Biohydrogen Production from Potato Waste Using Dark Fermentation

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for the Degree of Doctor of Philosophy

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DECLARATION STATEMENT

I hereby declare that:

1. I have not been enrolled for another award of the University, or other academic or professional organisation, whilst undertaking my research degree.
2. None of the material contained in the thesis has been used in any other submission for an academic award.
3. I am aware of and understand the University's policy on plagiarism and certify that this thesis is my own work. The use of all published or other sources of material consulted have been properly and fully acknowledged.
4. The work undertaken towards the thesis has been conducted in accordance with the SHU Principles of Integrity in Research and SHU Research Ethics Policy.
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ABSTRACT

The excessive use of fossil fuels as the primary energy source has resulted in significant environmental and economic challenges, including greenhouse gas emissions and the depletion of finite fossil fuel resources. To address these issues, there is a growing need for alternative and sustainable energy sources. Hydrogen holds promise as a clean and renewable energy option, and biohydrogen, produced through biological water splitting using microorganisms, emerges as a potential solution. Shifting towards biohydrogen production can help reduce greenhouse gas emissions, promote energy security, and lessen dependence on finite fossil fuels, making it a crucial step towards a more sustainable energy future.

The assessment of biohydrogen production was performed in the study using obligative facultative anaerobe *Clostridium butyricum* (NCTC 7423) in the batch experiments. The initial experiment tested the potential of the strain to produce hydrogen from pure carbohydrate sources glucose and starch. Under the inoculum to substrate ratio of 9%, the strain showed the yield of 1.23 mol H_2 / mol glucose and 0.73 mol H_2 / mol glucose from glucose and starch with the substrate degradation efficiency of 70% and 60% in glucose and starch. The theoretical yield of hydrogen from carbohydrate is 2 moles for butyrate pathway and 4 moles for acetate pathway and their respective efficiencies are 16.75% and 33.51%. The efficiency of the strain from the conducted experiments using glucose and starch were 60% and 36%. The effect of temperature was further tested for the provided strain which resulted in the improved substrate degradation efficiency from 70% to 90% but the hydrogen production reduced at higher temperature.

The strain has been reported to utilise starch as a substrate, this study further tested the use of natural waste rich in carbohydrate which in this case was potato waste. The potato waste was able to produce hydrogen with accompanied pre-treatment method which helps to improve the hydrolysis of carbohydrate present in the biomass. The potato was thermally and mechanically treated for biohydrogen production, three types of potato wastes were tested and the higher biohydrogen production was achieved in the boiled potato waste compared to raw and dried potato waste. The yield of the raw, dry and boiled potato waste achieved in the study are 65.05 ml/ g VS, 30.58 ml/ g VS, 103.39 ml/ g VS. The possibility of hydrogen production from all types of waste showed that potato in any form can be used to produce biohydrogen. The biohydrogen energy obtained by treating three types of potato waste are 9.3

kJ, 3.5 kJ, and 12.9 kJ from raw, dry and boil potato. The COD reduction obtained in the three types of waste were 17%, 11% and 23 %. The possibility of biohydrogen production from potato is a step towards making this process viable and energy efficient.

Biohydrogen production is affected by the process parameters, therefore response surface analysis was used to optimise the process parameter pH and temperature for biohydrogen production. The analysis showed that pH and temperature are significant factors for biohydrogen production and the optimum pH and temperature was found to be 4.5 and 39^{o}C which resulted in 129.50 ml H_2/g VS . The analysis of volatile fatty acid in the experiments also showed that the strain utilised butyrate pathway for biohydrogen production as the butyric acid production was dominant in all the experiments. By maintaining the pH and temperature the COD reduction further increase to 29% which shows that both pre-treatment and maintained process parameter can helps to improve the hydrogen yield.

Biohydrogen offers a promising alternative to fossil fuels, and Clostridium butyricum shows potential for hydrogen production from various carbohydrate sources, including potato waste. By optimizing process parameters, such as pH and temperature, biohydrogen production efficiency can be significantly improved. The findings highlight the versatility and renewable potential of potato waste for biohydrogen production, contributing to the development of sustainable energy solutions and addressing environmental concerns associated with traditional fossil fuel usage. Further research and investment in biohydrogen technology are essential for achieving a sustainable energy future. Low production of biohydrogen compared to the quantity of hydrogen produced by conventional is a main challenge which can be resolved by simultaneously replicating the process on pilot-scale.

Biohydrogen is a novel research area, it was intriguing working with the waste and the possibility of biohydrogen production gives the insight for the future how food industries can innovate and work on the infrastructure to utilise the waste generated on the site to add value to the waste. The statistical tool response surface methodology used for process optimisation was helpful and eliminated the hassle and huge number of experiments.

DEDICATION

To my beloved Nana, Dada, Nani, Dadi, parents, siblings and phupo for their immense prayers and financial support throughout my higher education.

To my dearest husband Taha, for being with me during tough times, his guidance and motivation which helped me to get through the challenging situations.

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I appreciate the encouragement, patience I got from my dearest husband Taha, and for the belief he had on me to complete this research. I want to thank my parents for selflessly letting me to endeavour new journeys in life and engage in the higher education. I am thankful to my mother-in-law who constantly prayed for me and blessed me with her kind words. Massive thanks to my siblings Abeera, Saleha , and Ashar for their continuous encouragement, love and support.

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List of Abbreviation

ATP	Adenosine Triphosphate
CoA	Coenzyme A
CCD	Central composite design
COD	Chemical oxygen demand
CO_x	Carbon oxide
C_xH_x	Hydrocarbon
Fd	Ferrodoxin
$Fd_{(ox)}$	Ferrodoxin oxidised
GtC	Gigatonnes of Carbon
MEC	Microbial Electrolysis Cell
NADH	Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H)
NO_x	Nitrogen oxide
OECD	Organisation for Economic Co-operation and Development
PEMFC	Proton-exchange-membrane fuel cell
PFL	Pyruvate-formate lyase
PFOR	Pyruvate ferredoxin oxidoreductase
PSB	Photo-synthetic bacteria
SDG	Sustainable Development Goals
SO_x	Sulfur oxides
TVS	Total volatile solid
TWh	Tera watt hour

List of Notation

F_{in}	Volumetric inflow
F_{out}	Volumetric outflow
dV/dT	Working volume variation
V	Working volume
D	Dilution rate
H(t)	Cumulative hydrogen production
P	Hydrogen potential
R_m	Maximum hydrogen production rate
λ	Lag phase
t	Incubation time

Chapter 1 **Introduction**

This chapter highlights the adverse effect of fossil fuel consumption and concerned carbon emission production in the environment. In past renewable energy sources were studied to reduce the carbon emission caused by the burning of fossil fuel but unpredictable times like COVID-19 emphasise on the exploration of various energy production route using renewable sources to achieve energy security. This chapter also establish the case for biohydrogen production from food waste and its contribution towards sustainable development goals.

1.1 Background

1.1.1 Impact of Fossil Fuels

Reserves of fossil fuels and its discovery has altered the power generation methods. Going back in time, its usage started to facilitate basic human needs and acted as main contributor for industrial revolution. Within 140 years, it changed the fate of different countries and the living style of humans (Peter, 2018). Fossil fuel consumption has increased exponentially and aided human lives since the beginning of 20^{th} century, and it continues to rise every passing year in significant parts of the world due to population growth and their respective energy demands (U.S. Energy Information Administration (EIA) - Total Energy Monthly Data, 2022). Petroleum, coal and natural gas are three main fossil fuels, Oil is the most consumed fossil fuel because of its cheap price followed by coal and natural gas, the consumption pattern is seen in the figure 1-1 which is seen to increase in the next years with few surges throughout. Fossil fuel consumption has caused irreversible damage to the planet's geography and environmental conditions which are evident in the form of global warming issues, air pollution, acid rain, drought, and typhoon (Perera, 2017). Fossil fuel distribution is uneven worldwide; for instance, the largest oil reserves are found in Venezuela, Saudi Arabia, Canada, Iran, Iraq, Russia, Kuwait, USA and Libya. Similarly, there are countries with fewer or no oil reserves like Norway, Japan, Sweden, Switzerland, Turkey, South Korea and Spain. This distribution is dependent on the climate and the organism that lived in that region million years ago (Ritchie & Roser, 2019). An obvious relation is seen in the adoption and development of renewable energy in the country with scarce fossil fuel sources like Sweden, Norway, and Iceland. China being the fastest growing economy and the developed country of

the world, uses most of the fossil fuel to meet their energy requirement of 33,512 TWh which is 25% of total 132,514 TWh global fossil consumption (Ma et al., 2019).

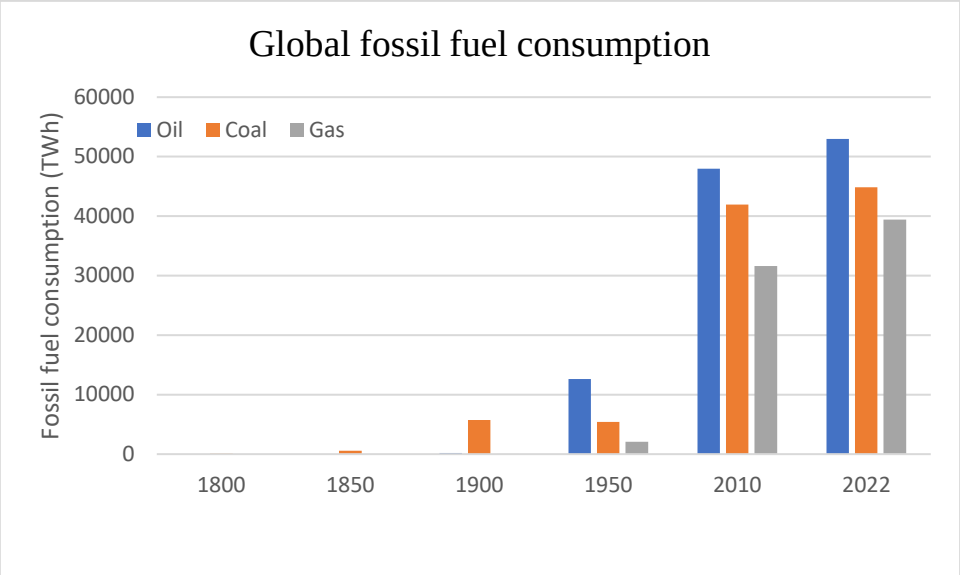


Figure 1-1 Global fossil fuel consumption (Ritchie & Roser, 2019)

With the world's population on the rise, there has been an increase in energy demands, and fossil fuels have become a significant source of energy generation both domestically and industrially. Currently, approximately 80% of the world relies on fossil fuels, particularly in key sectors such as electricity generation, food industry, transportation, and waste management. However, burning fossil fuels releases a substantial amount of carbon dioxide (approximately 5.5 GtC, or billions of metric tons of carbon) into the environment (U.S. Energy Information Administration (EIA) - Total Energy Monthly Data, 2022).

The combustion of fossil fuels also produces various pollutants, including carbon oxide (CO_x), nitrogen oxide (NO_x), sulphur oxide (SO_x), hydrocarbon (C_xH_x), and soot, which are released from vehicle exhaust, industrial processes, and residential heating. Moreover, incomplete combustion leads to the release of ash, droplets of tars, and other volatile organic compounds, which can contribute to the formation of ground-level ozone and smog (Prasad et al., 2019; Leliveld et al., 2019).

The accumulation of carbon dioxide in the atmosphere leads to the trapping of heat, resulting in global warming and subsequent geographical changes. The increased carbon emissions also negatively impact air quality, leading to respiratory illnesses and asthma due to the formation of air pollutants. Furthermore, global warming is associated with the melting of

glaciers, rising sea levels, and the occurrence of wildfires that disrupt natural habitats. Other disruptive effects of fossil fuel consumption include extreme weather events, smog, disruptions in food supply, and premature deaths, which have seen a rise in recent years (Freeman et al., 2018).

In an effort to address these environmental challenges, some OECD countries have been increasingly adopting renewable energy sources for electricity generation. For instance, the contribution of renewable energy has steadily increased from 17.6 TWh in 2008 to 27.4 TWh in 2018. To further compare the use of non-renewable and renewable sources, electricity production from coal is measured at 101,596.46 GWh, while electricity harnessed from wind stands at 127,340.9 GWh (US Energy Information Administration (EIA), 2017).

Apart from GHG emissions, another motivation towards renewable energy transition is unpredictable times like 2020 which has proven to be an eye-opener for the world to think about investing in alternatives at micro and macro levels. It is undeniable that additional energy generation sources are required to achieve sustainable goals and the earth's longevity. Fossil fuel dependence is important in terms of energy output, but when it comes to carbon emission production and exploitation of the ozone layer, this dependency is worrisome (Mohideen et al., 2021). The goal had never been to eliminate carbon emission but to reduce this rate by making small changes in routines and actions. The target is to go for the less and lesser emissions. Thus, to resolve environmental degradation, energy crises, and unpredictable global challenges, an alternative energy solution is exceptionally urgent. Few renewable sources are already investigated and implemented with large and small power generation capacity, like solar energy, wind energy, geothermal energy, hydropower and biomass. Renewable energy production share has increased globally with a lead in hydropower 4,222.21 TWh followed by wind power with 1429.62 TWh and solar with 724.09 TWh. Comparatively, these numbers are low because a total share of 136,761 TWh energy is produced from fossil fuels (Looney, 2021). In 2019, 88% of energy was produced using fossil fuels. Renewable energy is an area under exploration, and great potential is seen in its development, with a predicted rise of around 3% to 9% of its share in primary energy. The global penetration of modern renewable energy will reach 14% of final energy consumption by 2030, according to the policies of the International Renewable Energy Agency (IRENA).

Renewable energy is considered carbon-free because the actual generation of energy does not produce carbon emissions. However, it's important to note that the manufacturing of renewable energy equipment can still leave a carbon footprint, particularly when hazardous materials are used in the production of components like solar panels. Similar to fossil fuel power plants, renewable energy sources such as wind, ocean, and solar power can efficiently serve remote areas, but their effectiveness is highly dependent on specific climate conditions (Report, 2011).

Constructed renewable energy projects have a limited lifespan and require a significant amount of construction materials. While these concerns are valid, they become less significant when compared to the environmental impact of power generation from fossil fuels (Pehl et al., 2017). Figure 1-2 illustrates the carbon emissions resulting from the processes and manufacturing of renewable energy components, indicating that renewable processes generally impose a lower burden on the environment compared to fossil fuel-based methods (Amponsah et al., 2014).

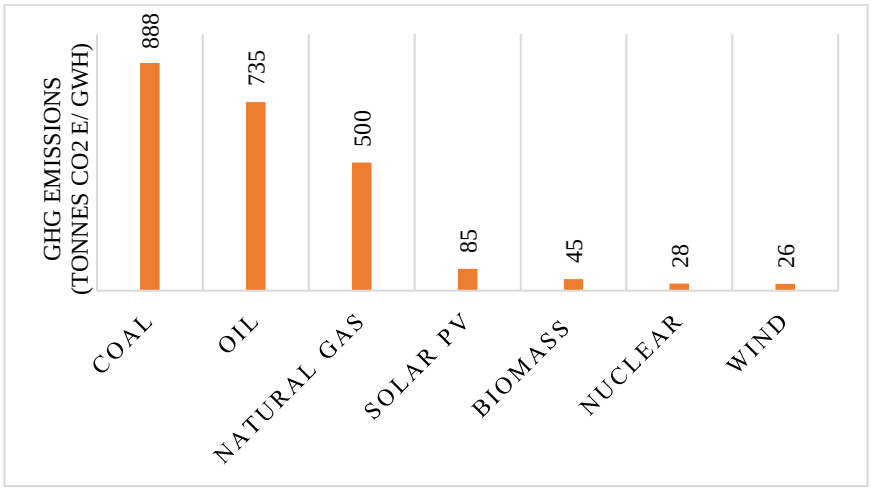


Figure 1-2 Average greenhouse gas emission by energy source (Ritchie, 2020)

Fossil fuels are burn in a plant and energy generation is highly dependent on the thermal efficiency of a plant itself. When fossil fuel is burn in a thermal plant most of the energy is lost in form of heat due to low conversion efficiency. Around 60% to 67 % of energy is lost in form of heat. Whereas when electricity is generated from renewable source, the direct output is measured, and no loss or waste is considered (Ritchie & Roser, 2020). This is explained by an example of energy mix. Consider a power requirement 50 terawatt-hours (TWh) of energy in three energy mix scenarios: only fossil fuel; renewable energy; and a

combination of both. Since the efficiency of plant is 38% 131TWh of energy is required to meet the demand of 50 (TWh). 81.57 TWh energy is lost which is more than the amount of energy required for consumption. In the case of renewable energy, the quantity of electricity generated is same as quantity which is used i.e. 50 Twh. In the last scenario if half of the energy is to be generated from each source that 25 TWh is required from renewable source and 65.7 TWh from fossil fuel, a total of approximately 90.7 TWh of energy input which accounts for 65.879 TWh of energy waste. Therefore, it is evident that the loss rate is much higher in fossil driven process when compared with renewable sources. Carbon emissions generates by fossil fuels are principally determined by their carbon content and hydrogen–carbon ratio. Over time, the trend in fossil fuel utilisation has shifted toward a higher hydrogen-to-carbon (H/C) ratio. Higher H/C ratio results in low carbon emission and has high energy efficiency from combustion. Wood contained twice as much carbon as coal. Coal, on the other hand, having a lower H/C ratio, is twice as energy efficient as wood. Later, coal was superseded by oil, which has a greater H/C ratio and hence benefited from higher energy efficiency and lower CO2 emissions than wood and coal both. Natural gas nevertheless has a lower carbon content than oil, coal and wood. Biofuels, on the other hand, have a lower carbon-to-hydrogen (C/H) ratio. In fact, biofuels like hydrogen have a C/H ratio zero (Hoffert et al., 1998). As a result, using hydrogen as a fuel could help reach the aim of total decarbonization. As a result, the current emphasis is on the development of efficient hydrogen production technologies as alternatives to depleting and carbon-intensive fossil fuels.

Energy security is one of the most crucial challenges needs to be addressed by today's world. Modernism and materialism have impacted human life at individual and collective basis (Ang et al., 2015). Human lives are powered by energy-consuming devices, and the need for energy is growing all the time. The procedures used to extract the energy from the Earth's crust have been shown to be harmful to the earth and its atmosphere. The only approach to meet and share the present energy demand is by developing new approach, infrastructure, policies, renewable, and ecologically friendly alternatives to the fossil fuels currently in use (Kariuki, 2018).

1.1.2 Importance of Renewable Energy

Importance

Renewable energy sources such as wind power, hydropower, nuclear power, and energy from biomass offer viable alternatives to rapidly depleting and carbon-intensive fossil fuels (Bull, 2001). Embracing a renewable energy economy is highly desirable as it promotes safety, accessibility, and sustainability. Wind power and solar energy are already making significant contributions, with the potential for further growth in the future. Bioenergy, harnessed from biomass, holds promise as a significant renewable energy source and has the potential to fulfill up to 50% of global energy needs in the 21st century, as claimed by the International Energy Agency.

Impact:

One of the most significant benefits of renewable energy lies in its lower emissions of pollutants compared to fossil fuels. Coal mining, oil exploration, and refinement are notorious for generating toxic waste and releasing harmful substances like mercury and heavy metals (Dincer, 1998). The consumption of coal for electricity generation also results in water contamination and the release of CO2, sulphur dioxide, nitrogen oxides, and mercury into the air. Petroleum products contribute to similar pollution issues. Renewable energy sources significantly mitigate these environmental hazards, reducing air pollution, acid rain, and damage to the ozone layer.

Challenges:

The accessibility and safety of fossil fuel reserves pose challenges as they are often located deep underground or under the sea, making extraction difficult and costly. Offshore drilling accidents, such as the Deepwater Horizon incident in the USA and the Gulf War oil spill in the UAE, can lead to devastating consequences for both the environment and human life. In contrast, renewable energy sources like wind and solar are abundant and easily accessible. Moreover, renewable energy systems offer enhanced safety as they avoid the risks associated with oil platform explosions and coal mining accidents.

Opportunities:

Fossil fuels' excessive use contributes significantly to greenhouse gas emissions, resulting in climate change and global warming with severe consequences for human health, agriculture, water supplies, biodiversity, and the spread of diseases (Full et al., 2021). Recognizing the immense threat posed by climate change, transitioning to renewable energy becomes an opportunity to combat this global health crisis. By embracing renewable energy sources, we can reduce our dependence on uncertain and fluctuating fossil fuel prices and improve energy stability. Additionally, renewable energy systems can be localized, making them less susceptible to disruptions caused by distant political changes (Kabeyi & Olanrewaju, 2022)

Environmental Sustainability:

The transition to renewable energy sources aligns with environmental sustainability objectives. Renewable energy systems produce minimal greenhouse gas emissions, which helps combat climate change and reduce the overall carbon footprint. By decreasing our reliance on fossil fuels, we can mitigate environmental degradation, preserve ecosystems, and protect biodiversity. Furthermore, renewable energy technologies, such as wind and solar power, have low water consumption, reducing the strain on limited water resources. (Kabeyi & Olanrewaju, 2022)

Economic Sustainability:

Embracing renewable energy presents significant economic advantages. Renewable energy technologies have experienced rapid advancements, leading to cost reductions and increased affordability. As these technologies become more accessible, they create new job opportunities and stimulate economic growth in the renewable energy sector. Moreover, investing in renewable energy infrastructure contributes to energy independence and shields economies from fluctuating fossil fuel prices. Long-term reliance on renewable energy promotes energy security and reduces vulnerability to geopolitical risks associated with fossil fuel imports (Kabeyi & Olanrewaju, 2022).

Social Sustainability:

Renewable energy adoption fosters social sustainability by addressing social equity and improving public health. Transitioning to cleaner energy sources benefits vulnerable communities, which are often disproportionately affected by the negative impacts of fossil fuel industries. Renewable energy projects can also provide localized energy solutions,

empowering communities and improving energy access in remote areas. Additionally, reducing air pollution through renewable energy utilization positively impacts public health by lowering respiratory illnesses and enhancing overall well-being (Singhal & Prashant, 2020).

In conclusion, sustainability is a fundamental pillar of the renewable energy paradigm, encompassing environmental, economic, and social considerations. Embracing renewable energy not only mitigates environmental degradation and combats climate change but also fosters economic growth, energy security, and social equity. By prioritizing sustainable energy practices, societies can chart a path towards a more resilient and harmonious future for generations to come.

1.1.3 Problem Statement

The exploitation of fossil fuels and its adverse effects, such as carbon emissions, have raised significant environmental concerns. As a result, there is a growing interest in exploring renewable energy sources and their potential to mitigate carbon emissions. "Hydrogen" has emerged as a promising alternative fuel, and various renewable and conventional methods are being tested to replace fossil fuels (IEA, 2019).

Among the renewable methods, biological processes for hydrogen production, such as dark fermentation, have garnered considerable attention. Dark fermentation has been extensively studied for its ability to utilize a wide range of waste materials rich in carbohydrates, proteins, fats, and lipids to produce hydrogen. This presents an opportunity to generate energy from organic waste in a less energy-intensive manner.

The yield of biohydrogen in dark fermentation is influenced by several factors, including process conditions, inoculum to substrate ratio, and pre-treatment methods. Crucially, the availability of carbohydrates in the waste material plays a vital role in the biohydrogen generation process.

In the food sector, abundant waste rich in organic matter is generated, and potatoes are a significant contributor to this waste stream. With global annual potato production reaching 400 million tonnes, a considerable amount of solid waste is obtained during potato processing, including potato wastewater, pulp, and peels. If not managed properly, this waste can pose environmental threats, such as microbiological or soil contamination (Pathak et al., 2018).

However, this waste also presents an opportunity for various applications, such as the generation of biopolymers, natural antioxidants, food additives, and biofuels like biohydrogen, biomethane, and bioethanol (Maroušek et al., 2018). In this study, we aim to explore the potential of biohydrogen generation from potato waste as an environmentally friendly and sustainable energy source. By utilizing this abundant waste material, we can contribute to both environmental protection and the production of valuable bioenergy.

1.2 Contribution Towards Sustainable Development Goals

The opportunity to treat waste which generates in food sector for hydrogen production is a targeted solution towards the problem of GHG emissions which results from fossil-based fuel, and environmental concerned practice (e.g. landfill & incineration). Hydrogen is gaining popularity, and demand is rising quickly (Venkata Mohan, 2009). The scrutiny and scope of hydrogen generation from various renewable source available in either bulk or scarce quantity will undoubtedly be the next big revolution in the energy transition. Biological hydrogen production is on baseline studies. Biohydrogen production is different from renewable energy when compared to energy generated from wind and solar energy in a way that efficiency of a wind turbine depends on its design, including factors such as the height of the pole, the width and shape of the blade, and the speed of the wind. When the wind turbine converts mechanical energy into electrical energy, it generates electricity. Similarly, solar panels convert photons into electrical energy, and their efficiency is influenced by their design, which includes factors like the materials used in the solar panel and the surrounding temperature (Chang et al., 2013). Current renewable energy is concerned with the design rather than the actual source because nature of the wind and sun rays stays the same. Food waste is diverse its composition varies therefore, to use it as a source for energy generation which can replicate results on large scale it is crucial to work with different kinds, conditions, and reactor designs (Khan et al., 2018a).

Bioprocess development identifies the robust design, space for a specific bioproduct which is achieved after a series of experimentation to understand the interaction of different parameters Bio-process focuses on the bioconversion of renewable resources into fuels and chemicals in the industrial setting. It also discusses the idea and concepts of integrated biorefineries for achieving sustainable food, energy, and industrial product production. The presumed green advantages of using biomass and bio-degradable matter are one reason for the uprising demand of bio-process (Doran, 2013). Current petroleum-based chemical, results in greenhouse gas emission and its predicted depletion in the future are all important factors in the sustainable transition toward bioproducts (Mohanakrishna & Mohan, 2013). Sustainability is typically defined as the processes and behaviours by which humans avoid consumption of natural resources to retain an ecological balance that does not degrade the quality of life in modern society (Scoones, 2007).

Three pillars make up sustainability: the economy, society, and the environment. Informally, these concepts are referred to as profit, people, and planet. The purpose was to create a list of global goals that address our world's critical environmental, political, and economic issues. That is why an agenda for Envision 2030 with 17 sustainable goals (SDGs) originated at the United Nations Conference on Sustainable Development in Rio de Janeiro in 2012. The current research addresses three Sustainable Development Goals (SDG) 7: Affordable and clean energy, 9: Industry innovation and infrastructure, 12: Responsible consumption and production. This research study the potential to utilise the food waste to produce clean energy "bio-hydrogen" by following the model of waste hierarchy which can be implemented in the food industry to ensure the responsible consumption and production, the idea is illustrated in figure 1-3. Climate change is widely considered as generation's one of the most pressing issues, so it is no surprise that one of the Sustainable Development Goals (SDG) focuses on 'urgent action to address climate change and its consequences' (United Nation, 2017) . Hydrogen is seen as a viable renewable energy and fuel source. The generation of (bio)hydrogen from renewable feedstocks or solid wastes (for example, food waste) is seen as a green, sustainable, and environmentally beneficial method. By bio converting carbon-neutral food waste to (bio)hydrogen, biohydrogen can play a critical role in decreasing greenhouse gas (Dahiya et al., 2018).

Waste hierarchy is designed by DEFRA Department for Environment, Food, and Rural Affairs. First and foremost, it puts high emphasis to waste prevention. When waste is generated, it is prepared for reuse, recycling, recovery and last but not least disposal takes precedence (e.g. landfill) (WRAP, 2017). Climate change is considered one of the critical challenges at this time; hence it is no wonder that one of the Sustainable Development Goals (SDG) focuses on "urgent climate change action and its impacts" (European Commission, 2019a). A promising source of clean energy and fuel has been explored for hydrogen. Proposal of biohydrogen production from renewable feedstocks or solid waste (e.g. food waste) is green, and can be seen as sustainable and environment-friendly approach to tackle ongoing challenges of waste management and carbon emission from fossil fuels. The opportunity to make hydrogen from food waste is one step close to getting carbon-neutral fuel (Dahiya et al., 2018).

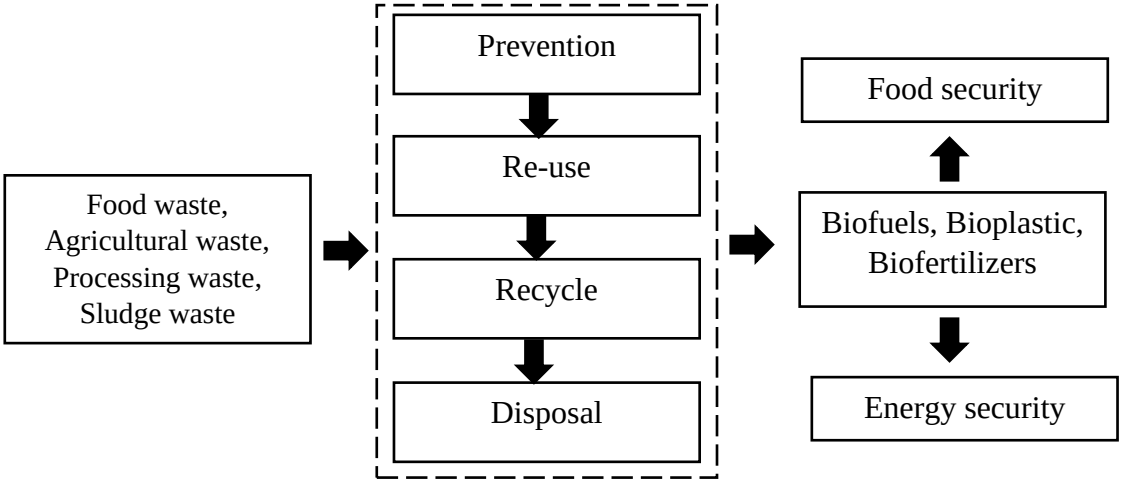
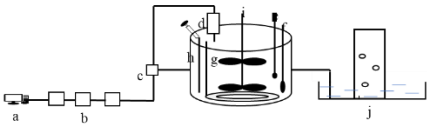


Figure 1-3 Targeted sustainable goal through food waste management & biohydrogen production

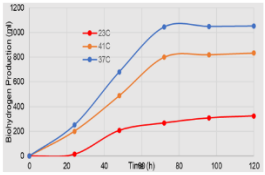
1.3 Research Aim

The research is carried out to utilise the potato waste for biohydrogen production in the dark fermentation using *Clostridium butyricum*. The following objectives were set to achieve the research aim in the thesis:

1) Setup a lab-based batch process for biohydrogen production, *C.butyricum* was grown and tested for biohydrogen production from pure carbohydrate source.
2) The effect of temperature was monitored for biohydrogen production in the reactor using pure carbohydrate source.
3) Pre-treatment methods were employed on potato waste for biohydrogen production.
4) Energy analysis were performed to determine the energy conversion efficiency of the process to make the system feasible.
5) Process optimisation technique was employed to enhance the biohydrogen production from two independent variable pH and temperature.
6) The analysis of liquid sample was performed to get the insight of metabolic pathway, substrate degradation, and COD reduction.

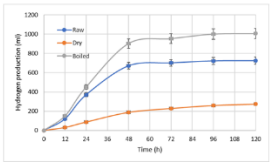


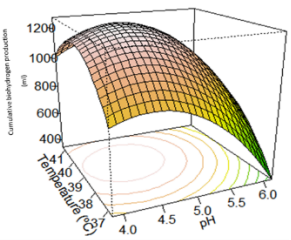
Setup a lab scale batch setup for Biohydrogen production



C.Butyricum tested on pure carbohydrate source to test the efficiency of the process based on energy yield, and molar yield. Effect of temperature was studied to see the changes in biohydrogen production

Three types of pre-treated potato was used to produce biohydrogen in the same lab setup which successfully accumulated biohydrogen from pure carbohydrates. Energy analysis were performed to predict the validity of the substrate if the utilisation of waste is employed at large scale





Statistical tool, Response surface methodology is employed to enhance biohydrogen production from boiled potato waste in the last study by varying the parameter pH and temperature

1.4 Thesis Layout

This thesis is divided in to seven chapters as follows:

I) Chapter 1 - Introduction: This chapter provides an overview of the background and motivation behind the study. It outlines the research aims, objectives, and the contribution of the research to the field.

II) Chapter 2 - Literature Review: Here, the need for biohydrogen as a renewable energy source is discussed, with a particular focus on utilizing potato waste through dark fermentation.

III) Chapter 3 - Methodology: This chapter details the methods and equipment used in the study to achieve the research objectives.

IV) Chapter 4 - Bio-Hydrogen Production from Pure Carbohydrate Sources: The findings of processing glucose and starch for bio-hydrogen production are presented in this chapter. Additionally, it also highlight the process efficiency and strain efficiency in terms of energy conversion efficiency of the process and the impact of temperature on bio-hydrogen production.

V) Chapter 5 - Pre-treatment of Potato Waste for Bio-Hydrogen Production: This chapter explores the effect of thermally and mechanically pre-treated waste on bio-hydrogen production. It also highlights changes in organic matter for the produced bio-hydrogen and provides an energy analysis of the potato waste.

