage, distinguishing it from traditional MFCs. According to the principle of thermodynamics, hydrogen synthesis requires a voltage higher than 0.110 V, as opposed to the -0.300 V generated by microorganisms. The redox value required for H^+ a decrease to H_2
(0.414 V) requires a substantially lower potential than the anticipated voltage of 1.230 V needed for water electrolysis.

However, the effectiveness is affected by microbial species, material of electrode, transmembrane type, voltage applied range, feedstock composition and concentration, and MEC configuration. MECs were initially designed in a two-chamber configuration before switching to a single-chamber configuration for greater simplicity, lower internal resistance, and avoidance of pH variations. The single chamber configuration addresses issues such as pH imbalance caused by H^+ accumulation in the anode chamber and H^+ consumption in the cathode chamber, as well as minimizing energy losses and ohmic losses associated with membrane use. This shift has effectively resolved significant challenges related to the two-chamber MEC setup [13].

Using MECs, equations depict the numerous chemical processes involved in the creation of hydrogen 2.19-2.21.

$$\text{Reactions at Anode: } CH_3COOH + H_2O = 2CO_2 + 8e^- + 8H^+ \quad (2.19)$$

$$\text{Reaction at Cathode: } 8e^- + 8H^+ = 4H_2 \quad (2.20)$$

$$\text{Overall Reaction: } CH_3COOH + H_2O = 2CO_2 + 4H_2 \quad (2.21)$$

Microbial electrolysis cells (MEC) are currently being used to produce hydrogen (H_2) from a variety of biological substances such as butyrate, sugar, acetate, or glycol, and solid wastes including waste-activated sewage and effluent from industries or residences. According to studies, combining MEC with anaerobic metabolism and dark fermentation procedures can help increase H_2 output. MEC efficiency is controlled by several factors, including the

substrate type, temperature, pH levels, and operating voltage. MEC devices had effectively operational from 0 to 45°C, with the highest efficiency recorded between 10°C and 20°C. Moreover, it has been investigated that elevating the externally applied voltage leads to higher H_2 yields in MEC systems. Electrogenic micro-organisms such as *Shewanella sp.* and *Geobacter sp.* play crucial roles in MEC operations, with *S. oneidensis* and *G. sulfurreducens* being the most extensively researched species [15]

2.1.5 Gasification

Natural resources for gasification is a feasible approach for transforming carbon-derived materials into valuable gaseous materials since it may utilize a wide range of raw materials. This thermochemical procedure necessitates extreme temperature varying from 700 to 1200°C, as well as with meticulous supervision throughout the oxidising substance. During gasification, biomass undergoes initial drying to reduce moisture content and then pyrolysis for thermal degradation, forming volatile compounds and char. Subsequent partial oxidation and reforming, facilitated by a gasifying agent, yield syngas. Deteriorating agents include oxygen, moisture, gas, and carbon dioxide, respectively. The syngas produced by gasification of biomass contains the composition of CO, CO_2, H_2, nitrogen, methane higher hydrocarbons, and small contaminants. Steam reforming and water-gas shift (WGS) processes can further purify syngas by increasing their hydrogen concentration. Catalytic steam reforming in two stages optimises the H_2/CO ratio and removes tar from syngas. Equations from 2.22 to 2.26 describe the chemical processes occurring during the gasification process [16].

$$C + CO_2 = 2CO \ (-164.9 \text{ MJ kg-mole}^{-1}) \quad (2.22)$$

$$C + H_2O = CO + H_2 \ (-122.6 \text{ MJkg-mole}^{-1}) \quad (2.23)$$

$$CO + H_2O = CO + H_2 \ (+42 \text{ MJkg-mole}^{-1}) \quad (2.24)$$

$$C + 2H_2 = CH_4 \ (+75 \text{ MJkg-mole}^{-1}) \quad (2.25)$$

$$CO_2 + H_2 = CO + H_2O \ (-42.3 \text{ MJ kg-mole}^{-1}) \quad (2.26)$$

2.1.6 SMR and Pyrolysis

(SMR) is a method that demands elevated temperatures and pressures. Hydrogen is produced through reactions (2.27) and (2.28) involving the participation of methane/CO and water with stresses that vary from 1.5 to 3 MPa.

$$CH_4 + H_2O \rightarrow CO + 3H_2 \quad \Delta H = 206.1 \text{ kJ/mol} \quad T = 700°C - 900°C \quad (2.27)$$

$$CO + H_2O \rightarrow CO_2 + H_2 \quad \Delta H = -41.1 \text{ kJ/mol} \quad T = 90°C - 230°C \quad (2.28)$$

The apparatus required for steam methane reforming has to compose of substances which could survive the variation in temperature across the initial and subsequent phases. To avoid catalyst poisoning, the procedure requires Ni or Ni alloys catalysts, and the gas must be de-sulfurize beforehand. The efficacy of reducing fuel to hydrogen varies between 65% and 85%. SMR produces a higher flow rate of hydrogen gas than the biological approach. This method produces CO_2 as an outcome, which leads to greenhouse gas emissions. Pyrolysis is the chemical breakdown of methane, as described in reaction (2.29), which occurs at temperatures that vary from 700 to 1100°C and pressures ranging from 0.5 to 2 MPa.

$$CH_4 \rightarrow C + 2H_2 \quad \Delta H = 75.6 \text{ [kJ/mol]} \quad (2.29)$$

Pyrolysis uses a smaller amount of hydrogen than the reforming of steam an individual supply of hydrogen but at a lower thermal energy approximately 37.8 kJ/mol against 63 kJ/mol for SMR. Each procedure needs a substantial amount of heat and are subject to poisoning of the catalyst due to sulphur impurities. Nonetheless, these were much more effective than ecological techniques. However, given rising energy demands and fossil fuel consumption, it is desirable to shift to more renewable sources [17].

2.2 PARAMETERS INFLUENCING DARK FERMENTATIVE HYDROGENSYNTHESIS

2.2.1 Inoculum Type

Further, they are two distinct forms of cultures utilised to generate H_2through DF: pure cultures and mixed cultures. Pure cultures, such as *Clostridium sp.* and *Enterobacter sp*., frequently get utilised because of their capacity to produce hydrogen at high rates (HPR) and yields (HY). However, one notable disadvantage of employing pure cultures is the need for sterile conditions during start-up and operation, which results in higher operational expenses owing to energy consumption. This issue, can be addressed by using mixed cultures, which

offer practical advantages, including simplified operation and easier process control. Moreover, mixed cultures demonstrate feasibility in utilizing complex organic wastes. Mixed cultures can be inoculated using a variety of sources, including animal manure, anaerobic sewage, and compost. However, there's a concern regarding mixed cultures, is the presence of H_2 absorbers such as methane-producing and homoacetogens, which can impede hydrogen generation. Pretreatment procedures are necessary to reduce the usage of these hydrogen consumers while increasing hydrogen generation. These procedures include processing with heat and acid, alkali treatment, sonication, aeration, freezing and thawing, and the addition of certain chemicals such as 2-bromoethanesulfonic acid and extended-chain fatty acids [18].

2.2.2 Feedstocks

Dark fermentation for hydrogen production utilizes various feedstocks, which may be split into 3 distinct generations. First-generation substrates include food crops such as sugarcane, sugar beetroot, maize and cassava, which are easily fermentable by microbes. Though, using food crops for biofuel production raises concerns about food competition and land use. Second-generation feedstocks, such as lignocellulosic biomass, have been developed as alternatives. Lignocellulosic biomass is challenging for microorganisms to digest, necessitating pretreatment and hydrolysis to obtain fermentable sugars before fermentation. Recently, attention has turned to third-generation feedstocks, particularly microalgae, for hydrogen production. Microalgae offer rapid growth rates and efficient CO_2 absorption and can be cultivated without soil with short harvesting cycles. Their biomass contains high levels of carbohydrates and lipids, which hydrogen-producing microorganisms can convert into hydrogen. The hydrogen yield (HY), hydrogen production rate (HPR), and overall process economy are influenced by differences in carbohydrate content, bioavailability, and biodegradation rates among these feedstock generations. Moreover, the concentrations of feedstocks must be carefully managed to prevent feedstock or product inhibition during fermentation [19].

Optimal feed concentrations maximise H_2 production implementing DF by reducing substrate inhibition. However, excessive substrate concentrations might cause unfavourable conditions, such as pH fluctuations caused by volatile fatty acid accumulation, which inhibits the growth of H_2 -generating bacteria. Scientists used a modified MTC medium to study [20].

2.2.3 Temperature

Operating temperature is a critical factor in the fermentation process, influencing microbial substrate utilization, specific growth rates, and the production of hydrogen and metabolic byproducts. Investigations on dark fermentative hydrogen production were carried out at a variety of temperatures, includes moderate (17-28°C), relatively mesophilic (31-46 °C), thermophilic (52-60 °C), and extremely thermophilic (>60 °C). Mesophilic microbes, such as *C. butyricum* and *E. cloacae*, and hyperthermophiles, such as *Pyrococcus furiosus* and *Caldicellulosiruptor bescii*, thrive best at temperatures that vary between 30 to 45 °C and 50 to 80 °C, accordingly. However, when using a combination of cultures, the best temperature for the generation of hydrogen could vary from the ideal development temperature for each strain. Most laboratory-scale studies on hydrogen production have focused on mesophilic microorganisms due to operational ease and maximum specific growth rates. Researchers have investigated the temperature impacts on hydrogen generation with a mixture of cultures, revealing that maximal hydrogen yields were acquired by elevating temperatures varying from 35 to 45 °C. However, yields reduced as temperatures rose from 45 to 55 °C. Other studies using chemostat-type H_2 bioreactors optimized the temperature for hydrogen generation with a mixture of microbial populations, achieving peak production at 45°C.

In recent times, there appears to be growing curiosity in thermophiles as promising alternatives for hydrogen production. Processes at elevated temperatures provide various benefits, notably reduced viscosity, improved mixing, reduced contamination risks, elevated reaction rates, and don't require the cooling of the bioreactor. Furthermore, thermophiles have shown the ability to produce maximal hypothetical H_2 outputs of 4 mol H_2/mol $C_6H_{12}O_6$, outperforming mesophiles, which normally generate a minimum of 2 mol H_2/mol $C_6H_{12}O_6$. High temperatures enhance entropy, making the system more active and lowering the possibility of invasion of H_2-using enzymes and bacteria. To summarise, the operational temperature is a critical determinant of hydrogen generation, which is regulated by the type of hydrogen-producing microorganism and substrate used [13].

2.2.4 pH

The pH of the fermentation media initiates a crucial function in influencing microbes metabolic pathways during dark fermentation, directly impacting the activity of hydrogenase enzymes are required in anaerobic fermentation. Together with temperature, operational pH determines the ideal biochemical routes in generating H_2 and helps control hydrogen-

consuming processes. Extremely acidic pH (at the level of 6) hinders the action of methanogenic bacteria in both mesophilic and thermally conductive environments due to its effect on hydrogenase enzyme activity, thus restraining methanogenic activities. Effective control of hydrogen-consuming microorganisms is achievable at high temperatures with an optimal pH of 5.5. However, it's important to notice the ideal working pH might differ throughout analysis, based on factors such as culture sources, feedstock characteristics, pre-treatment procedures, and organic loading rates. In general, the ideal pH range for bio-hydrogenation is 4.6–9.

In most investigations, CH_3COOH and $C_4H_8O_2$ are found as the primary contaminants of the optimal H_2 emitting route. According to research, acetic acid channels are preferred at balanced or near-neutral levels of pH, whereas $C_4H_8O_2$channels are more common in acidic pH limitations. For example, research was executed and it was found that acetic acid was the predominant biochemical output at pH 7, while butyrate prevailed at a baseline pH of 5.5 under mesophilic circumstances (37°C). A further investigation looked at the release of hydrogen from cheddar whey at pH values that varied between 5.5 to 7.7 at 39°C [21].

2.2.5 Hydraulic Retention Time

(HRT) serves a substantial part in dark fermentation (DF) processes by influencing substrate hydrolysis, intermediate, and product formation, thus impacting fermentative H_2 production. Additionally, subsequently may act as a crucial parameter for the activity of methanogens. Several investigations explored the effect of HRT on H_2generation in the DF method. Variation in growth patterns between hydrogen generators and end-users allows for the utilization of HRT as a means to suppress H_2 consumers in DF. Lower HRTs have been observed to enhance hydrogen production by washing out methanogens, leading to increased hydrogen yields as HRT reduces. Yet, the ideal HRT for biohydrogen synthesis relies on the substrate type and hydrolysis rates are influenced by substrate biodegradability. Nevertheless, HRT alone may not be adequate to inhibit methanogenic activity.

In this regard, Liu studied the impact of pH and HRT on the production of H_2 from residential garbage in a hyper-thermophilic (70°C) Constant Stirred Tank Reactor (CSTR). Assessments were conducted by using different HRTs with a steady pH level of 7, while the effect of at a steady HRT of three consecutive days was also investigated. When studies were conducted out at various HRTs with a constant pH of 7.0, the outcomes showed unstable production of

H_2 with additional methanogenic activity at the end. Nevertheless, a mixture of pH 5.5 and a three-day HRT resulted in ideal circumstances for biohydrogen synthesis [22].

2.2.6 Metal Ions

Biohydrogen synthesis is dependent on key metal ions such as Fe, Ni, and Mg, which serve as components for numerous enzymes involved in microbial biochemical and transport mechanisms. Hydrogenases, classified as FeFe, NiFe, and Fe hydrogenases. These kinds of enzymes contain sites of activity in which CO and CN groups bind to iron ions, forming an organised [4Fe-4S] subcluster joined to a 2Fe subcluster group by an intermediate cysteine thiolate, giving them an organometallic nature.

The catalytic mechanism of NiFe-hydrogenases during H_2 oxidation involves six steps, including H_2 molecule diffusion, splitting upon binding, hydride oxidation, proton transfer, electron transfer, and intermolecular electron transfer. However, an elevated concentration of metals can hinder nutrient availability and compromise membrane function, influencing the fermentative biohydrogen production process. For instance, higher Fe concentrations may lead to mass transfer limitations due to cell clump formation. Hydrogenase activity is also affected by these metals, with nickel promoting fermentative H_2generation by blocking nickel-iron hydrogenase activation.

Different bacterial strains respond differently to metal concentrations. For example, *Clostridium pasteurianum* primarily produces lactate at reduced Fe concentrations (less than 10mmol/L), while *Clostridium acetobutyricum* favours H_2 production at higher Fe concentrations (<approximately 25 mmol/L) at the time of glucose fermentation. Activation of NiFe hydrogenase occurs with the addition of Ni, with an optimal concentration of 0.1 mg/L Ni^{2+}, resulting in maximum H_2 production. The ideal levels of these metals vary depending on the bacteria and operational temperature, with 20 mg/L of iron ions yielding 3.35 mol H_2/mol $C_6H_{12}O_6$and 0.1 mg/L Ni^{2+} ions yielding 232 mg H_2/g glucose [23].

2.2.7 Partial Pressure of H_2

The partial pressure of H_2 (HPP) has a substantial impact on biological processes involved in H_2 synthesis, notably in dark fermentation, where hydrogenase activities can drop due to inhibition by feedback. Higher amounts of dissolved H_2 in the fermentation medium favour altered ferredoxin (Fd) reduction over reduced Fd oxidation, resulting in bidirectional hydrogenase oxidation and Fd reduction, eventually decreasing H_2 generation.

Under elevated Hpp conditions (exceeding 60 Pa), metabolic routes move towards solventogenesis, resulting in the genesis of reduced products like $C_3H_6O_3$, C_2H_5OH, C_3H_6O, $C_4H_{10}O$, and $C_3H_7NO_2$, consequently decreasing H_2 yield due to unfavourable thermodynamic conditions. To address this, transferring inert gases such as nitrogen and argon into the reactor headspace, together with gas elimination, effectively lowers Hpp, leading to a notable elevation in H_2 outputs by up to 68%. Thus, maintaining low HPP levels in H_2 bioreactors is essential for achieving high H_2 yields [24].

2.2.8 Nutrients

H_2-producing bacteria require both chemical and nitrogen-containing origins for growth, including molecules like NH_4Cl, CH_4N_2O, and NH_4CO_3, as well as biological components like yeast extract, peptone, and maize steep liquor. Research demonstrates that biological sources in fermentation produce more H_2. However, nitrogen-limiting circumstances can stymie H_2 synthesis in dark fermentation processes. Inorganic components such as nitrogen, sulphate, and phosphate are essential macronutrients for microbial development, but they can also hinder H_2 generation.

The feedstock contains high amounts of ammoniacal nitrogen and higher carbon-to-nitrogen (C/N) ratios, which hinder dark fermentation.. Surprisingly, H_2 production did not rise with varied sulphate concentrations; restricting sulphate concentrations reduced both optical density and H_2 production. Phosphate helps to maintain the medium's buffering capability. Bacteria can produce more H_2 at an intermediate phosphate concentration. The optimal phosphate content in N_2HPO_4 was 600 mg/L, while a 40% lower concentration of N_2HPO_4 resulted in a 30% decrease in hydrogen generation [25].

2.3 PRETREATMENT TECHNIQUES OF LIGNOCELLULOSIC BIOMASS

The complex and durable arrangement of lignin-rich biomass poses a significant challenge to second-generation biorefining. The complex network of biopolymers within lignin containing biomass, including cellulose, hemicellulose, and lignin, resists microbial and enzymatic breakdown. Permeability or the presence of proteins and acetyl groups has an additional impact on biomass resiliency. As a result, separating lignocellulosic components is critical for extracting monomeric sugars to make biopolymers, biofuels, and biochemicals. Pretreating lignocellulosic biomass is critical in biorefinery processes, enhancing biomass disintegration, increasing polysaccharide permeability, and enhancing enzymatic hydrolysis efficiency.

Appropriate processing minimises feedstock crystalline structure, enhances permeability, eliminates lignin, inhibits preventing formation, avoids sugar deterioration, consumes less energy, and is cost-effective. To disturb biomass structure, numerous traditional pretreatment technologies have been devised, including biological, physical, chemical, and physicochemical approaches. Nevertheless, these processes frequently have disadvantages, such as expensive maintenance, extreme conditions (pressure, pH, and temperature), the creation of harmful byproducts, and other constraints. To address the limitations of established procedures, novel, ecologically sound pretreatment approaches are required [26].

2.3.1 Physical Pretreatment

Mechanical techniques for pretreatment primarily aim to reduce the size of the particles of (LCB) while increasing the mobility of polysaccharides on its surface. Mechanical treatment (e.g., cutting, grinding, crushing), thawing, burning, microwave irradiation, pulsed electromagnetic radiation; ultrasonic stimulation, and decomposition are popular methods for disrupting biomass made from lignin. However, many of these processes demand substantial energy or power utilisation, costly machinery for processing, and may produce toxic chemical residues, rendering them unsuitable for massive operations and applications in industry. Nevertheless, some techniques, such as ultrasound and microwave irradiation, have shown promise in effectively decrystallizing and solubilizing cellulose and lignin from lignocellulosic feedstocks [27].

2.3.1.1 Pyrolysis

Throughout this procedure, the LB transforms into molecules of gas such as H_2, charred material, and CO. The charred wastes were generated, which was subsequently dealt with dilute acid and washed via H_2O. The water filtrates contain $C_6H_{12}O_6$ molecules, which are a primary source of carbon, utilised in the manufacturing of biofuels. It was processed *Azadirachta indica* timber bark, and a high of 49.5wt% bio-oil production was reported at 450 °C [28].

2.3.1.2 Microwave Irradiation

Microwave irradiation comprises using low-frequency electromagnetic waves, which are generated by cycling off a dipole. This method shows great promise in processing lignocellulosic biomass because its heat permeates the biomass, offering better energy efficiency than traditional heating methods. Temperature, initial biomass composition, irradiation period, microwave power, and catalyst presence all have a substantial influence on

radio-physicochemical biomass processing. Heating with a microwave causes heating in biomass's polar structure, resulting in hotspots and allowing cellulose fibrils to reorient themselves. Microwave irradiation has numerous benefits, comprising rapid reaction times, uniform heating, low activation energy, high product yield, few outcomes, ecological sustainability, and economical.

Studies demonstrated significant enhancement in total reducing sugars and methane yield, respectively from biomass pretreated with microwave irradiation. Microwave-assisted pretreatment with $FeCl_3$, H_2SO_4, and NaOH effectively removed lignin and selectively extracted fermentable sugars from sugarcane bagasse biomass in a short residence time. Considering the benefits, applications in industry of microwave heating for biomass processing remain limited, and optimization of microwave-assisted pretreatment conditions is necessary for the efficient extraction of polymeric sugars [29]

2.3.1.3 Pulsed-Electric Field

Using this technology, the lignin-containing feedstock is briefly treated at a high voltage of 5 - 20 kV/cm for a short period of time. This procedure creates gaps in the plasma membrane, exposing the cellulose, which is then digested by catalysts. This pretreatment process significantly increased the glucose content of lignocellulosic biomass and switchgrass [30]

2.3.1.4 Ultrasonication

Ultrasound-assisted pretreatment uses high-voltage electromechanical sound waves that usually vary from 20 to 500 MHz. These waves provide a significant shear force, effectively defibrillating and degrading lignocellulosic biomass. The approach is based on the 'cavitation effect,' in which ultrasonic waves break O-4 and O-4 lignin connections, resulting in the fragmentation of the ligno-polysaccharide complex inside the biomass. When these bubbles caused by cavitation reach a crucial size, they quickly collapse, causing a large increase in temperature (2000 to 5000 K) and pressure (up to 1800 atm). This technique provides various benefits, such as reduced residence times, high activation energy levels, and efficient mass transfer that efficiently decompose lignocellulosic biomass [31].

2.3.2 Chemical Pretreatment

Biochemical treatment is the administration of compounds such as alkaline substances, profound electrostatic chemicals, ILs, NH_3, O_3, and others to break the robust framework of lignin-based biomass. Chemical pretreatment aims to minimise feedstock crystal structure,

enhance permeability, and eradicate lignin, consequently improving the availability of biomass interfaces to enzymatic activity degradation. Most saturated and reduced acids approach the hemicellulose portion, partly eliminating lignin from lignin-rich biomass and increasing cellulose accessibility for hydrolysis by enzymes. Even so, the consumption of acids is frequently limited by disadvantages such as carbohydrate reduction, expensiveness, aggressiveness, and the formation of blockers. Few of the researchers had performed a pretreatment step of sugarcane bagasse with dilute phosphoric acid that, within ideal circumstances, yielded maximal lowering polymers.

Alkaline pretreatment procedures, on the other hand, seek to delignify feedstock by breaking aryl-ether, ester, and bonds of C-C, removing cellulose derivatives, and increasing surface area for effective hydrolysis and fermentation. Nonetheless, disadvantages such as extended response times and the costly expense of suppressing the effluent preclude extensive use of alkaline pretreatment. For example, sodium hydroxide was used in a moderate alkali-mediated pretreatment of cogon grass, resulting in increased saccharification productivity and maximum ethanol output during fermentation. Despite the advantages of traditional chemicals in enhancing decrystallization and cellulose accessibility, their extensive usage is hindered by challenges comprising costly recovery of acids/alkalis, the requirement for equipment tolerant to corrosion, and the generation of compounds. Consequently, a heightened focus has been on developing new environmentally friendly solvents in recent years [23].

2.3.2.1 Acidic

Sulfuric acid is often used for the processing of farm leftovers such as maize residue, poplar wood, tidy and switchgrass. According to studies, dilute hydrochloric acid is used for the enzymatic breakdown of wood needles (*Pinus roxburghii*), resulting in the maximum production of sugars at 0.43 grams per gram of material [32].

2.3.2.2 Alkaline

This approach employs alkaline substances like KOH, $Ca(OH)_2$, NH_4, and NaOH for pretreating lignocellulosic biomass. Sodium hydroxide was used to hydrolyze softwood pine and hardwood poplar, resulting in an improved bioethanol yield of 109.83 grams per kilogram of initial pinewood and 101.44 grams per kilogram of initial poplar wood [33].

2.3.2.3 Ozonolysis

This approach resulted in a significant degradation of lignin content. Various forest leftovers, including conifer and ebony sawdust, were treated to decrease their lignin percentage. After the ultraviolet pretreatment procedure, there was a rise in the release of monolignol chemicals, indicating the degradation of forest wastes [28].

2.3.2.4 Ionic-Liquids (ILs)

Ionic liquids, or ILs, have become known as highly sustainable and recyclable chemical solvents to prepare lignin-based biomass owing to their diverse and adjustable features. ILs are organic salts made up of cations and anions that have reduced melting temperatures, are non-flammable, have good chemical and thermal properties, and have moderate vapour pressure. Using ILs for treatment dissolves either the conductive or non-conductive elements in ligno-polysaccharides, lowers cellulose crystalline structure, enhances porosity, eliminates lignin, and improves cellulose mobility for digestion catalysts. Several imidazolium-based ILs have been used in the processing of lignin-based biomass.

As an instance, it was observed that lower temperatures (45°C) treatments with IL 1-ethyl-3-methylimidazolium acetate on oak trees sawdust, conifer wood chips, and prototype cellulose, yielding substantial glucose yields of 59.3%, 49.3%, and 68.2%, accordingly, during saccharification. IL 1-butyl-3-methylimidazolium chloride ([Bmim]Cl) was used to successfully delignify and bio-transform hardwood (hornbeam wood and spruce) and softwood biomass samples. Sugarcane tops biomass was pretreated with IL tris(2-hydroxyethyl) methylammonium methyl sulphate ([TMA]MeSO4) before being saccharified, resulting in an elevated sugar content of 182.18 mg/g biomass. However, despite their benefits, ILs has drawbacks such as high synthesis costs, possible toxicity, recycling problems, and non-biodegradability, which limit their extensive industrial use [34].

2.3.2.5 Deep Eutectic Solvents (DESs)

Deep eutectic solutions (DESs) are currently attracting substantial interest as an environmentally acceptable method of increasing the nutritional value of lignin-based biomass. These solvents, which are a combination of liquid eutectic materials with hydrogen-bond donors and acceptors in specified molar ratios, provide an additional benefit over their components. DESs, contrary to their components, remain in a liquid state with comparatively low melting temperatures. They have several similarities to ionic liquids (ILs), including

minimal vapour pressure, non-flammability, excellent thermo-chemical stability, and elevated miscibility, making them ideal for lignocellulosic biomass separation. However, DES stands out because of its simplicity of synthesis, low cost, non-toxicity, strong recyclability, and enhanced biodegradability. Under moderate processing conditions, DESs effectively dissolve lignin from lignocellulosic biomass and convert the crystalline cellulose fraction to amorphous cellulose [35].

2.3.2.6 Organosolv Pretreatment

The above method uses natural solvents, such as C_2H_5OH, CH_3OH, HCOOH, CH_3COOH, C_6H_5OH, $C_3H_8O_3$, CH_3COCH_3 or its solution form, to preferentially separate or eradicate lignin from lignin-based materials. Organosolv pretreatment is a more efficient way to produce ethanol, lignin, and other biochemicals using biomass substrates than conventional pretreatment methods. During this process, lignocellulosic biomass is combined with an organic solvent and water, with a solid-to-liquid ratio of 1:4 to 1:10 (w/w) and a solvent concentration of 35-70% (w/w). The inclusion of a catalyst can speedup the pretreatment reaction. This treatment degrades lignin connections and lignin-carbohydrate bonds, leaving a solid phase primarily consisting of both cellulose and hemicellulose.

To mitigate lignin accumulation, the substance being treated is flushed with a solvent made from organic matter and then rinsed with distilled water to eliminate the solvent that was used. The pretreated materials are then chemically saccharified and digested to create useful biochemicals, with the solvent extracted from the wasted liquor via distillation. Pretreatment of pine and beechwood biomass with moderate reactive organosolv solvents (tetrahydrofuran, acetone, and ethanol), produces cellulose-rich granular fractions appropriate for the growth of lactic acid fermentation from the *Lactobacillus delbruecki* strain. Beechwood biomass produced an increased lactic acid output of 62 g/L than pine biomass (37.4 g/L), demonstrating that organosolv pretreatment is suitable for both hardwood and softwood biomass. In the context of other recent investigations, sorghum feedstock was treated with a customised inexpensive organosolv including glycerol solvent and ammonia as an activator, resulting in considerable conversion (which decreases sugars of 422.35 mg/g feedstock) and fermentation outputs (36 g/L).

In addition, the growth of organosolv-based bioreactors will necessitate incorporating biodegradable components into treatment procedures while lowering processing expenses. It may be accomplished by reducing the amount of biological solvent utilised, improving the value of residues, and refining the entire procedure with computational techniques [36].

2.3.3 Physicochemical Pretreatment

This physical and chemical processing technique integrates both physical and chemical approaches and is thought to be more suitable in degrading lignin-containing compounds under a variety of procedure variables, including both pressure and temperature. The process includes a variety of procedures, such as CO_2 explosion, steam explosion, liquid hot water treatment, ammonia fibre explosion, wet oxidation, and many other combinations [26].

2.3.3.1 Steam Explosion

It offers a more cost-effective alternative than methods like grinding and soaking. This approach involves immersing dried biomass in vapour under elevated pressure and temperature, followed by a rapid pressure release as the biomass is forced through a small opening. This sudden pressure drop causes the water within the biomass to vaporize rapidly, imparting thermal-mechanical energy that breaks down the biomass. Scientists utilized hydrothermal and steam explosion methods to pretreat bamboo wood (*Bambus astenostachya*), decreasing lignin content from 21.7% to 14.7% [37].

2.3.3.2 Liquid Hot Water

The procedure requires controlling with elevated pressures and temperatures. Lignocellulosic biomass (LB) pretreatment often involves an extensive range of temperatures (125-320 °C) and pressures (5-200 bar). Yang and colleagues employed liquid hot water to prepare bamboo stems for bioethanol synthesis. After prior to treatment, xylan and lignin concentration decreased from 18.2% and 14.9% to 14.2% and 13%, accordingly. The best yields of glucose, ethanol, and xylose were obtained at 53.5%, 4.9 g/L, and 57.4%, correspondingly [28].

2.3.3.3 Ammonia Pretreatment

An ammonia-based or ammonia fibre explosion (AFEX) process involves treating lignin-based biomass with a solution of ammonia at elevated pressures (250-300 psi) and temperatures that vary from 60-100°C for a short period of time. Afterwards, the pressure is quickly relieved. This method, with its specific ability to cause swelling in the lignocellulose structure, improves enzymatic hydrolysis efficiency. The instantaneous pressure release induces mechanical damage in crystalline cellulose, resulting in lower biomass crystallinity. However, the breakdown efficiency of lignin and hemicellulose is quite poor. The AFEX method, with its low reaction temperatures and little inhibitor production, shows significant potential for the future of bioenergy and biomass conversion [18].