VI) Chapter 6 - Process Optimisation for Bio-Hydrogen Production using Response Surface Analysis: In this chapter, the experimental plan used to study the impact of independent variables on bio-hydrogen production is discussed, along with the identification of optimum conditions. Volatile fatty acid analysis is performed to understand the metabolic pathway taken by Clostridium butyricum in the study.

VII) Chapter 7 – Conclusion and Future Work: Highlight the achieved objective, findings and challenges overcome. This chapter also discusses the suggested future work to be able to make impactful changes in the presented study.

Chapter 2 **Literature Review**

2.1 Introduction

Food and energy security are the leading world issues. Both issues lack in the proper practice for management which is leading to the adverse effect on the environment. The world heavily relies on the fossil fuel which has damaged the environment for humans and animals. Hydrogen production from glucose back in 1981 the idea was invented by Sukomal Roychowdhury, which led to opportunities of targeting two leading problems with one solution “Bio-hydrogen”. Bio-hydrogen is a biological process as the name implies, it uses less energy intensive process. So far different types of food waste/loss generated in the upstream and downstream process have been studied for the scale up applications. This idea is still at its infant stages and discoveries are being made using different process, feedstock, micro-organisms, and end applications. Environmentalist has shown huge interest to adapt the concept of biorefinery which is done by adding value to the waste generates in the industry by either converting it into biofuels or biochemicals. This will not only increase the value of the waste, but it will also help to minimise the cost of waste disposal.

The biodegradable nature of the waste streams and biomass are the potential candidate of biofuels; however, a lot of issues are linked to damage food crops for biofuel production. Therefore, food waste stream can be an ideal candidate for considering the biohydrogen production and it also gives an option to use the energy on site or sell it to other vendors. Hydrogen fuel cell has reached a commercial scale which was invented back in 1839. Similarly, the variety of substrate with inoculum sources study is required to explore the potential of biohydrogen. This literature review covers the important aspect which address the need for valorising the waste by environmentally friendly process for biohydrogen production.

2.2 Hydrogen

Hydrogen is one unique fuel that does not produce carbon dioxide, carbon monoxide and hydrocarbons during combustion because when hydrogen undergoes combustion, it reacts with oxygen to form water vapor (H_2O) as the main byproduct. Since water vapor is a naturally occurring component of the atmosphere and does not contribute to the greenhouse effect, the combustion of hydrogen does not add additional greenhouse gases to the atmosphere which minimise greenhouse gas emission problem and it is known as clean fuel (*Hydrogen*, 2023). Hydrogen is found in abundance on the earth which makes up to three quarters of all matter (Jain, 2009). Although hydrogen is present in large quantity the problem it doesn't exist in molecular form therefore, it must be separated from other compounds. Hydrogen is now no more limited to the phrase "fuel of the future", the advancement and integration in the process are unbinding its potential to be used at large scale. Hydrogen is widely used in the transportation sector by fuel cell application (Bicer & Dincer, 2018). Taking account of fossil fuel consumption's constraints and effects, most governments have stated ambitious plans for a sustainable H_2 economy for the transportation and industrial sectors According to the Hydrogen Council (2020), global hydrogen consumption will rise to 15–18 percent by 2030, with delivery costs falling to 1.80 USD/kg. Furthermore, the use of hydrogen in NH_3 manufacturing (51%), oil refining (31%), and CH_3OH production (10%) has raised the need for hydrogen year after year. H2 demand has recently surged due to its attraction as a sulfur-free fuel for transportation and energy generation (Das, 2009). Hydrogen has meaningful advantages 1) Hydrogen has energy content of 122 kJ/kg, which is 2.75 folds higher than other conventional fuels on mass level. The comparison of energy content with different fuels is shown in table 2-1. 2) Hydrogen produces energy by producing water vapour as by product. 3) the combustion of hydrogen in automobiles is 50% more efficient than gasoline which is why hydrogen has been adopted as a fuel in transportation sector 4) Hydrogen can be easily stored as metal hydride (Peraldobicelli, 1986).

Hydrogen has a high energy storage capacity, and it has been demonstrated that 1 kilogram of hydrogen contains approximately 120 mega joules (MJ) of energy, which is equivalent to about 33.33 kilowatt-hours (kWh). This energy content in 1 kg of hydrogen exceeds that of many conventional fuels, making hydrogen a highly efficient and powerful energy source (Hwang & Varma, 2014) . The energy value of different fuel is mentioned in table 1 below. The National Aeronautics and Space Administration (NASA) began utilising liquid hydrogen

as rocket fuel in the 1950s, and NASA was among the first to employ hydrogen fuel cells to power spacecraft electrical systems (Jet Propulsion Laboratory (U.S.), 1975). Hydrogen is used in the United States for refining petroleum, treating metals, generating fertiliser, and processing foods. U.S. oil refineries employ hydrogen to reduce their fuel sulphur level. By combining hydrogen and oxygen atoms, hydrogen fuel cells generate electricity. Hydrogen interacts with oxygen in an electrochemical cell similar to a battery to create electricity, water, and little amounts of heat. Hydrogen has been pre-dominantly used as a basic raw material for technological processes: synthesis of aniline from nitrobenzene, hexa-methylene diamine synthesis, hydro cracking, synthesis gas generation, hydrogenation of coal, ammonia synthesis, methanol synthesis, hydrogenation of fats, oxo processes and major portion of manufactured hydrogen is used in ammonia synthesis. Hydrogen generation and its use in vehicles by the help of large fuel cell is a major focus of fuel cell research and development. Small fuel cells can power laptop computers as well as cell phones, as well as military uses. The application of hydrogen varies, to sum up this section hydrogen is used on its own in the form gas and as a secondary product in form of electricity. Hydrogen is not energy source itself it is an energy carrier, energy from another source is used to generate hydrogen. Hydrogen stores the energy from original source, and it is used to power the fuel cell (Møller et al., 2017).

Table 2-1 Energy content of various fuels in comparison to hydrogen (Astbury, 2008; H2 Tools, 2018)

Fuel types	**Energy contents [MJ/kg]**	
	Lower heating value	**Higher heating value**
Gaseous hydrogen	119.96	141.88
Liquid hydrogen	120.04	141.77
Natural gas	47.13	52.21
Liquified Natural Gas (LNG)	48.62	55.19
Crude oil	42.68	45.53
Liquefied Petroleum Gas (LPG)	46.60	50.14
Coal (wet basis)	22.73	23.96
Bituminous coal (wet basis)	26.12	27.26
Coking coal (wet basis)	28.60	29.86
Methanol	20.09	22.88
Ethanol	26.95	29.84

The Earth's crust is not naturally present with molecular hydrogen. Therefore, it must be separated before it can be used in practical applications. At the moment, the total yearly global hydrogen output is estimated to be over 368 trillion cubic metres (Pandu & Joseph, 2012). Out of the total worldwide hydrogen generation, steam methane reforming accounts for 48%, oil/naphtha reforming from refinery/chemical industrial off-gases accounts for 30%, coal gasification accounts for 18%, water electrolysis accounts for 3.9 percent, and other sources account for 0.1 percent (Baghchehsaraee et al., 2010). According to these estimates, fossil fuels account for 96% of global hydrogen production. These traditional procedures, however, are energy-intensive and not always environmentally beneficial because of extensive amount of carbon emissions. Because of clean properties of hydrogen once it is extracted from fossil fuel and for e.g. used by fuel cell to produce electricity it produces only water (Abánades, 2012).

2.2.1 The Concept of Hydrogen Economy

The major goal of a hydrogen economy is for hydrogen to be created primarily from readily available energy sources, with the goal of replacing present fossil fuels utilised in transportation, industry, residential, and commercial sectors. The hydrogen economy has been proposed as a highly refined and long-term solution to the world's interconnected problems, including (i) global environmental issues, (ii) natural resource depletion, (iii) the world's expanding population (iv) . Despite the obvious benefits, rapid conversion from a fossil fuel to a hydrogen-based energy system has been hampered by severe scientific, technological, and social challenges. The extraordinarily low density of hydrogen makes storage a major issue for transportation (Nejat Veziroglu, 2012, Bockris, 2012). Although refineries and chemical industries commonly employ hydrogen, the cost of generation, storage, and delivery is expensive and not feasible for most energy uses (Prachi R. et al., 2016). However, the enormous benefits of the hydrogen economy are so intriguing that governments from all over the world are spending heavily in improving the energy system's possibilities. The European Commission's High level Group on Hydrogen and Fuel Cell Technologies proposed in 2003 that the European Union achieve a hydrogen-based economy by 2050, and expects that by 2040, 35 percent of newly produced vehicles will be fuelled by zero carbon hydrogen (European Commission, 2003).. The U.S. Department of Energy's Energy Efficiency and Renewable Energy, Fossil Energy, Nuclear Energy, and Science Offices emphasized that the conversion to hydrogen-powered fuel cell vehicles should take place about 2020 (Durbin & Malardier-Jugroot, 2013) Figure 2-1 illustrate the concept of hydrogen economy for future energy generation and supply.

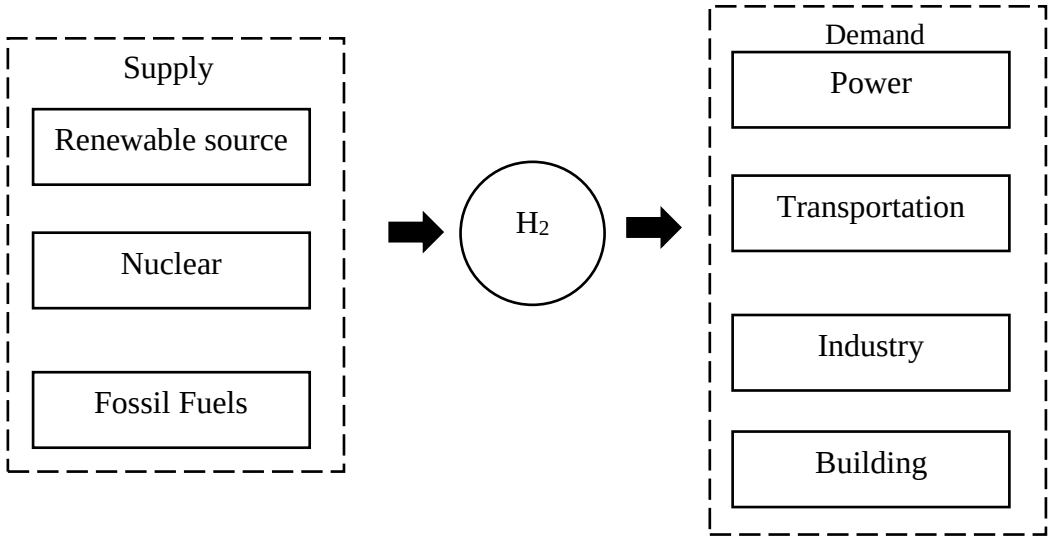


Figure 2-1 Concept of hydrogen economy

2.2.2 Biohydrogen Application

There are several ways to utilise the biohydrogen produced from the waste. The practical application of biohydrogen produced by dark fermentation in an internal combustion engine is not practical yet. Biohydrogen is not comprised of pure hydrogen. It has a hydrogen mixture (under 70 percent) and a CO_2 mixture and may include additional gases, including H_2S, NH_4, CH_4 and humidity. The purification of gases is essential prior to the actual use of hydrogen, and work has been examined on hydrogen separation and purification. Biohydrogen's commercial use is limited due to the difficulties of storing and transporting it. This issue can be overcome by integrating a biohydrogen-producing system with an electric fuel cell system. A study conducted by (Wei et al., 2010) work on the feasibility of combining proton-exchange-membrane fuel cell (PEMFC) for electricity generation . (LIN et al., 2007) found that it is very important to purify the hydrogen produced as a result of dark fermentation for optimum electricity production. (Rahman et al., 2016) found that the amount of hydrogen produced from dark fermentation is feasible to be integrated with fuel cell for on-site electricity production.

The utilization of biohydrogen produced through dark fermentation and explores its potential applications. Biohydrogen, although not pure hydrogen, can be used as an alternative fuel source due to its low carbon content and potential to minimize greenhouse gas emissions.

However, before using biohydrogen, it requires purification because it contains a mixture of gases, including hydrogen, carbon dioxide, methane, and ammonia. The presence of impurities can hinder its practical application in internal combustion engines. Therefore, researchers have been working on hydrogen separation and purification methods to make it suitable for use.

One promising solution to address the challenges of storing and transporting biohydrogen is integrating it with an electric fuel cell system. Several studies, such as the one conducted by Wei et al. (2010), have examined the feasibility of using proton-exchange-membrane fuel cells (PEMFC) for electricity generation from purified biohydrogen. Research by Lin et al. (2007) also emphasizes the importance of hydrogen purification to optimize electricity production using biohydrogen. Rahman et al. (2016) have demonstrated the practicality of integrating dark fermentation-produced hydrogen with fuel cells for on-site electricity generation.

The transportation sector is a significant contributor to global carbon emissions, with 23% of emissions attributed to vehicle fuels derived from fossil fuels. To address this issue, alternative fuels like hydrogen are gaining attention as a promising source to power vehicles, as noted by Brandon and Kurban (2017).

In summary, while biohydrogen has potential as a clean and renewable energy source, it requires purification for practical applications.

2.2.3 Biohydrogen Barriers & Challenges

Biohydrogen production faces the challenges of low yield which is the hindrance for the development on large scale. The full-scale application is not yet built anywhere due to low economic value and poor performance of the process because it is hard to replicate on large scale (Tian et al., 2019). However, researchers are intrigued to explore this technology and its integration with not only serial hydrogen production method but with the deployment of waste stream as free feedstock because the carbon emission from green hydrogen that is produced from renewables and nuclear sources is 43 g CO_2 e /kg hydrogen produced by electrolysis which is 0.46 % of carbon emission produced by steam reforming method (Reaño & Halog, 2020). Hydrogen is an attractive fuel as 1 kg of hydrogen has the same energy equivalent to one gallon of gasoline which produces 9.1 kg CO_2 during combustion. Apart from having high energy content, low carbon emission, carbon neutral property, green hydrogen deployment and its commercialisation is hindered by several challenges. Apart from production challenges that occurs at lab scale (Tian et al., 2019) which are optimised by bioprocess development, other challenges are well understood by considering all the phases involved in the green hydrogen production. The production of biohydrogen faces challenges of low yield, overall efficiency of the reactor, scaling up the reactor while maintaining the same production rates.

Once the hydrogen is produced, it is stored by one of the three methods compression, cooling, and hybrid. It can be stored on site, the challenges lie in the storage is high energy is requires to compress the hydrogen gas. There are safety concerns lies in the potential chemical reaction. In the phases of transportation and end use there is a need for weight, cost and volume minimisation of compressed hydrogen tanks for vehicle and fuel cell stacks. Fluctuation in temperature during fast transfer of compressed hydrogen can cause losses and thermal instability. Hydrogen is highly explosive gas because of high energy content therefore awareness for handling is crucial. It is to bring in notice that instead of overwhelmed challenges green hydrogen has the potential to act as a supportive pillar to decarbonise the energy sector (Full et al., 2021). The challenges in the transportation and storage phase can be overcome by exploring the option to use the hydrogen on site as a backup renewable energy source. The challenges at different stages are highlighted in figure 2-2. Current studies address the challenges occur at the first stage of biohydrogen production and will be the topic of concern throughout the report.

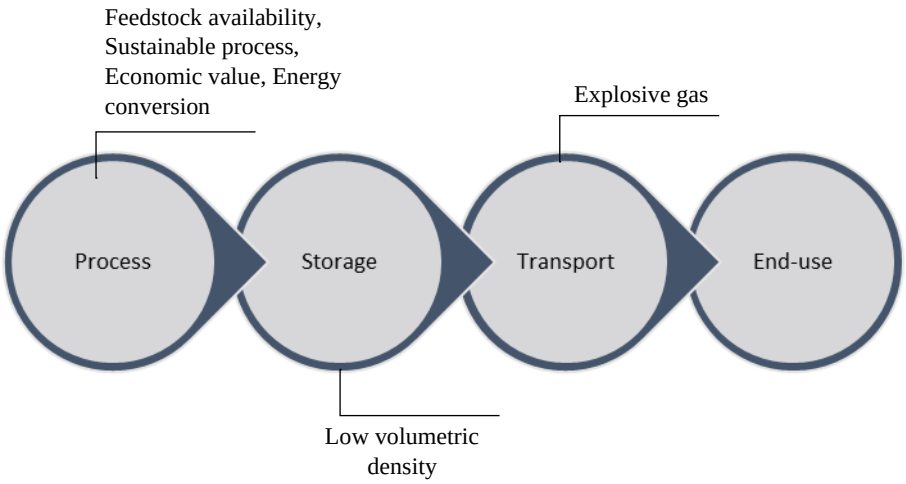


Figure 2-2 Identified challenges at different stages of biohydrogen production

2.2.4 Biological Hydrogen Production

The growing interest in hydrogen as a sustainable energy carrier across the world is tremendously increasing because of hydrogen attractive properties. Biohydrogen can be a potential alternative to fossil fuel and along with existing renewable method like electrolysis which is currently the major source of clean hydrogen (Scott, 2019). Biological hydrogen generation processes are likely to be less energy demanding than thermochemical hydrogen production methods since they operate at ambient temperatures and pressures. As carbon sources, these processes can employ a number of feedstocks. Waste substances can also be used as a carbon source, making waste recycling more efficient. Biophotolysis (direct and indirect), photo-fermentation, dark-fermentation, or combination of these process can create hydrogen (such as integration of dark- and photo-fermentation, or bio-catalyzed electrolysis, etc.). Biological hydrogen has been continually generated on a laboratory scale. Large scale production of biohydrogen is not yet reported (Eljack & Kazi, 2021).

Biohydrogen production reaction is fundamentally dependent upon the presence of a hydrogen producing enzyme. These enzymes facilitate the chemical reaction $2H^+ + 2e^- \leftrightarrow H_2$. A survey of all presently known enzymes capable of hydrogen evolution shows that they contain complex metallo-clusters as active sites. The main hydrogen producing enzymes are; nitrogenase, Fe-hydrogenase and NiFe-hydrogenase. Fe-hydrogenase enzyme is used in the biophotolysis processes whereas photo-fermentation processes utilize nitrogenase (D. H. Kim & Kim, 2011) .

Microbial Electrolysis Cell

The microbial electrolysis cell (MEC) is a new and emerging technology for producing biohydrogen from a diversity of wastewater feed stock. Biohydrogen is generated by microbial electrolysis cell It is also known as electro-fermentation or bio-catalyzed electrolysis cells. The MEC design is composed of two electrodes separated by an ion-exchange membrane in a two-chamber system. The most commonly used membrane is the Proton Exchange Membrane (PEM) to allow the separate entrapment of CO_2 at the anode from the captured H_2 capture at the cathode. Simple sugars like glucose, dark fermentation end product like acetate and fermentation effluent, and wastewater are all employed as substrates in this process. This implies that the MEC is just as capable of bioremediation and clean energy production as other biohydrogen fermentation methods. Therefore, it can be

integrated to other biological methods to utilise the effluent generated by dark or light fermentation (Marone et al., 2017).

MEC is a potential second-stage treatment technique for effluent from dark fermentation (Ding et al., 2016). MEC performance is dependent on factors like separator, substrate, microorganism, operational factors, reactor configurations, anode, and cathode material (Zhao & Ci, 2019). The financial feasibility of MECs using various scenarios for domestic wastewater treatment, the cost of producing 15 L of H_2 per cubic meter of influent per day at £5.09 (Aiken et al., 2019). The MEC technology to generate hydrogen deals with following challenges presented below.

1) Suppression of methanogens activity that leads to low biohydrogen yield (Chae et al., 2010).
2) The mechanisms for electron transfer between a microorganism and the electrode are not yet fully known (Kadier et al., 2019).
3) Cheaper yet operational material is required to be explored for electrodes. The plate price is accountable for 47-85% of the total cost (Rozendal et al., 2008).

Photo fermentation

Photo fermentation takes place in the presence sunlight as energy source, but it limits the biohydrogen production to day-time only. Although the sun is a cheap source of energy, but due to the limitation other sources like tungsten lamps are also used as energy source required to activate the photo-bacteria (Felipe Santos Moreira et al., 2022). However, application of tungsten lamp adds up the cost to overall process. Photo fermentative bacteria are divided in to two groups: Green and Purple. The green bacteria are further subdivided into green sulfur (e.g., *Chlorobium*) and gliding bacteria (e.g., *Chloroflexus*), while the purple bacteria can be further subdivided into purple sulfur (e.g., *Chromatium*) and purple non-sulfur bacteria (*Rhodobacter*). These photo fermentative bacteria have evolved light-harvesting complexes akin to photosynthetic organism. Light energy is converted to chemical energy via photophosphorylation Prominent micro-organism reported in literature for photo-fermentation are Purple non-sulfur photosynthetic bacteria (*Rhodobacter, Rhodopseudomonas, Rhodobium, and Rhodospirillum strains*) which absorb light energy and convert organic acids produced during anaerobic fermentation to Hydrogen and carbon dioxide in a nitrogen-deficient environment (Sağır & Hallenbeck, 2019).

The photo-synthetic bacteria (PSB) are able to utilise simple organic acid as electron donors. Electrons are transferred to nitrogenase by ferredoxin using Adenosine Triphosphate (ATP). These bacteria themselves are not powerful enough to split water. When nitrogen is not present, this nitrogenase enzyme can reduce proton into hydrogen gas again using extra energy in the form of ATP. The overall reaction of hydrogen production is represented in equation 2-1 (Akkerman, 2002).

$$C_6H_{12}O_6 + 6H_2O + \text{'light energy'} \rightarrow 12H_2 + 6CO_2 \ \Delta G_0 = +3.2\text{kJ} \qquad \text{Equation 2-1}$$

Photo fermentative hydrogen production appears promising because of the possibility of achieving hydrogen production from free solar light and organic wastes. However, its application is still far from being practical, low light conversion efficiencies, low hydrogen production rate, and the high-cost photo bioreactors. A lot of work is needed in enhancing hydrogen production rate and light absorption efficiency. It is particularly suited for hydrogen production from particular waste streams containing organic acids, and other metabolite product such as butyric acid ($C_4H_8O_2$), lactic acid ($C_3H_6O_3$), and acetic acid (CH_3COOH). Methanol (CH_3OH), butanol ($C_4H_{10}O$), or acetone (C_3H_6O) as attractive option for extracting additional hydrogen from effluents of dark hydrogen-producing fermentations (Sağır & Hallenbeck, 2019).

In contrast to dark fermentation, photo-fermentation can produce more hydrogen. However, the growth rate of anoxygenic PSB, on the other hand, is significantly slower than that of dark fermentative bacteria. Photo fermentative hydrogen production requires comparatively larger reactor size than dark fermentative hydrogen production (Zhang & Zhang, 2018). Photo-fermentation has relatively poor light-conversion efficiency which led to lower yield when compared to theoretical hydrogen production (Ding et al., 2016).

Biophotolysis

When the light source is used to split the water by the help of certain micro-organism, such process is termed as biophotolysis. Both water and light is plentiful on the planet which is the reason, it has cached the attention of many researchers. The green algae and cyanobacteria play important roles in biophotolysis. Bio-photolysis is divided in to two groups.

Direct biophotolysis

Direct biophotolysis is similar to the algal photosynthesis found in plants. In this process solar energy is directly converted to hydrogen via photosynthetic reactions represented by

equation. During photosynthesis, microalgae like green algae (*Chlamydomonas reinhardtii*) and cyanobacteria (*Synechocystis*) convert water (substrate) into hydrogen (H_2) and oxygen (O_2) in the presence of sunlight and carbon dioxide (CO_2) (Azwar et al., 2014). Hydrogen production is possible by this method under specific condition since Fe-hydrogenase activity is extremely oxygen sensitive. This process has restriction like light requirement, and production of the explosive H_2–O_2 mixture resulting from the process represented in equation 2-2 (Chen et al., 2016).

$$2H_2O + \text{'light energy'} \rightarrow 2H_2 + O_2 \qquad \text{Equation 2-2}$$

Indirect biophotolysis

Indirect biophotolysis involves separation of H_2 and CO_2 evolution reactions into separate stages which are coupled through fixation/evolution (Kossalbayev et al., 2020). The unique characteristic of cyanobacteria of using carbon dioxide in the air as a carbon source and solar energy as an energy source. Indirect biophotolysis comprises of two stages, the first stage involves photosynthesis of cyanobacteria, where CO_2 and H_2O are converted to organic substances and O_2. In the second stage a light-independent reaction occurs where the organic materials from the first stage are further broken down by the cyanobacteria into H_2, CO_2, and other soluble metabolites represented by the equation 2-3 and 2-4 (Huesemann et al., 2010)

$$12H_2O + 6CO_2 + \text{'light energy'} \rightarrow C_6H_{12}O_6 + 6O_2 \qquad \text{Equation 2-3}$$

$$C_6H_{12}O_6 + 12H_2O + \text{'light energy'} \rightarrow 12H_2 + 6CO_2 \qquad \text{Equation 2-4}$$

Figure 2-3 map the efficiency of different hydrogen production process and it can be seen that the process efficiency of hydrogen generation from fossil fuel dominates followed by the electrolysis with higher efficiency. However biological process faces the challenge of low efficiency.

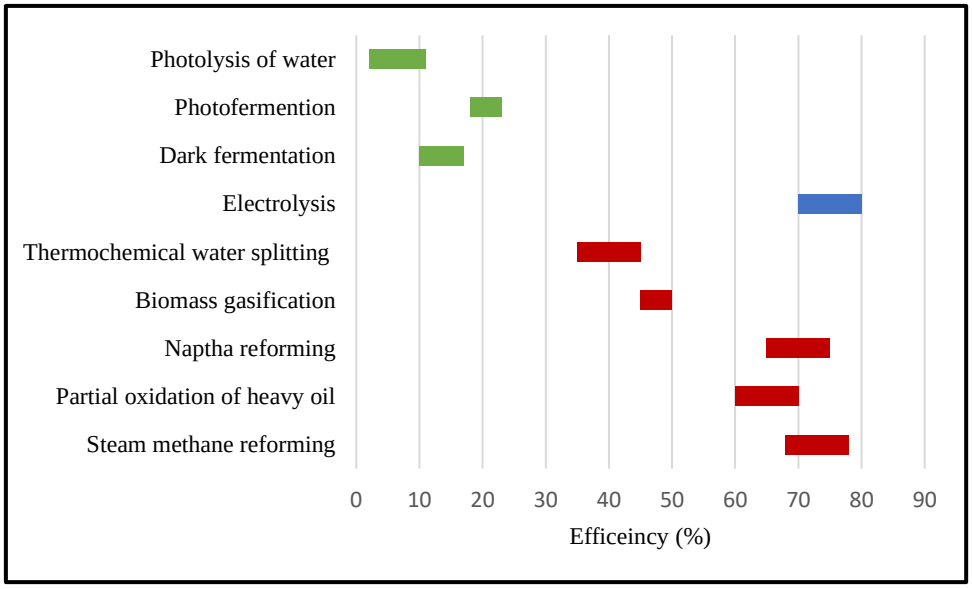


Figure 2-3 Hydrogen process efficiency (Holladay et al., 2009)

Figure 2-4 illustrates various hydrogen production routes based on industrial, renewable, and biological nature. Other than steam methane reforming method hydrogen is also produced from partial oxidation and auto-thermal reformation.

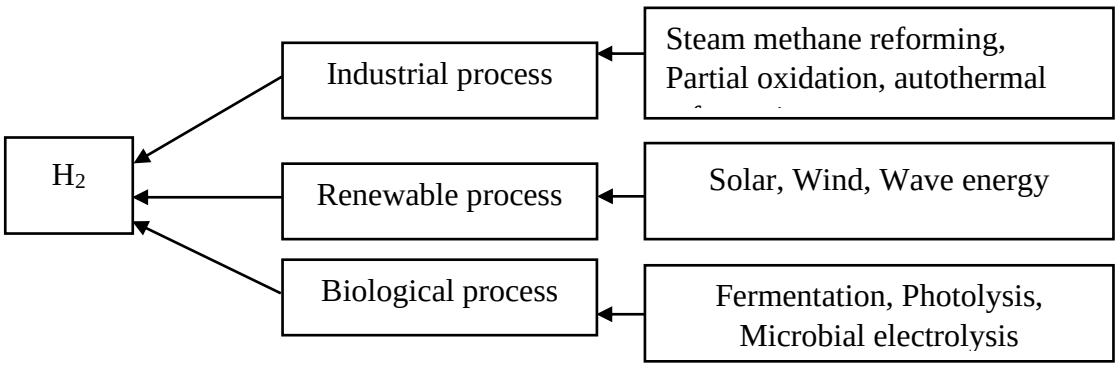


Figure 2-4 Hydrogen generation methods

A significant amount of research is conducted to find alternatives for hydrogen generation technology using renewable and environmentally friendly source of energy. Hydrogen can be produced from a variety of feedstock which unlike fossil fuel is distributed equally around the world. The availability of feedstock is an opportunity to explore the solution to produce

hydrogen from alternative sources. Though the efficiency of biological process is low, these methods are explored due to the utilisation of free and renewable nature of feedstock. Furthermore, once such technologies are developed all the countries will be able to produce their own energy increasing their economic and energy security.

Fossil fuel and the linked carbon emission from the derivation of fuels from them is one major issue on the face of the earth. However human lifestyle and food consumption patterns has led to the increase of problems related to food waste management, food insecurity, malnutrition in poor countries. According to the global estimates of the State of Food Insecurity in the World in 2018, 820 million people were affected by food insecurity worldwide and about 2 billion people experienced moderate or severe food insecurity, including 8% of the population in Northern America and Europe (FAO, 2019) . Today, food security and food waste prevention are continuously stressed due to the scarcity of natural resources, population growth, fluctuating food prices, dietary shifts, climate change, and food loss and waste (FAO, 2011b) (FAO, 2011a).

Disposal of food waste and loss was trivial thing until the end of 20th century and main focus was on food production and variation. But things changed in the 21st century, when escalating demands for processed foods raised which somehow tackled the depletion of natural resources, restrict energy demands, minimize economic costs, as well as reduced waste during production. Not all the waste generated on the processing factories can be re-used in the processed food therefore different methods are explored to tackle such waste to avoid carbon emission. Approach to deal with waste and energy production is to kill two birds with one stone because reduce and re-use food loss and waste results in GHG reduction (Hodges et al., 2011). A report published by (European Commission, 2019a) emphasise on policy making which can focus to make the world climate neutral continent by 2050. It is clear that GHG emission caused by either fossil fuel and food waste practices are trouble-some and harmful to the environment. To understand and identify the co-relation between two major world problem it is important to understand the different between food waste and food loss

2.3 Food Waste

2.3.1 Food Waste & Food Loss

Food waste and loss generation caused major environmental and societal problem across the world. It is disposed to landfills at a cost of money and its affect are harmful to human health. It leaves the air polluted and contaminate the soil quality. Landfills and incineration are the ultimate fate of food waste disposal. Amount of energy is extracted from incineration however it is not preferrable way but landfill has become a traditional anthropogenic source of methane (Adhikari et al., 2006). To date, an estimated 1.3 billion tonnes of global food waste is disposed of in landfills annually. Around the globe, over 30% of food is lost or wasted, which is equivalent to 1.32 billion tonnes of food generated for individual consumption costs the global economy over USD 900 billion (Gustavsson et al., 2011). Food waste in landfill goes through a series of bioconversions into biogas, which is an inflammable mixture of methane and carbon dioxide and trace amount of hydrogen. Biogas can be captured in modern engineered landfill sites and utilised for district heating or electricity generation. In 2007, over 3 Giga tonnes of carbon dioxide were released by food wastage that includes agricultural production, post-harvest handling and storage, processing, distribution, and consumption (FAO, 2011a). Global annual generation of food loss and waste amounts to 4.4 Giga tonnes equivalent of carbon dioxide, which is about 8% of total anthropogenic GHG emissions and only slightly less than that of global road transportation (Intergovernmental Panel on Climate Change, 2014).

It is found that post-consumer food waste was the highest overall loss in affluent economies (Parfitt et al., 2010), which are influenced by factors such as aesthetics and arbitrary sell-by dates. In countries with higher gross domestic product (GDP) per capita nominal such as Switzerland (USD$82,839) and Singapore (USD$64,582), food distribution and consumption accounted for the highest wastage in household food waste (World Bank, 2019). In the United Kingdom, the estimated amount of annual household food waste amounted to 25% by weight. In particular, bread was the greatest contributor to food waste, of which 32% of all bread procured was disposed. It is found that Food waste generation is dependent to income in some industrialized countries. Globally, food loss and waste constitute approximately over 20% of supplied food for individual consumption (Kummu et al., 2012).

Food waste refers to the waste generated during distribution and consumption stage at the end stage. Whereas Food loss is defined as losses in the processing during the preparation and post-harvest processing. There is no fix pattern identified for the waste generation, the food loss in developing countries in higher at post-harvest stages. However the quantity of waste reverse It is observed that food loss in developing countries was much higher at the immediate post-harvest stages than other stages. In contrast, countries with lower GDP per capita nominal, such as the Central African Republic (USD$510) (World Bank, 2019) ,had the highest food loss in the agricultural and post-harvest stages (Parfitt et al., 2010). With the huge amount of food loss, a substantial amount of variations occurred in different stages of the supply chain at which losses take place.