2.3.3.4 Carbon Dioxide Explosion Pretreatment

This approach is safe, cost-effective, non-combustible, and operates at low temperatures. Releasing fully pressurized explosive CO_2 directly into the lignocellulosic biomass (LB) enhances hydrolysis by increasing the surface area. The supercritical CO_2 (sCO_2) reaches liquid density but retains gaseous properties at specific temperature (31°C) and pressure (7.4 MPa) conditions. Sohni and colleagues applied sCO_2 to pretreat palm oil biomass, resulting in enzymatic hydrolysis that yielded a higher cellulose-based content of 61% compared to raw samples [38].

2.3.3.5 Wet Oxidation

This is a general procedure typically conducted under high temperature and pressure conditions. Throughout this method, numerous oxidation reactions occur, releasing organic acids that dissolve, remove lignin, and break down the hemi-cellulosic compounds. Biswas and collaborators applied the wet oxidation technique alongside proteins to effectively break down the lignocellulosic biomass (LB) derived from poplar sawdust, resulting in improved breakdown of hemicellulose (75%) and cellulose (83%) [39].

2.3.4 Biological Pretreatment

Biological pretreatment involves employment of the microbes, primarily fungi and bacteria, which can secrete lignocellulolytic enzymes externally to break down lignocellulosic biomass. This method is considered adequate, cost-efficient, and environmentally friendly because it requires lower energy inputs and generates fewer harmful by-products than chemical processes. In contrast, obstacles such as restricted accessible total area due to the complicated biomass structure, sluggish enzyme-mediated digestion rate, and the necessity of precise microbial development parameters impede the broad use of biological pretreatment on a large scale [40].

2.3.4.1 Microbial Consortium

Advantageous microbes are prevalent, and they exhibit desirable characteristics that encourage the formation of similar productive microbiological communities. Microbial communities tend to be eliminated from ecosystems in nature where decaying woody biomass is used as a substrate, including deteriorating straws in landfills or farmland. Many research investigating natural gas and bio-hydrogen generation have used microbial consortia for pretreatment, with considerable improvements in product outputs. For example, Zhong et

al. created a microbial consortium of bacteria and fungus for wheat straw pretreatment procedures, which led to increased degradation. Sustained anaerobic digestion with this prior treatment microbial consortia resulted in a 39.24% rise in total biogas and an 80.34% increase in total methane yield, as well as a faster start-up time than without treatment reactors throughout a 20-day working cycle. Researchers used lignocellulose-degrading microbial communities derived from the waste of horses and degraded wood and found increased biogas generation. According to another investigation concentrating on bio-hydrogen output, a microbial consortium known as OEM2 was used for lignin-based biomass pretreatment, leading to 85% hemicellulose disintegration within 12 days. The 16S rRNA analysis of the microbial consortia OEM2 found that Proteobacteria, Paenibacillus and Pseudomonas are the key producers to lignocellulose degradation [41].

2.3.4.2 Fungal Pretreatment

The fungi are utilised to breakdown both hemicellulose and lignin in recyclables using ecological pretreatment methods. Although brown rot fungi primarily impact cellulose, white and soft rot fungi can degrade both the two substances using the lignin-degrading compounds they produce. White-rot fungi are thought to be considered one of the finest at biologically pre-treating materials made from lignin. For example, pre-treating cornstalk with *Phanerochaete chrysosporium* resulted in a 34.3% drop in lignin content while losing less than 10% of holocellulose. Another investigation examined pre-treated cornstalk with Trichoderma resei Rut C-30, which resulted in a substantial rise in overall hydrogen output compared to direct fermentation of raw cornstalk, demonstrating the potential of fungal pre-treatment for improving the transformation of LCB waste to sustainable hydrogen energy. Additionally, it was reported an increase in bio-hydrogen generation with white-rot fungal pre-treatment processes of wheat straw, resulting in a lower lignin concentration and significantly greater hydrogen output than untreated straw. In a current investigation, pine needle litter pre-treated with white-rot fungus and then aerobically digested exhibited a significant decrease in holocellulose and total lignin material, demonstrating the benefits of fungal pre-treatment [42].

2.3.4.3 Enzymatic Pretreatment

Lignocellulose-degrading fungi possess enzymes that enable them to break down lignocellulose efficiently, utilizing two main categories of enzymes: oxidative and hydrolytic. Enzymes and chemical compounds are used in a variety of combinations to aid in

lignocellulose breakdown. Researchers studied how biochemical pre-treatment affected sugar beet pulp and wasted hops before methane generation. The resulting substrates were hydrolyzed for 24 hours using a variety of enzymatic agents including Celustar XL and Agro pectpomace (in a 3:1 v/v ratio), in addition to endoglucanase, xylanase, and pectinase. The findings indicated that the hydrolysates had significantly higher reducing sugar concentrations than the hydrolyzed feedstock.

Manganese peroxidase, a metabolic enzyme generated by white-rot fungus, initiates the oxidation of Mn^{2+} to Mn^{3+}, which aids in the breaking of lignin linkages. Current investigations additionally showed that the laccase enzyme catalyses the oxidation of phenols, anilines, and aromatic thiols found in lignocellulosic feedstock. Laccase treatment, which originates from phenol oxidase and contains various Cu^{2+} ions, has been proven to improve anaerobic digestion by increasing microbial growth [21].

2.4 BIOMASS FOR BIOHYDROGEN GENERATION

The optimum feedstock for hydrogen gas production should be both inexpensive and sustainable; with an abundance of carbohydrates that includes simple sugars. These sugars act as reliable sources for generating biohydrogen. Biohydrogen synthesis via biophotolysis of H_2O with cyanobacteria, microalgae, and photosynthetic anoxygenic bacteria is most suitable. The technique takes advantage of ample resources from nature, such as sunlight and water. These bacteria give electrons as their means of surviving under suboptimal conditions or to avoid electron transport chain decrease, hence acting as an antioxidant. In addition to these metabolic activities, nitrogen fixation can generate H_2 by the action of the nitrogenase enzyme. This mechanism is especially important in heterocyst-forming blue-green algae [43].

2.4.1 Agricultural Waste

The agricultural sector residues, which are mostly constituted of lignin-based substances, provide an environmentally friendly and economically viable source for the development of second-generation, environmentally friendly biofuel. These wastes are composed of residues from biomass that include photosynthetic products such as cellulose, hemicellulose, and lignin. They are made up of elements like straw, stover, peels, cobs, stalks, bagasse, and those who have been abandoned a side after harvesting economically useful crop products. In 2010, the global yearly production of farm leftovers was at 5.3 billion dry tonnes. As the population grows, so does debris produced by the agricultural, forestry, and aquaculture industries,

indicating that waste generation from these areas will increase in future generations. Discussion has been done related to the pros and cons of using agricultural waste as a substrate for biohydrogen generation. Here we review instances of crop leftovers as prospective substrate resources of dark fermentation processes and describe current improvements in their use [11].

2.4.2 Lignocellulose Waste

Lignocellulosic waste, comprising lignin, cellulose, and hemicellulose, is a complex macromolecule. Lignin, an insoluble polymer, forms covalent bonds with hemicellulose, while cellulose is enveloped within a complex containing both lignin and hemicellulose in the cell wall. This complex structure renders lignocellulosic waste resistant to degradation, including residues from plants, agricultural byproducts, and wood logging. Annually, approximately 179 million tons of lignocellulosic feedstocks are generated as agricultural residues, presenting an inexpensive resource for biofuel production. On the other hand, because of their poor permeability, diversity, and crystallisation, these substances cannot be easily metabolised and require pre-treatment to transform them into sugars that can be fermented.

Researchers are increasingly focusing on utilizing feedstocks for H_2 generation, considering them a potential feedstock for sugars that can be fermented. The process involves several steps:

- Pre-treatment of the heteropolymeric lignocellulosic substance to break down its complex structure.
- Hydrolysis of cellulose and hemicellulose to obtain a huge abundance of monomer glucose.
- Conversion of the acquired monomers into biofuels for utilizing microorganisms and bioprocess techniques.

Biohydrogen synthesis from lignin-based waste has received interest primarily because of its high efficacy. Scientists proved the efficacy of using several substrates made of lignin for biohydrogen generation and identified reasons of hindrance. The technique entails pre-treatment, hydrolysis, and dark fermentation to use various microbial cultures, resulting in increased biohydrogen generation yields and rates. DF is thought to be the most appropriate procedure for generating biohydrogen from diverse lignocellulosic substrates, with

photofermentation forthcoming in the next. Scientists obtained *Clostridium sp.* strain no. 2 from insects and used xylan from oat spelts to create biohydrogen at 18.6 mmol/g substrate. They also discovered that the microbe used 0.93 mmol of $C_6H_{12}O_6$ per hour while producing 4.3 mmol of H_2/hour. Furthermore, they discovered that greater temperatures resulted in increased cellulose hydrolysis into hydrogen, with considerable yields (e.g., 569 mL of H_2generated by 1 g of cellulose) [44].

2.4.3 Livestock Waste (Manure)

Cattle waste includes solid animal faeces, food waste (which frequently comprises a lignocellulosic percentage), and effluent-containing faeces and bodily waste. Cattle dung, poultry, and swine faeces are major sources of livestock manure, and they all have been known as pollutants that pose environmental concerns to the environment and oceans. Contemporary management approaches comprise its use in farmlands, as well as ecological restoration or treatment processes such as decomposition & anaerobic digestion. Even so, the former technique results in the unrestricted release of greenhouse gases, primarily methane (CH_4) from use of land. Effective waste control methods can reduce both immediate and subsequent GHGs by capturing. In advance of field implementation, fuel is extracted in the form of biogas from dung.

Nonetheless, faeces materials necessitate chemical and physical processing to inhibit methanogenic action, which uses hydrogen (H_2). Another disadvantage of using this feedstock is that ammonia's high nitrogen content may hinder biohydrogen synthesis. This can cause bioreactor failure, especially because cattle, poultry, and dairy manure possess low carbon-to-nitrogen (C/N) ratios and large quantities of ammoniacal nitrogen. It was observed that H_2 generation is possible for very high ammonia levels up to 7.9 g N/L in constant flow infrastructure, for as soon as the culture of bacteria is initially acclimatised reduced nitrogen content. However, biohydrogen synthesis declines as the total ammonia content rises above 2 g N/L.

Furthermore, excessive sulphate levels in pig dung can impede biohydrogen generation due to the inclusion of hydrogen-consuming acid-reducing agents. With its substantial amount of nitrogen, dung from animals can be utilised as a co-digestion medium to supplement other agricultural leftovers with nitrogen to maintain an acceptable carbon-to-nitrogen ratio. It was observed a hydrogen output of 1.18 to 1.65 mol H_2/mol glucose in a swine manure-based fermentation augmented by $C_6H_{12}O_6$. Yin observed an increased hydrogen output of 32.5

mL/volatile solids (VS) using acidification pre-treated dairy manures and a substrate pH of 5.0[45].

2.4.4 Industrial Waste

The worldwide economy is primarily reliant on industrial development, which frequently causes pollution due to greater consumption of water and wastewater output. Commercial pollutants, being non-biodegradable materials, cause significant environmental contamination; nonetheless, there is growing interest in using these wastes in road construction. Waste products created by companies, such as wastewater from factories and biodiesel manufacturing wastes, have the potential to produce biohydrogen. Although many studies focus on biohydrogen production via electrolysis along with other chemical procedures, the biochemical transformation of waste to hydrogen is considered the foremost cost-effective way.

Many starch- and cellulose-based compounds comprising elevated sugar content can be found in waste from the food and agriculture industries. Enzymatic or acid hydrolysis converts starch waste into maltose or glucose, which is then converted into sugars and biohydrogen. Interestingly, cellulose-containing residues are more difficult to eliminate, necessitating prior to treatment, the process of hydrolysis and later transformation into sugars and biohydrogen.

A number of investigations focused on the genesis of H_2 from food industry waste. Continuous reactor is used to manufacture biohydrogen from food industry waste, with varied yields depending on pH and hydraulic retention duration. Moreno-Andrade et al. examined hydrogen generation utilising various industrial wastes as feedstocks, and tequila vinasses produced the best output. Hydrogen was produced from pre-treated paper industry wastes by saccharifying and fermenting them simultaneously. Oceguera-Contreras et al. generated biohydrogen from agro-industry wastes, emphasising the importance of verminous-associated microbes in both biohydrogen production and lignocellulosic waste decomposition.

It was investigated how to produce hydrogen using agricultural and industrial wastes such as cheese ricotta and grain straw hydrolysis product, demonstrating efficient hydrogen production from both solo and mixed sources. Scientists created biohydrogen utilising tapioca effluent, dairy sewage, and citrus processed effluent, reporting high hydrogen yields. Biohydrogen was produced using fruit and vegetable wastes as well as crude cheese whey, resulting in high yields. Utilising starch wastes from agro-industries offers an effective and

inexpensive method of producing biohydrogen, with numerous input materials being potential possibilities [46].

2.4.5 Municipal Waste

Monitoring and processing waste materials presents substantial worldwide issues, which lead to deteriorating the environment. Sewage sludge, in specific, poses numerous ecological and medical problems. Although attempts to reuse solid waste have arisen as a long-term solution for reducing pollution, ecological preservation, and renewable energy generation. Biohydrogen generation from urban waste, such as sewage sludge, has gained popularity. Combined digestion of food and solid waste sludge produced a maximum hydrogen generation rate of 111.2 mL H_2/g VSS/hour. It was found that alkali-pretreated sewage sludge produced more hydrogen than dry waste.

Samrot investigated radiation and pretreatment with alkali for dissolving waste-activated sewage for biohydrogen generation. Furthermore, co-fermentation with leaf litter and sewage disposal waste yielded biohydrogen at a 20:80 integrating proportion, producing 37.8 mL/g VS-added. Organic sewage was used to serve as the culture for biohydrogen generation from maize stalks by anaerobic digestion, resulting in an ideal hydrogen output of 126.22 mL/g. Novel reactor layouts were additionally implemented. Considering certain circumstances, a (CMISR) utilising activated carbon produced a maximum of 353.9 mL/h/L of hydrogen. The preliminary treatments with sodium citrate and ultrasonic stimulation damaged the sludge sediment structure, which improved biohydrogen processing. Yang and Wang used electrostatic binders to dissolve the extracellular polymeric material in waste-activated sewage, resulting in much higher biohydrogen generation. In addition, biohydrogen was generated by waste-activated effluent from sugar in the processing factory using dark fermentation, generating 7.8 mmol of hydrogen [43].

2.5 MICROBIAL PATHWAYS

For imminent scarcity of energy sources prompted the massive production of biohydrogen. It isknown for over 70 years that cyanobacteria may produce bio-hydrogen when illuminated. Pre-incubation in dark conditions prompted the formation of hydrogen in the cells. Hydrogen is produced by the hydrogenase enzyme, which becomes active throughout the incubating phase. Fermentative output of hydrogen is determined by the form of the culture utilised, the reaction chamber type, and the temperature parameters. Many different forms of cultures are utilised for hydrogen generation, including pure cultures of hydrogen-producing microbes,

combined strains of anaerobic bacteria from organic matter piles, and anaerobic sewage. The changes in metabolism in fresh cultures are evident, and using unadulterated cultures allows us to gain an insight into the circumstances which promote a high production of hydrogen percentage and output [47].

Table 2.2. Hydrogen yield reported from various feedstocks from the dark fermentation process [13]

SUBSTRATE	**YIELD**
Cornstalks	58 ml/g
Rice straw	20 ml/g
Water hyacinth	51.7 ml/g
Laminaria japonica	115.2ml/g
Cassava wastewater	54.22ml/g
Microalgal biomass	135±3.11ml/g
Glucose	1.20mmol
Cheese whey wastewater	2.04 mol
Vegetable waste	12.61 mmol
Fruit juice industry wastewater	1.4 mol
Corn stover lignocellulose	1.67 mol
Cellobiose	1.64 mol
Distillery spent wash	39.8 L
Food waste	3.18 L
Olive oil mill effluent	196.2 ml/g
Molasses wastewater	130.57 mmol
Starch hydrolysate	5.40 mmol/g
Sucrose	0.98±0.32 mol
Glucose:xylose (9:1)	252 ml/l
Algae biomass	2.78ol

2.6 PILOT-SCALE APPLICATIONS

A vast majority of DF research is currently undertaken on a lab scale, using batch, semi-continuous, or continuous units. Yet there is a noteworthy lack of study on the commercial or large-scale deployment of DF procedures. There were only a few pilot-scale experiments. It was studied that a 1.48 m^3 constant flow anaerobic reactor for 200 days while feeding it sugarcane at an OLR ranging from 3.21 to 8457 kg COD/m^3/d. They obtained an ideal hydrogen output of 26.15 molH_2/kg COD eliminated under an OLR range of 34-55 kg COD/m^3/d, resulting in a maximal output rate of 5.59 m^3H_2/m^3 reactor/day. It was examined that a plug-flow angled DF unit having a capacity of 0.15 m, utilising household trash as a base material, and obtained a hydrogen production of 72mLH_2/GVS supplied. A different approach for pilot-scale investigation was undertaken, which included substrate tanks for storage, nutrient retention vessels, mixing tanks, an agitator granular sludge bed fermenter, and a gas-liquid-solid separator. This high-rate system ran for 67 days underneath mesophilic conditions, with an OLR of 40-240 kg COD/m^3/d and glucose as the base material. Under these conditions, an OLR of 240 kg COD/m^3/d produced 15.59 m^3/m^3d of hydrogen and yielded 1.04 molH_2/mol sucrose. Lee carried out a two-step pilot-scale thermophilic DF and anaerobic digestion procedure with food residue. In DF stage, they retained an OLR of 16.3 kg TVS/m^3d and a (HRT) of 3.3 days, resulting in a yield of 66.9 L H_2/kg Total Volatile Solids. In this process, AD effluents were propagated to the DF stage to keep the pH between 5 and 6 [48].

2.7 CHALLENGES AND FUTURE PROSPECTIVES

Over the past twenty years, several efforts have been made to enhance the economic viability of the hydrogen production process. Nevertheless, certain significant technical obstacles persist, and surmounting these challenges would lead to an improvement in the overall efficacy of H_2 generation through the biological routes outlined in table 2.3. These issues may be solved by optimising H_2-producing bioreactor designs, making process changes, carefully selecting appropriate raw materials, and identifying effective microbial strains. In the metabolic pathways involved in H_2 production, intermediary molecules contend for identical reductants as H_2, resulting in lower H_2 yield. As a result, researchers are investigating strategies to divert those pathways away from creating inexpensive compounds. To get around the stoichiometric limitations of dark fermentation procedures, initiatives are being conducted to construct strong biocatalysts via biological engineering. H_2 yield can be enhanced by deploying appropriate bioreactor designs and improving process variables. Even

so, weak molecular rates of conversion have an impact on the process's economics, necessitating further study to overcome the 4 mol H_2/mol glucose constraint. The most recent study has focused on hybridization methods, such as combining dark fermentation and photofermentation, in which volatile fatty acid compounds obtained during the initial phase are used as feedstock in the subsequent phase, which could increase the expected H_2 production to 12 mol H_2/mol glucose. Furthermore, novel methods involving the production of methane or electro-fermentation have been researched to improve the utilisation of energy. While much study has already been done in these areas, more work is needed to increase H_2 synthesis through natural processes. Integrated hydrogen production with standard effluent treatment strategies provides benefits such as waste rehabilitation and renewable power generation [49].

Table 2.3. Technological obstacles in different process of biohydrogen production

Types of process	**Technological obstacles**
DF	• Inadequate transformation efficacy of the substrate • Reduced yield of hydrogen • Thermodynamic constraints • Synthesis of a gas mixture including both hydrogen and carbon dioxide, requiring purification.
Photofermentation	• Dependence on an additional source of light is necessary • The method is constrained to both daylight and darkness cycles, relying on sunlight for illumination • Reduced hydrogen yield attributed to exceedingly low infrared conversion effectiveness
Direct biophotolysis	• Oxygen production triggered by the action of PS II • Need customised biological reactors • Reduced hydrogen yields due to exceptionally poor radiation conversion effectiveness
Indirect biophotolysis	• Decreased hydrogen output attributed to hydrogenase(s) • Necessity for an additionalsource of light • Overall daylight conversion rate was noticeably low.

Sustainable hydrogen is the purest form of energy around the world, releasing just water vapour when ignited. Expanding up its economic usage reduces emissions while simultaneously improving the ecology. After years of studies, implementation of lignin-based biomass is used for fermentation into ethanol has been a slow process. This protracted process is caused by issues involving a lack of established technology at pilot scales, insufficient funding from the government, and a narrow bridge linking laboratory studies and production. Although advances in genetic and fundamental research have enhanced the knowledge of lignocellulosic materials, several elements remain obscure. The financial assessments of lignocellulosic ethanol and hydrogen, for example, did not begin until the turn of the century, and data on bioenergy distribution dynamics is limited. These studies are important to conduct laboratory based experiments for pretreatment procedures.

Producing lignocellulosic biohydrogen necessitates a risky technique, but merged ecological refinery technologies provide a feasible answer, as stated by Valdez-Vazquez and Sanchez in 2018. Production is a major difficulty in this method, as observed by Grabarczyk et al. in 2019, with present results in the scientific community proving inadequate. Major modifications must be made to increase efficiency and obtain sustainable outputs from biomass. Although using many component activities can reduce the method's viability from a financial aspect. Recently, integrated bioprocessing/single-pot bioreactor technologies have emerged, demonstrating technological potential, albeit extensive financial assessments are required to determine their affordability. Furthermore, combining hydrogen generation and carbon capture can reduce biogenic emissions, resulting in an environmentally friendly energy system having zero greenhouse gases [20].

2.8 TECHNO-ECONOMIC ANALYSIS

The economic viability of lignocellulosic bio-hydrogen is pivotal for its commercialization, as many biofuels and products derived from lignocellulosic materials face challenges in competing with fossil fuel sources in terms of energy production. Early identification of bottlenecks through economic viability assessments is crucial for resource conservation. While feasibility assessments typically occur during the project's pilot phase, energy from renewable sources requires a comprehensive end-to-end solution for large-scale implementation. While much research focuses on understanding basic phenomena and smaller-scale systems, gaining insight into the industrial landscape is essential for researchers.

Techno-economic analysis facilitated by process simulators like AspenPlus or Superpro Designer plays a vital role in this regard. The process begins by adding necessary components to the simulation, including carbohydrates, gases, chemicals, and solutions, and estimating their physical properties to ensure accurate simulation. Stream-level information is then inputted for unit operations, and a comprehensive flowsheet is developed by integrating different streams and unit operations to replicate real-world operating conditions. Mass and energy balances are assessed for the completed flowsheet. The economic evaluation is then performed to establish essential characteristics including investment cost, operational cost, and return on investment. Flowsheet progress is data-driven, having unit activities necessitating accurate data like working conditions. In circumstances when information is unavailable, estimates derived from current research are implemented.

Biohydrogen production from lignocellulosic sources entails various costs, including growing and reaping expenses for feedstock. Expenses for lignocellulosic materials range from 22 to 85 $/ton, depending on biomass type and availability. There exists a direct linear relationship between biomass procurement costs and biofuel production expenses. The annual increase in biomass costs is attributed to factors such as rising fertilizer and labour costs, inflation, and insufficient support to farmers. Additionally, transportation costs are incurred for moving biomass, with shipping to the European countries costing about 0.005 – 0.015 $/dry ton-km and trucking expenses amounting to 0.42 $/ton-km for distances up to 35 km.

Biohydrogen production incurs additional costs due to analysing within the facility, which includes capital expenditures and operational charges. Capital expenditures comprise construction of the premises, equipment purchase and setting up, and operating capital, whereas operational costs include raw materials, labour, utilities, and taxes. These costs vary depending on the biomass source, pretreatment procedures, reactors operating parameters, hydrogen yield, and output. In this regard, scientists evaluated the integrated dark and fluorescent photo-fermentation of barley straw for the output of hydrogen vs ethanol. The expense of producing ethanol from barley straw was 23.1 $/GJ (energy basis), where as the price of producing biohydrogen was 503.10 $/GJ, making hydrogen generation around twenty times more costly. Lower effectiveness, worse environmental performance, and reservoir expenditures all lead to higher hydrogen prices. Photo-fermentation and its accompanying unit maintenance donate to 90% of these expenditures, demonstrating how producing hydrogen exclusively through photo-fermentation cannot be financially feasible. In comparison, hydrogen obtained from a combination technique comprising bio-oil and fast

pyrolysis had an average cost of production of 45.26 $/GJ, making it less than one-tenth of the expenses connected with these combined dark- and photo-fermentation processes. Additional estimates suggest that the manufacturing expense of hydrogen through a bi-phase process involves H_2 and CH_4 ranges from 60 to 405 $/GJ. Profitability as well as production cost of hydrogen are influenced by various factors, including hydrogen productivity, efficient sugar conversion, and acetic acid concentration. For instance, a 20% increase in hydrogen productivity leads to an 8% reduction in production costs [50].

2.9 CURRENT STATUS OF BIOHYDROGEN PRODUCTION IN INDIA

With growing worldwide endorsement from politicians, environmentally friendly hydrogen accounts for just over one per cent of all worldwide hydrogen generation and utilisation in accordance with the International Energy Agency's (IEA) Global Hydrogen Review 2023 was released in September. To achieve the organization's Net Zero Emissions (NZE) Scenario, sustainable production of hydrogen must expand more than 100 times by 2030. On January 4, 2023, the Indian Union Cabinet approved ₹19,744 crore in investments for the National Green Hydrogen Mission (NGHM). The project intends to establish India as an international centre for green hydrogen production, consumption, and export. It intends to achieve the exceedingly ambitious goal of producing five million tonnes of renewable hydrogen annually by 2030.

The current state of the implementation of environmentally friendly hydrogen in the country is as follows:

i. GAIL Limited has launched India's first project for integrating hydrogen into the town's gas distribution grid. At Avantika Gas Limited (AGL)'s City Gas Station in Indore, Madhya Pradesh, 2% by volume of hydrogen is incorporated into the CNG pipeline and 5% into the PNG pipeline.

ii. Beginning January 2023, NTPC Limited will incorporate up to 8% Green Hydrogen (vol/vol) in its PNG Pipeline at NTPC Kawas Township in Surat, Gujarat.

iii. Other PSUs have undertaken other projects, including:
   a. NTPC launches hydrogen-powered fuel cell electric vehicle (FCEV) buses in Leh.
   b. NTPC launches hydrogen-powered fuel cell electric vehicle (FCEV) buses in Greater Noida.

c. Oil India Limited has created a 60kW hydrogen fuel cell bus that combines an electric powertrain and a fuel cell.

d. Indian Oil will use experimental pilot plants to produce Green Hydrogen using electrolysis of water using solar power, biomass oxy steam gasification, and CBG reforming to fuel 15 hydrogen fuel cell buses.

CHAPTER 3

MATERIALS AND METHODS

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3.1 ASSESSMENT OF SAMPLING SITES

The Tattapani hot spring is situated at a height of 655 metres absl on the shores along the Satluj River in the Mandi district of Himachal Pradesh, India, can be pinpointed using geographical coordinates 31°14’ 56” N, 77° 5’ 10’’E (Fig.3.1). Despite the Satluj River's temperature measuring around 5°C–6°C, the hot spring's reservoir water reaches a significantly higher temperature of 60°C. To investigate microbial diversity, samples were collected from various points where hot water emerged along the riverbanks. These samples were then mixed for the extraction of metagenomic DNA. Notably, the area surrounding the hot springs exhibited salt and sulphur deposits of varying hues, including white and light yellow.

The Chir-pine tree is the most extensively distributed throughout Himachal Pradesh (*Pinus roxburghii*). Pine trees are typically located at high altitudes, and the shape of their leaves makes it possible for them to develop easily by preventing snow and rain from staying on top of them (Fig. 3.2). In Himachal Pradesh, forests encompass almost 67% of the state's total land area, providing a habitat for a staggering variety of flora and fauna. Among all the region1,25,885 acres of land were covered with Pine trees. The primary characteristics of pine needles, namely their high flammability and acidic nature, are the essential characteristics required in biomass to manufacture biofuel.

Fig. 3.1 Tattapani Hot Spring (area of Collection of water samples)

Fig. 3.2 Pine Needles at JUIT (area of of pine needles)

3.2 PHYSIO-CHEMICAL ANALYSIS OF WATER SAMPLE

Physiochemical tests of the Tattapani water sample were performed using certain instruments and standard protocols for analysis.

Table 3.1. Physio-chemical analysis of water sample

Property	Composition
TDS	2950 mg/L
EC	20 mhos/cm
pH	7
Cl^-	1000 mg/L
Dissolved oxygen	9.62 mg/L
Hardness	480 mg/L
Temperature	60°C
Odour	Rotten egg (qualitative)
Colour	Normal (Hazen scale unit)
Appearance	Clear (qualitative)
Taste	Salty (qualitative)

3.3 MICRO AND MACRONUTRIENT ANALYSIS OF WATER SAMPLE

Micro and macro nutrient analysis was done of the Tatapani water sample to know the composition of nutrients present in the water. Nutrients which were analysed are Cl^-, N, P, Ca, K, Mg, S, Fe, Mn, Cu, Zn, and B using the standard methods for estimation.

3.4 PRETREATMENT OF PINE NEEDLES

The entire pretreatment method was categorised into three sections:
Stage 1: Grinding preparation

Stage 2: The preliminary treatment for steam explosion

Stage 3: Acids and bases pretreatment method

Pine twigs were gathered, processed in an electric blender, and filtered. Technically milled litter was sterilised with a steam explosion at 121°C for 30 minutes, using pine needle in small amounts. The physically prepared substrates were treated with 1N HCl for 30 minutes at 70°C, then with 1N NaOH for a further 30 minutes at the identical temperature. The NaOH procedure continued until a transparent solution was obtained. The material was treated for additional 30 minutes with 1N HCl at 70°C, prior to 10 minutes of water washing at 70°C [42].