Food waste is categorised into avoidable food waste (edible) and unavoidable food waste (non-edible). The generation of avoidable food waste can be reduced by performing precautionary measures at each stage from cradle to grave. The reduction of unavoidable food waste can only be achieved with proper waste management and recycling strategies (Dahiya et al., 2018). Food waste mainly consists of organic fractions, carbohydrates, proteins, lipids and inorganic components. Food waste can be converted into bio-commodity chemicals and bioenergy by applying various chemical and biological processes (Dahiya et al., 2015). Plenty of scientific research studies on food waste valorisation are emerging, which prove the technological feasibility of converting food waste into a diverse range of value-added chemicals and biofuels. For example, hydroxyl methyl furfural (HMF), which is one of the top building blocks and important precursors for the manufacturing of various derivatives, can be synthesized via catalytic thermal conversion from starchy, cellulosic and sugary food waste. Production of other chemicals with high commercial values such as glucose (Yu et al., 2016) and levulinic acid (Chen et al., 2017) are also proved viable through valorisation of source-separated food waste. Reaction kinetics and operating conditions would act as significant research prospects for technological advances (Yu & Tsang, 2017).

Researchers are highly interested in producing biogas with food waste as a substrate in anaerobic digestion. Recent research studies on food waste valorisation generate products such as liquid biofuels, commodity chemicals, biohydrogen, and bioelectricity (Pham et al., 2015). Electroactive bacteria can also be generated by food waste with an abundant source of electrons, which can generate bioelectricity from waste treatment. In order to enhance production of biogas and biochemicals from the hydrolysis of food waste, different pre-treatment approaches including physical, chemical, physio-chemical and enzymatic

approaches can be adopted, and integrated into the food waste collection and recycling systems. In order to actualise the benefits in implementing circular bioeconomy, the synergies and conflicts of the existing national policies should be evaluated and discussed (Sen et al., 2016).

Nearly 54% of food loss and waste occurs at upstream process which includes production and post-harvesting. 46% accounts for downstream process which includes distribution, processing, and consumption (Food and Agriculture Organization, 2013) which has a good potential to be utilised for valuable materials, bioenergy and biofuel (Gustavsson et al., 2014). Defined food waste by using the resource flows of the agri-food system. Food waste is defined as "any food, and inedible parts of food, removed from (lost to or diverted from) the food supply chain to be recovered or disposed (including composted, crops ploughed in/not harvested, anaerobic digestion, bio-energy production, co-generation, incineration, disposal to sewer, landfill or discarded to sea)." Any food being produced for human consumption, but which leaves the food supply chain, is considered FW while organic materials produced for the non-food production chain are not considered FW. Therefore the definitions of food loss and food waste overlap. These terms are used in literature for material discharged at both the manufacturing and retail stages and the consumption or household levels, highlighting the need for commonly agreed and improved definitions (Williams et al., 2015), For the convenience food waste is the term used in the thesis to address food waste and loss as shown in figure 2-5. Developing countries have relatively high food loss 30% as compared to developed countries 21%. On the other hand, developed countries have higher portion of food waste 35% compared to developing countries 14%. This difference could be because of the decision made by managerial bodies, priority, standards of quality and production criteria (Ishangulyyev et al., 2019).

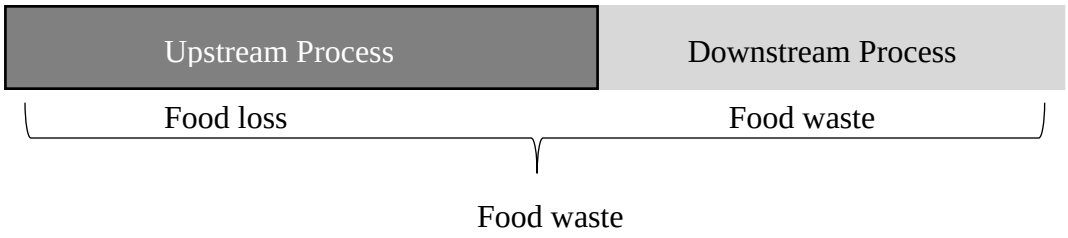


Figure 2-5 Food waste v/s Food loss

Table 2-2 lists few examples of recovered material from food waste. Food waste recovery is among the most researched area in the food industry. The development of sustainable utilisation of waste is one of the main challenges for society and discovery of such solution is only viable if they can extract the valuable material from the waste to achieve social, economic, political, and environmental benefits. The feasibility of the process is examined by the efficiency the input and the output gained, carbon emission, reduction in organic matter and other considerable parameters depending on the selection of process (Cecilia et al., 2019).

Table 2-2 Food waste type and potential recovery from added process (Cecilia et al., 2019).

Waste Material	**Products**	**Process**
Fruit waste	Fertiliser, fodder	Pre-treatment
Vegetable waste	Biopolymer, food additive, bioactive compound	Extraction
Brewery waste	Biogas	Anaerobic digestion
Kitchen waste	Biogas, dye	Anaerobic digestion
Sewage sludge	Biogas	Anaerobic digestion
Industrial wastewater	Biogas, fertiliser	Anaerobic digestion
Organic waste	Biogas	Anaerobic digestion
Oil mil waste	Biogas, Hydrogen	Anaerobic digestion, fermentation

2.3.2 Food Waste Impact

Today, food security is continuously stressed due to the scarcity of natural resources, population growth, fluctuating food prices, dietary shifts, climate change, and food loss and waste. Food is responsible for 26% of global greenhouse gas emission (Dahiya et al., 2018). The food waste is generated at different stages such as harvesting, transportation, storage, and processing. Food waste has important implications for food safety, security of nutrition, food quality and safety, natural resources, and protection of the environment. It has consequences for the sustainability of food systems and economic growth (Sen et al., 2016). These factors have grabbed the attention of food scientists and the food business to food loss, food waste, coproducts, and by-product management throughout the previous decades. Food waste management is another dilemma the world encounters and its management addresses two sustainable development goals: zero hunger and ensure sustainable consumption and production patterns. As quoted by United Nation food, energy and water is ‘nexuses to sustainable development goals (United Nation, 2019). Along with United Nation other leading organisation emphasize on the importance of recovery of maximum nutrients before the waste and loss is discarded and use the most of it to generate valuable by product.

To understand the potential targeted sector of different renewable hydrogen production methods and how such methods can be implemented to curb carbon emission by providing sustainable energy solutions. A reference study is analysed based on the amount of carbon emission which comes from utilisation of renewable and non-renewable sources in different economical sector. In 2019, around 6,558 million metric tons of CO_2 equivalent was produced in result of human activities. Transportation is accounted for the largest share of greenhouse gas emission which accounted for 29% in 2019, which means majority transportation ran on petroleum-based fuel either gasoline or diesel. Electricity production holds the second number with a share of 25%, which indicates that it must have produced from coal and natural gas. 23% emission were produced by industry that could be from burning fossil fuel for energy, chemical reactions, material used to produce good, & disposal of waste. The other noticeable sector includes commercial & residential and agriculture which is responsible for 13% and 10% greenhouse gas emissions. Represent the future renewable energy generation pathways with the addition of biological methods

(Environmental Protection Agency, 2019). The first two sectors with the highest GHG emissions are already targeted by other renewable energy generation projects like solar, wind, hydro power and so on. However more advancement, policies and diverse methods are required to produce renewable energy by the help of unwanted products which produce as a result of normal operation in other sectors like food industries.

2.3.3 Circular Bioeconomy in Food Sector

A bioeconomy utilizes the potential of bioscience and biotechnology to tackle various challenges by offering food, feed, wood-products, furniture, paper, bio-based textiles, bio-chemicals, bioplastics, bio-pharmaceuticals, and bio-energy to meet the needs of a growing population, all while safeguarding our natural resources (*Sustainable and Circular Bioeconomy for Food Systems Transformation | Food and Agriculture Organization of the United Nations*, 2020). The present environmental consequences of food production are unsustainable, with around one-third of all food lost or squandered. Therefore, working on integrated solutions to convert the old linear 'produce-use-dispose' strategy into a circular bioeconomy is a need of the hour. A circular Bioeconomy implies re-incorporating manufacturing by-products and residues as secondary raw materials, i.e., finding new uses for waste products. Circular economy is founded on the core concept of "closing the loop," which entails collecting trash from various processes, recycling it, and reusing it to generate new products (Lieder et al., 2017). This allows to enhance sustainability by establishing alternate sourcing options and reducing agricultural, transportation, and consumer-driven losses, as well as energy use. Investigating the incorporation of novel efficient technologies for the extraction, fractionation, conversion, and purification of heterogeneous waste and by-products into operational value chains. This includes side-stream analysis in selected food value chains, as well as the development of complementary partnerships and technological solutions for disassembly, reassembly, recycling, and logistical strategies to mobilise new scalable value chains from side-stream supply to commercially successful products (Vishwakarma et al., 2022). The essential concepts of both the bio-based and circular economies are intertwined, highlighting the need for practical integrated measures to increase material reuse and recycling. A transition to a circular bioeconomy allows for a more efficient use of biomass's many components in order to generate profitable bio stream production of high-value biochemicals and bioenergy. As a result, this notion could have a good impact on long-term economic development by providing new organisational employment and encouraging environmentally friendly product design with minimum environmental impact. Food waste utilisation is important for the advancement of a circular economy, as the usage of it can boost income, increase food safety and provide energy in the poorest countries of the globe. Food waste has a strong impact on food security, food quality and economic development, as well as on the exploitation of food waste to preserve the

natural environment (Moreau et al., 2017). The use of this approach is predicted to result in a circular bioeconomy that is low-carbon, resource-efficient, and sustainable. The circular bioeconomy aligns with the green chemistry approach, which focuses on the intriguing possibility of using accumulated organic wastes created by various activities as a renewable feedstock for the manufacture of bioenergy such as biofuels. This concept's main considerations are biodegradability, reusability, and recyclability (Ghisellini et al., 2016).

Food waste management is merely not a choice but an obligation because of its continuous negative impact on climate change. Despite of the pacts like Montreal Protocol, UN Framework Convention on Climate Change, the Kyoto Protocol and ongoing the Paris agreement, climate change and its consequences are treated as hoax in both developed countries and developing countries. The earth global condition is aggravated compared to last eight hundred thousand year, because of excessive human activities in past 150 years achieved by burning fossil fuel and deforestation. IPCC predicted that the world will be hit by 1.5^{o}C of warming between 2030 and 2052 if the world will continue to produce greater carbon emissions. There stays a scrutiny of who is responsible to produce more carbon emissions. In today's globalised world, strategies are also interconnected; so, CE packages should be linked with the Paris Agreement on Climate Change and the Sustainable Development Goals to ensure that future systematic methods are established in close collaboration to achieve a better future for all. Renewable energy development is dependent on several factors policies, politics of the country, shareholders, local energy generation plants. The challenges are different and vary with the scale, size and nature of the project (OECD, 2016).

In today's energy environment, diversification of energy sources is essential (Yilanci et al., 2021). The development of renewable, carbon-neutral, alternative, and eco-friendly fuels is critical to meeting the world's growing energy demands. Global energy markets are in transition, with the energy mix changing toward cleaner, lower-carbon fuels as a result of environmental concerns and technical advancements. Although renewable energy's percentage of total energy remains tiny, at approximately 4%, it accounted for over a third of the growth in primary energy last year. "Hydrogen Economy," is a system which promotes Hydrogen as a primary fuel to address some negative consequences of hydrocarbon (Carlozzi et al., 2019). The economy based on H2 is guaranteed to be less polluting than an economy based on fossil fuel. H2-powered cars on the road are currently and hydrogen, which will have great potential in the future, are thought to be very promising fuel for both stationery

and transportation purposes. Bioenergy is considered to have the ability to produce renewable, carbon-neutral energy via sustainable pathways. They provide a method for diversifying energy sources in order to decrease supply concerns while simultaneously promoting domestic rural economies. Because of its renewability, bioenergy generated from microbes is of considerable importance in today's energy environment. Microorganisms have adaptable and diversified metabolic machinery that allows them to produce a wide range of biobased products, including bioenergy/fuels. This transition from the old growth-model (based on fossil-fuels) to a circular economy (CE) target to cut emissions and ultimately promote sustainable agriculture, food production, and bioeconomy (D'Amato et al., 2017). Sustainable bioeconomy can turn residues, food-processing by-products, and food waste into valuable resources and ultimately help reducing food waste by 50% up to 2030 (European Commission, 2019b). However, food business operators need to carefully consider the enablers and barriers of CE principles implementation. Utilization of food waste as feedstock for biofuel production omits the use with the resources (e.g., corn, sugarcane) having food values as well. Food waste can be utilized through a microbial route to obtain biohydrogen (S. Han, 2004), biomethane (Yang et al., 2007), bioethanol , biobutanol (H. Huang et al., 2015) and biodiesel (Pleissner et al., 2013) Furthermore, it can also be used as the starting material for value-added products such as furfural, volatile fatty acids, and citrus derivatives (Hong & Haiyun, 2010). Circular bioeconomy is economic policy initiated to add value to the processes by defining the use of waste generated at different stage of manufacturing a product.

2.4 Biohydrogen feedstock assessment

Fossil fuels hold an important place for hydrogen production; currently, 88% of hydrogen is generated using fossil fuels, with 40%, 30%, and 18% from natural gas, oil, and coal, respectively. 4% is generated by electrolysis, and only 1% comes from biomass by using gasification, pyrolysis, and combustion—none through biological ways yet (Hydrogen, 2023). Although fossil fuel usage still holds a strong place to produce hydrogen, it results in greater carbon emission as fossil fuel is non-renewable. Carbon capture storage technology is developed to store the carbon dioxide released in the process. This is not widely implemented at commercial scale because of impurities and low concentration (Salvi & Jindal, 2019). Electrolysis is costly, in the areas where electricity rate is high, but it is feasible to make hydrogen where electricity is available at cheaper rate. Table 2-3 highlights the production cost per kg of hydrogen by using conventional and biological methods. The price is estimated in the case of dark and photo-fermentation based on the lab scale experiments (Khan et al., 2018b). Biomass gasification for hydrogen production uses renewable source but this process is highly energy intensive and categorised as thermo-chemical and not included in biochemical production method. The main cost involved in the current methods to produce hydrogen is dependent on the supply and price of raw material (coal, oil, and gas) and feedstock (waste, processing waste, dairy waste, cellulose waste) for biological hydrogen, power supply, transportation, and storage. Currently fossil fuel can generate the major portion of hydrogen to meet the global demand, but its usage is not environmentally friendly without carbon capture storage (Dowaki et al., 2007). The supply of raw material plays a role in the production process, the price are surge greatly in unforeseen circumstances (COVID-19) (Global Data Energy, 2022), due to delay and insufficient supply which is one factor to ponder on and another reason for transition towards biohydrogen production to maintain energy security.

Table 2-3 Hydrogen production cost via different methods (Yukesh Kannah et al., 2021)

Hydrogen production process	**Production cost (£)**
Photo biolysis	1.43–1.77 £/kg H_2
Dark fermentation	0.79–2.49 £/kg H_2
Gasification	0.72–2.20 £/kg H_2
Pyrolysis	1.14–2.00 £/kg H_2
Steam reforming	0.97–2.72 £/kg H_2
Waster electrolysis	2.34–3.51 £/kg H_2

Not only raw material, but the cost is also highly affected by the process used to produce the energy for example the cost increased from £ 2.33, £5.6-5.99 and £7.08 through photoelectron chemical, electrolysis and photovoltaic cells using solar as source/raw material (Wang et al., 2010; Hwang, 2013; Bhandari et al., 2014; Acar et al., 2015).

Different types of waste rich in organic matter are generated around the globe which is the potential feedstock and studies using all the biological methods are tested to explore the biohydrogen production to lower the environmental impact by re-routing that waste from landfill and incineration (Brentner et al., 2010). The transition towards bioenergy could be intentional or because of the super imposed environmental legislation like landfill is discouraged because of environmental pollution and to avoid the practice The European council directive on the landfill of wastes 1999/31/EC imposed the rates per ton required to landfill and the rates of landfill have increased in the past years which has lowered the amount of waste which goes to landfill (Directive 1999/31/EC, 1999). Organic waste is rich in protein, carbohydrates lipids which can be broken down into hydrogen methane, carbon dioxide and soluble metabolites by the help of micro-organism which breaks complex organic matter into soluble matter. Utilising the waste is another approach to promote renewable generation of energy thus such waste is considered as ideal feedstock for the fermentative biohydrogen production through dark fermentation, and it is a well-studied substrate in the literature. Biohydrogen is reported to be produced by both solid and liquid wate in form of agricultural waste, municipal waste, dairy wastewater and mixed waste ((Nath & Das, 2003; Valdezvazquez et al., 2005; Sabaratnam, et al., 2009; Arantes et al., 2017).

In United Kingdom Hydrogen Strategy adopts a comprehensive perspective in building a robust hydrogen sector within the country. It outlines the necessary steps to facilitate the production, distribution, storage, and utilization of hydrogen, aiming to create economic

opportunities across various industrial regions in the UK. With well-defined objectives and guiding principles, the strategy offers a roadmap for the gradual expansion and growth of the hydrogen economy over the next decade. Balancing immediate action with long-term vision, the strategy aims to stimulate innovation and attract investments essential to achieving the UK's ambitious hydrogen goals. Most of the attention is given to integrating conventional process of hydrogen generation with carbon capture storage (HM Government, 2021).

Pure carbohydrate such as glucose, sucrose and starch are widely studied material for biohydrogen production. Nevertheless, this pure carbohydrate is expensive (300-1000/ton) which can increase the cost of biohydrogen production and the process overall. This issue is overcome by the surplus amount of waste generates as food and starch-based waste, cellulosic materials, dairy waste, palm oil mill effluent wastewater and glycerol (Show et al., 2012). The composition of such waste makes it a potential resource to be utilise for biohydrogen production if processed under suitable conditions. Apart from synthetic waste which pure carbohydrate, circulating the sustainable waste (corn stalk, cassava wastewater, wood fibres, starch waste water, dairy waste, carrot pulp and waste water) as a feedstock for biohydrogen production is not only economical but also minimize the environmental pollution issues by extracting biofuels and useful material. The characteristic of dark fermentation is it can process a wide variety of renewable organic wastes which was a remarkable gap in bioenergy generation (Sabaratnam et al., 2009) and it is the value-added process to generate energy from the waste. Apart from positive outlook of dark fermentation for biohydrogen production, low yield is still a challenge to address which is impacted by several factors in the process. One of them is the selection of feedstock and its properties, it is important to choose the feedstock that is widely available at cheaper price to increase efficiency of the process. Biohydrogen is also dependent on the treatment of feedstock. Due to complex nature of organic waste, it is required to pass the waste through different treatment methods so that micro-organisms can effectively react with the hydrolysed material. During the pre-treatment process cell walls and membranes are solubilised which transforms macromolecule to micro molecule for hydrogen producers. Organic waste has also gained popularity because of the amount of energy content present in it. In this section various organic wastes are discussed (Haiza et al., 2013).

2.4.1 Food Waste

Food industry has evolved in the recent years, and it is known as the most innovative industry due to increase in demand of food variation in terms of taste, contents, and composition. Food is the basic need of human and animals and with the time people have developed their taste and due to globalisation food production has expanded its horizon at production level. With the ongoing demand of vegan and gluten free food, food industries have managed to cater the needs from producing different kind of products. With the increase in the food variety, few challenges have added up at both upstream and downstream stages that caters with the waste handling. 20-60% of waste stream is consisted of food waste (Kim et al., 2008). Landfilling and dumping the waste in to ocean has been the most common practice for disposing the waste. Food wastes consist of beans, grains, flour, rice, meat, vegetable, fruits, and fish. It contains carbohydrates (starch, glucose, fructose, xylose, glucose, cellulose, hemicellulose), proteins, lipids, and organic acids. Decomposing such rich material in the landfill may cause severe environmental problem like greenhouse gas emission, global warming, and odour problem. Therefore, solutions other than landfill were explored and implemented (Mohd Yasin et al., 2011). One of the solutions is to use it as animal feed however the study conducted by (Djomo & Blumberga, 2011) suggested that potato peel can be use as animal fodder after hydrogen is extracted from it and found it as a better value added step.

Food waste has been widely used in anaerobic digestion to derive methane gas from it and use it to meet energy demand with the integration of combined heat and power engine to produce gas and electricity. Anaerobic digestion is a process which can produce multiple useful products like methane, hydrogen, carbon dioxide. Depending on the composition of the waste the liquid contains volatile fatty acids. The solid waste can be used as fertiliser in the farming land (Zhang et al., 2007). Food waste containing carbohydrate, lipid, protein , cellulose follow different metabolic pathway for biohydrogen production (Evvyernie et al., 2001; N. Kumar & Das, 2000; Xu et al., 2008). Utilising food waste and food processing waste through a biological route for biohydrogen production can be beneficial to implement because of less energy consumption (Lin et al., 2013). Energy from food waste will not be able to replace the conventional fossil fuel because the quantity of food waste generated is not enough to produce the energy which is enough to meet the requirement, but it is an additional source of energy coming from waste and at the same time it can solve the issue of waste disposal. A project will initiated by BayoTech and IBMS Group next year as a part of

the UK's first renewable hydrogen project which will use biomethane from food waste as a feedstock. It claims to produce 1000 kg of hydrogen per day to power vehicle for zero emission form the food waste the quantity is not defined. The project will setup next year in this way food waste can help powering vehicles and contribute towards the concept of energy mix ("UK Hydrogen Project to Use Food Waste-Derived Biomethane," 2021). Pilot scale study reported using food waste by (Cavinato et al., 2012) showed long term stable performance without parameter variation. Hydrogen production was 66.7 l/kgTVS with 0.72 m^3/ kgTVS of biogas which consisted of 58% CH_4 6.9 H_2 and 36% CO_2. Mostly researched substrate studied for biohydrogen production from food waste consist of kitchen waste from restaurant and canteens consist of discarded cooked food, salad, peelings (Girotto et al., 2015) . Different methods are studied by (Pham et al., 2015) to utilise food waste fermentation, anerobic digestion, dark fermentation, pyrolysis and gasification , and incineration. Incineration is a mature technology to reduce waste volume and supply heating and power for domestic usage however on the account of carbon emission generation because the process is not pollutant free. Among different types production process for biofuel production, dark fermentation is considered to be the most viable approach (Linke, 2006; Zhang et al., 2007; Linder, 2019) reported the energy content of 21.6 MJ/m^3 27.2 MJ/m^3 from potato processing waste and food waste using continuous stirred and batch reactor from CH_4 recovery. Anaerobic digestion of food waste to methane is becoming a popular and being adopted quickly for large scale application (Clarke & Alibardi, 2010).

To produce hydrogen from food waste various pre-treatment methods are employed to enhance the rate of production, not only pre-treatment methods the effect of process parameters is also studied on the hydrogen production. Thermal, alkaline, acidification, ultrasonic, microwave pretreatment are some of the methods studied to increase the production of biohydrogen. Where one pre-treatment method has worked for one substrate has not necessarily worked for different substrate with altered composition which is why experimental work is continued to provide knowledge on process behaviour. The widely adopted pre-treatment method is thermal because of low power consumption and no extra requirement of chemicals. Kitchen wastes is comprised of high amount of carbohydrate, proteins, and starch which acts as a useful substance for the biohydrogen production using dark fermentation. Addition of sludge, sewage waste, enzymes can increase the production of biohydrogen because the 80:20 addition facilitate degradation process (Cappai et al., 2018).

studied the effect of substrate/inoculum ratio and found out 0.14 g performed well with maximum hydrogen production 88.8 L H_2/Kg food waste.

Table 2-4 shows the composition of food waste obtained from kitchen and canteens, (Vavouraki et al., 2013) performed biohydrogen production, in the study solids are comprised of 94.1% volatile solid is the combination of 55 % of total sugar, 25.0% soluble sugar, 4.99% nitrogen, 16.9% protein, 24 % starch, 1.7% soluble starch, all these components were beneficial for biohydrogen production . (He et al., 2012) used canteen waste with 48.25% carbon , 0.76% nitrogen, 35.47% total sugar. (Hyoun et al., 2004) found out 158.4 g/l total COD, 50.3 g/lCOD soluble COD 84.9 gCOD/l total carbohydrate, 4.4 gN/l in food waste. Sewage sludge comprised of 31.9 g/l total COD, 0.14 g/lCOD soluble COD 5.0 gCOD/l total carbohydrate, 2.3 g N/l.

Table 2-4 Composition of food waste

Substrate	Moisture (%)	Total Solid (%)	Ash (%)	Volatile Solid (%)	Reference
Kitchen waste	81.5	18.5%	5.9	94.1	(Vavouraki et al., 2013)
Canteen waste	81.7	18.3	-	87.48	(He et al., 2012)
Food waste, sewage sludge	84.1, 95.0	15.9,5.0	-	15.2,2.5	(Hyoun et al., 2004)

2.4.2 Waste Water

Industrial wastewater is a cheap substrate for valuable biohydrogen production. Industrial waste water from sugar, beverage, chemical, potato and distillery industry have been studies by numerous researchers as a potential substrate for biohydrogen production. Huge amount of wastewater is released from domestic, agriculture and industries around the world (Lucas et al., 2015). The presence of multiple compounds in it is considered as environmental concern and on other hand it is also seen as the opportunity for that compound recovery. It is said that wastewater contains more amount of energy than energy which is required to treat it. The energy present in the waste water ranges from 17.8-28.7 kJ/gm of chemical oxygen demand (Angenent et al., 2004). There is a potential to recover thermal, kinetic, thermal, and kinetic energy from wastewater in the form of hydrogen, methane, metal recovery (Sivagurunathan

et al., 2017). Many technologies are explored for the resource recovery from wastewater So far biological methods have attracted more interest because of less energy requirement. Similar to food waste, waste water is consist of different organic fraction which is categorised by means of a variable elemental composition which is dependent on the type of sector . Wastewater mainly comprise of carbohydrate, lipids and proteins. To enhance the hydrogen production it is required to pre-treat the waste water to balance the carbon-nitrogen ratio (Yu, 2002). (Ramprakash & Muthukumar, 2015) worked on the optimisation of the hydrogen production from rice winery wastewater by changing reactor configuration to 2.1 mol H_2/ mol hexose. Studied rice mill water to produce hydrogen by using pure culture Enterobactor aerogenes RM08 in a batch reactor with operating condition of pH 6.7 temperature 33^oC and organic loading rate (OLR) of 10.2 g starch /L. (Sivagurunathan & Lin, 2016) studied the biohydrogen production from beverage waste water using mixed culture at operating condition of pH 6.3 temperature 37^oC and hydraulic retention time (HRT) in a continuous stirred tank reactor with the result hydrogen production of 1.05 mol/mol substrate. The hydrogen yield from the pure culture is slightly higher than using mixed culture. There could be a lot of reasons for that as hydrogen production is dependent on the operating condition, design of the reactor and process parameters (Mota et al., 2018). Utilizing mixed cultures could enhance the biohydrogen at large scale industrial (Ntaikou et al., 2010). The presence of sugar molecule like glucose, sucrose, maltose is vital for biohydrogen production Therefore, wastewater originating from food processing industries are considered as ideal candidate for biohydrogen production since they are easily biodegradable, contains highly hydrolysable materials like sugars, carbohydrates (Ntaikou et al., 2010; Veeramalini et al., 2019). Biohydrogen production can be a valuable add-on to the wastewaters come from sugar industry which is rich in sugar and carbohydrates can be easily metabolized by the microbes. The sugar rich industrial wastewater was considered to be ultimate substrate for production of biohydrogen (Arimi et al., 2015). The carbohydrate rich wastewater originating from food processing industries have more hydrogen potential than the protein and fat rich wastewaters originating from dairy industry wastewater because of low protein degradation, therefore substrate conversion rate is low. Most of the food processing waste has to be diluted to decrease the OLR so that it can result in better hydrogen production (Cappelletti et al., 2011) utilise cassava waste water and found the increase in biohydrogen production by decreasing the OLR to 2.41 mol H_2/ mol glucose. In pure culture clostridium, Enterobacter, Rhodobacter, Bacillus and Citrobacter has been utilised as micro-organism to treat industrial waste (Rupprecht et al., 2006; Ntaikou et al., 2010; Cappelletti et al., 2011; Ozmihci & Kargi,

2011). The mixed culture requires additional pre-treatment to inhibit methane production and increase the activities of hydrogen producing bacteria (Venkata Mohan et al., 2008). (Stanislaus et al., 2018) found out that on increasing the substrate to inoculum ratio from 2 to 8 the H_2 concentration increased from 23.4% to 69.6%. But on further increasing the substrate to inoculum ratio from 8 to 14 the H_2 concentration decreased to 59.3% by using digested sludge and microalgal biomass.

Process integration is a good approach to achieve maximum COD removal (Khongkliang et al., 2017) achieved the COD removal of 81% by integrating dark fermentation and microbial electrolysis method. Hydrogen production reported to improve by the integration of dark and photo fermentation from synthetic waste water to 15.16 mol/kg $COD_{removed}$ and 13.70 mol/kg $COD_{removed}$ from dairy wastewater. On comparing synthetic and dairy wastewater, a greater COD removal efficiency was reported in synthetic wastewater. The COD removal efficiency of synthetic wastewater in acidogenic were 47.50% and COD removal efficiency of dairy wastewater were 37.18% (Özkan et al., 2012). Even after maximum COD removal, excellent biodegradability, availability of feedstock none of the batch and continuous lab scale experiment are replicated large scale. There are few pilot scale studies been done using molasses (Ren et al., 2006).

2.4.3 Agro-Industrial Residue

Lignocellulosic resource consist of agricultural and forestry waste which is also a studied feedstock for biohydrogen production. Around 220 billion tons of lignocellulosic waste is produced which makes it the most abundant raw material in the world. It consists of three components which are cellulose 40% (a linear glucose polymer), hemicellulose 25% (a heteropolymer consisting of C_5 and C_6 sugars) and lignin 20% (an aromatic macromolecule) (Sun et al., 2015). The high cellulose content can be used as substrate for biohydrogen generation but the complex structure of ligno-cellulosic bioass is bottleneck to efficiently produce biofuel (Akhtar et al., 2016). Its complex structure requires an additional step for treatment which can ease the process to derive energy from it. Substrate like sugarcane baggase, corn stover, corn cobs, corn bran, rice bran, rice husk, sorghum leaves, sorghum stover and wheat straw are studied for biohydrogen production through dark fermentation (Datar et al., 2007; Pattra et al., 2008; Heredia-Olea et al., 2015; Kumar et al., 2017; Fillat et al., 2017). The hydrogen yield from one process is very low reported so , agricultural residue

is feasible for biohydrogen production with integration of process (Fatma et al., 2018) . There are physical, chemical, physiochemical, and biological methods are available for pre-treatment (Park et al., 2015; Loow et al., 2016; Dahadha et al., 2017; Kumar & Sharma, 2017). The physical pre-treatment involves reducing the size of material requires extensive energy 11-27.6 kWh/metric ton and this range increases in case of hard woods which is 85.4-118.5 kWh/ tonn (Rajendran et al., 2018). Cellulose pre-treatment results in the production of toxic compound such as furfural and phenolic compound which inhibit the hydrogen production. Therefore, it is very important to choose the correct method for treatment that balance the energy requirement and the carbohydrate degradation in the waste (Jönsson & Martín, 2016). Until now, a cost-effective and environmentally benign pre-treatment method that can completely delignify biomass is yet to be established. The challenge with ligno-cellulosic material is the conversion of carbohydrate into fermentable sugars, until now, there is a lacking a cost-effective and environmentally friendly way for the pre-treatment method that can completely delignify biomass is yet to be established.

2.4.4 Waste Activated Sludge

Waste activated sludge is a type of liquid and solid mixture with moisture content 95-99.5%, 35-61% protein and 7-1% carbohydrate (Cheng & Logan, 2007). It is formed during the process of biological wastewater treatment leftover after the organic matter is converted into carbon dioxide and microbial biomass. Disposal and treatment of activated sludge is expensive process it takes 60% of the operational cost of the plant (Weemaes & Verstraete, 1998). It adds up to environmental problem because of the presence of poisonous substance and high amount of water content. Currently more attention has been given to anaerobic digestion to utilise it for methane recovery 60-70% of methane volume the residue is used as agricultural compost (Appels et al., 2008). With the presence of rich content of organic matter in the sludge, it has received attention bio-oil and biodiesel.

Waste activated sludge is studied for feasible large-scale implementation of biohydrogen production. Waste activated sludge is preferred inoculum to ferment hydrogen producing bacteria. Natural sources like anaerobic sludge, waste activated sludge, compost, cow dung have been studied for this purpose (Cai et al., 2004; Ren et al., 2008; Assawamongkholsiri et al., 2013). The waste activated sludge is used to study for hydrogen producing micro-organism because while using it as a substrate the biohydrogen production was very low due

to low biodegradability. The main components of activated sludge are 1) microbial biomass which is a measure of the mass of the living component of soil organic matter 2) biodegradable organics which is enclosed in the microbial cell membranes. Therefor it is extremely important to disrupt the cell membrane so that biodegradable matter is accessible which supports hydrogen production (Li & Noike, 1992). When the composition of sludge is compared to food waste and industrial waste water it is rich in protein matter as compared to carbohydrate 20:80 (carbon: protein) (Yin & Wang, 2015). Due to this characteristic, waste activated sludge must undergo hydrolysis processes do that organic in the waste sludge can be degraded for better hydrogen production. In the hydrolysis and disintegration process the large weight molecule such as protein and carbohydrate are degraded in to small weight molecule such as amino acid and glucose (Hu et al., 2016).