3.5 PHYSIOCHEMICAL ANALYSIS OF PINE NEEDLES AND ITS CHARACTERIZATION

Biomass made from lignin has been proposed as a substrate for biofuel generation, comprising H_2, CH_4, C_2H_5OH, and C_4H_9OH because it is environmentally friendly, sustainable, and abundant. This type of biomass is the most prevalent raw material, with a complicated structure that consists mostly of three polymers (cellulose, hemicellulose, and lignin), as well as ash and solvents.

Table 3.2 Composition of Pine needles

Cellulose	38%
Hemicellulose	20%
Lignin	25%

For the characterization of pine needles (cellulose, hemicellulose and lignin) insoluble pine needles were obtained. Water-soluble extractives from pine needles have been eliminated by immersing them in deionized water at 2% (w/v) and stirring at 70°C overnight to produce obstinate pine needles. The soluble extractives were filtered, and the pine needles were rinsed twice with deionized water at room temperature to eliminate any leftover soluble extractives. The recalcitrant pine needles were then obtained and dried at 60°C overnight for further experimentation [51].

3.5.1 Cellulose

The cellulose content of the substrate (pine needles) was determined using the method developed by Crampton and Maynard.

3.5.1.1 Sample Collection and Maintenance

Overnight dried pine needles were used for the estimation. The sample was stored for future use, ensuring it remained free from moisture.

3.5.1.2 Reagent Preparation

The reagents were prepared for a volume of 250 ml by mixing 185 ml of acetic acid, 23 ml of nitric acid and 42 ml of distilled water in a beaker.

3.5.1.3 Methodology for Cellulose Estimation

1g of the dried in an oven sample was added in a 250 ml beaker with the reagents, and the mixture was cooked until brown vapours were formed. The remaining material was passed through with filter paper. It was subsequently rinsed several times with water, alcohol, and acetone until all residue was removed. The remaining material was placed in a pre-weighed crucible and cooked in a hot oven at 150°C overnight. Upon freezing in a desiccator, the specimen was calibrated (W1). The crucible was then heated in a muffle furnace at 450°C for one hour preceding being weighed again (W2). The measurement of weight reduction was used to determine the total quantity of cellulose in the material being stu

3.5.2 Hemicellulose

The percentage of NDF and ADF was assessed using the methodology outlined by Goering and Van Soest.

3.5.2.1 Estimation of NDF (Neutral Detergent Fibre)

3.5.2.1.1 Reagents required for the preparation of NDS

NDS, or natural detergent solution, consists of the following components per 100 ml: 6gSDS, 3.72gEDTA, 1.36gsodium borate decahydrate, 2.28g disodium hydrogen phosphate, and 10 ml 2-ethoxyethanol.

Preparation of NDS and its methodology

In a glass beaker, 50 ml of DW was mixed with EDTA and Sodium Borate Decahydrate, and the resulting mixture was heated until mixed. SDS and 2-ethoxy ethanol were first dispersed in boiling distilled water before being mixed with the preceding solution. Disodium Hydrogen Phosphate had been dissolve individually in hot water before being added to the mixed

solution. To achieve thorough solvent dissolution, the pH was set to 7, and the total amount was adjusted to 100 ml with distilled water.

1g of oven-dried sample (W) was put in a glass beaker. Then, in order, 100 ml of NDS, 2 ml decaline, and 0.5g Sodium Sulphite were added. The contents were cooked for around 5-10 mins before slowly refluxing for an hour. Following refluxing, the material was passed through filters and placed to a pre-weighed crucible (A1). The filtered material was washed three times: with hot water, 100% ethanol, and acetone. The crucible was subsequently dried at 105°C for 12 hours and weighed (A2). The NDF (%) was determined as follows:

$$\text{NDF (\%)} = \frac{\text{A2-A1}}{\text{W}} \times 100 \qquad (3.1)$$

3.5.2.2 Estimation of (ADF) Acid Detergent Fibre

3.5.2.2.1 Reagent Preparation

An AD is prepared by mixing 2 gof CTAB in 100 ml of solution. For 1N Sulphuric Acid, 2.75 ml of Sulphuric Acid is mixed with 97.25 ml of distilled water.

3.5.2.2.2 Methodology for ADF Estimation

1g of oven-dried sample (W) was placed in a beaker. The beaker was filled sequentially with 100 ml of ADS and 2 ml of decaline. The combination was then heated for around 5-10 minutes before gradually refluxing for an entire hour. After refluxing, the material was filtered and put to a pre-weighed crucible (designated A3). The sample in the crucible was then rinsed three times with hot water, 100% ethanol, and acetone. The sample-containing crucible was dried at 105°C for twelve hours before being weighed (designated A4). The ADF (%) was determined as follows:

$$\text{ADF (\%)} = \frac{\text{A4} - \text{A3}}{\text{W}} \times 100 \qquad (3.2)$$

$$\text{Hemicellulose (\%)} = \text{NDF (\%)} - \text{ADF (\%)} \qquad (3.3)$$

3.5.3 Lignin Estimation

The crucible containing the residue from the ADF procedure was positioned on a 500 ml flask filled with water and covered with 20 ml of 72% H_2SO_4. The contents were agitated with a glass rod until a smooth paste formed. The crucible was then replaced with acid and stored in an ice bucket. After three hours, the surplus acid was filtered using filter paper. The remaining material was rinsed three times with hot water, ethanol, and acetone until it was

acid-free. The crucible was subsequently dried in an oven at 100°C before being cooled in a desiccator and weighed (denoted as A5). Following that, the crucible was heated in a muffle furnace at 500°C for three hours before being weighed again (denoted as A6)[52].

$$\text{Lignin Content (\%)} = \frac{A5-A6}{W} X100 \qquad (3.4)$$

3.6 PREPARATION OF SERUM BOTTLES BY USING DIFFERENT MEDIA

3.6.1 Bushnell Haas Broth

Serum bottles of 120 ml were taken, washed properly and dried in a hot air oven for further experiments. Bottles are filled with 80 ml of Bushnell Haas broth and 1% pine needles.The serum vials were capped and crimped using silicone septa and metal caps. The serum vials had been sterilised and purified with ultrapure, devoid of oxygen air utilising a 22-inch gauge syringe at 15 psi for 15 minutes. The serum vials were infused with 2 ml of the water sample collected from the Tatapani hot spring and kept for two weeks at 37°C and 60°C in an incubator agitator set at 150 rpm. All tests were carried out in replicas, with suitable controls [51].

3.6.2 Nutrient Broth

Serum vials containing 120 ml were thoroughly cleansed and dried out in an oven with hot air for future studies. The vials are filled with approximately 80 mL of nutrient broth. The serum bottles were capped and compressed using silicone septa and metal caps. These serum vials had been sterilised and evacuated with ultrapure, oxygen-deprived nitrogen using a 22-inch gauge syringe at 15 psi for 15 minutes. The serum vials were inoculated with 2 ml of water sample collected from the Tattapani hot spring and placed for two weeks at 37°C and 60°C in an incubator agitator adjusted at 150 rpm. All tests were carried out in duplicate, with suitable controls.

Table 3.3 Concentration of media used

Name of Media	Concentration of media used	Carbon source
Bushnell Haas Broth	Accurate	Pine needles
Nutrient Broth	¼ of the accurate	Nutrients present in media
Nutrient Broth	½ of the accurate	Nutrients present in media
Nutrient Broth	Accurate	Nutrients present in media

3.7 METAGENOMIC DNA EXTRACTION

To evaluate the metagenomics of the obtained consortia, complete DNA has been extracted from the sample with an Xploregen kit (Xploregen Discoveries Private Limited, India). To begin, a large sample was inserted in a 2 ml Eppendorf tube, followed by 60 µl of lysis buffer (C1) and repeated inversions. The tube was subsequently vortexed at full speed for ten minutes. The tube was spun up at 10,000 x g for 30 seconds, and the remaining liquid was transferred to a clean 2 ml Eppendorf tube, giving approximately 400-500 µl. To extract proteins and RNA, add 250 µl of buffer (C2), vortex for 5 seconds, and incubated at 4°C for 5 minutes. Following the incubation process the vial was spun down for 1 minute at 10,000 x g. The pellet of cells was discarded, and 600 µl of residual had been moved to a new, sterilised 2 ml Eppendorf tube. To remove remaining residues, add 200 µl of buffer (C3), briefly vortex, and agitate at 4°C for 5 minutes. After the incubation process the vial was spun down at 10,000 x g for 1 minute. The debris was eliminated and 600 µl of the residual was added to a sterilised 2 ml Eppendorf tube. Adhesion buffer (C4) has been added and stirred with 1200 µl of residue before vortexing for 5 seconds. Next, 675 µl of the resulting solution was put onto an MB spin column and spun at 10,000 x g for 1 minute. The supernatant was then eliminated. The process was carried out twice until the entire sample was analysed. For washing the DNA, add 500 µl of wash buffer (C5) to the column and agitate at 10,000 x g for 30 seconds. The supernatant was eliminated and the process of centrifugation continued at 10,000 x g for one minute. The MB spin column was carefully inserted in a sterilised 2 ml Eppendorf tube so as to avoid spilling the C5 solution on the column. Next, 100 µl of buffer (C6) was poured to the middle of the white filter membrane for DNA extraction. On the other hand, sterilised DNA-free PCR-grade water can be utilised for this procedure. [For DNA elution, buffer C6 is 10 mM Tris-HCl, pH 8.5, EDTA-free]. The tube was subsequently spun at ambient temperature for 30 seconds at 10,000 x g before discarding the MB spin column. The DNA was now suitable for use in downstream uses and should be kept at -20°C to -80°C.

3.8 TARGETED 16S rRNA CLONE LIBRARY PREPARATION

To replicate the incomplete 16S rRNA gene, PCR was performed using a TAQ master mix and diluted metagenomic DNA as the base sequence in an Eppendorf master cycler. The oligonucleotide primer sets utilised in this study are listed in Table 4. For amplifying, 40 ng of DNA obtained from the sample was mixed with 10 pM of each primer. A primer sets 16sF and 16sR were subjected to thermal cycling that included a first denaturation step at 95°C, which was followed by 25 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C,

elongation at 72°C for 2 minutes, and a final extension at 72°C for 10 minutes, with a hold at 4°C.

Table 3.4 The Primer Sequence Used for Amplification of Partial 16S rRNA Gene

Primer Name	**Primer Sequence**	**V Region**
16sF	5'AGAGTTTGATGMTGGCTCAG3'	V3-V4
16sR	5' TTACCGCGGCMGCSGGCAC3'	V3-V4

Prior to amplification by PCR, the DNA that was taken from the samples was analysed with NanoDrop and agarose gel. NanoDrop measurements were used to test DNA quantity, with 260/280 ratios ranging between 1.8 and 2. The amplified 16S PCR product was then purified and tested for quality using gel electrophoresis and NanoDrop. To generate the sequencing libraries, each sample's amplicons were cleaned with Ampure beads to eliminate unneeded primers, followed by 8 cycles of PCR with Illumina barcoded connectors. The resulting libraries have been purified with Ampure beads and measured using a Qubit dsDNA High Sensitivity assay kit. Sequencing was performed utilising the Illumina MiSeq platform using a 2x300PE v3-v4 sequencing kit(Illumina Private Limited, Singapore).

3.9 OPTIMISATION OF MEDIA FOR THE ISOLATION OF THERMOPHILIC FACULTATIVE ANAEROBIC BACTERIA

The water samples from Tatapani hot spring underwent processing to enumerate and isolate cultivable mild thermophiles (50-60°C) and extremophiles (70°C). Nutrient Agar and Bushnell Hass Agar were utilized as isolation media at temperatures of 60°C and 70°C. While preparing Bushnell Haas Agar 1% pine needle extract was added to it which will act as a carbon source as Bushnell Haas is a carbon-free media. After 48h hours of incubation, colonies exhibiting a white-cream colouration were identified, and further observation of agar plates was conducted to discern various morphotypes and phenotypic characteristics. Colonies showing distinct morphological differences were selected and streaked continuously using the identical separation media to produce clean cultures. These pure colonies were preserved at 4°C and subjected to repeated revival to ensure their reproducibility.

3.10 SUBCULTURING

To obtain pure colonies of the isolated microorganisms and subculturing, quadrant streaking was done.

3.11 GRAM STAINING

For preliminary identification, Gram Staining was performed. A neat transparent slide was taken and washed with detergent. Clean it with a muslin cloth rather than cotton, as cotton fibres can stick to the slide and produce incorrect results. A smear of bacterial culture was made, and just a few drops of normal saline were added. It was left to dry naturally and then repaired with heat. I added crystal violet dropwise and waited 1 minute. Added several drops of iodine (mordant) about one minute and cleansed over with purified water. Wash with 95% alcohol (decolorizer) for 20 seconds, then with distilled water. Added a few drops of safranin (counter stain) for 35 to 45 seconds, then rinsed with purified water. The slides were air-dried and viewed under a microscope.

3.12 TEMPERATURE TOLERANCE OF THE ISOLATED MICROBE

Microbe was initially isolated at 60°C at the Bushnell agar plate and was checked for temperature tolerance at 60°C, 70°C, and 80°C at the nutrient agar plate.Nutrient broth was prepared of volume 100 ml in which a few colonies from the Bushnell agar plate were inoculated and incubated at 60°C for 3-4 days. When turbidity was observed nutrient agar plates were prepared and 100 uL of the specimen had been distributed on a petri plate.

Afterwards, the plates were incubated at three different temperatures along with their control.

3.13 IDENTIFICATION OF THE ISOLATED MICROBE

This work related to sequencing and identification was supported by Biokart Genomic Lab, Bangalore. Certain experiments were performed for the identification of the isolated microbe.

3.13.1 DNA Extraction

For the extraction of gDNA of the microbe Xploregen gDNA kit was used. 1 ml of Xploregen gDNA extraction buffer solution 1 was putted to the beaded vial. The sample was addedto the beaded vial containing Xploregen gDNA extraction buffer solution 1. The vials were horizontally vortexed at maximum speed for 10 minutes.300 µl of Xploregen gDNA Extraction Buffer solution 2 was puttedto the beaded vial containing solution and horizontally vortexed the vial at maximum speed for 7 minutes. The instrument was spun at 10,000 rpm for three minutes at ambient temperature(RT). 800µl of residue was transferred to a 2 ml sterile vial. Then, 200µl of Xploregen gDNA Extraction Buffer solution 3 was added and agitated for 5 seconds. The vials have been spun at 10,000 rpm for a period of two minutes. 800µl of residue was transferred to a clean sterile 2ml vial. 1 ml of Xploregen gDNA

Extraction Buffer solution4 was put to the residue and agitated for 5 seconds.700µl of lysate was transferred to the spin column and spun down at 10,000 rpm for 2 mins, remaining liquid was discarded. The above two steps were repeated to collect all the lysate and ensure that the entire lysate was processed via the spin column. 600µl of Xploregen gDNA Extraction Buffer solution 5 was added to the spin column and and spun down at 10,000 rpm for 2 minutes, the supernatant was discarded. Then, 600µl of Xploregen gDNA Extraction Buffer solution 6 was added to the spin column and centrifuged at 10,000 rpm for 2 minutes, the supernatant was discarded. The empty spin column was spun down for 5 minutes at 10,000 rpm. The spin column was placed into a sterile 1.5 ml vial.30µl of Xploregen gDNA Extraction Buffer solution 7 was added to the centre area of the spin column and spunned for 5 minutes at 10,000 rpm. The spin column was placed to a new1.5ml sterile vial. The spin column was eliminated, and two elution tubes were kept at -20°C for additional analysis.