2.4.5 Potato Waste

Potato is the most commonly consumed crop throughout the world, and it is known as the "king of vegetable", because of its consumption it is grown all over the world. Potato tubers are rich in vitamin C, niacin and vitamin B6, and they provide high value of food per unit area. The potato-based products like chips, mased potato, wedges, and hash browns are not only consumed at domestic level but they are popular among local restaurants, takeaways, and international food chains (Mohammadi et al., 2008). High demand of potato products adds up to more potato waste generation at different levels in the industry during the manufacturing of those products. Approximately 0.16 tonnes of solid waste per tonne of treated potato is obtained. Such waste comprises potato wastewater, pulp and peel generated for food purposes during the industrial processing of potatoes. Under poor management practices, such waste offers environmental threats, e.g. a danger of microbiological or soil contamination) (Pathak et al., 2018). Potato waste characteristics, quantity and its conventional recovery possibilities are presented in table 2-5. Biohydrogen from potato is not only justified because of the amount of it generates but the research has also concluded that carbohydrate rich feedstock like potato could be a potential feedstock for biohydrogen production (Okamoto et al., 2000).

Table 2-5 Types of potato waste and the value added products

Waste characterisation	**Quantity**	**Standardized value**	**Without recovery**	**Value added products**
Potato wastewater	1 tonne = 0.7 m^3 waste	-	Drench into fields	Biogas and yeast
Potato pulp	100 kg = 42–54 kg wet pulp waste	1 kg = 420 gm	Animal feed	Chips and fries
Potato peel	1 tonne chips = 50 kg of peel	1 kg = 50 gm	Composting	Lactic acid formation

In the study conducted by (Wu, 2016), 0.25 g of lactic acid per g potato peel was achieved. They used leftovers from potato peel fermentation for production of biogas as the raw material with a dry matter concentration of 6.4 to 7.9% anoxic fermentation resulted, around 60-70% methane was produced within 8 to 10 days of the procedure. In the instance of raw material with a dry matter content of 9.1 percent, lactic acid waste fermentation has been

extended to 14 days to yield 65 percent methane. The investigation showed that greater levels of dry matter in the raw material had hindered methanogenesis. Potato pulp , when subjected to enzymatic processing its condensed version is used to produce chips and fries. Different types of potato waste are studied to produce value added product prior to dumping the waste in the land and incineration to promote circular waste hierarchy. Where potato waste is considered to produce various products like yeast, phenol, propionic acid, lactic acid for bioplastic. It can be an ideal substrate for biohydrogen production due to its composition (80–95% volatile solids and 75–85% moisture). (Panagiotopoulos et al., 2015) conducted research to find the suitability of feedstock for biohydrogen production. They used technical suitability map (TSM) using four parameters: yield potential, sugar mobilization efficiency, fermentability and coproduct yield and value as shown in table 2-6. Sugar beet juice and sweet sorghum juice were found to be excellent raw materials for biological hydrogen production mainly due to their high content of easily fermentable sugars

Table 2-6 Technical suitability analysis parameters

Parameter	**Definition**
Yield potential	Maximum hydrogen yield based on two-step stoichiometric hydrogen fermentation, assuming 80% conversion to hydrogen and 20% to microbial biomass production and other by products refer equation above
Mobilization efficiency	Percentage of all carbohydrates in the raw material that can be converted to fermentable sugars
Fermentability	Ability of pre-treated raw material to improve or inhibit fermentation
Coproduct yield and value	Characterization of both the volume and the value of the coproduct from pre-treatment/hydrolysis

The suitability of the studied raw materials was ranked in the order with respect to the surface area calculated from the rhombic graph plotted by using TSM: sugar beet juice > sweet sorghum juice > potato steam peels > barley straw > miscanthus > sweet sorghum bagasse > carrot press cake > wheat grains > wheat straw > wheat bran. This study facilitates the selection of substrate for biohydrogen production in this study. Although bio-hydrogen produced from renewable sources has the potential to be an alternative production approach, its contribution is currently less than 1%. Large-scale hydrogen generation from biomass or carbohydrates, i.e. hydrogen via fermentation, is unquestionably desirable and preferred, but only on a laboratory or pilot scale. As hydrogen yields are known to be low, it is necessary to

use a low-cost feedstock desirably rejected material (waste) rather than pure carbohydrate-rich raw materials with high economic value that contribute to human dietary requirements, such as starch, maize, or sugars. Waste biomass streams with lower yet degradable carbohydrate contents and supplied at a low or no cost (due to the necessity for treatment before to discharge) are economically the preferred feedstock for bio-hydrogen generation.

Various studies are reported that have used different forms of potato to produce biohydrogen and biomethane. It is because the potato is classified as a carbohydrate rich product. Clostridium and Thermoanaerobacterium are reported in various studies to produce biohydrogen from carbohydrate (Zhang et al., 2003). Re-using the waste can be an opportunity to analyse various carbohydrate as a substrate for biohydrogen production. (Chen et al., 2008) described the production of hydrogen from starch and starch hydrolysates by five pure cultures of different mesophilic Clostridium strains. All five strains produced hydrogen from starch hydrolysates, but only two of them fermented raw starch at highly reduced hydrogen production rates. Hydrogen production from starch is possible by individual microbial species like *Clostridium butyricum* ,C. acetobutylicum , Thermotoga neapolitana and Thermococcus kodakaraensis (Argun et al., 2009). Waste generated from potato is rich in starch and approx. 80% of the dry matter is carbohydrate, which are readily converted anaerobically into hydrogen and ethanol. Moreover, sweet potato contains indigenous bacteria which may aid in the bioconversion of the sweet potato starch into hydrogen and ethanol before it is directed for animal fodder and compost (Djomo & Blumberga, 2011).

In spite of having excellent potential potato waste is not widely studied for biohydrogen production a few studies are reported in the literature related to biohydrogen production from potato waste (Yokoi et al., 2001; Belokopytov et al., 2009; Mars et al., 2010; Ghimire et al., 2015).Few studies have been reported using extreme thermophile. A study conducted by (Mars et al., 2010) used extreme thermophiles Caldicellulosiruptor saccharolyticus and Thermotoga neapolitana used glucose, hydrolysed and untreated potato steam peels as carbon source for bio hydrogen production both strains performed well and resulted in the biohydrogen production of 2.4 to 3.8 mol H_2 mol^{-1} glucose. Yokoi et al., 2001) studied the biohydrogen production from sweet potato starch residue as a carbon source and obtained a biohydrogen yield of 2.7 mol H_2/mol glucose was attained by a mixed culture of C. butyricum and E. aerogenes HO-39.

(Mishra, 2004) used the potato waste water generated from chips factory to produce ethanol by using cultures of A. foetidus MTCC 508 and A. niger ITCC 2012 strains, it reduced the COD index by approximately 60%. Mixed culture resulted in 90% reduction of COD during 60 h of incubation. The chips production requires considerable amount of water during the washing, peeling and blanching of the raw material while cooking. Wastewater generated as a result of these operations is characterized by high organic matter load, which results in their high BOD and COD. After draining the waste from chips production to water channels or rivers, it contributes to their pollution due to the high chemical oxygen demand and high sulphate and ammoniacal nitrogen concentrations. Purification of such waste through conventional processes of active sediment requires considerable energy input, thus leading to the high cost of waste management. The high starch value in that wastewater can be used as a substrate for biohydrogen production. The overall composition of the potato wastewater in shown in table 2-7.

Table 2-7 Composition of potato wastewater from chips factory (Mishra, 2004)

Component	**Quantity (g/L)**
Starch	19.47
Reducing sugars	0.04
Nitrogen	0.46
Chemical Oxygen Demand	8.1

Potato pulp which is another type of waste generates in the potato industry, it consists of both peel and pulp. The composition of the potato pulp is mentioned in the table 2-8. Because of high viscosity and rich composition, it is subjected to dry to reduce the moisture content so that the residual can be used as a feed for cattle's (Djomo & Blumberga, 2011) But due to rich starch content of potato steam peels, and potato pulp it can be profitable to divert the waste through bioprocess routes for the production of biofuels.

Table 2-8 Characterisation of potato pulp (Kurnik et al., 2015)

Component	Per wet weight (% w/w)
Dry matter	13.0
Ash	0.5
Starch	4.9
Cellulose	2.2
Hemicellulose	1.8
Protein	0.5
Nitrogen	0.8
Phosphorus	0.1
Potassium	0.3
Magnesium	0.1
Calcium	3.0
Sulfur	1.0

2.5 Feasibility of Biohydrogen from Potato waste

The annual production of potatoes has risen to almost 400 million tons globally in 2021, which is ten times more than recorded in 1960 (FAO, 2022). Potato when used in the food industries produce by-product which is rich in organic matter that need to be managed to tackle environmental pollution. The use of such waste can contribute towards economic growth and waste utilisation (Wu, 2016) . Different form of wastes generate from potato food industries are valuable and economical to be utilised for the production of value-added substance. The potato waste can be used to generate biopolymers, natural anti-oxidant, food additive and bio-fuel in form of biohydrogen, biomethane and bioethanol (Maroušek et al., 2018) . Several studies have been conducted to test the potential of potato waste for biofuel and then directing the waste to animal fodder or discharge into lands (Mohammadi et al., 2008). Almost 12-20% of the material goes to waste in potato industries, in the form of wastewater, pulp, and potato peel. The potential to utilize the waste for biohydrogen production by dark fermentation can be calculated by estimating the hydrogen production using stoichiometry. According to this, each gram of polysaccharides theoretically produces a maximum of 553 mL of hydrogen, assuming acetate as the sole by-product (Fang et al., 2006). The theoretical potential of hydrogen production from different types of potato waste is calculated in Table 2-9.

Table 2-9 Theoretical potential of hydrogen production from different categories of potato waste

Types of waste generates in the industry	**Peel**	**pulp**	**wastewater**
Starch per kg of waste	78 gm	44 gm	300 gm
For 1 kg of waste **Hydrogen production 1 gm starch = 553 ml H_2**	43134 ml	24332 ml	165900 ml
Conversion to litre	43.1	24.3	165.9

Rich carbohydrate content in the potato has encouraged researchers to study the potential of biohydrogen production by potato waste , (Dong et al., 2009) produced hydrogen by using potato waste using organic fraction of municipal waste at 37^{o}C and obtained yield of 106 ml/g VS. By harvesting clostridium species from the sludge of wastewater (Salem et al., 2018) produced the biohydrogen of 150 ml/g VS using potato wastewater as a substrate.

2.6 Dark Fermentation

General Explanation

Dark fermentation is the most well-researched method for bio-hydrogen generation. A significant number of researchers have opted for this method, leading to numerous publications to date (Lopez-Hidalgo et al., 2022). This process utilizes a wide range of waste materials, such as food waste, agricultural waste, domestic waste, and industrial waste, for hydrogen generation. Dark fermentation is a biological way to produce hydrogen. It also uses organic and inorganic waste feedstocks as well as stable hydrogen-evolving enzymes and requires less energy input for the system compared to other hydrogen production methods, such as electrolysis and thermochemical processes like steam methane reforming, makes it more energy efficient process. Carbohydrate is the preferred carbon source which results in volatile fatty acid (acetic acid and butyric acid formation), alcohols a mixture of gases composed of carbon dioxide, hydrogen. Several microorganisms are engaged in this process, which involves converting various biochemical monosaccharide and polysaccharide substrate into biohydrogen and other products such as carbondioxide, acetic acid and butyric acid (Kapdan & Kargi, 2006).

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

Glucose Acetic acid

$$C_6H_{12}O_6 \rightarrow 2H_2 + CH_3CH_2CH_2OH + 2CO_2$$

Glucose Butyric acid

As demonstrated in figure 2-6, in the first step the in case of complex sugar (oligo-saccharide) (polysaccharide) are converted in to simple sugar (mono-sugar) glucose. In the next step the simple sugar is completed via acidogenesis in to gases, carbon dioxide , hydrogen, volatile fatty acid, alcohols and other metabolite products (Łukajtis et al., 2018).

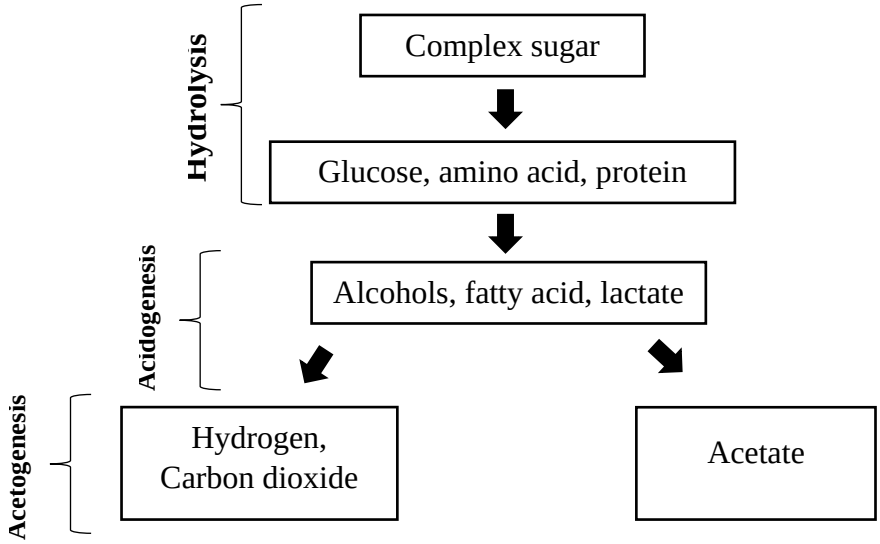


Figure 2-6 Dark fermentation stages

Theoretical Yield

When acetic acid is produced as a by-product in the above metabolic pathway the yield of hydrogen is 4 mole for 1 mole of glucose (544 ml H_2/ g hexose 25^{o}C) and 2 moles of hydrogen for 1 mole of glucose with butyric acid as a by-product (272 ml H_2 / g hexose at 25^{o}C) (Vardar-Schara et al., 2008; Guwy et al., 2011; Singh & Rathore, 2017). Under unfavourable conditions metabolic pathways lead to ethanol and acetic acid formation which results in a lower hydrogen gas production of 2 moles per mole of glucose as shown in equation 2-5

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2H_2 + 2CH_3COOH + CH_3CH_2OH + 2CO_2 \qquad \text{Equation 2-5}$$

The accumulation of acetic acid in the fermentation medium does not correspond to more hydrogen production because some micro-organisms can convert hydrogen and carbon dioxide into acetic acid as shown in equation 2-6.

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \qquad \text{Equation 2-6}$$

The organic acids which are produced during dark fermentation can be utilised as a substrate for photo fermentation. Hydrogen and carbon dioxide are produced as a result of oxidation of these organic acids by the help of photo fermentative bacteria. Ideally 12 mol of hydrogen per mole of dextrose can be produced when dark fermentation and light fermentation is merged together as a hybrid system as shown in the equation 2-7 when acetic acid is used in second stage fermentation to extract hydrogen (Nath & Das, 2009; Singh & Rathore, 2017).

$$CH_3COOH + 4H_2O \rightarrow 8H_2 + 4CO_2 \qquad \text{Equation 2-7}$$

The system is not favourable if the end product of the pathway is lactic acid, ethanol, or propionate then no hydrogen is produced. This is caused when facultative anaerobes undergo anaerobic respiration by consuming nitrate and fumarate as terminal electron acceptors. Hence, consideration should be made while choosing the media so that it does not contain these electron acceptors to promote hydrogen production (Alibardi & Cossu, 2016).

Biochemistry of Dark Fermentation

Fermentation is a metabolic process to obtain energy from molecules. Fermentation helps re-generate ATP which provides energy to derive different processes in a living cell. Dark fermentation is the conversion of organic substrate to biohydrogen catalysed by diverse group of microbes in the absence of light. Biohydrogen is a transient and natural by-product of a variety of metabolic processes catalysed by microorganisms. Dark fermentation is oxygen-free process and conducted in presence of nitrogen to create anaerobic environment. Alcohol and acids dispose of as end product and metabolite due to the absence of oxygen and kerb cycle. Dark fermentation process has the advantages of continuous biohydrogen generation in the absence of light, high biohydrogen production efficiency, and yield (Li & Fang, 2007; Das & Veziroglu, 2008)

Anaerobic pyruvate metabolism is a major process for most microbe-mediated hydrogen generation during substrate catabolism. The mechanism of glycolysis fermentations occurs in which metabolic activities lead to the creation of hydrogen from glucose, is well understood by figure 10. In the initial step of glycolysis, glucose is converted to pyruvate by hydrogen producing bacteria, producing adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and a critical intermediate in the synthesis of nicotinamide adenine dinucleotide and hydrogen in its reduced form (NADH) and flavin adenine dinucleotide in the case of Escherichia coli ($FADH_2$) are formed. Pyruvate is converted to coenzyme A (acetyl-CoA) in

absence of oxygen. In addition to acetyl-CoA , carbon dioxide (CO_2), hydrogen(H_2) via a mechanism mediated by pyruvate ferredoxin oxidoreductase (PFOR) and hydrogenase. Acetyl-CoA subsequently converts to acetyl phosphate with a concomitant generation of ATP and acetate. Oxidation of pyruvate to acetyl-CoA requires a reduction of ferredoxin (Fd). Reduced Fd is oxidized by [FeFe]-hydrogenase and catalyses the formation of H_2. This reaction occurs in clostridia, the overall reaction is shown in the reaction below.

$$\text{Glucose} + \text{ATP} \rightarrow \text{Glucose 6 Phosphate} + \text{ADP}$$

$$\text{PFOR : pyruvate} + \text{CoA} + 2\text{Fd}_{(\text{ox})} \rightarrow \text{acetyl} - \text{CoA} + \text{CO}_2 + 2\text{Fd}_{(\text{red})}$$

$$2\text{H}^+ + \text{Fd(red)} \rightarrow \text{H}_2 + \text{Fd(ox)}$$

where Fd(ox) and Fd(red) are oxidised and reduced ferredoxin.

The pyruvic acid used to produced hydrogen by undergoes another pathways catalysed by the enzyme pyruvate-formate lyase (PFL) as indicated in reaction pathway. In the presence of [NiFe]-hydrogenases or [FeFe]-hydrogenases, formate may be easily converted to hydrogen and carbon dioxide. With the simultaneous oxidation of NADH and/or synthesis of ATP, acetyl coenzyme A can be transformed into numerous organic molecules that are fermentation value-added products (ethanol, butanol, butyric acid, or acetic acid) (Benemann, 1996; Khanna et al., 2011; Balachandar et al., 2013).

$$\text{PFL: pyruvate} + \text{CoA} \rightarrow \text{acetyl} - \text{CoA} + \text{formate}$$

$$\text{formate} \rightarrow \text{CO}_2 + \text{H}_2$$

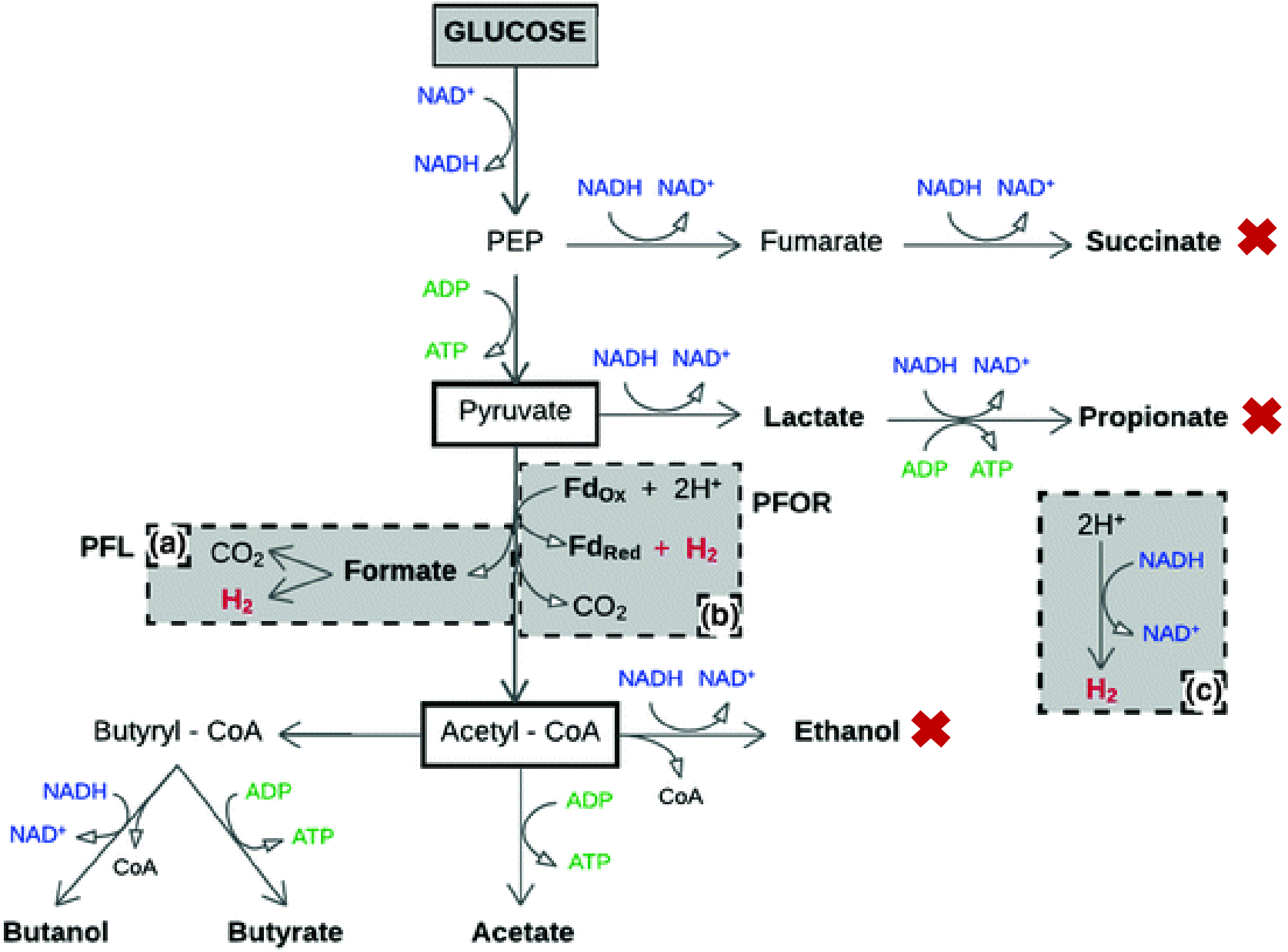


Figure 2-7 Metabolic pathway of dark fermentation adopted from (Toledo-Alarcón et al., 2018) a) Clostridia pathway b) Escherichia coli pathway c) pathway for hydrogen evolution called the NADH pathway, hydrogen is evolved by the re-oxidation of NADH ($H_2 \leftrightarrow 2H+ + 2e-$), red crossed are reactions that diminish the production of H_2

Table 2-10 provides the information about the reaction which contributes to hydrogen production and the product formation which inhibit hydrogen formation.

Table 2-10 Summary of reactions involved in the hydrogen production and inhibition

Comments	**Reactions**
H_2 production	$C_6H_{12}O_6 + 2H_2O \rightarrow 2\ CH_3COOH + 2CO_2 + 4H_2$
	$C_6H_{12}O_6 + H_2O \rightarrow CH_3COCH_3 + 3CO_2 + 4H_2$
	$C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2CO_2 + 2H_2$
No production	$C_6H_{12}O_6 \rightarrow 2\ C_3H_6O_3$
	$C_6H_{12}O_6 \rightarrow C_4H_9OH + 2CO_2 + H_2O$
	$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$
H_2 consumption	$C_6H_{12}O_6 + 2H_2 \rightarrow 2\ C_3H_6O_2 + 2H_2O$
	$C_6H_{12}O_6 + 2CO_2 + 2H_2 \rightarrow 2\ C_4H_6O_4 + 2H_2O$
	$H_2 + CO_2 \rightarrow HCOOH$

Hydrogen production in dark fermentation is mediated by hydrogenase using electron from reduced ferredoxin (F_{red}) and reduced nicotinamide adenine dinucleotide (NADH) to reduce protons to dihydrogen. The maximum hydrogen yield obtained from dark fermentation is 4 moles of hydrogen for 1 mole of glucose. As mentioned above this reaction can only occur when glucose is fully oxidised into acetic acid. This is not possible in practice because of Thauer limit, yield of H_2 is only possible if all the reducing equivalents are converted into hydrogen. The Thauer limit, also known as the thermodynamic minimum for hydrogen production, is a theoretical thermodynamic limit that sets the maximum yield of hydrogen gas during anaerobic fermentation. It is based on the principle of thermodynamic favourability, and it suggests that certain microbial reactions tend to reach equilibrium when the energy released or consumed is minimized. In the context of biohydrogen production, the Thauer limit represents the maximum yield of hydrogen that can be obtained from a given substrate under anaerobic conditions. Hydrogen partial pressure is of great importance to divert the practical condition closer to theoretical values by taking out the gas at regular intervals (Thauer et al., 1977). Overcoming the Thauer limit in biohydrogen production and favouring H2 over other reduction reactions can be challenging due to thermodynamic constraints. The principle suggests that once the system reaches the Thauer limit, further shifting the equilibrium toward hydrogen production becomes energetically unfavourable.

2.6.1 Energy Conversion Efficiency

Several methods are reported to calculate the energy efficiency from dark fermentation to check the feasibility of the process. (Z. Zhang et al., 2017) Reported the way to calculate energy efficiency, the conversion efficiency is calculated for co-production which is calculated by the equation 2-1:

$$E = \frac{V_{H_2} \times Q_{H_2} + V_{CH_4} \times Q_{CH_4}}{Q_{\text{substrate}} \times m} \times 100\% \qquad \textit{Equation 2-8}$$

Where energy conversion efficiency (%), V_{H_2} is H_2 gas volume (ml) , Q_{H_2} is the heat value of hydrogen gas 12.86 J/ml, V_{CH_4} is the heat value of methane 35.82 J/ml , Q_{CH_4} is the heat value of substrate (J/g) , is the dry weight (g).

In case of zero methane production the above equation can be re-written as;

$$E = \frac{V_{H_2} \times Q_{H_2}}{Q_{\text{substrate}} \times m} \times 100\% \qquad \textit{Equation 2-9}$$

The energy conversion calculation is similar to reported by (Kumar & Das, 2000; Das, 2001), this calculation can be done by equation 2-3 when pure carbohydrate source is used.

$$\text{ECE}(\%) = \frac{\text{LHV } H_2 \times H_2 \ yield}{\text{LHV substrate}} \times 100\% \qquad \textit{Equation 2-10}$$

2.6.2 Dark Fermentation Commercialisation

To evaluate the opportunity of dark fermentation to treat the food waste or processing wastewater, It is important to analyse the feasibility of the process if implemented in future on large scale, either integrated with current industrial process or established on its own. Previous studies (Chang & Hsu, 2012 ;Chang et al., 2013) discussed both the scenarios of hybrid renewable energy system (HRES) and commercialisation of biohydrogen from biomass. Their findings suggest that biohydrogen production from waste would increase the value of waste treatment, but fermentative hydrogen production has to be integrated into the process to take most of the material from the effluent of the first stage process. Whereas in the other study the findings were positive to integrate the biohydrogen fermentation process with other renewable process wind, solar and fuel cell to meet the requirement of electric power and thermal energy requirement in remote areas to harness wind and solar energy.

As quoted by A.Einstein "The world as we have created it is a process of our thinking. It cannot be changed without changing our thinking". Other than low conversion efficiency of overall process, commercialisation of biohydrogen faces challenges from the government policies, infrastructure , lack of motivation and awareness for renewable energy. Figure 2-8 shows the stages that will be needed in the commercialisation of hydrogen production. The preferred utilisation methods for biohydrogen are as follows:

1) Separate the hydrogen gas and purify it to 99.99% for reselling
2) Directly resell the low-grade hydrogen i.e. purity <99.99%
3) Generate electricity from pure biohydrogen using fuel cell
4) Blend low purity hydrogen with methane in CHP to produce thermal energy

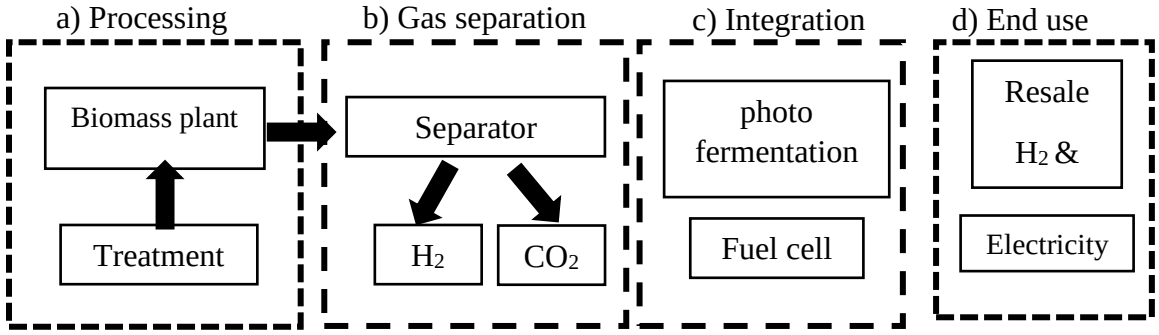


Figure 2-8 Stages in commercialisation

From the pilot study conducted by Han et al.(2016), the cost for each stages can be estimated. While implementing dark fermentation for hydrogen production from waste the cost division is mentioned in the table 2-11.

Table 2-11 Cost distribution of biohydrogen from dark fermentation Han et al.(2016).

Component	**Description**	**Cost (US$)**
Equipment cost	Fermenter, stirrer, centrifuge, pre-treatment plant, inoculum plant, purification system	178,990
Indirect/ direct cost	Installation, land, electrical installation, legal expenses	368,514
Fixed capital investment	Equipment cost + Indirect / direct cost	547,504
Working capital cost	6.5% Fixed capital investment	355,88
Total capital cost	Fixed capital investment + working capital cost	583092
Utilities	Involves the cost of electricity and water to hydrolyse the waste in case of solid waste.	6410
Raw material cost	Feedstock, inoculum, and chemicals	7408.4
Operating labour	Engineers and operators	60,000
Maintenance and repair	1% of FCI	5475
Laboratory charges	8% of operating labour cost	4800
Other costs	5% of total production cost	4204.7
Annual production cost	Raw material cost + utilities + labour + maintenance and repair + laboratory cost + necessary cost	88298.1

This study shows that the highest share of cost in annual production cost is incurred by the operating labour cost which is 68% of the total annual production cost. As mentioned in the previous sections biohydrogen production is dependent on the feedstock nature & availability. This study involved purchasing the waste if the biohydrogen plant is installed in the industry which already generates wastewater it would be more beneficial as it will lower the cost of raw materials (Han et al., 2016).

2.6.3 Integration with Other Renewable Energy Systems

The popularity of hybrid renewable energy system provides another alternative to meet renewable energy requirement. With the discovery of biohydrogen production from food waste, industrial processing wastewater, and biomass there is an opportunity to combine wind, solar and fermentation to generate power (Bajpai & Dash, 2012). Conventionally hybrid wind and solar system are provided backup by diesel engine. The integration of biohydrogen through fermentation, solar and wind system is another area of research and development which can is explored to uncover the potential in hybrid renewable energy system (Zhi et al., 2010).

Reported that by combining biohydrogen fermentation process with renewable energy system wind and solar can provide electricity to external system when the there is no power generation by wind turbine and PV. Another finding in the report also suggested the reduced cost of biohydrogen fermentation process. The system is carbon neutral because the carbon generates during dark fermentation process can be used in food and beverages industries (Chang et al., 2013). The energy calculation for thermal energy and electric power are tabulated in the table 2-12. The production volume figures are taken from (Chang & Hsu, 2012).

Table 2-12 Energy analysis for biohydrogen production through dark fermentation (Chang et al., 2013).

Consideration	Calculations	Values
Annual production of hydrogen	1 m^3 × 18 m^3/(m^3-day) × 365 day = 6570 m^3	591.0 kg
Power generation efficiency of CHP 40% heating efficiency 50%	591 kg x 33.31kWh x 0.4 (electric power) 591 kg x 33.31kWh x 0.5 (thermal power)	7874.0 kWh 9843.0 kWh
Power consumed by 1 kWh of H_2 generation		4.1 kWh
Power consumed by pre-treatment for 1kWh of H_2 generation		2.0 kWh
Overall electric power consumed	(4.1 + 2) kWh x 591 kg (electric power)	3605.1 kWh
Available electric power by dark fermentation process	(7874-3605.1) kWh	4268.9 kWh

It is concluded from this section that biohydrogen production from dark fermentation is a feasible option to investigate. Biohydrogen production is on the research and development stage that is why the opportunity lies in selecting the right feedstock. The different scenarios are discussed to produce the biohydrogen and the most viable is the production of biohydrogen on the site where there is waste production and the integration of biohydrogen from dark fermentation and other renewable energy system. The cost of overall biohydrogen production is dependent on operating cost and the second dominant cost comes from raw material cost which not only includes the feedstock cost but also the cost of chemicals, and inoculum.