Quantification of DNA

The isolated DNA from the specimen was treated with NanoDrop and GEL Check previous to being utilised for amplification by PCR. NanoDrop values of 260/280 between 1.8 and 2 are utilised to assess DNA purity.

3.13.2 PCR Amplification of 16S Gene

179 ng of DNA collected is utilised during amplification, in addition to 10 pM for each primer.After extraction of genomic DNA, 1.2% agarose gel was prepared and gel electrophoresis was performed. Then, the gel was viewed under gel doc and later PCR was done of the obtained nucleic acid.

3.13.2.1 Cycling conditions

Table 3.5 Cycling conditions for PCR (30 cycles)

Initial Denaturation	3 minutes at 94°C
Denaturation	1 minutes at 94°C
Annealing	1 minutes at 50°C
Extension	2 minutes at 72°C
Final Extension	7 minutes at 72°C

3.13.2.2 PCR Amplification Conditions

Table 3.6 Volume of chemicals added in PCR

PCR Amplification Conditions	Volume
DNA	1 µl
16S Forward Primer	2 µl
16S Reverse Primer	2 µl
dNTPs (2.5 mM each)	4 µl
10X Taq DNA polymerase Assay Buffer	10 µl
Taq DNA Polymerase Enzyme (3U/ ml)	1 µl
Water	30 µl
Total reaction volume	50 µl

3.13.3.3 Composition of TAQ Master Mix

1) High-Fidelity DNA Polymerase

2) 2.5mM dNTPs

3) 3.2mM $MgCl_2$

4) PCR Enzyme Buffer

3.13.3.4 Primer details

Table 3.7 Primers used in PCR

S. No.	Oligo name	Sequence (5' to 3')	Tm (°C)	GC- Content
1	16S Forward	GGATGAGCCCGCGGCCTA	57	72.22%
2	16S Reverse	CGGTGTGTACAAGGCCCGG	58	68.42%

The amplified gene was loaded in 1.2% agarose gel and visualized under gel-doc.

3.14 BIOINFORMATICS ANALYSIS

The obtained amplified gene was sequenced using the ABI 3130xl platform and the obtained the whole sequence had been submitted in NCBI, and the nucleotide accession number was obtained and the BLAST was performed of the first ten similar sequences and the phylogenetic tree was constructed.

3.15 BACTERIAL PRESERVATION

To do the long-term preservation of the isolated microbe glycerol stocks were prepared and the culture was lyophilized and stored at -80°C, Slants of agar were also produced for short-term retention and kept at 4 degrees Celsius.

3.16 BIOHYDROGEN ESTIMATION

For the estimation of hydrogen in serum bottles qualitative and quantitative tests were done.

3.16.1 Qualitative Estimation

Qualitative estimation of hydrogen gas was done using Keithley's Picometer & Voltage Source Hydrogen Gas Sensor Setup. In this instrument, we can analyse the results if resistance change is observed in the sample. If there is a change in the resistance it implies that hydrogen gas is present in our sample.

3.16.2 Quantitative Estimation

Quantitative analysis of hydrogen gas was done using gas chromatography at Farelabs Private Limited; Gurugram. The gas chromatograph was configured along with a TCD, or thermal conductivity detector, and a Porapak Q column. An air-tight injector was used to remove 100 µL of sample from the bottles and transfer it into the GC sampling port. The carrier gas (N_2) flow rate was 10ml/min, and the nozzle and detectors had heat of 70°C and 100°C, accordingly. The temperature of the oven was set at 35°C for a minute, then scaled up to 50°C at an average of 5°C per minute, and finally kept at 50°Cfor 2 minutes (total run duration 4.5 min). The % of hydrogen contained in the specimen was estimated utilising a formula:

$$\text{Hydrogen \%} = \frac{\text{Sample area} \times \text{Standard concentration}}{\text{Standard area}}$$

CHAPTER 4

RESULTS AND DISCUSSION

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4.1 PRETREATMENT OF PINE NEEDLES

For the, effective generation of biohydrogen from LCB, pine needles were pretreated. If the raw pine needles were used directly, then the α-terpineol present in the pine needles would release acids, inhibiting the growth of microorganisms, making it unable to degrade the complex structures present in the pine needles i.e cellulose, hemicellulose and lignin. Pretreatment of pine needles will enhance the total surface area of the lignocellulosic biomass by the process of milling, making it easier for the microbe to utilise the substrate easily and can degrade the complex structures present in pine needles. Afterwards, acid-base pretreatment was done by using HCl and NaOH which will remove the acid-producing α-terpineol, a chemical substance from the pine needles and making easier for the microbes to utilise and thus breakdown the complex structures of cellulose, hemicellulose and lignin. Pretreatment is an essential step as it promotes increased surface area, digestibility, improved reactivity and the dissociation of cellulosic in biomass to increase fermentable monomer sugars for better yield of hydrogen generation. Initially 54g of pine needles was taken and after chemical pretreatment process, the final weight observed was 14g.

Fig. 4.1 Dried Pine Needles

Fig. 4.2 Pretreated Pine Needles

4.2 PREPARATION OF SERUM BOTTLES

Serum bottles containing Bushnell Haas Broth along with pine needles and nutrient broth with different concentrations of media were prepared and purged to maintain anaerobic conditions, making it oxygen-free along with their controls and incubated for two weeks at two different temperatures i.e. 37°C and 60°C for further observations. After the time period of two weeks, it was observed that maximum turbidity was observed in the serum bottle in which ½ the concentration of nutrient broth media was used and less turbidity was observed in 60°C. It was observed that the temperature range for growth of the bacteria is between 35°C to 60°C and further preliminary tests were performed for the identification of the bacteria.

Fig. 4.3 Purging of N_2e in serum bottle containing Bushnell Haas broth

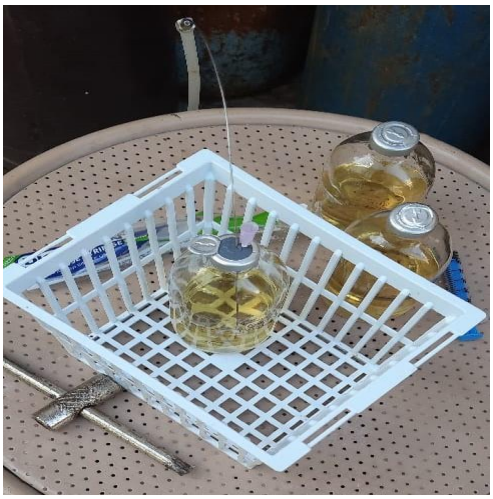


Fig. 4.4 Purging of N_2 in serum bottle containing Nutrient Broth

4.3 METAGENOMIC ANALYSIS

The total GC content was found 53.4% in the water sample. As a part of the metabolic community analysis, we performed a detailed community analysis of the microbiota present in the Tattapani hot springs, and the taxonomic link at different levels was explored. Among the bacterial population, the members were found to be represented by a total of 20 phyla, which were further subdivided into a total of 40 classes, 79 orders, 145 families and 155 genera. However, in our study, the Proteobacteria phylum (55%) appeared as the most dominant phylum, followed by Bacteroidetes (15%), Actinobacteria (12%), and Firmicutes (12%), respectively (Fig. 4.5). According to the literature, Proteobacteria is the most abundant phylum found in most of the hot springs as it represents a substantial positive correlation with the physical properties of water. These bacteria are commonly referred to as

thermophiles, and their enzymes have significant biotechnological and industrial application prospects [53].

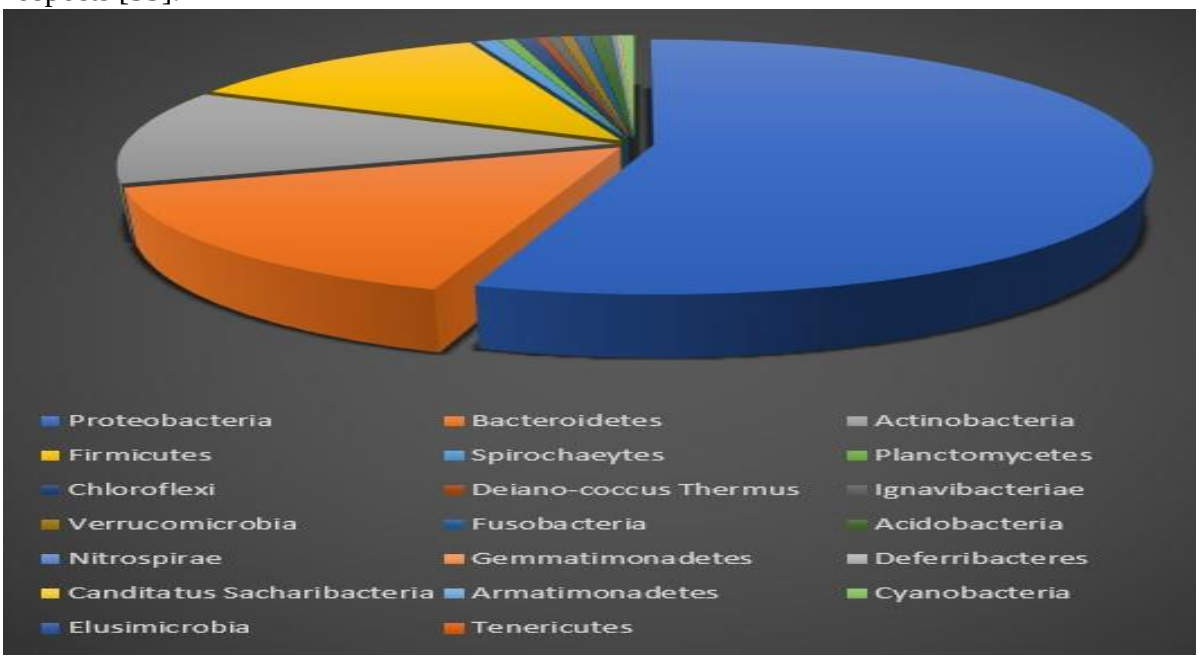


Fig. 4.5 Phylum levels found in the sample.

The most abundant genera that were found during metagenomic analysis were *Chryseobacterium, Unclassified Micrococcaceae, Labrenzia, Magnetospirillum, Unclassified Bradyrhizobiaceae, Pseudomonas, Acinetobacter, Prevotella, Unclassified Comamonadaceae and Unclassified Moraxellaceae* (Fig. 4.6).

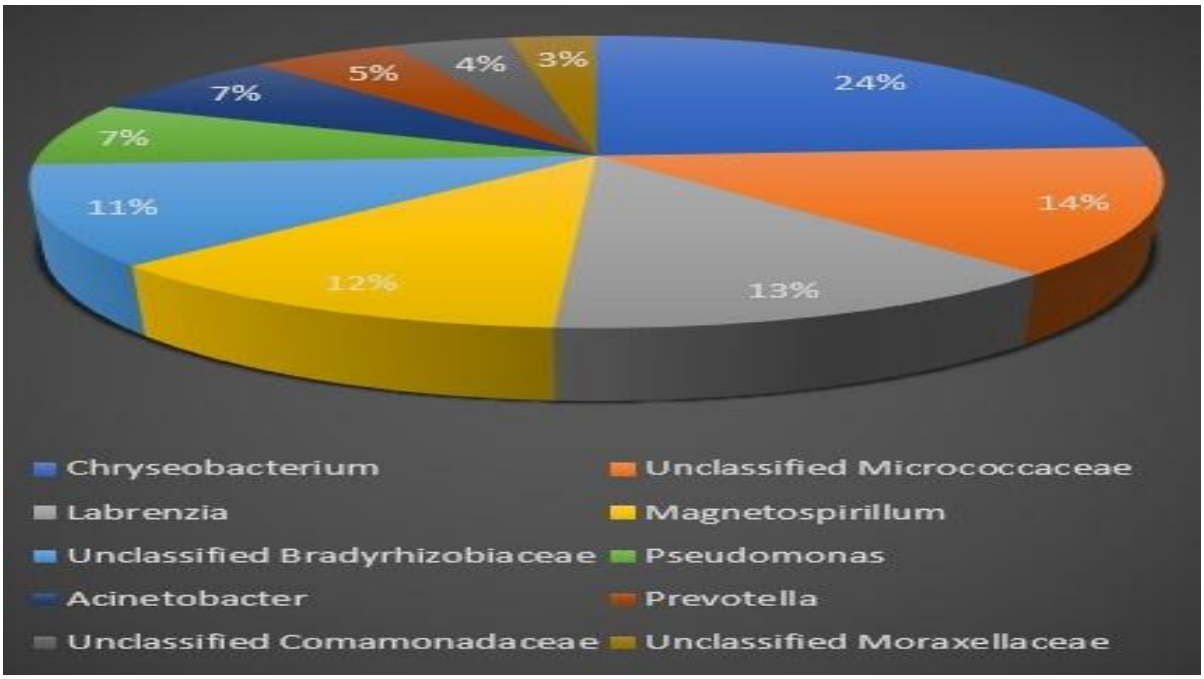


Fig. 4.6 Ten most abundant genera levels found in the sample

Metagenomics allows accessibility to the operational gene profile of microbial communities, providing a considerably wider characterization than phylogenetic analysis, which is frequently focused on the diversity of a single gene, such as the 16S rRNA gene. After metagenomics (Fig. 4.7), microbial community analysis was done. During analysis, it was found that several of the taxa were identified to be involved in the decomposition or degeneration of carbohydrates or organic complex compounds during the investigation, such as *Clostridium*, *Acetobacter*, *Rubrobacter* (resistant to gamma radiation), *Ruminococcus*, *Collinsella*, and *Morganella* [54] [55]. *Desulfovibrio*, *Sphingobium*, *Stenotrophomonas*, *Pseudomonas*, *Halomonas*, *Shewanella*, *Paracoccus*, *Mycobacterium*, *Hydrogenphaga*, *Sphingomonas*, *Rhodococcus*, *Morganella*, and *Methylobacterium* are among the many microorganisms that participate in bioremediation processes [56] [57].

Turicibacter, *Acinetobacter*, *Brevundimonas*, *Bacteroides*, *Prevotella*, *Ruminococcus Bacillus*, and *Clostridium* were among the genera capable of generating hydrogen [58] [59] [60]. Few genera were discovered in the water sample, which were primarily found in the human gut, indicating that human involvement is likely in the Tattapani water sample. They were identified as *Faecalibacterium*, *Dysosmobacter*, *Ruminococcus*, *Anaerostipes*, *Lachnoclostridium, and Veillonella* [61].

From an agricultural view, many of the genera were found to possess plant growth-promoting properties, such as *Azospirillum*, *Sphingobium*, *Arachidicoccus*, *Hydrogenphaga*, *Stenotrophomonas*, *Methylobacterium*, *Pseudomonas*, *Curtobacteria*, *Rhanella* [56][62]. Many of them show antibiotic-resistant properties as well as antibiotic production properties which are of great importance from a pharmaceutical point of view. Genera which show antibiotic-resistant properties, were *Chryseobacterium*, *Pseudomonas*, *Halomonas*, *Acinetobacter*, *Staphylococcus*, *Bacteroides*, *Enterococcus*, *Sorangium*, *Bdellovibrio*, *Brevibacterium*, *Ignavibacterium*, *Akkermansia*, *Corynebacterium* **[**63][64] whereas genera that show antibiotic producing properties were *Kytococcus*, *Streptomyces*, *Pseudomonas chlororaphis*, which has a phenazine-type antibiotic active agent against specific fungal plant pathogens, and the closely related species *Pseudomonas aurantiaca*, which produces di-2,4-diacetylfluoroglucylmethane, a compound antibiotically active against Gram-positive organisms. *Lysobacter* is also used in the production of novel antibiotics [65][66].