2.7 Batch Process

The bioreactor operating modes cover a wide range of characteristics, such as substrate conversion, product concentrations and sustainable and dependable performance, is a major component in implementing the biotechnological process. Various bioreactor configurations are considered to produce biohydrogen such as; Batch, continuous, semi-continuous. Bioreactor sizes are classified as small scale (100-400ml), semi-pilot (2-10 L) and pilot scale(>20 L). The selection of operating mode is directly linked to the test parameters, including volumetric input and outflow, volume of operation and dilution rate. Batch processes are a constant volume fermentation in which all fermentation medium components are initially introduced There have been numerous studies into microbial fermentation engineering involving continuous fermentation (Crooke et al., 1989). The fermenter content is continually supplied and taken out in the continuous manner, whereas the volume is usually maintained throughout the experiments. The detailed kinetics of these processes are described in table 2-13 (Xin et al., 2019).

Table 2-13 Batch and continuous mode of operation

Parameter[a]	**Mode of Operation**	
	Batch	**Continuous**
F_{in}	0	$F_{in}(t) = F_{out}(t)$
F_{out}	0	$F_{out}(t) = F_{in}(t)$
dV/dT	0	0
V	Constant	Constant
D	0	Constant

F_{in} (volumetric inflow); F_{out} (volumetric outflow); dV/dT (working volume variation); V (working volume); D (dilution rate).

The primary goal of the batch experiments is to enhance H_2 production, which is measured via three parameters: mol H_2/mol hexose, mL H_2/g volatile solids (VS), and mL H_2/g chemical oxygen demand (COD). Batch fermentation is commonly used in conventional biological fermentation, and procedure parameter, including temperature, pH, and ventilation, is needed to be controlled (Gong, 2010). In this fermentation process, no feeding broth is added except acid and alkali solution, which are used to adjust the pH values. The production stage is typically carried out in a single bioreactor which is dependent on a number of inoculation stage. The time required for batch bioprocessing ranges from hours to weeks,

depending on the biocatalyst and substrate utilised. Batch-mode reactors are easy and flexible to operate. Batch process have been used to determine the biohydrogen potential from organic and synthetic substrate.

Continuous process is employed after conducting test on batch process for optimum value but, not necessarily the continuous process increases the yield of biohydrogen production copying the similar substrate and process condition. In a study tested hydrogen production in batch and continuous reactor and found out that hydrogen yield was more in a batch process with 2.53 mol H_2 / mol sucrose while in continuous reactor it reduced to 1.77 mol H_2 / mol sucrose at 60^o C and pH 6.25 and 5.5. In this case pH change could have resulted in more hydrogen production (O-Thong et al., 2008; Othong et al., 2008). Most of the studies reported positive result in the hydrogen production whenever the system was replicated at continuous process. A study conducted by (Chen et al., 2008) produced biohydrogen from raw and hydrolysed starch using continuous culture of *C.butyricum* CGS2. The volumetric and specific hydrogen production rate increased with the decrease in HRT from 12 to 2 h. Whereas the hydrogen yield decreased with the decrease in the HRT from 2.03–1.50 mol H_2/mol glucose (i.e., 10.6–7.8 mmol H_2/g COD or 12.5–9.2 mmol H_2/g starch). In continuous system the highest hydrogen production rate was obtained (1.5 l/h/l or 534 ml/g VSS/h).

A lot of studies are reported for biohydrogen production in a batch process using cassava, corn, beet root, oil palm, wheat. soybean, barley, sorghum, and sugarcane (Bundhoo, 2019). However, a very few researches have been conducted using potato in any form as a substrate (Vanginkel et al., 2005; Laurinavichene et al., 2010; Özgür et al., 2010). Table 2-14 highlights the difference in batch and continuous system.

Table 2-14 Batch v/s Continuous system

Parameters	**Batch**	**Continuous**
Growth	Conditions do not remain constant	Conditions are kept constant
Fermentation Setup	Once initiated, outside system conditions remain same	System condition can change even after initiating the setup
System Type	Closed system	Open system
Microbial Growth	Microbial growth is shown in lag, log, and stationary phase	Microbial growth stays in exponential phase at all time
Media & nutrient addition	Media is added once in the beginning. Nutrients are consumed at slower rate.	Fresh media is added at regular intervals. Nutrients are quickly utilised by the micro-organisms.

2.8 Overview of *Clostridium species* for Bio-hydrogen Production

Various substrates have been considered for the production of hydrogen by pure cultures. Among different microbes Clostridium and Enterobacter were most widely used as inoculum for fermentative hydrogen production as shown in table 2-15. The temperature range of 30°C to 40°C has received significant attention in the research on biohydrogen production due to its favourable conditions for the activity of mesophilic microorganisms, however fewer studies are reported which have used thermophilic (high temperature) and psychrophilic (low temperature) range (Hu et al., 2013; Gopalakrishnan et al., 2019). The commonly used substrate is glucose and starch however xylose, glycerol, molasses, and sucrose are also studied for biohydrogen production. *butyricum* strains were frequently chosen and had the highest bio-hydrogen potential (Beckers et al., 2015). Other Clostridium spp., such as *Clostridium beijerinckii* and *Clostridium pasteurianum*, also showed good hydrogen generation capacity. When compared to *Clostridium* spp., *Enterobacter* spp. and *Bacillus* spp. produced less hydrogen. Among the dark fermentative hydrogen producers, *Clostridium* sp. and Enterobacter sp. have attracted more attention due to their high growth rate (Zhang et al., 2011). Clostridia are capable of performing diverse metabolic functions, including the conversion of starch, protein, and purines into organic acids (i.e., acetic, butyric, and caproic acids), alcohols, CO_2, and hydrogen (Zou et al., 2018).

Clostridia are extensively studied micro-organisms for higher hydrogen yields and the opportunity to co-culturing Clostridium spp. with other bacteria is a potential technique for utilising renewable feed sources, which has been widely employed in biotechnology to make bio-fuels and bio-solvents (Du et al., 2020). The anaerobic nature of *Clostridium* species and their availability and extraction from natural habitats make them an attractive choice for studies on biohydrogen production. These properties hold promise for the potential implementation of large-scale biohydrogen production using organic waste as a substrate.. It is also the dominant species existing in microflora of anaerobic hydrogen fermentation processes, especially in the inoculum sludge that can be easily enriched by heat, acid/base, aeration-pre-treatment. The hydrogen producing clostridium species are reportedly extracted by treating the sludge with 3-4 Volts of electric shock. The heat temperature reported in the literature range from 75^oC – 121^oC for 10 min- 2 hours (Wang & Wan, 2008). The parameters for acid/base treatments lies in the range of pH 3/4 (acid) and 10 for base for 24

hours. The mixed culture is incubated in the air for 24 hours for aeration pre-treatment (Wang & Wan, 2008; Xiao & Liu, 2009; Yin et al., 2014a). Treatment like gamma irradiation, ultra-sound, microwave radiation are also studied to isolate hydrogen producers (Yin et al., 2014b; Patel et al., 2015). Clostridium sp. can get energy from carbohydrates through a variety of metabolic pathways that are affected by culture conditions. As a result, controlling culture conditions can boost hydrogen production (Cai et al., 2013). Table 2-15 shows the isolated strain of clostridia sp. from natural sources. Clostridium sp. spore-forming bacteria are the most important industrial microorganisms of choice in anaerobic hydrogen fermentation. They have been utilized to recover carbohydrates such as glucose, starch, xylose, sucrose to hydrogen gas and solvent (Yin & Wang, 2017). The observation of multiple metabolic phases is a distinguishing feature of dark fermentation. Hydrogen and volatile fatty acids (VFA) are produced during the exponential growth phase of clostridia. In normal batch culture, the metabolism shifts to fast solvent generation only during the late development phase (J. Lay, 2003). When acetic or butyric acid is produced as the end metabolite, fermentative bacteria utilize glucose to make hydrogen with a potential yield of 4 or 2 mol H_2/mol glucose. In reality, hydrogen yields are lower than theoretical yields because some carbohydrate is converted to end products other than acetic acid. To date, low yields and production rates have been key impediments to commercialization of biohydrogen production technologies in the laboratory by fermentative bacteria. Improving the level of hydrogen production is required for practical application. The study conducted by (Yin & Wang, 2017) found that *Clostridium butyricum* INET1 strain was able to hydrolyse monosaccharide faster than the polysaccharide and resulted in the better hydrogen production.

The majority of studies are conducted using pure bacteria cultures for fermentative hydrogen production and glucose is used as a model substrate; however, to explore the potential of the process it is preferable to produce hydrogen from organic wastes using pure cultures because it is more feasible for industrialization to achieve the goal of waste reduction. More studies with pure culture and organic wastes are encouraged.

Table 2-15 Psychrophiles, Thermophiles, Mesophiles Micro-organisms

Micro-organism	**Hydrogen producer species**	**References**
Mesophiles		
Clostridium species	*C.butyricum, C.beijerinckii, C.pasteurianum. C.tyrobutyricum*	(Lin et al., 2007; Chmiel & Yargeau, 2008; Hu et al., 2013; Seelert et al., 2015; Wong et al., 2018; Mahato et al., 2020)
Enterobacter species	*E.aerogens, E.asburiae, E.cloacae*	(Tanisho, 1998; Zhang et al., 2011b; Joseph & Arun, 2012; Hu et al., 2013; Zhang et al., 2011b; Pachapur et al., 2017; Abd-Alla et al., 2019; Boshagh et al., 2019)
Bacillus species	*B.firmus, B.amyloliquefaciens, B. tequilensis*	(Kotay & Das, 2007; Das & Veziroglu, 2008; Mazareli et al., 2019)
Thermophiles		
Klebsiella species	*K.Pneumoniae, K. oxytoca*	(Liu & Fang, 2007; Niu et al., 2010 Maintinguer et al., 2011; Yu et al., 2012)
Thermoanaerobacterium species	*T.thermosaccharolyticum W16, T.thermosaccharolyticum PSU-2*	(OTHONG et al., 2008b; Cao et al., 2010; Ren et al., 2010; Kumar et al., 2018)
Psychrophiles		
Polaromonas species	*P. rhizosphaerae*	(Alvarez-Guzmán et al., 2016; Alvarez-Guzmán et al., 2017)
Rahnella species	*R. aquatilis*	(Dębowski et al., 2014)

Table 2-16 Isolated *Clostridium. sp*

Isolated strain	**Source**	**reference**
Clostridium butyricum CWBI 1009	anaerobic sludge	(Calusinska et al., 2015)
Clostridium butyricum W5	sludge	(Wang & Jin, 2009)
Clostridium butyricum EB6	anaerobic digested palm oil mill effluent sludge	(Chong et al., 2009)
Clostridium sp.	waste activated sludge	(Lay et al., 2010)
Clostridium beijerinckii Fanp3	anaerobic sludge	(Pan et al., 2008)
Clostridium beijerinckii	anaerobic sludge	(Zhao et al., 2011)
Clostridium sp.	wastewater sludge	(Wang et al., 2003)
Clostridium sp.	pig manure digester	(Lay, 2003)

2.9 Factors Affecting Dark Fermentation Process

2.9.1Nutrients

Nutrient are the key supporter for microbial growth. Nitrogen and phosphate, metal ions are needed in the fermentation process. The deficiency varies depending on the carbon to nitrogen ratio of each substrate. The availability of nutrients like carbon, nitrogen, vitamins, phosphates and trace elements are essential to maintain the optimum microbial growth. Peptone a nitrogen source from corn starch waste enhances hydrogen production. Hydrogen production is also influenced by micronutrients (Hay et al., 2013) studied the influence of Ni^{2+}, Mg^{2+}, and Fe^{2+}), yeast extract, glutamate, albumin, and molybdenum for enhanced H_2 recovery from effluent of dark fermentation. For the stabilization of the fermentation reaction, maintaining an optimum C/N is important. The optimal range of C/N ratio 13 to 25 was reported. Extreme ratios could develop a negative effect on biohydrogen production (Li & Fang, 2007; Rughoonundun et al., 2012) recorded that high concentrations of metal ions has negative effects on the hydrogen producers.

2.9.2 pH

Acidic pH range (5-6) favours biohydrogen production and very low pH can inhibit biohydrogen production. However, research published by (Mota et al., 2018). observed higher hydrogen production at pH lower than 4 not only this, but the study also observed stable hydrogen yield at pH of 2.7. This study helped to build a case stronger for the feasibility of biohydrogen production because low pH leads to the elimination of using excessive acid or base mediums which adds extra cost and leads to the unfavoured economic requirements. (Lee et al., 2002) reported the optimum pH in the range 4.5-9. The cell structure, metabolic pathway, microbial growth and yields are powerfully concurrent with the pH (Sivagurunathan et al., 2016).

(Khanal, 2003) observed the dependence of the acetate and butyrate levels from different initial pH ranges (4.5–7.5). The low biohydrogen production at pH<4.5 is because of the formation of solvent in the liquid medium (Van Ginkel & Logan, 2005). In the study conducted by (Chen et al., 2015) found the optimum conditions for the small-scale fermentative hydrogen production system were at pH 7.0 using industrial waste. For clostridium species the optimum pH lies in the range of 4-6 (Masset et al., 2012; Sheng et al., 2015) found the optimum pH 7.5 at temperature 55^{o}C for producing hydrogen from

lignocellulosic waste. (Vi et al., 2017) found the optimum pH 6.05 for biohydrogen production from sweet potato starch at starch concentration of 27.63 g/L. (Lay et al., 2012) reported the optimal biohydrogen production at pH 6.7–7.0, sweet potato concentration 150 g COD/l heat-treated sewage sludge seed. The reason for the different behaviour at optimum pH can be due to the selection of feedstock, microbes used, pre-treatment methods and the initial pH of the substrate.

2.9.3 Temperature

Temperature is an important variable parameter in hydrogen production which effect the substrate degradation, microbial growth, and metabolic activity. Many researches have been performed on studying the effect of temperature, there is not a fixed optimum temperature point for increased biohydrogen production. However the optimum range is found to be around 37^{o}C and 55^{o}C (Shin, 2004). A very few studies have been published using extreme thermophiles, (Kongjan & Angelidaki, 2010) studied the biohydrogen production at 70^{o}C although the biohydrogen was produced but the study conducted by (Perera et al., 2010; Lin et al., 2012) suggested that high temperature range is uneconomical and unfavourable for the energy production. Temperature not only affect the hydrogen production it has impact on the metabolic pathways. The relation between temperature and biohydrogen production is not linear increasing the temperature can result in higher hydrogen production but it can result in the inhibition if not kept at optimum level. Temperature has effect on the VFA formation, microbial communities and conversion of substrate. (Zhang et al., 2009) found that temperature also effect hydrolysis process . (İnce et al., 2017) used processing wastewater to produce methane and found the higher COD removal rate for thermophilic temperature range reported temperature regulation during the fermentation process is dependent on the nature of the feedstock for extremely complex substrate like cellulose mesophile holds the hydrogen production and thermophilic operation can be used to produce hydrogen which will also result in the higher hydrolysis rate.

(Foglia et al., 2011) Conducted a study using juice water at thermophilic and mesophilic range, their finding indicate that mesophilic operation was 10 times higher in reducing the capital cost for dark fermentation, but better economic performance was achieved in the thermophilic operation conditions applied is while going for the feedstock which requires pre-treatment. Similar observations were recorded for solid waste (Li & Fang, 2007).

2.9.4 Hydrogen partial pressure

Production of hydrogen and hydrogen partial pressure are inversely proportional. Hydrogen in the reactor is found in two states gas and liquid. High partial pressure of hydrogen in the reactor is the high concentration of liquid inside the reactor. High partial pressure results in the inhibitation of hydrogen production because of the diversion of metabolic pathways to lactate, ethanol, butanol, and acetone formation while compromising on the production of hydrogen (Levin, 2004). High pressure also results as a barrier for the conversion of long chain fatty acid into acetate and hydrogen (Niel et al., 2003).

Biohydrogen is a result of reduction of proton by F_{red} and NADH, at higher H_2 concentration in the liquid phase the reduction becomes thermodynamically unfavoured (Ntaikou et al., 2010). Therefore, it is important to decrease the hydrogen partial pressure during the process which can be done by removing the gas in the headspace at continuous intervals (Kim et al., 2006; S. Chang et al., 2012; Esquivel-Elizondo et al., 2014). On decreasing the partial pressure hydrogen production is found to improve by 13.3 % using clostridium as hydrogen producer micro-organism (Laurent et al., 2012) similar observations were recorded by (Oh et al., 2009). (Ding & Zhao, 2018) observed the change in biohydrogen production by reducing the partial pressure to 20% the hydrogen production increased by 54%. Other than continuous release of gasses in the reactor, hydrogen partial pressure can be lowered by having large head space area (W. Park et al., 2005) and by sparging with the nitrogen and carbon dioxide gas the studies were performed on continuous stirred tank reactor (Kim et al., 2006). It was found by (Nguyen et al., 2010) suggested the ratio of 2:1 for headspace: liquid volume.

2.9.5 VFA production

The short volatile fatty acids like acetic acid, butyric, lactic acid and propionic acids are formed as intermediate by-products during hydrogen production in dark fermentation, However, no hydrogen is produced if lactic acid and propionic acid are produced as intermediates. A series of substrate pre-treatment steps are followed to prevent the influence of lactic acid bacteria and other hydrogen consuming bacteria during the fermentation of hydrogen production. The VFA production is not discouraged in the effluent as in commercial biohydrogen application it can be used in several industrial applications including chemicals the most prominent one is in bioplastics (Sekoai et al., 2018), biofuels

and chemical industry (Dahiya et al., 2015). For optimum biohydrogen production (Kim et al., 2006) found a relation in hydrogen production and ratio of butyric acid to acetic acid when B/A has value of 3:2 it results in H_2 yield of 2.5 mol / mol glucose this relation can be well understood by the reaction:

$$4C_6H_{12}O_6 + 2H_2O \rightarrow 3CH_3CH_2CH_2COOH + 2CH_3COOH + 8CO_2 + 10H_2$$

This relation is tentative and does not apply to all type of process and substrate. Some clostridia species can lower the hydrogen production, certain species of *clostridia* have the capability to reduce hydrogen production by converting homoacetogens, which are microorganisms that produce acetic acid, and this can affect the overall biohydrogen production. Analysis of soluble metabolites produced during the fermentation process can provide valuable insights into the pathways and products involved in biohydrogen production.

2.9.6 Total Solid Content

The state of the matter inside the reactor can be categorised in to wet (<10% TS) , semi dry (10-20% TS) and dry (>20%TS) process (Karthikeyan & Visvanathan, 2013). The challenges in the dry fermentation is the lower hydrogen yield, mixing of substance (Robledo-Narváez et al., 2013). The decision is dependent on the type of feedstock used to produce hydrogen, high amount of water supply is required to operate the dark fermentation on dry process. Total solid (TS) content higher than 19% resulted in the formation of lactic acid during the metabolic shift and introduced a limit to hydrogen production (Motte et al., 2014).

Clostridium species, the main hydrogen-producing bacteria are sensitive to dissolved oxygen concentration that is a potential threat for reactor operational stability. Addition of L-cysteine in fermentation broth can significantly improve the hydrogen generation process and reduce the start-up time when compared with the process lack of this component. Nonetheless, the addition of L-cysteine into the reactor increases the operational cost and therefore, biological deoxygenation by employing facultative anaerobes along with their strict anaerobic counterparts seems to be a preferred way (Guo et al., 2013) . The addition of reducing agents, appropriate culture supplementation as well as the optimization of fermentation conditions e.g. adjusting substrate to inoculum ratio can help to enhance hydrogen production and lead to beneficial changes in hydrogen production.

2.10 Substrate Pre-treatment Methods

Pre-treatment method is dependent on the type of the substrate. The complex structure like lignocellulosic waste, mixed kitchen waste and food waste is reported to go through severe pre-treatment methods which includes the use of acid, heat and ultrasonic method to hydrolyse the substrate. It is important to disturb the structure of cell to enhance hydrogen production (Monlau et al., 2013). The pre-treatment prior to utilisation for biohydrogen production reduce the crystallinity of the cellulose and increase the surface area of the materials to improve the separation of the lignin and hemicellulose fractions. Other than lignocellulosic waste, simple organic waste and industrial waste go through simple pre-treatment process result in a higher proportion of readily available feedstocks for the fermentation process by microorganisms for hydrogen production. In few studies a combination of different pre-treatment is employed based on the feedstock to attain superior results compared to single pre-treatment methods. Just like every other factor the pre-treatment cost adds in the expense of overall process of biohydrogen production. Feedstock can be treated physically, chemically, biologically to maintain the low cost of the process (Turner et al., 2008).

Common pre-treatment approaches for biohydrogen production include i) physical, mechanical, thermal, autoclave ultrasonication ii) biological : enzymes iii) chemical : acid/alkali.

2.10.1 Physical

Thermal

Physical method caters a variety of methods unlike other method some of the aspect of physical pre-treatment can be useful to different feedstock not just for lignocellulosic waste. Blending, milling, chopping comes under the category of mechanical treatment. Thermally treated feedstock is converted into simpler substrate from it complex structure due to the disruption of chemical bonds of the cell wall and membrane. The high temperature application also inhibits methanogens in the feedstock if any present. The temperature range varies from 121 ^{o}C to 175^{o}C, 15 min to 60 min maximum, 50^{o}C to 220^{o}C for large scale application for 20 minutes to 24 hours (Orozco et al., 2012). Thermal treatment also reduces supplementary inoculum dosage by deactivating hydrogen consumers (Parthiba Karthikeyan et al., 2018). A study done by (Kim et al., 2009) shows that by the thermal treatment of 90^{o}C

for 20 min hydrogen yield mainly increased because of the presence of clostridium sp. , thermal treatment favours its growth condition. The effect of thermal treatment is positive because it increases the solubilisation of substrate. Thermal treatment is not efficient for treating cellulosic waste, no change in the decomposition was found. It was observed that the efficiency of the process is positively correlated with the temperature until 140 °C. Pre-treatment after 150 °C–200 °C clearly shows an insignificant improvement in H2 production for kitchen waste (Ma et al., 2011).

Autoclave

It is sterilization technique; it employs the principle of developing steam under pressure in a vessel this process leads to disrupt the microbial structure which occurs at 121^{o}C at 15 psi. When applied to waste such as food waste, the temperature range 121^{o}C to 127^{o}C for a time of 1 hour is required and changes the physical characteristics of the food waste like size and surface area (Hu et al., 2014). Autoclaving is highly energy intense process so the temperature and duration is selected in order to balance the energy consumption and productivity of the process.

Mechanical

Mechanical pre-treatment process is employed to increase the surface area of the feedstock which can be achieved by chopping, milling and grinding. Another technique is ultrasonication (Gadhe et al., 2014) found out 97% in biohydrogen production in the CSTR reactor . (Elbeshbishy et al. 2011) conducted a study using sonication outside and inside the reactor in a continuous stirred tank reactor. Their findings were the hydrogen production increased with a built in sonication reactor.

2.10.2 Chemical

Hydrochloric acid, sulfuric acid , acetic acid , hydrogen peroxide are mainly used oxidising agent treatment leads to increase in the internal surface area and reduction in the degree of polymerisation (Kumar et al., 2009). (Vavouraki et al., 2013) reported the increase in the concentration of soluble sugar to 120% after using HCl. In the alkaline pre-treatment aqueous ammonia , sodium hydroxide , calcium hydroxide are used to enhance biohydrogen production. Alkali pre-treatment also reported to increase the biohydrogen production by increasing the solubilisation rates for carbohydrate, proteins and SCOD. The optimum pH

reported in the literature for alkali pre-treatment is in the range of 11-13 (Zhao et al., 2011) where as for acid it is 2-3 (Elbeshbishy et al. 2011)

2.10.3 Biological

The application of biological pre-treatment is not abundantly reported in the literature. The reason could be this is a slow process and the outcome achieved by biological pre-treatment can be quickly achieved by other pre-treatment process like chemical, physical and mechanical. Biological treatment is commonly used for treating cellulose based feedstock and algal biomass, followed by physical or chemical pre-treatment (Xie et al., 2008a). (Rafieenia et al., 2017) concluded biological treatment technologies are inexpensive compared to other methods however they are time consuming.

The best pre-treatment method has not been identified yet because if one method has worked for one type of substrate not necessarily has worked for other due to the composition of the feedstock. By overviewing the literature different authors have been able to increase the yield. The only challenge lies in the economics of the method. From the mechanical treatment milling, chopping and grinding has worked positively for almost all types of substrates (Sołowski et al., 2019).

2.11 Summary

The literature review covered the impact of fossil fuel and its adverse effect of environment which led to the studies on current renewable energy generation methods and its advantages. Most of the renewable energy methods solar, wind, hydro does contribute to the energy generation but their high reliance on climate condition is under exploration. The use of fossil fuel cannot eradicate completely without investing on multiple renewable energy generation methods. This is the reason energy mix concept is preferred until the complete disappearance of fossil fuels. Right now, researches are emphasised towards hydrogen economy because of the attractive property of this element. Conventional methods to generate hydrogen are energy intensive and damage the air quality. Different biological methods were reviewed to understand the process and their potential to treat the waste. It was also discussed in the literature review that how the use of food waste and loss can be beneficial for both the owner and the policy designers. The re-direction of food waste for energy is an opportunity to get away from landfill and incineration taxes by following circular waste hierarchy which also leads to the achievement of circular bioeconomy to the food sectors.

Different substrate rice, wheat, cassava, palm mill effluent, paper effluent, cheese whey have been studied for biohydrogen production by dark fermentation but there are a very few studies reported from potato waste. This research aims to explore the potential of potato waste for biohydrogen production under different conditions. There are very few researches reported which have addressed the energy conversion efficiency of the overall process. The research aims to address these gaps and come up with the conclusion of how feasible it is to use potato waste for biohydrogen production.

Chapter 3 **-Methodology**

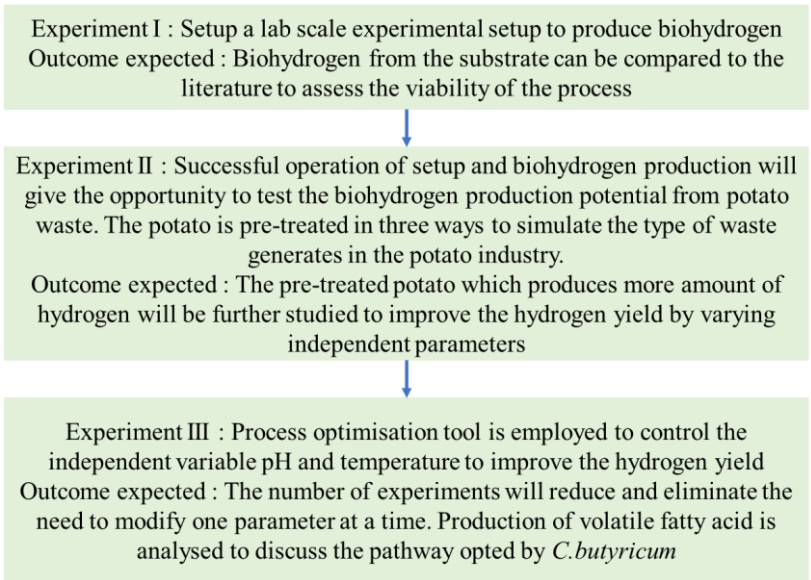
This chapter details the methods employed for biohydrogen production from pure carbon sources and with potential food waste substrate (potato waste) on lab-scale using *Clostridium butyricum*. *C.butyricum* was grown in an anaerobic environment by following appropriate aseptic techniques. The growth stage involves the selection of growth media to provide sufficient nutrient. Biohydrogen production composition is analysed by sensors equipped with thermal conductivity detector and infrared sensors. The hydrogen volumetric analysis is done by water displacement method. The liquid analysis is done to understand the effect of reaction occurred in the reactor on biohydrogen production and inhibition. Modelling is done by fitting the result in the modified gompertz equation to get the values P, R_m and λ which are hydrogen potential (ml), maximum hydrogen production rate (ml/h) and the lag time (h). The goodness of the fit is determined by the R value.

Experimental Plan

Experiment I : Biohydrogen production in a batch reactor with *Clostridium butyricum* using pure carbohydrate source glucose and starch

Experiment II : The effect of pre-treatment method on biohydrogen production from potato waste

Experiment III : Process optimisation using two independent variable pH and temperature for biohydrogen production using potato waste.



3.1 Growth of *Clostridium butyricum*

C. butyricum is a biosafety level 2 pathogen strictly anaerobe endospore forming gram positive microbe. *C. butyricum* is normally found in abundance in nature, it is isolated from wastewater, soil, animal digestive organs and contaminated dairy product. It is heterophobic and strict anaerobe bacteria. The suitable temperature for the activation of the culture lies within the mesophilic range, typically between 30°C to 40°C. Additionally, it can grow in a pH range from neutral to 6.5. To ensure proper growth and development of the microbes, aseptic techniques and practices were strictly followed. The microbes were cultured in an anaerobic chamber, which provided an oxygen-free environment and replaced it with nitrogen to create the preferred optimum conditions for their growth. Essential nutrients were also provided to support their development during the culturing process.

The growth kinetics was determined by observing the turbidity and measuring the optical density at 540 nm by taking out the culture media after 2 hours of interval. Measuring OD_{540} at regular intervals is the most common and easiest way of monitoring bacterial growth. OD_{540} measurements are used to determine the phases in microbial growth in the culture. The stationary value indicates that microbes are in the stationary phase and ready to be used for inoculation with a carbon source in the reactor. Figure 3-1 illustrates the basic principle of light absorbance in the medium with added microbes, the production causes the light to diffract. In contrast, the blank is set as the Reinforced clostridia medium (RCM) purchased from Sigma Aldrich without the microbes addition. The RCM consist of of (g/L) meat extract 10.0; peptones 10.0; yeast extract 3.0; D(+)glucose 5.0; starch 1.0; sodium chloride 5.0; Sodium acetate 3.0; L-Cysteinium chloride 0.5; Agar-agar 0.5. This blank sample served as a control to account for any background or non-microbial-related changes that could occur in the medium during the experiment. By including the blank, we could differentiate the specific effects caused by the microbial activity from other factors that might influence the medium's properties or composition. This approach helped ensure the accuracy and reliability of the results by providing a baseline reference for comparison with the actual experimental samples containing the microbes.

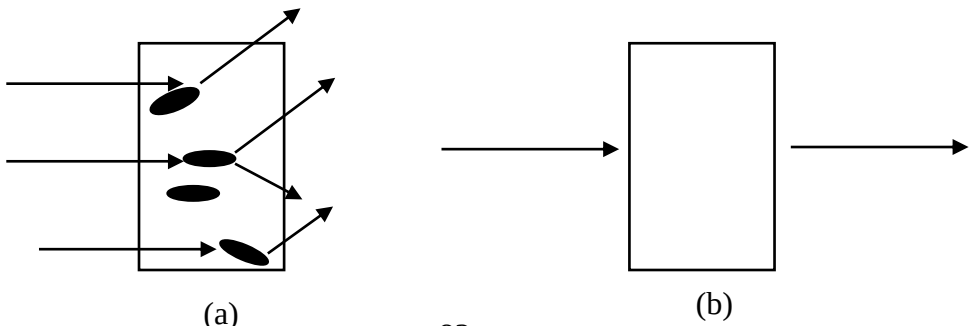


Figure 3-1 Light scattering and absorbing in (a) medium with microbes added (b) clear medium

Freeze dried stock

The process of cultivating fresh Clostridium butyricum involved the use of Reinforced Clostridial Medium (RCM) supplemented with a nutrient solution to support the growth of the microbes for the batch experiments. To ensure the preservation of the microbes for future experimental work, it is crucial to follow proper storage procedures. After the bacterial growth was achieved, 500 µl of the culture was transferred into a cryovial containing 2ml of 30% glycerol. The cryovial was then gently mixed to ensure proper distribution of the glycerol. The glycerol stock tube, containing the mixed culture and glycerol, was immediately frozen at -80°C. This glycerol stock serves as a long-term storage solution, allowing the microbes to remain viable for years.

When it becomes necessary to recover the bacteria from the glycerol stock for further experimentation, the cryovial should be removed from the freezer and defrosted at room temperature. Once thawed, the contents of the cryovial can be suspended in Hungate tube filled with fresh growth medium to revive the microbes and prepare them for use in the experiment. By following these storage and revival procedures, the *Clostridium butyricum* can be preserved effectively for extended periods and can be readily retrieved and utilized when needed in future experimental studies.

3.2 Batch Setup for Biohydrogen Production

Minifors 2 by INFORS is used as a batch reactor/ fermenter in the current research, with a total volume of 2 litre and working volume of 1.5 litre. The fermenter consists of glass vial, pH probe, temperature probe, inlet for nitrogen gas sparging, outlet to analyse the liquid samples and for releasing the gas from headspace passed through condenser to avoid the moisture. The nitrogen flow was controlled through a touch screen display. The fermenter is equipped with two shafted stirrers for agitation powered by the motor. Temperature, pH, nitrogen gas flow, batch time was monitored on a digital display connected to a fermenter. The fermenter is connected with a cooling agent which serves a dual purpose of regulating the temperature of the reactor and maintain the cooling temperature in the condenser. Inoculation was done using a syringe filled with inoculum and injected by using the septum provided on the top of the reactor. The inoculation is performed using aseptic technique to avoid contamination. The fermenter is also supplied with 4 feed bottles and automatic controller on the side to regulate the pH both acidic and alkaline when required to perform experiments at regulated pH. In order to create the dark environment, the glass vessel was wrapped in aluminium foil with the covered edges on the top.