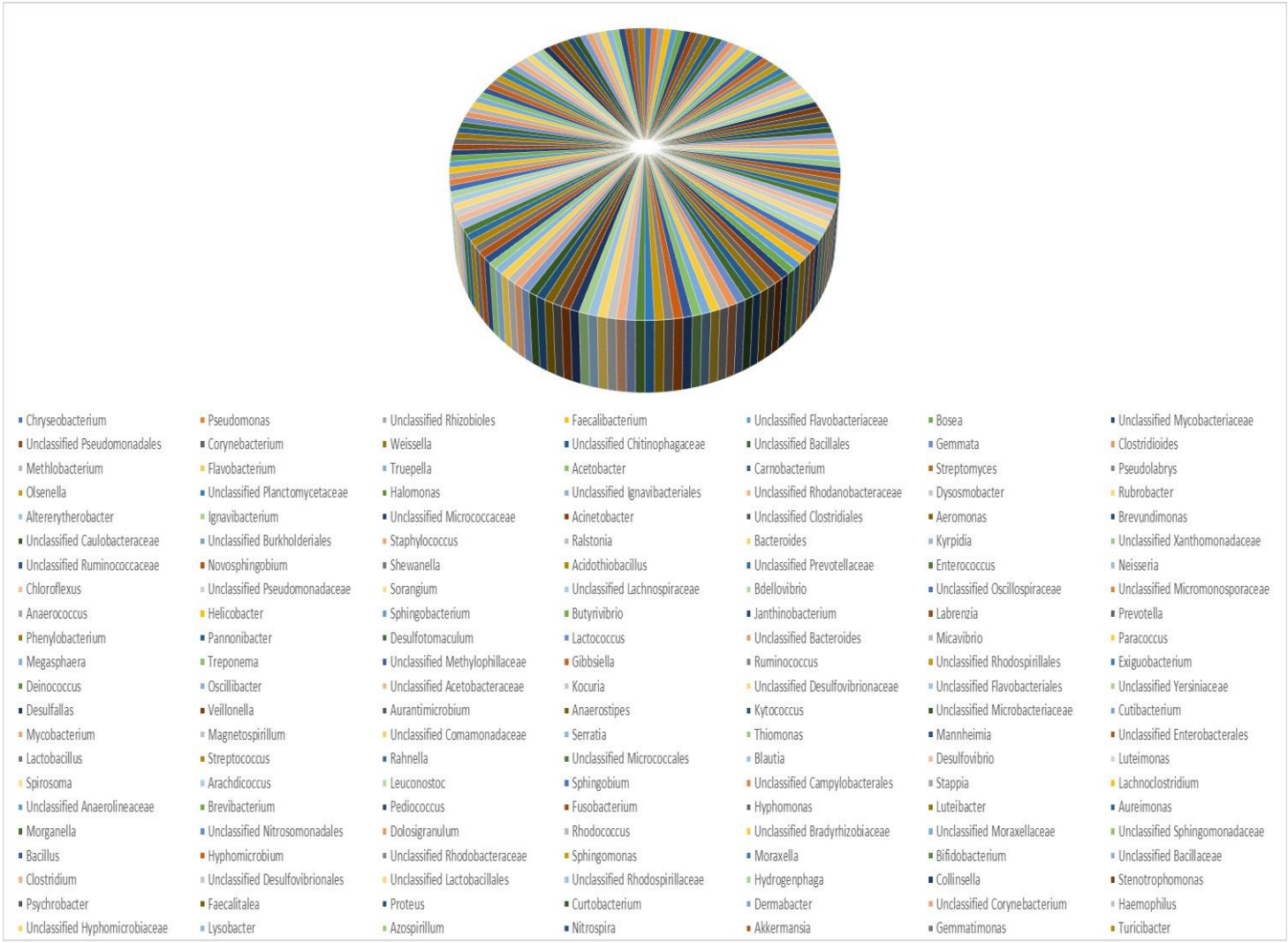


Fig. 4.7 Genera found in the Tattapani water sample

4.4 MICRO AND MACRONUTRIENT ANALYSIS OF TATTAPANI WATER SAMPLE

Nitrogen, Phosphorous, Potassium, Calcium, Magnesium, Sulphur and Iron are categorised as macronutrients present in the water sample whereas Manganese, Copper, Zinc and Boron are categorised as micronutrients. The microorganisms necessitate substantial quantities of these nutrients for their survival. These nutrients are found in the cells of bacteria to grow, promote its growth and carry out certain functions according to Aryal et al. Potassium has an impact on the action of multiple enzymes, Calcium is an essential part of bacterial endospores, Magnesium is engaged as an essential cofactor of various enzymes, and so on. Nitrogen is an integral component for the breakdown of amino acids, purines, pyrimidines, some carbohydrates, and lipids. Phosphorus can be found in nucleic acids, phospholipids, nucleotides such as ATP, cofactors, proteins, and other constituents of cells. Sulphur is required for the formation of several compounds, including the amino acids cysteine and methionine, as well as certain carbohydrates, biotin and thiamine. On the other hand, microorganisms require a number of additional substances on tiny amounts, known as microelements, micronutrients, or trace components. Manganese, zinc, and copper are among the nutrients in question. These are not required for microbial growth, but they play a role in a variety of biological functions. Zinc is present at the active site of numerous enzymes, while manganese catalyses the transfer of the phosphate group. The presence of these nutrients in the Tattapani water sample indicates that the water itself is acting as a good source of media required by the microbes for their survival.

Table 4.1 Nutrients present in Tattapani water sample

Nitrogen	3.5 ppm
Phosphorus	0.015 ppm
Potassium	69.2 ppm
Calcium	383 ppm
Magnesium	56.1 ppm
Sulphur	33.2 ppm
Iron	0.236 ppm
Manganese	0.017 ppm
Copper	0.041 ppm
Zinc	0.006 ppm

4.5 CHARACTERIZATION OF PINE NEEDLES

According to Wawro et al., the composition of dried pine needles was found as cellulose (38%), hemicellulose (20%) and lignin (20%) [67]. In our results, the composition observed was cellulose (35%), hemicellulose (15%) and lignin (30%) and the loss in composition will be stated as the soluble extractives present in it and other components which were not further tested. For the characterization, pine needles of size 14-16 cm were taken crushed and sieved with 44 BSS sieve size for further experiments.

The observed results for the characterization of pretreated pine needles were cellulose (31%), hemicellulose (8%) and lignin (21%). The size reduction and pretreatment led to the much loss in composition of pine needles.

Fig. 4.8 Dried Pine needles

4.5.1 Characterization of Raw Pine Needles

Property	Composition
Cellulose	35%
Hemicellulose	15%
Lignin	30%

Fig. 4.9 Steps of Cellulose Estimation

Fig. 4.10 Steps of Hemicellulose Estimation

Fig.4.11 Steps of Lignin Estimation

4.5.2 Characterization of Pretreated Pine Needles

Property	Composition
Cellulose	**31%**
Hemicellulose	**8%**
Lignin	**21%**

Fig. 4.12 Steps of Cellulose Estimation

Fig. 4.14 Steps of Lignin Estimation

Fig. 4.13 Steps of Hemicellulose Estimation

4.6 MAINTENANCE OF PURE CULTURE OF ANOXYBACILLUS

These Bushnell and Nutrient agar plates show us that the obtained microbe *Anoxybacillus kestanbolensis*was preserved for its medium shortage for further experiments

4.6.1 Simple Streaking4.6.2 Quadrant Streaking

Fig. 4.15*Anoxybacillus kestanbolensis* strain K1

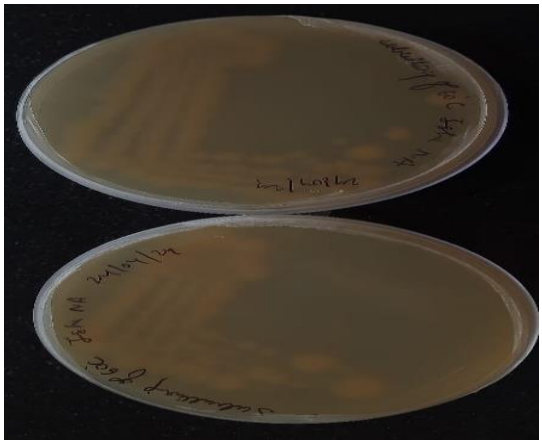


Fig. 4.16*Anoxybacillus kestanbolensis* strain K1

4.7 GRAM STAINING

Gram-staining of the isolated bacteria was performed to know the preliminary and morphological features of the isolated bacteria. It was observed that the isolated bacteria was Gram-positive with rod -shaped morphology.

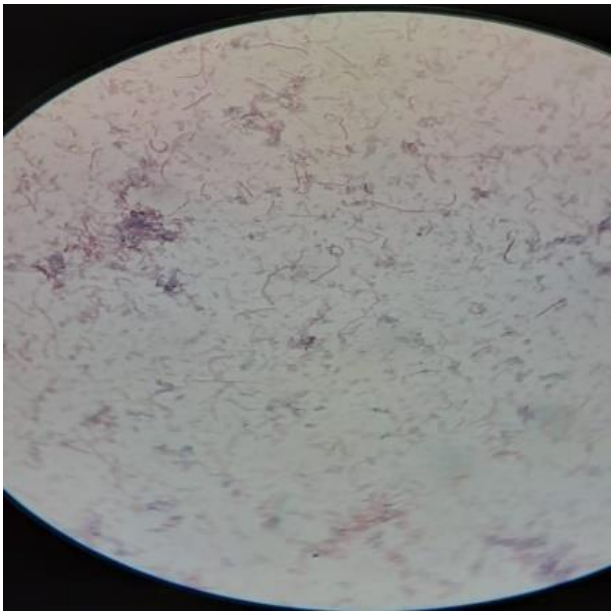


Fig. 4.17 Gram-Positive bacteria

4.8 TEMPERATURE TOLERANCE

As the isolated bacteria is a thermophile, to know its maximum range of temperature tolerance, nutrient agar plates were prepared and incubated at three different temperatures i.e. 60°C, 70°C, and 80°C. Growth was observed at 60°C and 70°C and white-cream coloured colonies was observed.

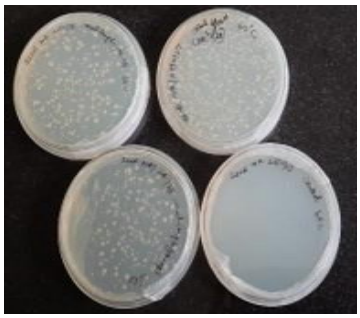


Fig. 4.18 Pure colonies were observed at 60°C

Fig. 4.19 Pure colonies were observed at 70°C

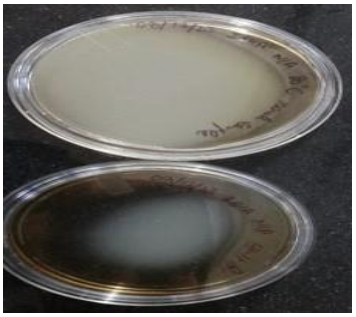


Fig. 4.20 No observation was detected at 80°C

4.9 MICROBIAL IDENTIFICATION

In the 1st well sample was loaded and the well naming L indicates the ladder. A clear band of 1500 bp was observed.

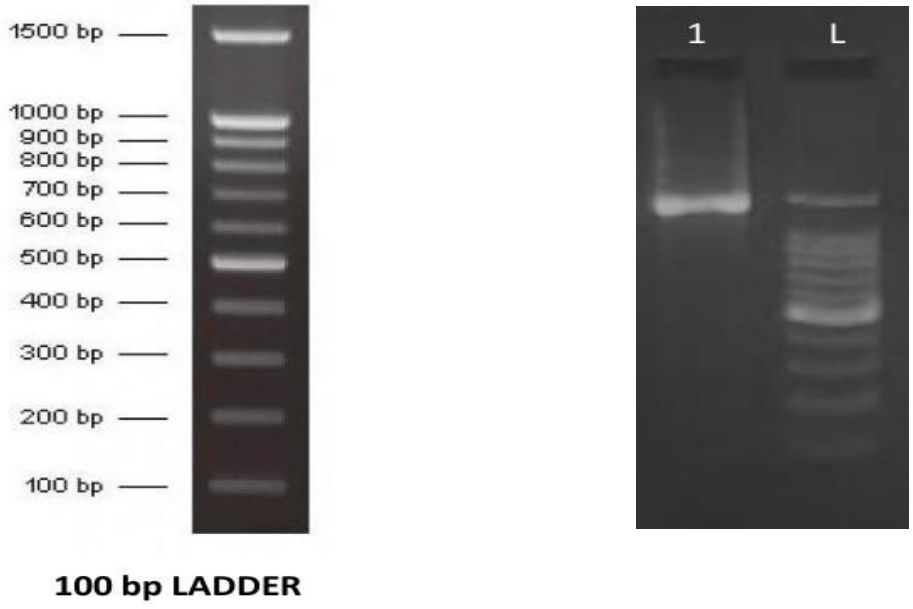
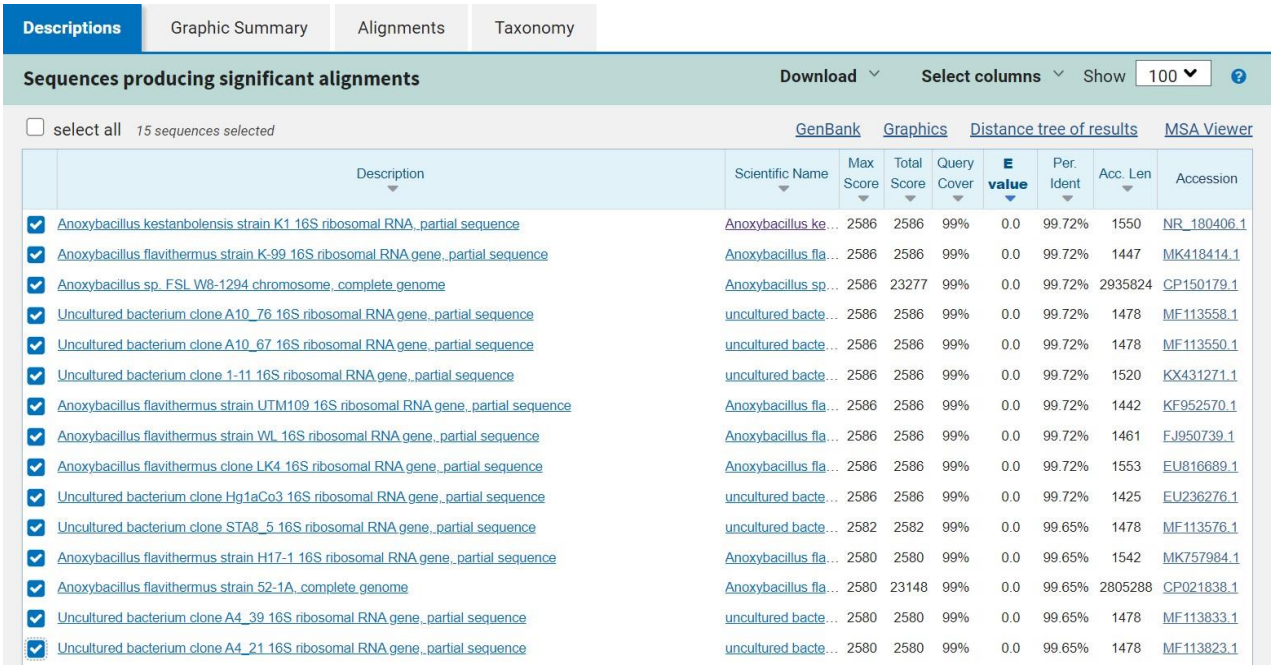


Fig. 4.21 PCR Gel Amplified images

4.10 BIOINFORMATICS ANALYSIS

The sequence of the identified bacteria was submitted at NCBI under the accession number PP710358 and identified as *Anoxybacillus kestanbolensis* strain K1 and the BLAST was performed of the first 10 similar sequences and Phylogenetic tree was constructed.