The outlet of the gas is connected to the sensors BCP- H_2 , BCP- CO_2 and BCP- CH_4 purchased from BlueSens, Germany . For the volumetric analysis of produced gas, the gas was released through water displacement method 3% NaOH solution to capture hydrogen gas from the reactor. A thermal conductivity detector (TCD) was installed with the H_2 sensor, the detectors for CO_2 and CH_4 were Infra-red sensors The composition of the gas is determined by the sensors attached to the reactor the composition data was transmitted to monitor by the help of Bluesens (GUI interface) provided with the sensors.

Figure 3-2 represent the schematic diagram of batch setup for biohydrogen production which includes (a)Data acquisition (b) Gas sensors (c) Three-valve controller to release gas (d) Condenser (e) pH (f) Temperature probe (g) Gas sparger (h) sampling port (i) Stirrer (j) Water displacement unit.

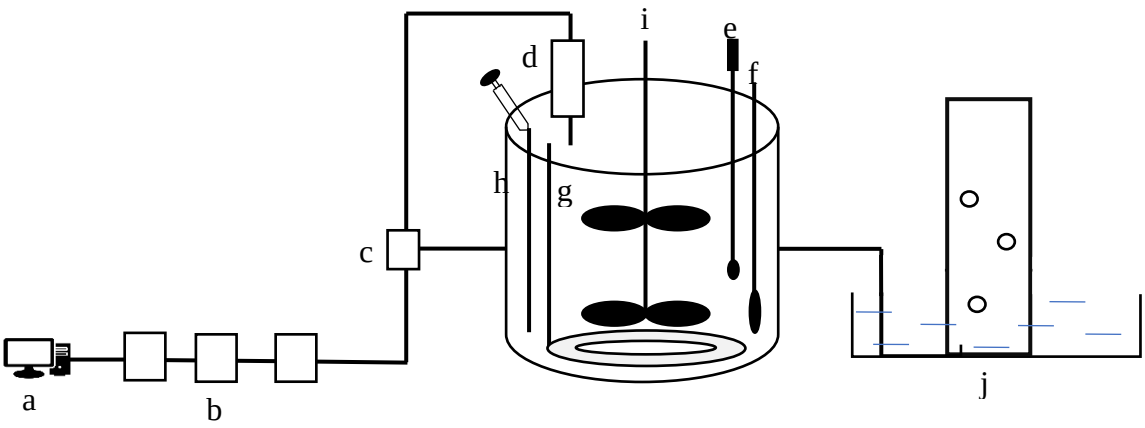


Figure 3-2 Schematic diagram of experimental setup

Figure 3-3 shows the lab setup for biohydrogen production with the sensors in the fume hood, which are shown in figure 3-4. The individual parts in the reactor are numbered and explanation is as follows:

1) Condenser

It is the gas outlet equipped with a condenser to avoid moisture trapping in the gas pipe. The gas can be stored in the sampling bag and pass through the sensor for composition. Volume analysis is done by releasing the gas through the water displacement method.

2) pH Probe

The Hamilton pH electrode was supplied with the Minifors2; the electrically controlled electrode serves pH monitoring and pH regulation when required in the system. To ensure the proper working of the pH probe it was calibrated by the buffer solution at pH 4, 7 and 10.

3) Temperature Probe

The temperature probe was inserted inside the rod chamber connected to the lid of the fermenter. The temperature regulation is made by setting the temperature on the digital display provided in the Minifors2.

4) Air in-flow

The reactor is provided with gaseous input for creating anaerobic environment. The nitrogen gas cylinder was placed near the system and the supply was connected to the bioreactor. The flowrate was controlled by adjusting the amount in the digital display. The nitrogen gas pipe was connected to the air filter and through the sparger.

5) Gas Sparger

The gas sparger was emersed in the liquid medium, once the nitrogen gas supply was turned on the bubbles came out from the sparger assured the supply of gas inside the fermenter.

6) Feed Bottles

The bioreactor is also supplied with the feed bottles, it was used to stock the acidic and alkaline solution to maintain the pH. The feed bottles were connected to the pumps that would automatically activate if the pH is to be maintained in certain experiments. The pH was maintained by pumping either the acidic and basic solution during the fermentation and it was monitored by using the pH probe.

7) Stirrer

To ensure the proper mixing of substrate nutrient and microbes stirring speed was also adjusted and monitored.

8) Sampling port

The sampling port helped to inject out the liquid sample from the reactor during the fermentation. The three valve was attached to the sampling port to avoid contamination after inoculation.

9) Inoculation Septum

The inoculation septum hole was present in the lid of the fermenter. The inoculation was done through injection. The batch time started as soon as the inoculation is done to monitor the total time.

10) Gas outlet

Gas outlet was provided with the condensation unit. The gas output was released in two ways one to bag to do volumetric analysis and to the sensors for composition analysis

11) Cooling unit

The cooling unit Julabo (FL-300) serves two purposes. It maintains the temperature in the condenser and water is circulated in the heating jacket when the temperature-controlled unit is selected.

An autoclave (prior clave serial no 4369) is a separate unit used to sterilise the medium before starting the experiment. It is also used to sterilise the bioreactor before and after every use. After the end of the process the liquid waste was bleached and wasted as hazardous waste.

Figure 3-3 Lab setup for biohydrogen production

Figure 3-4 Gas sensors

3.3 Biohydrogen Yield Calculation

In a batch process biohydrogen production is monitored by continuously releasing the gas, it can be done by collecting the gas into syringe, gas collection bag and release the gas through water displacement setup. The sensor gives the hydrogen composition, and the total volume of the gas is calculated by biogas fraction consisting of hydrogen, carbon dioxide, and methane is daily monitored at 1-day interval using BCP-H_2, BCP-CO_2, and BCP-CH_4 sensors (Bluesens GmbH, Germany) with a measuring range of 0–50%, 0-100%, 0-100% respectively. The volume of biogas was measured with the volume displaced. The cumulative hydrogen volume was determined according to the equation below. Although the sensibility of hydrogen sensor is 0-50% as stated by the manufacturer which is mentioned in terms of purity of the gas. However, when the known composition of gaseous mixture (H_2, Co_2, and CH_4) was passed, it was able to detect hydrogen up to the concentration of 99.99%. This was tested during the calibration of the sensor with standard biogas mixture and by-passing pure hydrogen through the sensor. The cumulative hydrogen production is calculated by equation 3-1.

$$V_{H,i} = V_{H,i-1} + C_{H,i}\left(V_{G,i} - V_{G,i-1}\right) + V_H\left(C_{H,i} - C_{H,i-1}\right) \qquad \text{Equation 3-1}$$

where, $V_{H,i}$ and $V_{H,i-1}$ represent the cumulative hydrogen gas volume at current (i) and previous time interval (i − 1), respectively. $C_{H,i}$ and $C_{H,i-1}$ are the fraction of hydrogen at current (i) and previous (i − 1) time interval. $V_{G,i}$ and $V_{G,i-1}$ are the total biogas at current (G, i) and previous (G, i − 1) time interval, and V_H represents the total volume of headspace in the reactor.

The hydrogen yield is calculated by equation 3-2

$$H_2 \text{ yield } = \frac{\text{Amount of } H_2 \text{ produced } (mol)}{\text{Amount of glucose consumed (mol)}} \qquad \text{Equation 3-2}$$

where the amount of hydrogen produced (mol) was obtained through the cumulative hydrogen production (L) at the end of fermentation divided the molar volume of gas (22.4 L/mol). The initial measured value of substrate (mol) was obtained as the ratio of the initial measured concentration of sugar (g/L) in the feed and the molecular weight of glucose

(180 g/mol). The hydrogen yield was calculated as the ratio of the moles of produced H_2 to the moles of substrate at the start of fermentation.

Substrate conversion efficiency is calculated by equation 3-3, which gives the pattern of glucose consumption rate and the dependent biohydrogen production.

$$\text{Substrate consumption rate } (\%) = \frac{(C_0 - C_t)\left(\frac{g}{L}\right)}{(C_0)\left(\frac{g}{L}\right)} \times 100\% \qquad \text{Equation 3-3}$$

where C_0 is the sugar concentration at the beginning of fermentation; C is the sugar concentration at the end of the fermentation.

There are different ways to calculate the hydrogen yield; when the carbon source used in the experiments is not pure, the hydrogen yield equation can be modified. The final yield is calculated as a function of volatile solid represented in equation 3-4.

$$H_2 \text{ yield } = \frac{\text{Amount of } H_2 \text{ produced } (ml)}{\text{Volatile solid (g)}} \qquad \text{Equation 3-4}$$

3.4 Liquid Sample Analysis

Liquid samples were collected from the bioreactors for the analysis of sugar concentration, chemical oxygen demand (COD), volatile fatty acids (VFAs), total solid (TS) and volatile solid (VS).

3.4.1 Estimation of Carbohydrate by Anthrone Method

For pure carbon source the glucose concentration is determined by the anthrone method. This method is rapid and convenient for the determination of hexoses, aldopentoses and hexuronic acid present in either monosaccharide or polysaccharide by acid hydrolysis. In this method carbohydrates are hydrolysed into simple sugar by diluting it with strong acid, hydrochloric acid. Glucose is dehydrated in to hydroxymethyl furfural, when furfural is mixed with anthrone reagent a green-blue colour solution is formed the standard curve is plotted with known concentration of carbohydrate against the absorbance at 620nm. A UV-Vis spectrophotometer (GENESYS 50) was used to determine the absorbance at 620 nm This method is adopted from (Plummer, 1978), The concentration of unknown sample can be find using equation 3-5.

$$Unknown\ concentration = \frac{Absorbance\ of\ unknown * Concentration\ of\ standard}{Absorbance\ from\ standard} \quad \text{Equation 3-5}$$

The concentration of unknown sample can also be calculated by finding out the intercept of the standard curve. The given value of absorbance can be automatically calculated using excel trend function.

3.4.2 Moisture Content

The method to determine the moisture content present in potato waste was found following standard method (Telliard, 2001). The sample is weight on weighing scale (KERN AES) with the precision of (precision ± 0.1 mg).The moisture content (%) is found by drying the sample in the oven (LTE OP100), at 103 ± 2 °C, for 2 hours. The sample is allowed to cool in the desiccator until it reach the room temperature for further measurements.

The moisture content was determined using equation 3-6.

$$M\ (\%)\ = \frac{W_{sample} - (W_{total} - W_{dish}) * 100}{W_{sample}} \qquad \text{Equation 3-6}$$

M : Moisture content

W_{sample}: weight of sample (mg)

W_{total}: Dry weight of residue and dish (mg)

3.4.3 Total Solid

Following the method mentioned in 3.4.2. The total solid content are determined by equation 3-7.

$$\%TS\ = \frac{(W_{total} - W_{dish}) * 100}{W_{sample} - W_{dish}} \qquad \text{Equation 3-7}$$

TS :Total solid content

W_{sample}: weight of wet sample and dish (mg)

W_{dish}: weight of dish (mg)

W_{total}: Dry weight of residue and dish (mg)

3.4.4 Volatile Solid

Transfer dried residue in the evaporating dish, and heat the furnace to 550°C and ignite the sample for 2 hours in griffin electric furnace (K81). Cool the residue in a desiccator to balance the temperature. Calculate the final weight as $W_{volatile}$. The fixed solids and volatile solids are determined by using equations 3-8 and 3-9.

$$\%Fixed\ solids\ = \frac{(W_{volatile} - W_{dish}) * 100}{W_{total} - W_{dish}}$$ Equation 3-8

FS :Fixed solid content

W_{dish}:Weight of the dish (mg)

W_{total}: Weight of the dried residue and dish (mg)

$W_{volatile}$: Weight of residue and dish after ignition (mg)

$$\%Volatile\ solids\ = \frac{(W_{total} - W_{volatile}) * 100}{W_{total} - W_{dish}}$$ Equation 3-9

VS : Volatile solid content

W_{dish}:Weight of the dish (mg)

W_{total}: Weight of the dried residue and dish (mg)

$W_{volatile}$: Weight of residue and dish after ignition (mg)

3.4.5 Volatile Fatty Acid Quantification

The composition of volatile fatty acid formed during the process of biohydrogen process is very important to estimate the pathway opted by the microbes. Therefore, it is very important to quantify the VFA formation and the pH change during the process. In this study gas chromatography (Agilent Technologies 7890A) equipped with a mass spectrometer detector (Agilent Technologies 5975C) is used to quantify the short-chain volatile fatty acids. Standard VFA solution was bought from Sigma Aldrich. The liquid sample was injected out from the sampling port and stored at 2^{o}C. For analysis the sample was filtered through a cellulose acetate membrane 0.45 µm and acidified using 2% formic acid. VFA analysis was conducted using 30 m × 250 µm × 0.25 µm HP-5MS 5% phenyl Methyl Silox column. The temperature of the GC oven range from 80^{o}C to 200^{o}C ,injection port temperature was set at 250^{o}C. Helium was used as a carrier gas with a flow rate of 1 ml/min.

3.4.6 Chemical Oxygen Demand

Chemical oxygen demand is tested in the later experiments in the fermentation process but not extensively by following a protocol mentioned by (Method 410 . 3 : Chemical Oxygen Demand (Titrimetric , High Level for Saline Waters) by Titration, 1978). The liquid sample was taken out from the reactor and standard method was followed to analyse the chemical oxygen demand of the influent and effluent for each run. The efficiency of the fermentation process was assessed by determining the COD concentrations of the influent and effluent sample then the COD reduction is calculated by equation 3-10.

$$\text{COD reduction} = \left[\frac{COD_{inf} - COD_{eff}}{COD_{inf}}\right] \times 100 \qquad \text{Equation 3-10}$$

where, COD_{inf} = influent chemical oxygen demand (g/L), COD_{eff} = effluent chemical oxygen

demand (g/L).

Chemical oxygen demand gives the information about chemically oxidisable material present in the substrate and it is used to calculate the energy content of the feedstock. The value represent the maximum value present in the feedstock. The chemical composition of the waste determines the hydrogen yield in terms of per unit volatile solid (VS) or chemical oxygen demand (COD) (Alibardi and Cossu, 2016) by knowing the COD of the waste, Hydrogen production can be predicted based on theoretical relation. In the example of ideal fermentation all the COD is removed and is converted in to hydrogen but in real scenario this efficiency is not attained and a part of organic matter is converted in to gas and the rest of it is transformed in to alcohols, metabolites. Given that 1 gm of COD can produce 0.46 lH_2 (Paudel et al., 2015). The relationship between COD value of influent and effluent is illustrated in the figure 3-5. It gives the information of the amount of organic matter transformed into hydrogen during the fermentation, it can be seen that 47% COD removal is achieved.

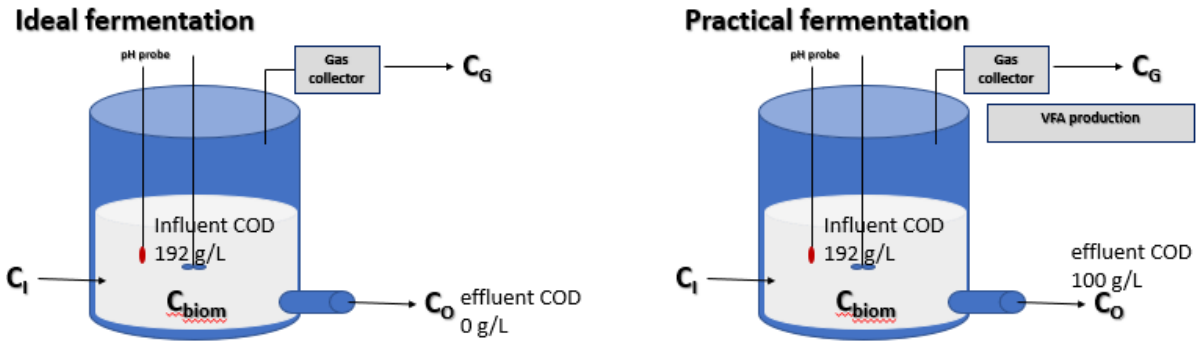


Figure 3-5 COD conversion in fermentation

3.5 Energy Efficiency of The Process

The heat of combustion of substrate is find out by drying the sample in the desiccator and burn the sample in the bomb calorimeter. The sample was weight in the crucible before combustion and after the combustion. The initial and final temperature was noted until the substrate was burned completely equation 3-11 is used to calculate the heat absorbed by the system by burning the amount of waste.

$$q_{\text{absorbed}} = m_{\text{water}} \times C_g \times \Delta T \qquad \text{Equation 3-11}$$

m_{water} assuming the density of water is 1 gml^{-1} ,mass of water is the mass of water in the combustion chamber, C_g Specific heat capacity of water, ΔT is the change in temperature.

Assuming no heat is lost, the energy released by the combustion is the energy absorbed by the water then heat of combustion is calculated by equation 3-12.

$$q_{\text{absorbed}} = q_{\text{released}}$$

$$\text{heat of combustion} = q_{\text{released}}\,(j) \div \text{ mass of substrate } (g) \qquad \text{Equation 3-12}$$

Several methods are reported to calculate the energy efficiency from dark fermentation to check the feasibility of the process. (Zhang et al., 2017) Reported the way to calculate energy efficiency, the conversion efficiency is calculated for co-production which is calculated by equation 3-13:

$$E = \frac{V_{H_2} \times Q_{H_2}}{Q_{\text{substrate}} \times m} \times 100\% \qquad \text{Equation 3-13}$$

Where energy conversion efficiency (%), V_{H_2} is H_2 gas volume (ml) , Q_{H_2} is the heat value of hydrogen gas 12.86 J/ml, $Q_{\text{substrate}}$ is the heating value of the substrate (J/gm). m is the weight of substrate (g).

For the pure carbohydrate sources, the energy conversion calculation can be calculated by the equation 3-14 reported by (Kumar & Das, 2000, Das, 2001).

$$ECE(\%) = \frac{\text{LHV } H_2 \text{ (kJ/mol)} \times H_2 \text{ yield (mol)}}{\text{LHV substrate (kJ/mol)}} \times 100\%$$ Equation 3-14

3.6 Errors

To have meaningful analysis and interpretation it was important to ensure accurate and reliable results. To achieve this, several important aspects were considered, including experimental error and repeatability, instrument accuracy and calibration, and random error test standard deviation.

Experimental Error and Repeatability:

The room temperature, where the experiment was carried out was kept constant throughout the year. The fermenter temperature was regularly monitored. To minimize experimental error, careful planning and execution of experiment were ensured. Each experiment was replicated thrice. The liquid sample and gas samples were taken out in triplicates to evaluate the repeatability of the experiments and assess the consistency of the findings. The samples taken out for energy calculation was done in triplicates and average value was presented. The lab equipment including fermenter, gas sensors were calibrated after every six months while autoclave and cooling unit was serviced once a year.

Instrument Accuracy and Calibration:

Accurate measurement instruments are essential for obtaining reliable data in a biohydrogen experiment. Ensuring the accuracy of the instruments involves regular calibration using known standards. Calibration helps to establish the relationship between the measured values and the true values, reducing measurement uncertainties. Periodic calibration checks and adjustments of the instruments throughout the experiment are necessary to maintain accuracy and enhance the validity of the results.

Random Error Test Standard Deviation:

To avoid the random error and variability in the data, in all the experimental work random error calculation involves analyzing the variability in data obtained from repeated measurements. The process includes conducting replicate measurements, calculating the mean, determining the deviation from the mean, squaring the deviations, summing the squared deviations, calculating variance and standard deviation. The standard deviation represents the spread or variability of data points around the mean, indicating the precision and reliability of the measurements. The error bars in the scatter plot are represented in form of standard deviation which represent the variability of data point around the mean.

3.7 Biohydrogen Production Modelling

In the dark fermentation batch process biohydrogen is produced as a result of degraded substrate and with the production of metabolites. The modified Gompertz equation is by far the most commonly recognised model for describing the kinetics of hydrogen generation in the batch process. It is applied to estimate the cumulative hydrogen production over time. The modified Gompertz equation 3-15 was developed by (Zwietering et al., 1990).

$$H(t) = Pexp\left\{-exp\left[\frac{R_m \times 2.718}{P}(\lambda - t) + 1\right]\right\} \qquad \text{Equation 3-15}$$

Where $H(t)$ represents the cumulative hydrogen production, P is the hydrogen potential (ml H_2), R_m is the maximum hydrogen production rate (ml-H_2/h), t is the incubation time (h), and λ is the lag-phase (h). The values of P, R_m and λ for each batch experiment were determined by fitting the hydrogen production data into the above equation using curve fitting tool in the MATLAB. The three parameters identified by fitting the experimental data into the equation gives the value which can be compared with different studies conducted using different substrate, process parameters by converting into specific terms like hydrogen production per unit of COD, TS, VS and hexose unit. The majority of the researchers use this model to estimate the hydrogen production in batch testing utilising various substrates and inoculum (Antonopoulou et al., 2011).

3.7 Summary

The experimental setup and analytical methods were employed to produce biohydrogen and delivered results at different operating conditions with the pure carbohydrate source and potato waste. The liquid sample analysis helped to analyse the consumption of the substrate (e.g., glucose, starch) over time. This information is essential for assessing substrate utilization rates and efficiency of the process carried out in the fermenter. Identification and quantification of various metabolites (acetic acid, butyric acid, valeric acid and propionic acid) produced during the fermentation process, Chemical oxygen demand (COD) was also calculated from the liquid samples taken out in triplicates at regular intervals.

Chapter 4 **Biohydrogen Production from Pure Carbohydrate Sources**

4.1 Introduction

The results presented in the chapter were conducted as a preliminary study of a batch reactor. Clostridia species are well studied for biohydrogen production, *Clostridium butyricum, Clostridium beijerinckii, Clostridium pasteurianum, Clostridium tyrobutyricum* have been isolated to produce biohydrogen (Lin et al., 2007; Chmiel & Yargeau, 2008; Masset et al., 2012; Hu et al., 2013; Seelert et al., 2015; Wong et al., 2018; Mahato et al., 2020). These strains of clostridia have been employed to produce hydrogen from pure carbohydrate sources and industrial solvents that contain carbohydrate. Although pure carbohydrate is an expensive raw material for industrialisation, it has been used in biohydrogen production studies to validate the process, examine the kinetics, nutrients composition and effect of bioprocess conditions (Kong et al., 2006; Lin & Chang, 2004; Sinha & Pandey, 2011; Vendruscolo, 2014 Litti et al., 2021). The Reinforced Clostridium medium (RCM) used in the research has sufficient nutrients that can provide sustainable growth of *Clostridium butyricum*; the nutrient composition is similar to Mannitol, Deoxycholate, and Tryptose (MDT) medium used in the study conducted by (Savichtcheva et al., 2011).

Heterotrophic bacteria require a supply of carbon sources, salts, amino acids, and nitrogen to sustain microbial growth. A study conducted by (Ogino et al., 2005) observed a significant increase in hydrogen production when using both peptone and reducing agents. Consequently, tryptone yeast extract was utilized in the fermentative hydrogen production process during the batch operation.Several pure carbon sources, namely glucose, starch, and sucrose, have been subject to investigation for biohydrogen production from C. butyricum (Masset et al., 2010; Cai et al., 2010; Junghare et al., 2012). In this study, glucose was employed to assess the efficiency of the strain, while starch was selected as a substrate to explore the potential for biohydrogen production, considering the substantial volume of starch wastewater generated at an industrial scale (Fernandes et al., 2010).

In this thesis, glucose with a concentration of 10 g/L was selected as a model substrate to investigate the influence of temperature on biohydrogen production, with the provision of necessary nutrients. The relationship between biohydrogen production and fermentation temperature was found to be non-linear, suggesting that an increase in temperature does not always result in a proportional increase in hydrogen yield. However, the study revealed that optimal hydrogen production occurs at lower and ambient temperatures. Specifically, the optimal temperature range for biohydrogen production was observed to be between 37°C and 55°C, as reported by Shin (2004). It is worth noting that some other researchers have identified an alternative temperature range of 20-40°C, which is more suitable for mesophilic bacteria. This chapter aims to contribute to a better understanding of the functionality of fermenter, *C.butyricum* potential to produce biohydrogen temperature dependency in biohydrogen production using glucose and starch as a model substrate and shed light on the potential applicability of these findings for optimizing hydrogen yield in different temperature conditions.

4.2 Material and methods

4.2.1 Cultivation of *C.butyricum*

Clostridium butyricum (NCTC 7423) was purchased from American Type Culture Collection (ATCC). To activate the bacteria, *C.butyricum* was activated in the sterile environment by mixing 1 ml of reinforced clostridium medium in to the ampule. The activated bacteria were then transferred into 15 ml of Reinforced Clostridial Medium (RCM) purchased from Sigma Aldrich. The RCM consisted of (g/L) meat extract 10.0; peptones 10.0; yeast extract 3.0; D(+)glucose 5.0; starch 1.0; sodium chloride 5.0; Sodium acetate 3.0; L-Cysteinium chloride 0.5; Agar-agar 0.5. The culture was prepared in Hungate tubes and placed in an anaerobic chamber after good shaking for 48 hours at 37^{o}C, pH 6.5. This culture is provided with other nutrients to enhance the growth of media by inoculating 10% (v/v) in 50 ml of tryptone yeast extract, 1 litre of culture contains (g/L), tryptone 5.0, and yeast extract 3.0 and supplemented with K_2HPO_4 ,1.0 for maintaining the pH. The turbidity started to change in the first 12 hours, and the optical density at 540 nm was measured to be 0.635, which ensured the growth of bacteria. This culture was used in all the experiments as inoculum for biohydrogen production.

4.2.2 Batch Setup

In experiment two pure carbohydrate source brought from Sigma Aldrich were utilised starch and glucose. The fermentation medium comprised of 10 g/l starch or 10g/l glucose, and nutrients (g/l) ; NaCl, 5, peptone, 2, KH_2PO_4 ,1. When starch was used in the fermenter it was first mixed with warm water then the volume was adjusted accordingly. The fermenter was cleaned and filled with the following medium with working volume of 1.5l. The fermenter was filled with 800 ml of fermented medium with controlled temperature at 37^oC and agitation speed of 250 rpm. The pH was uncontrolled. The nitrogen gas was purged in the fermenter to create an anaerobic environment. After the temperature control was achieved, the fermenter was inoculated with 70 ml C. Butyricum. The fermenter was inoculated with 9% (v/v),72 ml *Clostridium butyricum* prepared as mentioned in 4.2.1,it was transferred with a sterile hypodermic disposable syringe. The fermentation lasted for seven days until no further biohydrogen was produced.

In Experiment Two, pure carbohydrate sources, namely starch and glucose, were obtained from the reputable supplier Sigma Aldrich. The fermentation medium was formulated with 10 g/l of either starch or glucose, along with the following nutrient concentrations per liter: NaCl (5 g), peptone (2 g), and KH2PO4 (1 g). For the preparation of the fermenter using starch, the substrate was initially mixed with warm water and adjusted to the desired volume. The fermenter was then meticulously cleaned before being filled with the specified medium, with a working volume of 1.5 liters. Subsequently, 800 ml of the fermented medium was carefully introduced into the fermenter, and the temperature was tightly controlled at 37°C, with an agitation speed of 250 rpm. However, it is important to note that pH control was not implemented during this process. To create an anaerobic environment conducive to the experiment, nitrogen gas was purged into the fermenter.

Upon achieving the desired temperature control, the fermenter was inoculated with 70 ml of *C.butyricum* culture, prepared according to the methodology outlined in section 4.2.1, and it was transferred using a sterile hypodermic disposable syringe.. The substrate to inoculum ratio was 9% (v/v). The fermentation process continued for a duration of seven days, during which biohydrogen production was closely monitored. The experiment was concluded when no further biohydrogen was observed to be generated.

The experimental conditions and procedures were carried out with utmost precision to ensure reliable and accurate results in accordance with established scientific protocols. The number of experiments were replicated three times at the end of each run by maintaining the same conditions. The analysis of each liquid and gas sample was performed in triplicates to determine the repeatability of the results.

4.2.3 Analytical Method

The hydrogen and carbon dioxide production composition was monitored using BCP-H_2 , BCP- CO_2 sensor. In all the experiments, the produced biogas comprised 20-45% hydrogen and carbon dioxide 25-50%. The volume of the gas was measured by the water displacement method, and the composition of hydrogen gas was calculated by the cumulative hydrogen gas equation 3-1 mentioned in chapter 3 section 3.3. The reactor was equipped with a pH electrode connected to the monitor of the reactor, which recorded the pH profile inside the reactor throughout the experiment. The concentration of glucose was determined by the anthrone method on a daily basis.

4.3 Results & Discussion

4.3.1 Biohydrogen production from *Clostridium butyricum* using glucose and starch as a model substrate

Clostridium butyricum, a strict anaerobe known for its ability to degrade diverse carbon sources, was investigated for its biohydrogen production potential in this study. Hydrogen production from starch and glucose was quantified, yielding 90 ml and 154 ml, respectively, with cumulative hydrogen production reaching 915 ml and 1080 ml, as evidenced by figure 4-1 and 4-2. The corresponding yields were determined to be 0.73 mol H2 / mol glucose for starch and 1.23 mol H2 / mol glucose for glucose. The successful biohydrogen production from starch reinforces the strain's capacity to utilize starch and glucose as a carbon source, leading to hydrogen generation. However, despite the achievement of hydrogen production, the observed yield is relatively low, which may be attributed to uncontrolled pH conditions during the process. Additionally, the decrease in the pH and low hydrogen production during the fermentation is due to the formation of volatile fatty acid in the reactor.

Notably, the study conducted by Beckers et al. (2010) reported a comparable yield of 0.73 mol H_2 / mol glucose; however, this yield was obtained through co-culturing *C. butyricum* and *C. freundii* in a 48-hour fermentation. In contrast, the present study achieved a similar yield over a longer period of 120 hours. This disparity may indicate the strain's efficiency in consuming the carbohydrate source, contributing to the relatively low yield of hydrogen observed.

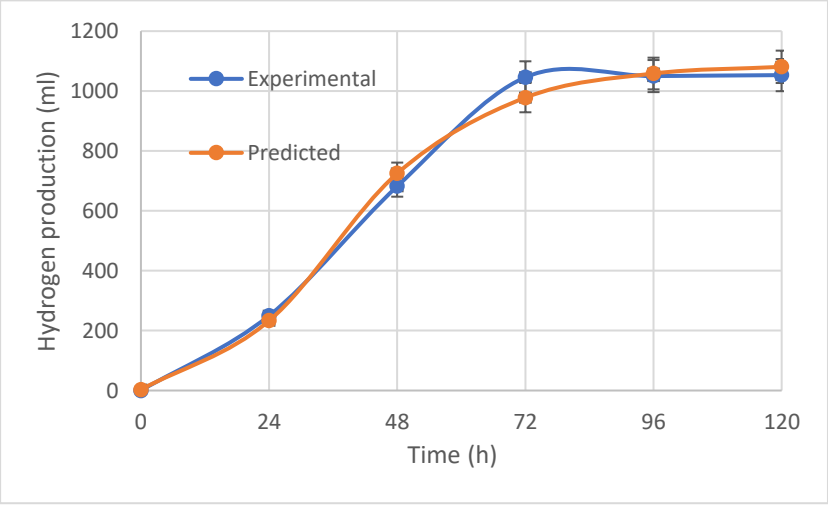


Figure 4-1 Cumulative hydrogen production from glucose

In light of these findings, it is imperative to comprehend the underlying factors influencing hydrogen production and yield in *C. butyricum*. Such understanding is crucial in optimizing the biohydrogen production process, enhancing its efficiency, and positioning it as a promising and sustainable energy source for the future. As a result, the outcomes of this study contribute significantly to the development of biohydrogen production technologies with the potential to address energy challenges while promoting environmental sustainability.