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 100

select all *15 sequences selected* — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Anoxybacillus kestanbolensis strain K1 16S ribosomal RNA, partial sequence	Anoxybacillus ke…	2586	2586	99%	0.0	99.72%	1550	NR_180406.1
Anoxybacillus flavithermus strain K-99 16S ribosomal RNA gene, partial sequence	Anoxybacillus fla…	2586	2586	99%	0.0	99.72%	1447	MK418414.1
Anoxybacillus sp. FSL W8-1294 chromosome, complete genome	Anoxybacillus sp…	2586	23277	99%	0.0	99.72%	2935824	CP150179.1
Uncultured bacterium clone A10_76 16S ribosomal RNA gene, partial sequence	uncultured bacte…	2586	2586	99%	0.0	99.72%	1478	MF113558.1
Uncultured bacterium clone A10_67 16S ribosomal RNA gene, partial sequence	uncultured bacte…	2586	2586	99%	0.0	99.72%	1478	MF113550.1
Uncultured bacterium clone 1-11 16S ribosomal RNA gene, partial sequence	uncultured bacte…	2586	2586	99%	0.0	99.72%	1520	KX431271.1
Anoxybacillus flavithermus strain UTM109 16S ribosomal RNA gene, partial sequence	Anoxybacillus fla…	2586	2586	99%	0.0	99.72%	1442	KF952570.1
Anoxybacillus flavithermus strain WL 16S ribosomal RNA gene, partial sequence	Anoxybacillus fla…	2586	2586	99%	0.0	99.72%	1461	FJ950739.1
Anoxybacillus flavithermus clone LK4 16S ribosomal RNA gene, partial sequence	Anoxybacillus fla…	2586	2586	99%	0.0	99.72%	1553	EU816689.1
Uncultured bacterium clone Hg1aCo3 16S ribosomal RNA gene, partial sequence	uncultured bacte…	2586	2586	99%	0.0	99.72%	1425	EU236276.1
Uncultured bacterium clone STA8_5 16S ribosomal RNA gene, partial sequence	uncultured bacte…	2582	2582	99%	0.0	99.65%	1478	MF113576.1
Anoxybacillus flavithermus strain H17-1 16S ribosomal RNA gene, partial sequence	Anoxybacillus fla…	2580	2580	99%	0.0	99.65%	1542	MK757984.1
Anoxybacillus flavithermus strain 52-1A, complete genome	Anoxybacillus fla…	2580	23148	99%	0.0	99.65%	2805288	CP021838.1
Uncultured bacterium clone A4_39 16S ribosomal RNA gene, partial sequence	uncultured bacte…	2580	2580	99%	0.0	99.65%	1478	MF113833.1
Uncultured bacterium clone A4_21 16S ribosomal RNA gene, partial sequence	uncultured bacte…	2580	2580	99%	0.0	99.65%	1478	MF113823.1

Fig. 4.22 BLAST data of first 10 similar sequences

To know the similarity among the sequences phylogenetic tree was constructed (Fig. 4.23). As, the isolated microbe was identified as *Anoxybacillus kestanbolensis* strain K1. According to literature, it can be a potential thermophile for the production of biohydrogen. *Anoxybacillus* is a genus of the Bacillaceae family, which belongs to the Firmicutes phylum, class Bacilli, order Bacillales. Alkali-thermophiles are *Anoxybacillus* species that thrive in temperatures ranges from 50°C to 65°C and pH levels of 5.6 to 9.7 and are commonly found in hot springs. These organisms are Gram-positive spore-forming rods with facultative anaerobic characteristics. The *Anoxybacillus* genus now includes 24 recognised species and two subspecies. Notably, *Anoxybacillus* species have been proposed as agents for bioremediation of several contaminants, such as Hg^{2+}, Cr^{2+}, Al^{3+}, As^{3+}, and nitrogen oxide ions, as well as possible options for biohydrogen generation. Recent study has emphasised the wide range of thermostable enzymes available in this newly formed species, suggesting tremendous potential for both industrial and environmental applications [68] [69].

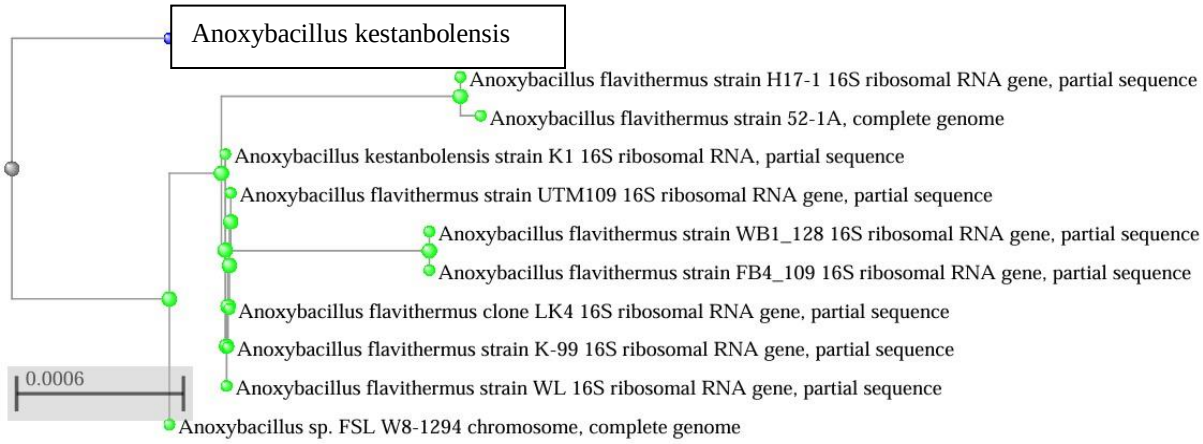


Fig. 4.23 Phylogenetic relationships of *Anoxybacillus* variety strains determined by 16S rRNA genes

4.11 PRESERVATION OF BACTERIA

For preserving the bacteria for long and short durations, lyophilized cultures, glycerol stocks and nutrient agar slants were prepared.

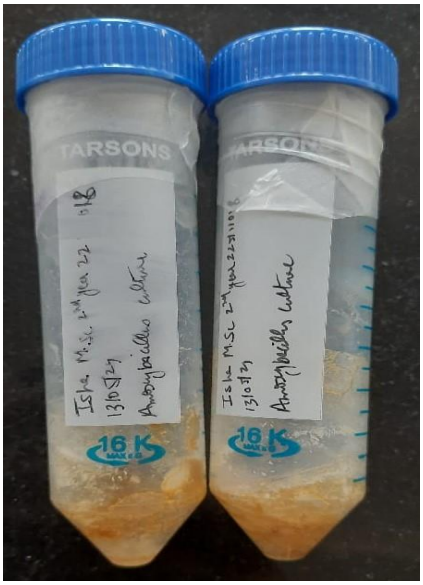


Fig. 4.24 Lyophilized enzyme

Fig. 4.25 Nutrient agar slants

4.12 BIOHYDROGEN ESTIMATION

For the estimation, qualitative and quantitative tests were done.

4.12.1 Qualitative Estimation

It was done using Keithley's Picometer & Voltage Source Hydrogen Gas Sensor Setup. During the qualitative analysis of biohydrogen, three peaks were observed naming P1, P2, P3 with respect to standard as it indicates the change in resistance to show the presence of biohydrogen in the sample. Qualitative test only determines the quantity. Positive results were observed. For quantitative estimation Gas Chromatography was performed.

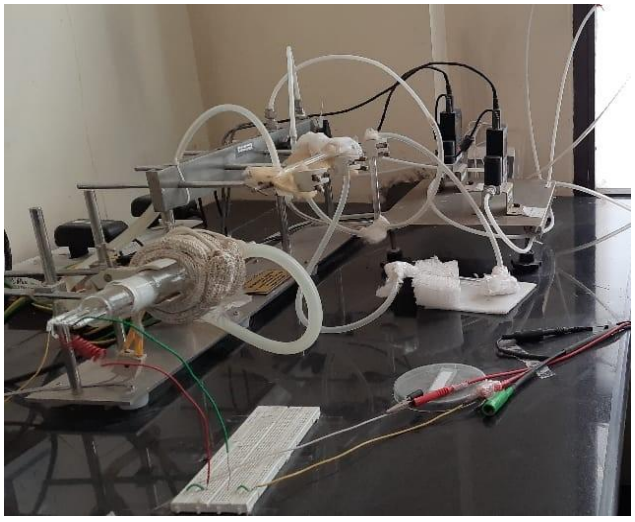


Fig. 4.26 Keithley's Picometer & Voltage Source Hydrogen Gas Sensor Setup

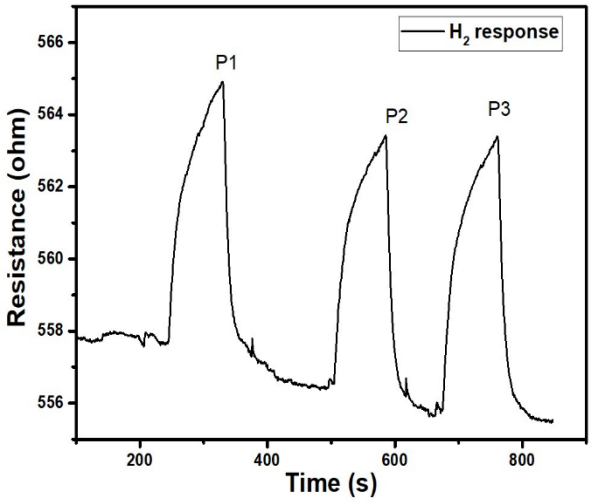


Fig. 4.27 Change in resistance was observed

4.12.2 Quantitative Estimation

The hydrogen concentration was found to be detected in the serum bottle in which ½ of the content of the nutrient broth was used as media, and at a temperature of 37°C. The concentration of hydrogen present is 14.26 ml/L. During the procedure, the dark fermentation procedure was used to obtain biohydrogen yield in the sample as this process occurs in the absence of light and is cost-effective. According to Zhang et al. reported the concentrations of biohydrogen by using different substrates in this unit. 91.2 ml/L of H_2 was reported when sorghum stover was used as the substrate, 88.5 ml/L in corn stover and 84.2 ml/L in rice straw. During the procedure, organic substrates which were used for biohydrogen yield are broken down by the anaerobic bacteria. The major metabolic pathway involved, converts organic compounds (such as sugars, starches, and lignocellulosic materials) into hydrogen and other byproducts [70].

It is well known that pine needles are found in abundance in Himachal Pradesh which can be a great source of substrate which can be utilised for biohydrogen production using dark fermentation process. The microbial consortium which was collected from Tattpani hot spring was acting as a source of inoculum in our experiments. Using dark fermentation process for the production of biohydrogen has certain limitations such as low hydrogen yield and production of other gases. During experiments, for better yield of biohydrogen certain parameters were determined such as modifying the substrate composition, fermentation conditions such as pH, temperature and HRT.

In our sample biohydrogen generating bacteria is *Anoxybacillus kestanbolensis* strain K1. According to literature, it has been said that it can be a prominent bacteria for biohydrogen production but no one has reported yet in the world for biohydrogen production [68]. It has been reported firstly for biohydrogen production. Consequently, various Anoxybacillus strains isolated from different environments have been extensively investigated for their biotechnological and commercial applications, including enzyme production, bioremediation, and the degradation of hazardous chemicals. Some strains have the ability to produce exopolysaccharides with beneficial biological properties such as antibacterial, antioxidant, and anticancer effects [71] The microorganism which had been reported for maximum biohydrogen generation is *Caldicellulosiruptor saccharolytics* DSM8703 yields 11.2 mmol H_2/g [51]. Others are *Thermoanaerobacterium thermosaccharolyticum* M18 yields 3.53 mmol H_2/g, *Caldicellulosiruptor bescii* yields 22 mmol H_2/L and *Thermotaga neapolitana* DSM 4359 yields 3.8 mol H_2/ mol of glucose [72].

CHAPTER 5

CONCLUSION

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- The main goal of this thesis is to produce biohydrogen from pine needles using dark fermentation process. In our research, extremophilic consortia is collected from Tattapani hot spring, Mandi, Himachal Pradesh.
- Extremophilic consortium will be acting as a source for inoculum and pine needles will be acting as substrate for the production of biohydrogen.
- Experiments were conducted such as metagenomic analysis to study the whole microbial community present in the hot spring, isolation was done of the bacteria that was found to produce biohydrogen.
- Its characterization, identification and sequencing was performed and the obtained sequence was submitted to NCBI with the accession number.
- Several variables such as temp, pressure, pH, hydrogen partial pressure and HRT was optimised for the better yield of biohydrogen.
- For analysis of biohydrogen, qualitative and quantitative tests were done. Quantitative test was done by using gas chromatography. For pilot scale production of biohydrogen from this sample more research can be done.

CHAPTER 6

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CONFERENCE PUBLICATIONS

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1. **BIOHYDROGEN PRODUCTION FROM PINE NEEDLES USING DARK FERMENTATION**
   Isha Agarwal, Vijay Kumar Garlapati, Sudhir Kumar
   Poster Presentation at 2nd International Conference on Biotechnology and Bioinformatics (ICBAB-2023) organised by JUIT, Solan from July 11-13, 2023.

2. **EXTREMOPHILIC MICROBIAL MACHINERY TOWARDS BIOHYDROGEN PRODUCTION THROUGH PINE NEEDLE-BASED DARK FERMENTATION**
   Isha Agarwal, Vijay Kumar Garlapati, Sudhir Kumar
   Abstract submitted at IIT Mandi for the 4th Himachal Pradesh Science Congress (HPSC) on "Role of Science, Technology & Innovation in Achieving Sustainable Development Goals"

3. **BIOHYDROGEN PRODUCTION FROM PINE NEEDLES USING DARK FERMENTATION**
   Isha Agarwal, Vijay Kumar Garlapati, Sudhir Kumar
   Abstract submitted at 2nd International Conference on Biotechnology and Bioinformatics (ICBAB-2023) organised by JUIT, Solan from July 11-13, 2023.

BOOK CHAPTER

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HYDROGEN PRODUCTION BY ELECTROCHEMICAL PROCESS
Isha Agarwal, Swati Sharma, Deepak Sharma, Sudhir Kumar, Vijay Kumar Garlapati
Book chapter under press in Renewable Hydrogen Opportunities and Challenges in Commercial Success, Elsevier. [ISBN: 9780323953795]

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