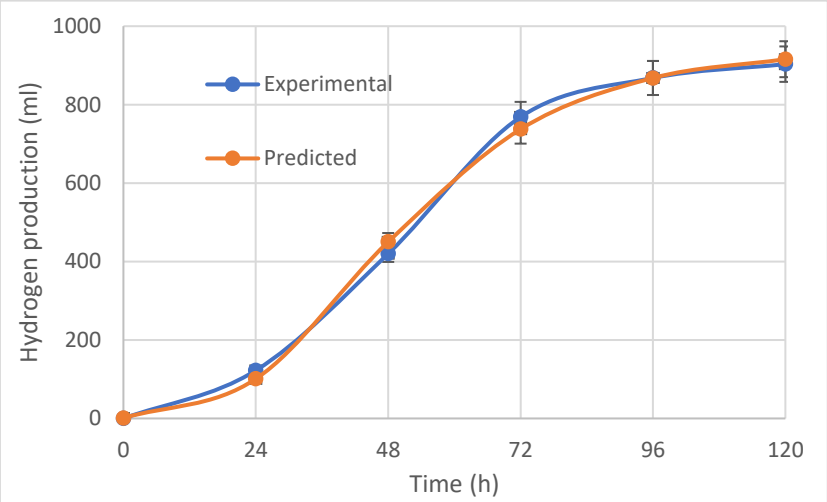


Figure 4-2 Cumulative hydrogen production from starch

Clostridium butyricum demonstrated the capability to efficiently hydrolyze starch and initiate hydrogen production within the initial 24 hours of incubation without requiring prior treatment. However, the relatively low rate of hydrogen production from starch suggests that pre-treatment could serve as an effective approach to enhance the process. Notably, some clostridia strains have been reported to be unable to consume starch for biohydrogen production without prior hydrolysis, as observed in the study by Chen et al. (2008). In this study, the substrate degradation efficiency for glucose and starch was found to be 70% and 60%, respectively. Most of the glucose was consumed within 72 hours of fermentation, resulting in higher hydrogen production. In contrast, *Clostridium butyricum* took a longer time to degrade the starch and utilize it as a carbon source for biohydrogen production. This prolonged degradation process led to the accumulation of biogas in the reactor, even after 96 hours of fermentation. Although 60% of the starch was consumed, it did not result in biohydrogen production, potentially due to its conversion into solvents, as evident from the

decrease in pH from 6.5 to 5, indicating the accumulation of volatile fatty acids in the reactor. The error bars in figure 4-1 and 4-2 indicates the fermenter was effectively able to maintain the process parameters during the first 24 hours and the results were replicable however the error slightly increased after 24 hours which could be due to several reasons: prolonged fermentation times leading to the accumulation of inhibitory compounds or changes in microbial physiology, affecting overall hydrogen production. Figure 4-3 shows the cumulative biohydrogen production from glucose and starch and it is evident from the graph that glucose was able to produce more hydrogen because of its simple structure and it is also a monomer which reduce the hydrolysis time for the microbes to be able to produce biohydrogen.

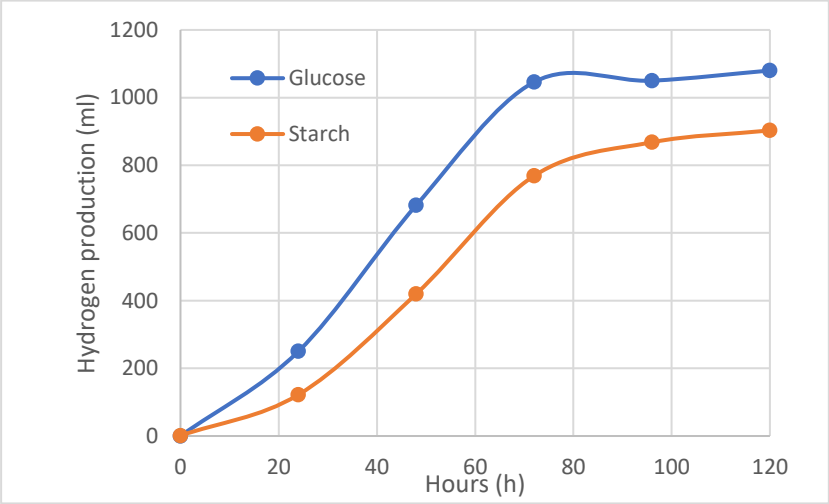


Figure 4-3 Cumulative hydrogen production from glucose and starch

According to the butyrate pathway, the calculated amount of hydrogen resulting from glucose is 2 mol / mol glucose, as the majority of the solvent primarily constitutes butyric acid formation, as reported by Wang et al. (2008). The isolated strain of clostridia species used in this study was first discovered in 2013 and has since been considered a promising producer of biofuels and biochemicals, as stated by Xin et al. (2013). Another study by Ortigueira et al. (2015) utilized this strain to investigate various carbon sources for biohydrogen production and observed successful biohydrogen production from glucose and starch, while no biohydrogen production was observed from xylan and cellulose. These findings emphasize the potential of *Clostridium butyricum* as a biohydrogen producer and the significance of substrate selection and pre-treatment strategies in optimizing biohydrogen production processes. The characterization of this strain's metabolic pathways and carbon source

preferences is instrumental in harnessing its biofuel-producing capabilities for future sustainable energy and industrial applications.

4.3.2 The lag time in biohydrogen production

The modified Gompertz equation is used to fit the experimental data for glucose and starch. The values of hydrogen production potential (P), hydrogen production rate (R_m) and lag phase (λ) were determined by best fitting the cumulative hydrogen production by using the curve fitting tool in MATLAB. The regression value was in the range of 0.996-0.998, the determination coefficient (R^2) of over 0.96 for all the regressions confirms the applicability of the modified Gompertz model. Table 4-1 shows that the lag time is affected by the type of model substrate with a lower lag time of 13.78 hours in glucose and 19.73 hours in starch. The justification of the lag time can be glucose a monosaccharide, is an easily accessible carbon source available to microbes to start the fermentation and produce biohydrogen production. In contrast, the time took to degrade the starch was slightly higher, which resulted in increased lag time. A similar pattern in the lag time can be observed in (Yin & Wang, 2017), where the lag time for glucose was 10.4 hours and starch was 16.8 hours from newly isolated strain *Clostridium butyricum* INET1. The energy yield obtained from biohydrogen production is 10.61% from glucose and 6.23 % from starch. Considering the theoretical yield of biohydrogen from butyrate pathway the maximum energy yield calculated by equation 3-14 in section 3.5 are 17.25% and 17.07% with LHV of glucose, starch and hydrogen 2805 kJ/mol, 2835 kJ/mol, 242 kJ/mol. The energy yield can improve with better biohydrogen production. The energy yield would further degrade if other than dark fermentation used in the study as they account for additional energy requirement.

Table 4-1 Parameters predicted by the modified Gompertz model

Substrate	**H_2 production**	***P*** **(ml)**	$\boldsymbol{R_m}$ **(ml/h)**	$\boldsymbol{\lambda}$ **(h)**	**R value**	**Energy yield**
Starch	915.83	940.40	15.98	19.73	0.99	6.23%
Glucose	1080.87	1089.00	22.23	13.78	0.99	10.61%

4.3.3 Effect of temperature on biohydrogen production from glucose

The aim of biohydrogen production from renewable feedstock pure and other waste sources is to provide renewable energy from a less energy-intensive process. The energy gain of fermentation is dependent on the difference between initial and the final fermentation temperature. Therefore, it is essential to keep the difference low for improved net energy gain (Perera et al., 2010; Lin et al., 2012). The temperature was controlled by a heating jacket connected to the fermenter for temperature control. The thermophilic range offers the advantages to prevent contamination; and less volatile fatty acids are formed, but the highest biohydrogen productivities are reported for mesophilic range 20-45^{o}C. As shown in figure 4-4 increasing the temperature from 37 ^{o}C to 41 ^{o}C improved substrate degradation efficiency to 90%, but the yield did not improve, and the hydrogen production per gram of glucose fell from 150 ml to 92 ml the substrate degradation due to increase in temperature was also reported by (Zhang et al., 2009) due to improved hydrolysis rate. A similar observation was made by (Wang & Wan, 2008) using the mixed culture and reported that an increase in the temperature helped the carbon intake of the microbes due to better acidogenesis occurred at higher temperature.

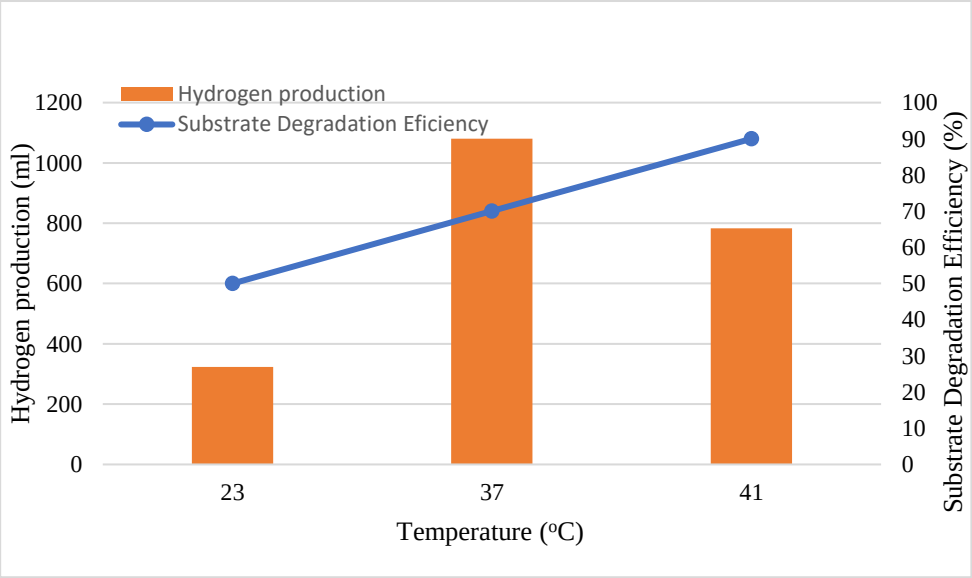


Figure 4-4 Effect of temperature on substrate degradation efficiency and hydrogen production using glucose

As shown in figure 4-5 that the lag time for hydrogen production at higher temperature is less, which shows that the microbial activities initiated as the fermentation started, whereas the time required by glucose to initiate the hydrogen production was higher, which led to the small hydrogen production and low substrate conversion efficiency. Biohydrogen production can be effective at high temperatures when the complex substrate is incubated with mixed and co-culture. High temperature can be utilised for complex substrate because it can facilitate in the hydrolysis to increase the substrate degradation efficiency (Nazlina et al., 2009), used food waste to produce biohydrogen and the highest biohydrogen production 593 ml H_2 / g carbohydrate was observed at 55^0 C but for that pH was maintained at 7. Not enough work has been done on biohydrogen production at low temperatures except for one species *Enterobacter*. The biohydrogen production temperature range is 30-37ºC (Pandey et al., 2009), where the highest hydrogen production was reported at 30ºC. *Enterobacter* is mainly employed with other species for biohydrogen production at lower temperature ranges.

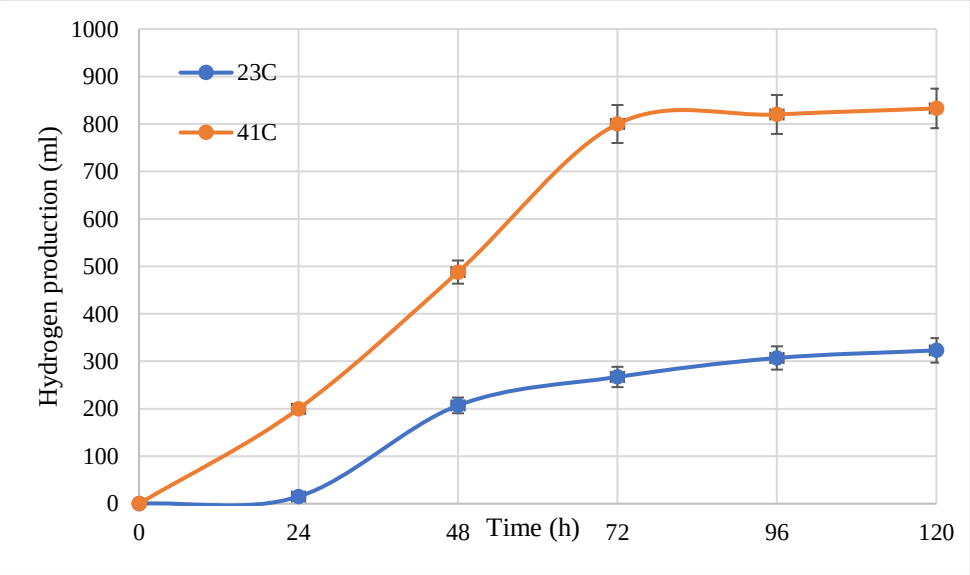


Figure 4-5 Effect of temperature 23ºC and 41ºC on hydrogen production using glucose

4.4 Summary

In the biohydrogen production experiments, the choice of substrate type, glucose, and starch had a significant impact on the yield of hydrogen. *Clostridium butyricum* exhibited the ability to utilize both glucose and starch as substrates for biohydrogen production. The results indicated that glucose was more efficiently utilized by the microorganism, as evidenced by a higher yield of 1.23 mol H_2 / mol glucose compared to 0.73 mol H_2 / mol glucose from starch. However, it was observed that the consumption of starch by the microorganism was lower than that of glucose, with only 60% of the starch being consumed during fermentation. This consumption did not result in the biohydrogen because the substrate must be converting in to volatile fatty acids due to long hydrolysis time.

The differences in hydrogen production between glucose and starch can be attributed to their distinct molecular structures. Glucose is a monosaccharide, readily available for direct utilization by the microorganism, whereas starch is a polysaccharide that requires prior hydrolysis to break it down into simpler sugars before it can be utilized for biohydrogen production. The lower yield of hydrogen from starch suggests that the hydrolysis process might have limitations in the current experimental setup.

Furthermore, the fermentation temperature was found to have a considerable impact on biohydrogen production. Higher temperatures increased the substrate degradation efficiency, indicating enhanced breakdown of glucose and starch. However, despite the improved substrate degradation efficiency, the hydrogen yield did not increase at higher temperatures, indicating that other factors might be limiting the overall biohydrogen production process. At lower temperatures, both the hydrogen yield and substrate degradation efficiency decreased, suggesting that the microorganism's metabolic activity might be reduced under these conditions.

Overall, the findings of this study highlight the importance of substrate selection and fermentation temperature in biohydrogen production. Understanding the impact of different substrates and process conditions on hydrogen yield and efficiency is crucial for optimizing biohydrogen production processes. The ability of *Clostridium butyricum* to effectively hydrolyze both glucose and starch opens up possibilities for utilizing other substrates in future biohydrogen production studies.

Chapter 5 **Pre-treatment of Potato Waste for Biohydrogen Production**

5.1 Introduction

The commonly employed pre-treatment method for natural waste include i) physical : mechanical, thermal , autoclave ultrasonication ii) biological : enzymes iii) chemical : acid/alkali. To avoid the extra cost of chemical for pre-treatment physically pre-treated potato waste is used in the study for the biohydrogen production. This chapter presents the results obtained from the experimental work conducted to produce biohydrogen from potato waste using the dark fermentation. The *Clostridium butyricum* which was able to hydrolyse the starch in the previous study is used to produce hydrogen from natural waste. The energy analysis is also performed to evaluate the energy efficiency from each type of pre-treated waste.

Pure carbohydrate source is easily degradable by the pure cultures. But the plenty of waste generation from fruit and vegetable in the food industry during processing can be used as an opportunity to produce biohydrogen from naturally occurred substrate. Compared to all the types of waste generates in different sectors food waste accounts for a large proportion globally (Thi et al., 2015). Amongst this type of waste is the material which is rich in carbohydrate content like potato. Potato waste is a cheap vegetable, and it is used in this study because of the high carbohydrate content, it is grown almost everywhere in the world in bulk amounts (Pathak et al., 2018). Potato is a cheap vegetable, which is consumed domestically, and it is used in large amounts in food industries to produce chips, mashed potatoes, hashbrowns. The production of all these products also produces starch rich waste which is already been used to produce yeast, starch, ethanol and butyric acid. However, in this work potato is chosen as a substrate to test the potential of biohydrogen production. Since different strains of *Clostridium butyricum* are used to produce biohydrogen from pure and hydrolyse starch, it will be further reacted with potato to analyse the efficiency of the strain.

Clostridium butyricum (NCTC 7423) was able to produce hydrogen from a pure carbohydrate source. Different strains of Clostridium are used to ferment pure carbohydrate sources and naturally occurred carbohydrates in the form of waste streams originating from the industrial process. To make the system economically viable, it is important to test the substrate, which

is commercially available. Produced hydrogen from starch with a co-culture of *Clostridium butyricum* and enertobacteor and *Clostridium butyricum* and rhodobacter sp M-19. *Clostridium butyricum* is a starch fermenting isolate (Chen et al., 2008) tested the hydrogen production from raw starch and starch hydrolysates by pure culture of clostridium species found out that *Clostridium butyricum* was able to produce hydrogen even from raw starch. Even with hydrogen accumulation of raw starch, it is essential to pre-treat the substrate when pure cultures are employed to enhance the yield.

Biohydrogen production is dependent on various process factors like pH, temperature, nutrients and composition of waste in the reactor. Additionally, in the dark fermentation, the first step hydrolysis the hydrolysis also plays a crucial role the final biohydrogen outcome from the substrate. The pre-treatment method is employed to improve the solubility of organic compounds to increase the biodegradability of the substrate (Deepanraj et al., 2017). Biohydrogen production was evident without pre-treatment from a study conducted by (Chen et al., 2008a). However, even with hydrogen accumulation of raw starch, it is essential to pre-treat the substrate when pure cultures are employed to enhance the yield. Various other studies emphasise the importance of pre-treatment for biohydrogen production (Turner et al., 2008; Parthiba Karthikeyan et al., 2018). Different treatment methods like thermal, chemical, ultrasonication and BESA pre-treatment are used to enhance the biohydrogen production from a variety of waste that contain potato. The hydrogen production increased 60% when thermally pre-treated potato was used in the study at 100 ^{o}C (Salem et al., 2018) . They also observed that the lag time was significantly reduced by employing the pre-treatment method though the reported lag time was 2 hours for all the pre-treatment. Biological pre-treatment method is also used to pre-treat the potato waste, like using alpha-amylase (Xie et al., 2008) . When using the natural waste, it is essential to maintain the optimum total solid percentage; biohydrogen production decrease with the increasing amount of solids in the fermentation. The increased amount of total solid can stop the substrate utilisation due to increased load on microbes. As reported by (Fernandez et al., 2008), when the reactor is loaded with more total solid, the performance degrades.

5.2 Material and Methods

5.2.1 Preparation of Substrate

The potato was purchased from the local grocery store; it was washed, cut into small pieces. I) The pieces were dried in the oven at 110^{o}C until the moisture was gone and grind in powder form in the chopper to produce fine powder. II) the pieces were boiled at 70^{o}C and blended using home blender by the addition of water 1:2 (w/v) III) The raw and boiled potato waste was blended by the addition of water with the ratio of 1:2 (w/v). Following optimised nutrient medium was added to make a healthy environment for pure *Clostridium butyricum* to grow in the fermenter (mg/L): 125 K_2HPO_4, 15 $MgCl_2.6H_2O$, 25 $FeSO_4.7H_2O$, 5 $CuSO_4.5H_2O$, 0.125 $CoCl_2.5H_2O$, 5,240 NH_4HCO_3 and 6,720 $NaHCO_3$ and 2% peptone (Nualsri et al., 2017). The three types of pre-treated waste were tested for biohydrogen production. The moisture content, TS and VS of the raw, dry and boiled potato waste are mentioned in table 5-1. All types of pre-treated waste were further diluted with distilled water to 10% TS for biohydrogen production.

Table 5-1 Total solid and Volatile solid of waste

Waste type	**TS**	**VS**	**Moisture**
Raw Potato	23.6%	91%	76.4%
Dry Potato	94.5%	85%	5.5%
Boil Potato	17.7%	89%	82.3%

5.2.2 Batch Setup

The substrate was prepared as mentioned in the previous section; the waste volume used in the fermenter is 700 ml with 30 ml nutrient solution and inoculated with 70 ml *Clostridium butyricum* in a reactor with a working volume of 1.5l. The pH was adjusted to 7 for the study of the effect of pre-treatment on the substrate. The substrate to microbe ratio was 9%. The fermentation was carried out at 37^{o}C stirring speed 250 rpm. The inoculation was done when the temperature was maintained in the reactor The fermentation lasted for seven days, until no biohydrogen was produced.

5.3.2 Analytical Method

The moisture content ,TS, VS were measured by the standard methods (Telliard, 2001). The chemical oxygen demand was calculated by taking out the liquid sample at the beginning and end of fermentation by titrimetric high level (Method 410 . 3 : Chemical Oxygen Demand (Titrimetric , High Level for Saline Waters) by Titration, 1978). The soluble chemical oxygen demand is obtained by filtering the sample through the filter membrane 0.45µm and following the standard method for sCOD determination. The carbohydrates in liquid were examined using the anthrone method. A UV-Vis spectrophotometer (GENESYS 50) was used to determine the absorbance at 620 nm. The biogas composition was measured by the thermal conductivity detector and infrared sensors BCP-H_2 , BCP-CH_4 , BCP-CO_2 . The biogas volume was measured by the water displacement method, and the volume of hydrogen was determined by multiplying the composition of hydrogen with total biogas volume. Table 5-2 shows the property of the waste before the start of fermentation.

Table 5-2 Properties after pre-treatment

Waste type	**COD (mg/l)**	**sCOD (mg/l)**	**VS (g/l)**	**Carbohydrate (g/l)**
Raw potato	12,150	1,458	15.9	17.4
Dry potato	10,920	900.52	12.8	14.5
Boil potato	14,020	2,523	13.9	22.1

Energy analysis

The energy content per gram of pre-treated potato (raw, dry and boiled)were determined using bomb calorimeter. The amount of energy in each gram of raw, dry, and boiled potato waste was 1.79, 2.11 and 1.95 kJ. The energy efficiency of biohydrogen produced from 100 gm of potato waste was calculated by equation 3-13 in chapter 3 section 3.5 which was also used by (Zhang et al., 2017) to calculate the energy efficiency of biohydrogen production from latanus orientalis leaves as carbon source.

5.4 Results and Discussion

5.4.1 Effect of pre-treatment on initial soluble cod of the waste and final cod of the effluent

The initial soluble cod values of all the treated waste varied in raw, dry and boiled potato waste suggested that thermally treating the waste was the effective way to increase the degradability of the substrate. The substrate degradability was achieved because the physical pre-treatment helped weaken the substrate's cell wall and facilitate releasing the organic matter. The COD reduction in the pre-treated waste was 17%, 11% and 23% for raw, dry and boiled potato. A study conducted by (Salem et al., 2018) applied control, thermal, base, acid, ultrasonic, H_2O_2 and thermal acid treatment on potato waste water. The reduction in the cod value in the effluent was 34%, 55%, 56%, 60%, 50%,63% and 62% respectively. It can be noticed that the majority of reduction occurred in hydrogen peroxide pre-treatment, which was 8% less from thermal pre-treatment. However, the COD reduction obtained in the current study is 23% in thermally treated potato waste is less as compared to the aforementioned study, It can be because of the micro-organism used, the biohydrogen production increase when sludge is used as inoculum as it contains mixed culture that can enhance biohydrogen production rate and substrate degradability (Elsharnouby et al., 2013). In this study, the choice of pre-treatment method was selected so that the energy required to treat the waste like electricity and heat can be generated from renewable energy source if the process has to be replicated on a large scale. The use of a chemical would add extra cost to the overall process and difficult to develop into fully renewable process. The remaining COD values are shown in table 5-3.

Table 5-3 COD value of effluent

Waste type	**Initial COD (mg/l)**	**Final COD (mg/l)**	**COD Reduction (%)**
Raw Potato	12,150	10084.5	17%
Dry Potato	10,920	9718.8	11%
Boil Potato	14,020	10795.4	23%

5.4.2 Effect of pre-treatment on lag time, biohydrogen production and biohydrogen production rate

The biohydrogen production started to occur within the first 8 hours of fermentation in all treated waste. The lag time in all the raw, dry, and boiled potato waste were 7.31, 7.89 and 7.62 hours. There is not much difference in the lag time of the biohydrogen production because a similar pre-treatment nature was applied to the potato waste. However, the lag time was comparatively higher in the dry potato waste. All the treatment methods were able to produced hydrogen in the short lag time. However, there was significant improvement in hydrogen production and hydrogen production rate. For raw potato, the hydrogen production was 724.1 ml, and the hydrogen production rate was 22.63 ml/h. For dry potato waste the hydrogen production was 274 ml and the hydrogen production rate was 4.69 ml/h. For boil potato waste, the hydrogen production was 1006 ml, and the hydrogen production rate was 28.05 ml/h. Boiled potato waste was able to produce more hydrogen the low hydrogen production in dried potato waste suggested that drying and grinding the waste did not help in the hydrogen accumulation.

5.4.3 Effect of pre-treatment on biohydrogen yield

Figure 5-1 illustrates the cumulative hydrogen production for three types of waste used in the study: raw, dry, and boiled potato waste. The yields for each waste type were as follows: 65.05 ml/g VS (volatile solids) for raw potato waste, 30.58 ml/g VS for dry potato waste, and 103.39 ml/g VS for boiled potato waste. However, biohydrogen production in dehydrated potato waste ceased prematurely, resulting in lower biohydrogen yields compared to raw and boiled potato wastes. The error bars at higher temperature shows conditions become unfavourable at high temperature as the results were repeatable at lower temperature. Another reason could be the hydrolysis rate is higher at high temperature resulting in more biohydrogen and volatile fatty acid production where as at low temperature conversion in to hydrogen is slow with slow hydrolysis rate.

The successful occurrence of biohydrogen production from raw and boiled potato waste indicates the potential for direct utilization of potatoes in hydrogen production, eliminating the need for an additional drying step. Furthermore, the presence of moisture in the waste was found to enhance biohydrogen production.

The potato industry generates various types of waste, which could be harnessed for biohydrogen production through thermal treatment. Nevertheless, pre-treatment of the substrate is essential to eliminate impurities and inhibit the growth of hydrogen inhibitors in the waste.

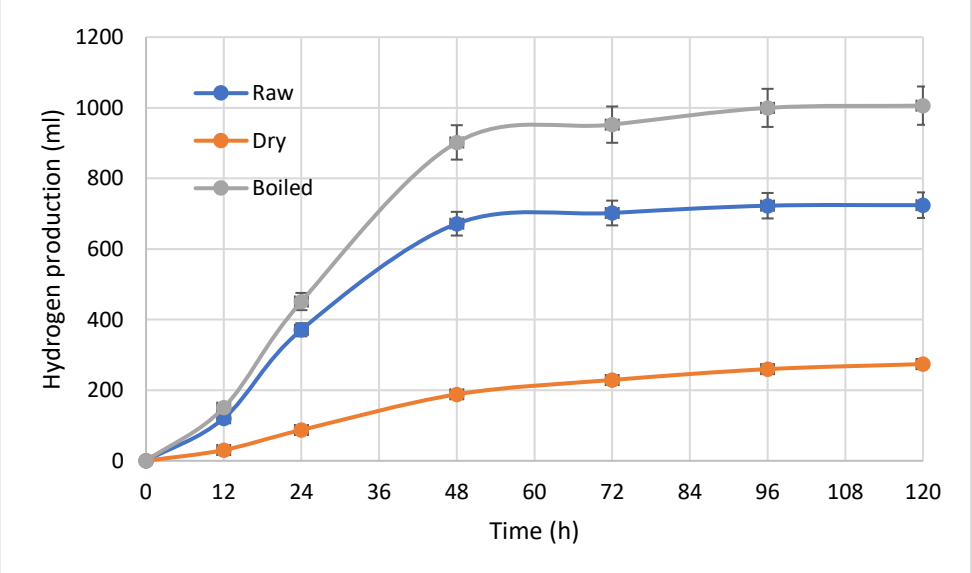


Figure 5-1 Cumulative hydrogen production from raw, dry and boiled potato waste

The experimental results obtained from the studies indicate that pre-treatment methods were effective in improving the degradation efficiencies for biohydrogen production, with the exception of the drying method. The study also revealed that biohydrogen production could occur at 37℃ without pH control, presenting an opportunity to treat food waste generated worldwide without the need for chemical treatment (Kumar & Mohan, 2018).

Robust pre-treatment methods such as alkaline, thermal, microwave, thermal-alkaline, and microwave-alkaline processes can be employed for complex waste, such as lignocellulosic waste (Ozkan et al., 2011). In this study, less energy-intensive and cost-effective pre-treatment methods were employed since potato waste primarily consists of carbohydrates.

Limited studies have been conducted on biohydrogen production from potato waste, with the maximum yield obtained being 300 ml/gVSS by Sekoai et al. (2019). Salem et al. (2018) obtained a hydrogen yield of 150 ml/g VS from thermally treating potato wastewater in a batch reactor, while Xie et al. (2008) produced a biohydrogen yield of 200.4 and 217.5 ml/g VS in a batch reactor from potato waste. The available literature suggests minimal pre-treatment requirements, such as milling, neutral pH, and thermal treatment, for potato waste to achieve biohydrogen production from all types of waste (Sołowski et al., 2019).

5.4.4 Energy analysis of the process

Utilising potatoes for hydrogen generation is an effective way of creating renewable energy. To evaluate the energy efficiency of the process. The energy content of all three types of waste raw, dry and boiled potato waste was calculated with their respective hydrogen production using the energy equation, the energy efficiency is 5.2% from raw potato waste, 1.7% from dry potato waste and 6.6% from boiled potato waste. Overall cumulative hydrogen production from the experiment resulted in 9.3 kJ, 3.5 kJ, and 12.9 kJ energy from raw, dry and boiled potato waste. The energy efficiency calculated by (Zhang et al., 2017b) using latanus orientalis leaves as substrate was comparable to the first stage; dark fermentation of their process which was equivalent to 4.3%. Their energy efficiency improved by combining the fuels obtained from dark and photo fermentation for hydrogen and methane production, which reached 15.17% and 22.28%.

5.5 Summary

In the biohydrogen production experiments, natural potato waste was utilized as a potential substrate. However, to improve the efficiency of biohydrogen production, pre-treatment of the waste was necessary. Three distinct pre-treatment methods were employed, namely blending for raw potato waste, drying and grinding for dry potato waste, and boiling followed by blending for boiled potato waste. These pre-treatment methods were chosen to investigate the impact of physical treatment on biohydrogen production.

The results from the batch experiments demonstrated that all three types of pre-treated potato waste were capable of producing biohydrogen using Clostridium butyricum. This finding suggests that potato waste shows promise as a potential substrate for biohydrogen production.

Quantitative analysis of the biohydrogen production revealed varying yields for each type of pre-treated waste. The raw potato waste yielded the highest amount of biohydrogen, with a value of 65.05 ml/ g VS. The dry potato waste produced a lower yield of 30.58 ml/ g VS, while the boiled potato waste resulted in the highest biohydrogen yield of 103.39 ml/ g VS.

The cumulative energy equivalent obtained from the three pre-treated potato wastes was 9.3 kJ for raw potato, 3.5 kJ for dry potato, and 12.9 kJ for boiled potato. These values provide insights into the energy potential of each pre-treated waste and can be valuable for further evaluations and applications in biohydrogen-based energy systems.

Furthermore, chemical oxygen demand (COD) reduction was analyzed to assess the effectiveness of each pre-treatment method in breaking down the organic matter and enhancing biohydrogen production. The results showed that the boiled potato waste exhibited the highest COD reduction of 23%, followed by 17% for raw potato waste and 11% for dry potato waste. This suggests that boiling the waste was the most effective pre-treatment method in terms of organic matter degradation and subsequent biohydrogen production.

In terms of lag time, it was observed that the dry potato waste exhibited a relatively longer lag time compared to raw and boiled waste. However, overall, the differences in lag time among the pre-treated wastes were not significant, indicating that the pre-treatment methods had a limited impact on the initiation of biohydrogen production.

In conclusion, this study demonstrates the potential of utilizing potato waste as a substrate for biohydrogen production. The different pre-treatment methods explored in the experiments revealed varying levels of biohydrogen yield and COD reduction, with boiling the waste showing the most promising results. These findings contribute to the scientific understanding of biohydrogen production from potato waste and pave the way for further optimization and utilization of this renewable and environmentally friendly energy source.

Chapter 6 **Process Optimisation for Bio-Hydrogen Production using Response Surface Analysis**

6.1 Introduction

This study aims to investigate the impact of pH and temperature on biohydrogen production by employing response surface analysis for experimental design. The optimum biohydrogen production conditions are determined using *Clostridium butyricum* and potato waste derived from previous experiments. It is known that biohydrogen production is highly dependent on environmental conditions, such as temperature and pH. These values can vary based on the substrate, process type, utilized strain, and pre-treatment methods employed (Alibardi & Cossu, 2016).

Hydrogen production from clostridia follows the acetate/butyrate pathway. Pyruvate is broken down into acetyl-CoA, leading to the production of hydrogen, butyrate, acetate, and ethanol as major end products. Optimal conditions need to be maintained for hydrogen production to occur during the exponential phase and continue until reaching the stationary phase. In uncontrolled pH conditions, hydrogen production ceases, and the metabolic pathway shifts towards solvent production (Kumari & Das, 2017).

Assessing the feasibility of the process requires increased biohydrogen production. Therefore, it is necessary to study the combined effects of various process parameters on biohydrogen production. In previous studies, numerous experiments have been conducted to enhance biohydrogen production using Clostridium species, focusing on individual factors such as temperature, pH, and inoculum effects (Fang et al., 2006; Lin et al., 2011). To overcome the complexity arising from an increased number of experiments and to study the interactions among factors, response surface analysis is employed to determine the optimum conditions for biohydrogen production. Response surface analysis is a widely used mathematical and statistical modelling tool for process optimization (Aydar, 2018). Several studies have utilized this method to investigate the effects of process parameters on biohydrogen production using different substrates and inoculums (Dan Jiang et al., 2016; Shaterzadeh & Ataei, 2017). However, there are limited studies on potato waste by *Clostridium butyricum*

focusing on the interaction between pH and temperature for determining the optimum conditions.

6.2 Design of Experiment

The factors chosen in the study are temperature and pH, it has been widely reported to be the most influential factors (Lin et al., 2011). The optimum pH reported for clostridium species lies in the range of 4-6 and the temperature in 37-41^{o}C because of its mesophilic nature (Zhang & Shen, 2006; Masset et al., 2012). Biohydrogen optimisation from potato waste using *Clostridium butyricum* as inoculum source was carried out using central composite design. The results are analysed in the R.Studio for response surface analysis. The factors pH and temperature are coded as x_1 and x_2 as coded values the factors are tested at high, low, and centre level +α, 0 and -α. The relation between coded and natural variables is described by equation 6-1 and 6-2.

$$pH = \frac{pH - centre\ point}{\frac{1}{2}\ (range)} \qquad \text{Equation 6-1}$$

Similarly for temperature

$$Temperature = \frac{Temperature - centre\ point}{\frac{1}{2}\ (range)} \qquad \text{Equation 6-2}$$

The relation between coded and natural variables is shown in table 6-1

Table 6-1: Coded values for two independent variables for central composite design

Variables	Coded symbol	-1	0	1	-1.414	+1.414
pH	x_1	4	5	6	3.58	6.41
Temperature (ºC)	x_2	37	39	41	37.58	40.41

For two factors k=2, the α value is determined by $\sqrt{k}$, the centre point is determined by the average of the highest and lowest level. The central composite design matrix of two independent variables for cumulative biohydrogen production is coded and natural value is shown in the table 6-2.

Table 6-2 Central composite design for three level

Points	Run	x1	x2	pH	Temp
Factorial point	1	-1	-1	4.0	37.0
	2	-1	1	4.0	41.0
	3	1	-1	6.0	37.0
	4	1	1	6.0	41.0
Central point	5	0	0	5.0	39.0
	6	0	0	5.0	39.0
	7	0	0	5.0	39.0
	8	0	0	5.0	39.0
	9	0	0	5.0	39.0
Axial point	10	1.141	0	6.4	39.0
	11	-1.141	0	3.5	39.0
	12	0	1.141	5.0	40.4
	13	0	-1.141	5.0	37.5

6.3 Material and Methods

6.3.1 Batch setup

The substrate was prepared as mentioned in the chapter 5; boiled waste was selected for this study as it resulted in higher biohydrogen production. The waste volume used in the fermenter is 700 ml with 30 ml nutrient solution and inoculated with 70 ml *Clostridium butyricum*,, making the substrate to inoculum ratio 9%. The initial pH of the experiment and the temperature was maintained according to the experimental plan derived from CCD. The pH was maintained by supplying 1 M of NaOH and 1 M H_3PO_4. The nitrogen gas was purged in the fermenter to create an anaerobic environment. The inoculation was done when temperature and the pH was maintained in the reactor The fermentation lasted for seven days, until no biohydrogen was produced.

6.3.2 Analytical Method

The TS, VS were measured by the standard methods (Telliard, 2001). The chemical oxygen demand was calculated by taking out the liquid sample at the beginning and endpoint of fermentation by titrimetric high level (Method 410 . 3 : Chemical Oxygen Demand (Titrimetric , High Level for Saline Waters) by Titration, 1978). The liquid samples were also analysed for volatile fatty acid formation. The liquid sample was injected out from the sampling port and stored at 2^o C. For analysis the sample was filtered through a cellulose acetate membrane 0.45 µm and acidified using 2% formic acid. VFA analysis was conducted using 30 m × 250 µm × 0.25 µm HP-5MS 5% phenyl Methyl Silox column. The temperature of the GC oven range from 80^{o}C to 200^{o}C, injection port temperature was set at 250^{o}C. Helium was used as a carrier gas with a flow rate of 1 ml/min. The biogas composition was measured from the thermal conductivity and infrared sensors BCP-H_2, BCP-CO_2 and BCP-CH_4; the volume of biogas was measured by the water displacement method. The hydrogen volume was obtained by multiplying the composition of hydrogen gas with the total volume of biogas obtained from the experiment.

6.3.3 Central Composite Design

The experiments were conducted in the order shown in table 6-2 with five replications at a central point, and altogether 13 runs were performed. The second-order polynomial equation 6-3 was used to express the effect of the independent variable on the response, which is the cumulative biohydrogen production.

$$B = \beta o + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \beta_{11} {x_1}^2 + \beta_{22} {x_2}^2 \qquad \text{Equation 6-3}$$

Where B is the cumulative biohydrogen production, βo is the model intercept, β_1 , β_2 are linear and β_{11} , β_{22} are quadratic terms. β_{12} is the interaction between the two variables. ANOVA was applied for the diagnostic checking of the appropriateness of the proposed model.

6.4 Results and Discussion

6.4.1 Effect of variables on biohydrogen production

In Figure 6-1, the contour plot presents a comprehensive illustration of the impact of variables, pH, and temperature on biohydrogen production. The plot reveals a clear optimum point, situated towards the left in terms of pH and in the middle concerning temperature. The significance of pH and temperature is found in the ANOVA table 6-3, providing further validation for the chosen optimization direction. Moreover, the contour plot reinforces the appropriateness of the selected range for the central composite design, as the optimum point falls within the plotted range, affirming the reliability of the experimental setup.

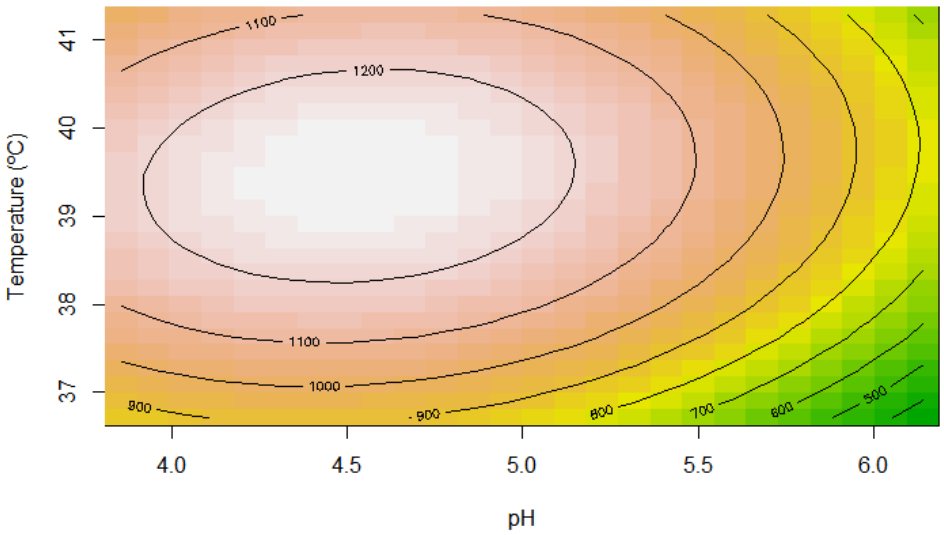


Figure 6-1 Contour plot illustrating the impact of pH and temperature

The cumulative hydrogen production was found to be influenced by both parameters (pH and temperature). The results were analysed using an ANOVA table, and a quadratic model was selected for optimization. The R-squared value of the model was determined to be 0.9355, indicating that 93.55% of the variability in the response can be explained by the model. The adjusted R-squared value, which considers the number of predictors in the model, was 0.8895.

To determine the significance of the model terms, a significance level (α) of 0.05 was used. Terms with p-values less than α are considered significant, while those with p-values greater than α are deemed insignificant. The results of the ANOVA, shown in Table 6-3, indicate that both the linear and quadratic variables are significant in relation to cumulative hydrogen production. However, the two-way interaction term was found to be non-significant.

Table 6-3 Significance of coefficient of regression

Factor	**Estimate**	**Std error**	**t value**	**p value**	**Remark**
Intercept	1215.527	34.16	35.58	$3.597e^{-09}$	Significant
$\mathbf{x_1}$	-182.534	30.728	-5.9404	0.00057	Significant
$\mathbf{x_2}$	105.711	30.728	3.4403	0.0108332	Significant
$\mathbf{x_1 x_2}$	-186.395	39.482	0.9927	0.3539	Non-significant
$\mathbf{x_1^2}$	-191.049	39.822	-4.6807	0.00225	Significant
$\mathbf{x_2^2}$	-191.049	39.822	-4.7976	0.00197	Significant

The model was polished after removing the two-way interaction, and the equation for the model for cumulative biohydrogen production is.

$$B = 1215.52 - 182.534x_1 + 105.711x_2 - 186.395{x_1}^2 - 191.049{x_2}^2 \qquad \text{Equation 6-4}$$

In the equation 6-4, B is the cumulative biohydrogen production, and x_1 and x_2 are the coded value for pH and temperature, respectively. The summary of the second-order model in Table 3 provides the results of the canonical analysis of the surface rather than the steepest ascent. The analysis reveals that the stationary point obtained from RStudio x_1 = -0.48 and x_2 = 0.27 in coded unit falls within the experimental region that is close to the optimal conditions. Both eigenvalues are negative -186.39 and -191.0494 which indicates that the stationary point is maximum (Lenth, 2020).

Figure 6-2 shows the three-dimensional response surface of pH and temperature variation on cumulative biohydrogen production. The response surface shows the clear optimum point which falls inside the boundary range.

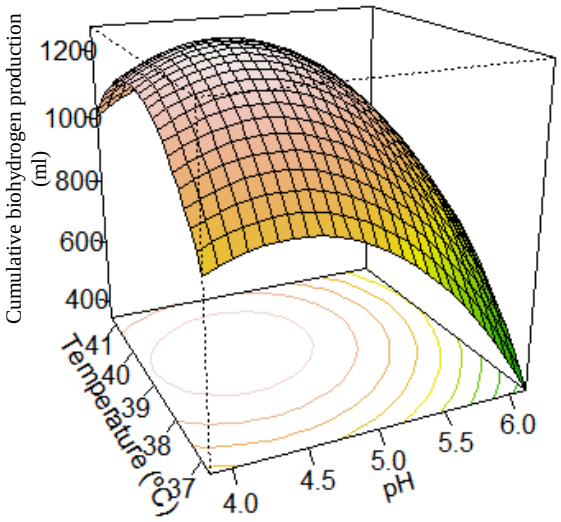


Figure 6-2 Three-dimensional response surface plot showing the effect of pH and temperature

6.4.2 Effect of pH

The anaerobic fermentation favours acidic pH as reported in the literature, and low pH facilitates the hydrogenase enzyme activity and the metabolic pathways of biohydrogen production (Shin, 2004). Similar observations were made in the experimental values. The pH range studied in this study was from 4-6. The reason for choosing this range was found in the literature where biohydrogen production is observed at the pH lower than 4.5 and even at pH lower than 3.5 (Mota et al., 2018) . A study found no biohydrogen production at 4.0 (Chong et al., 2009) using *Clostridium butyricum* from palm oil mill effluent. The different optimum pH is based on the nature of the substrate and inoculum used.

6.4.3 Effect of Temperature

Temperature is another critical parameter for biohydrogen as it facilitates the hydrolysis of the substrate. It can be seen in the figure that the optimum biohydrogen temperature point lies near 39^{o}C . The biohydrogen production increase with the increase in the temperature and it decreases when the temperature rises above 39^{o}C which shows that biohydrogen production

is also affected by the temperature. Optimum temperature 37ºC was obtained by observed (Shaterzadeh & Ataei, 2017) using Clostridium acetobutylicum. In some cases, the high temperature can result in better hydrogen production (Lee et al., 2006; Dessì et al., 2017). Clostridia is a mesophilic species therefore the optimum temperature 39ºC is justified (Elsharnouby et al., 2013).

6.4.4 Verification of the optimum point

To verify the model, an additional experiment was performed at the optimum points of pH 4.5 and a temperature of 39°C, as calculated by the model. This experiment resulted in a hydrogen (H2) production of 1256.78 ml, which is close to the predicted value of 1274.81 ml. The experimental and predicted results are presented in Table 6-4. Additionally, the summary includes the lowest and highest range studied in this chapter, as well as the optimum point obtained from the response surface analysis.

Table 6-4 Response surface analysis summary parameters

Factor	Higher	Lower	Optimum
pH	6	4	4.5
Temperature (°C)	41	37	39

Response surface analysis proved beneficial in reducing the number of experiments and avoiding the need for analyzing one factor at a time. In this study, the optimum pH and temperature for biohydrogen production were determined to be 4.5 and 39°C, respectively, resulting in a 29% reduction in chemical oxygen demand (COD). These optimal values were verified through biohydrogen production experiments, which yielded higher results compared to all other conditions. The relationship between the observed and predicted values for cumulative biohydrogen production is illustrated in Figure 6-3, the linear relationships among these values indicate a strong correlation among the results obtained with the experimental data and data generated with the model.

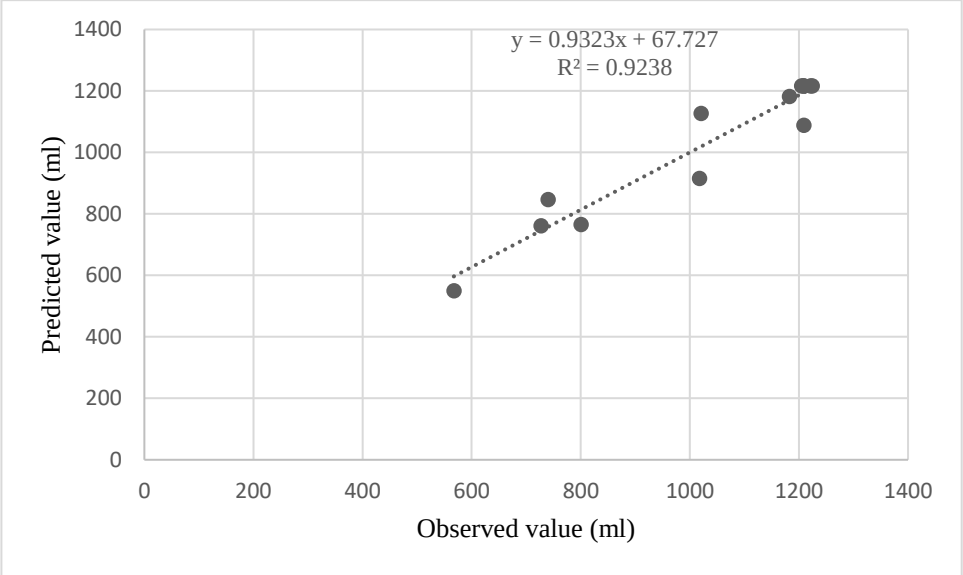


Figure 6-3: Observed and predicted values of cumulative biohydrogen production

6.4.5 Volatile fatty analysis

Volatile fatty acid (VFA) production is closely associated with hydrogen production. A series of experiments were conducted at the optimum temperature of 41°C, within the pH range of 4-6. Table 6-5 displays the VFA accumulation at the end of fermentation, indicating concentrations ranging from 3254-3672 mg/l. Acetic acid and butyric acid were found to be dominant compared to valeric acid and propionic acid, providing valuable insights into the metabolic pathways involved in biohydrogen production.

The production of acetic and butyric acids is highly favourable for biohydrogen generation, as these acids serve as essential precursors. Propionic acid remained negligible within the pH range of 4 to 4.5, increased to 6% at pH 5.0, and then decreased to 3% at pH 6.0. The shift in pH influences the metabolic pathways within the microbial environment, promoting propionic acid production. The higher pH levels facilitate the conversion of intermediates, such as succinic acid, to propionic acid, which is further supported by the reduced biohydrogen production at higher pH levels.

Throughout all pH levels, butyric acid production exceeded that of acetic acid. The ratio of butyric acid to acetic acid ranged from 0.54, 0.43, 0.86, and 0.67 for pH values of 4, 4.5, 5.0, and 6, respectively, highlighting the dominance of the butyrate-type metabolic pathway during fermentation. Notably, an observation made by Zhou et al. (2018) aligns with our findings, as an increase in pH from 5.0 to 6.0 resulted in a decrease in the concentration of butyric acid.

Table 6-5: Volatile fatty acid accumulation at different pH

pH	Acetic acid (mg/l)	Butyric acid (mg/l)	Propionic acid (mg/l)	Valeric acid (mg/l)	Total VFA (mg/l)
4.0	1138.9	2093.7	-	21.4	3254.0
4.5	1015.8	2344.5	-	25.7	3386.0
5.0	1505.5	1730.9	215.3	137.2	3589.0
6.0	1578.9	1652.4	113.8	77.1	3672.

Despite the reduction in butyric acid concentration from pH 4.0 to 6.0, it remained higher than the concentration of acetic acid at pH 5.0 and 6.0. This observation aligns with the

findings of Huang et al. (2016), who reported a higher concentration of acetic acid at alkaline pH, as observed in our experiment. However, acetic acid did not dominate over butyric acid formation, as found in a study by Fang et al. (2006).

The cessation of hydrogen production in the reactor was due to the accumulation of volatile fatty acids (VFA). The increased VFA levels could explain the reduced biohydrogen production at higher pH levels, as a significant portion of the organic matter is converted into volatile fatty acids. The optimized conditions, with controlled operating parameters, resulted in a 29% reduction in chemical oxygen demand (COD) compared to the biohydrogen production obtained from potato waste pre-treatment alone. This indicates that both pre-treatment and maintaining optimal operating conditions have a significant impact on biohydrogen production.

6.5 Summary

This study employed response surface methodology (RSM) as a valuable tool to optimize the experimental design for biohydrogen production using potato waste. RSM allowed for the systematic exploration of the effects of pH and temperature at different levels, leading to the identification of the optimal conditions. The high correlation coefficient (R value of 0.92) between the predicted and experimental values indicates the reliability of the RSM model.

The results revealed that both pH and temperature significantly influenced biohydrogen production, as indicated by the p-value less than 0.05. The increase in temperature up to 39°C positively impacted biohydrogen production, while further elevation in temperature resulted in decreased biohydrogen yields. Similarly, maintaining a pH value of 4.5 proved to be optimal for achieving higher biohydrogen production, and deviations from this pH level led to reduced yields.

The dominance of butyric acid in the reactor confirmed that *Clostridium butyricum* predominantly followed the butyrate pathway for biohydrogen production. This insight into the metabolic pathway provides valuable information for optimizing the bioprocess and enhancing biohydrogen yields.

Furthermore, the study demonstrated that boiled potato waste, when subjected to the maintained operating conditions, exhibited increased biohydrogen production and a 29% reduction in Chemical Oxygen Demand (COD). This highlights the effectiveness of pre-treatment methods and the importance of maintaining process parameters for improving hydrogen yield and waste reduction.

The use of RSM, understanding of metabolic pathways, and optimization of operating conditions are key scientific aspects that contribute to the successful production of biohydrogen from boiled potato.

Chapter 7 **Conclusion and Future Work**

In this research endeavour, a batch process was methodically employed to explore the biohydrogen production potential of *Clostridium butyricum* through fermentability testing. The strain's efficiency in generating biohydrogen from glucose and starch substrates was found to be 36.5% and 60%, respectively, with corresponding substrate degradation rates of 70% and 60%. These results offer valuable insights into the strain's performance in utilizing different carbon sources for biohydrogen production.

Of particular significance were the reduced lag times observed during biohydrogen production from glucose and starch, corroborating findings reported in the existing literature. This phenomenon is attributed to the limiting hydrolysis rate within the fermenter, impacting the kinetics of the process. The hydrogen yield obtained from glucose and starch, measuring 1.23 mol H2/mol glucose and 0.73 mol H2/mol glucose, further underscores the strain's capacity to produce biohydrogen from these substrates.

Moreover, the conversion efficiency of glucose and starch into biohydrogen was evaluated at 61.5% and 36.5% when considering the theoretical value of 2 moles. These assessments provide valuable benchmarks for evaluating the overall efficiency of biohydrogen production from these specific substrates. The successful repeatability of the laboratory-scale experiments instils confidence in the viability of transitioning to pilot-scale studies for biohydrogen production. Such scalability opens promising prospects for potential industrial applications of the biohydrogen production process.

Furthermore, temperature's influence on biohydrogen production, specifically using glucose as a model substrate, was investigated. The findings revealed increased substrate degradation efficiency at higher temperatures. However, it was noted that the strain efficiency reduced to 32.5% at elevated temperatures, indicating the need for carefully considering the temperature during the process.

The energy yield of the biohydrogen production process was found to be 10.65% and 6.23% from glucose and starch, respectively, with calculated energy potentials of 297.66 kJ and 176.66 kJ. The impact of temperature was also evident, with energy potential reaching 166.98 kJ at 41°C, and a reduction to 125.62 kJ at a lower temperature. These energy potential assessments offer critical insights into the process's energy efficiency and sustainability.

In light of these observations, it is recommended that, for large-scale replication of the experiments, operating the reactor at ambient temperature may prove to be a feasible approach. To address the issue of reduced substrate degradation efficiency, incorporating a hydrolysis step before the inoculation start is suggested. This approach enables the provision of higher temperatures for shorter durations during hydrolysis, rather than maintaining higher temperatures throughout the entire experiment for an extended period.

These research findings contribute significantly to the understanding of biohydrogen production using different substrates, strain efficiency, energy yields, and the influence of temperature. The exploration of optimization strategies to enhance the process efficiency and sustainability holds great promise for the advancement of biohydrogen production technology, offering environmentally friendly and renewable energy solutions for the future.

The conducted experimentation represents a notable effort in the domain of biohydrogen production, employing potato waste as a sustainable and abundant substrate for renewable energy generation. The strategic selection of potato waste as the primary feedstock stems from its wide availability and the significant quantities of waste generated during potato processing across both industrial and domestic sectors. The inclusion of physical pre-treatment methods in the biohydrogen production process was a crucial aspect, contributing to the successful optimization of hydrogen yields from the waste material. A noteworthy consideration pertains to waste disposal regulations, as stipulated by Directive 1999/31/EC. The stringent measures imposed on industries generating waste with high organic content underscore the imperative to mitigate environmental impacts. The research highlights the importance of reducing the organic matter in potato waste prior to disposal, a vital step in adhering to waste disposal guidelines and promoting responsible waste management practices.

The experimental findings, subsequent to the pre-treatment of various types of potato waste, demonstrated a remarkable enhancement in hydrogen production from boiled potato waste. The cumulative hydrogen production of 1006 ml per 100 gm of potato waste exemplifies the potential of this particular waste type as a viable source for biohydrogen generation. The pre-treatment process also exerted a notable impact on the volatile solid content of the waste, thus accentuating the biohydrogen production potential by enhancing the availability of organic matter for conversion. An essential insight gathered from the study pertains to the swift

hydrolysis of carbohydrates in the potato waste, evidenced by the relatively short lag time range of 7.39-7.81. This expedited hydrolysis facilitated the prompt conversion of complex carbohydrates into biohydrogen and other soluble metabolites, thus accelerating the overall biohydrogen production process. The evaluation of energy conversion efficiency further emphasizes the viability of utilizing potato waste for biohydrogen production. The energy conversion efficiencies of 5.2%, 1.7%, and 6.6% for raw, dry, and boiled potato waste, respectively, are indicative of the potential for substantial energy yield from this renewable source. Notably, the energy conversion efficiency of boiled potato waste, at 6.6%, surpasses that of pure starch, exemplifying the advantageous properties of this waste material for sustainable energy production. The experimental work on biohydrogen production from potato waste yields valuable insights and promising avenues for renewable energy generation. Potato is among the top consumed and produced crops in the world. The results obtained in the study to produce biohydrogen from different types of potato waste can help to develop better waste management practices at upstream and downstream stages. By harnessing natural waste resources through pre-treatment strategies, industries can contribute significantly to sustainable waste management practices while simultaneously unlocking the immense potential of biohydrogen as a clean and renewable energy source. This study serves as a testament to the feasibility of adopting innovative approaches that align with environmental preservation and energy sustainability goals, ultimately paving the way for a greener and more resilient energy future.

In the last chapter the optimization of biohydrogen production from potato waste was pursued by controlling two independent variables, namely pH and temperature. Employing a three-level factorial design, a total of 13 experimental runs were carefully conducted, encompassing a range of desired pH and temperature conditions. The response surface analysis emerged as a valuable tool, enabling the visualization of the intricate interplay between pH and temperature through a comprehensive 3-dimensional plot. The contour plot derived from the experimental results further bolstered the justification of the optimum pH and temperature range identified for clostridia species in existing literature. As a result of the analysis, the optimal pH was determined to be 4.5, with a temperature of 39°C representing the most conducive conditions for enhanced biohydrogen production. Subsequently, an additional experiment was executed at these optimal points, culminating in a notable increase in cumulative hydrogen production, amounting to 1256.78 ml. Moreover, the Chemical

Oxygen Demand (COD) reduction was further enhanced, reaching 29%, as a direct consequence of maintaining the optimal pH and temperature conditions.

The application of response surface analysis emerged as a pivotal strategy in maximizing biohydrogen yield by precisely controlling the two independent variables, pH, and temperature. As evidenced, the process yielded an impressive 11% increase in biohydrogen production when conducted under the defined optimum conditions of 4.5 pH and 39°C temperature. These findings represent a significant contribution towards establishing an efficient and sustainable biohydrogen production process, thereby promoting the utilization of natural waste as a valuable resource for renewable energy generation. The selection of physical pre-treatment methods in this study bears paramount importance, as these techniques can readily fulfil the external energy requirements using renewable energy sources. Furthermore, the absence of chemical usage in the pre-treatment process renders it economically viable, ensuring a more cost-effective and environmentally friendly approach.

Additionally, the mesophilic nature of *Clostridium butyricum* plays a pivotal role in the success of this biohydrogen production process. The strain's ability to produce biohydrogen at relatively low temperatures (39°C) from potato waste offers a remarkable opportunity to employ biological dark fermentation for waste treatment, as opposed to the energy-intensive gasification process that operates at much higher temperatures (700-900°C). This presents a significant advantage in terms of energy efficiency and resource conservation. It is noteworthy that the carbon dioxide generated in the experiments exhibited a relatively low grade, amounting to less than 50%. Leveraging absorption and stripping technology presents a viable means to enhance the purity of carbon dioxide, making it suitable for utilization in various industries, particularly in the food and beverage sector.

In conclusion, the systematic optimization of biohydrogen production from potato waste, driven by the control of pH and temperature, showcases the potential for sustainable and eco-friendly energy solutions. The judicious selection of pre-treatment methods and the utilization of mesophilic *Clostridium butyricum* further contribute to the feasibility of replicating the process on a larger scale, with reduced energy consumption and cost-effectiveness. This research highlights the promising prospects of harnessing biological dark fermentation as a viable alternative to conventional energy-intensive processes, underscoring the transformative potential of bioenergy for a greener and more sustainable future.

The cumulative biohydrogen production attained through the process optimization presents an opportune occasion to assess the power generation potential for treating $1m^3$ of waste, adopting a methodology similar to that employed by Chang et al. in their 2013 study. The integration of dark fermentation with renewable energy sources allows for an insightful calculation. Upon thorough evaluation, it is revealed that processing $1m^3$ of potato waste can yield an electric power generation of 15.06 kWh. This substantial electricity output demonstrates the promising prospects of biohydrogen production as an efficient and viable means to harness energy from organic waste streams.

This significant finding offers valuable learnings and implications for the sustainable utilization of waste materials for energy generation. It underlines the substantial potential for integrating biohydrogen production with renewable energy sources to achieve an environmentally friendly and economically feasible energy production process. Furthermore, the assessment underscores the importance of optimizing bioenergy conversion processes, which can transform waste management practices and contribute to a greener and more sustainable future.

The calculated electric power generation of 15.06 kWh for processing $1m^3$ of potato waste reveals the tangible energy benefits attainable through the integration of dark fermentation with renewable energy systems. This finding encourages further exploration of innovative approaches to maximize energy recovery from waste materials, effectively converting them into valuable resources. Moreover, the knowledge gained from this assessment fosters the development of novel and scalable bioenergy technologies, which hold great promise for waste-to-energy conversion on a larger scale. Such advancements can significantly contribute to addressing energy demands while mitigating environmental impacts associated with waste disposal.

As the pursuit of renewable and sustainable energy sources becomes increasingly imperative, this research outcome emerges as a valuable addition to the field of bioenergy. By showcasing the energy potential that can be harnessed from biohydrogen production, the study inspires continued investigations into optimizing waste-to-energy processes, offering a compelling pathway towards a more circular and resource-efficient energy paradigm. In conclusion, the evaluation of power generation potential from biohydrogen production signifies a significant milestone in the quest for sustainable development goals.

The target values of a viable process for the research are as follows:

1. High Biohydrogen Yield: The primary target is to achieve a high biohydrogen yield from the fermentation of potato waste. The research seeks to optimize the process conditions, such as pH and temperature, to maximize the production of biohydrogen.

2. Efficient Substrate Utilization: The process should efficiently utilize the carbohydrate-rich content of the potato waste. This involves investigating different pre-treatment methods to enhance the hydrolysis of polysaccharides and improve substrate degradation efficiency.

3. Energy Conversion Efficiency: The research aims to determine the energy conversion efficiency of the biohydrogen production process. This parameter reflects the effectiveness of converting the available energy in the substrate into biohydrogen.

4. Chemical Oxygen Demand (COD) Reduction: Another target is to achieve a significant reduction in the COD of the potato waste during the biohydrogen production process. This indicates the extent to which organic matter in the waste is broken down and utilized.

5. Comparison of Pre-Treatment Methods: The research aims to compare the effectiveness of different pre-treatment methods for potato waste, such as blending, drying, and boiling. The objective is to identify the most efficient pre-treatment strategy to enhance biohydrogen production.

6. Use of Response Surface Analysis: Successful implementation of RSM, which allowed for the optimization of process parameters. The obtained results demonstrate a good fit between the predicted and experimental values, indicating the reliability of the model. The contour plot further illustrates the significance of pH and temperature in influencing biohydrogen production, supporting the accuracy of the chosen optimization direction.

7. Sustainability and Feasibility: The research evaluates the overall sustainability and feasibility of utilizing potato waste as a potential substrate for biohydrogen production. This includes assessing the energy output, waste management, and economic viability of the process.

8. Scalability and Reproducibility: The process should be scalable and reproducible on a larger scale, making it applicable for potential industrial implementation. The research investigates the practicality of scaling up the biohydrogen production process.

To address the challenge of less hydrogen production in dark fermentation and pave the way for successful technology commercialization and feasibility, the following recommendations are proposed:

1. Optimize Substrate Selection: Explore a wide range of organic substrates to identify the most suitable feedstock for dark fermentation. By selecting substrates with higher hydrogen yields and better biodegradability, the overall hydrogen production efficiency can be significantly enhanced.

2. Process Parameter Optimization: Conduct thorough investigations into the impact of various process parameters such as temperature, pH, hydraulic retention time, and organic loading rate. Fine-tuning these parameters based on experimental findings can lead to improved hydrogen production rates and yields.

3. Inoculum Source Enhancement: Pay close attention to the source and characteristics of the inoculum used in dark fermentation. Utilizing enriched microbial consortia or specific hydrogen-producing strains can boost hydrogen production rates and facilitate a more efficient biogas production process.

4. Co-Digestion and Pretreatment: Explore the benefits of co-digestion with other organic wastes or pretreatment techniques to enhance the degradability of the feedstock. Co-digestion can result in synergistic effects, leading to increased hydrogen yields and improved overall efficiency.

5. Microbial Metabolic Engineering: Investigate the potential of microbial metabolic engineering to develop hydrogen-producing microorganisms with enhanced metabolic pathways. Genetically modifying key hydrogen-producing microbes can potentially increase their activity and hydrogen production capabilities.

6. Process Integration and Scale-Up: Focus on integrating dark fermentation with other biotechnological processes or wastewater treatment systems. Synergistic process integration can enhance resource utilization and maximize hydrogen production efficiency. Additionally, consider pilot-scale studies to validate the feasibility of the technology at a larger scale.

7. Economic Viability and Market Assessment: Conduct a comprehensive economic analysis to evaluate the commercial viability of the dark fermentation technology. Identify potential markets, assess the competitive landscape, and develop a robust business plan to attract investment and support commercialization efforts.

8. Public Awareness and Stakeholder Engagement: Raise public awareness about the importance and benefits of hydrogen production through dark fermentation. Engage relevant stakeholders, policymakers, and industry partners to garner support and funding for technology development and deployment.

By implementing these recommendations, the technology's hydrogen production efficiency can be significantly enhanced, making it more commercially viable and feasible for broader adoption. Continuous research, optimization, and collaboration will be key to unlocking the full potential of dark fermentation as a sustainable and viable source of hydrogen production.

Future Work

Based on the results achieved in this research the following future work can be recommended:

1) Integration with other process and application of continuous flow system should help in building the strong ground for deployment of biohydrogen production from potato waste at pilot scale.

2) In depth analysis of enzymes involved in the biohydrogen production should help in increasing the efficiency of the currently used strain *Clostridium butyricum*.

3) The mixture of carbohydrate rich and nitrogen rich substrate should be tested to eliminate the need for nitrogen sparging.

4) More independent variable should be studied for the relevance and improve biohydrogen yield from potato waste.

5) The divergence from pure culture to mixed culture from natural habitat should be explored as it can result in the better economic value of the process.

6) The effluent generates from biohydrogen production contains volatile fatty acid, which can be an excellent source for bioplastic. This should be explored to improve overall waste recovery.

7) Food waste with naturally low pH should be explored to avoid the pH maintenance from chemicals.

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