VALORIZATION OF WASTE ACTIVATED SLUDGE: BIOHYDROGEN PRODUCTION AND RECOVERY OF VOLATILE FATTY ACIDS

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DECLARATION

I hereby declared that the work presented in this thesis report title "***Valorization of waste activated sludge: biohydrogen production and recovery of volatile fatty acids***" submitted to Jadavpur University, Kolkata in partial fulfilment of the requirements for the award of the degree of M Tech is a bonafide record of the research work carried out under the supervision of Prof. Joydeep Mukherjee. The contents of thesis report in parts, have not been submitted to and will not be submitted by me to any other Institute or University in India or abroad for the award of any degree or diploma.

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ABSTRACT

Over the course of the industrial revolution, over 80% of the world's energy has been produced by burning fossil fuels. Moreover, over the past 10 years, the global energy consumption has increased dramatically and is expected to do so for the next fifty years. Therefore, Energy is crucial for ensuring world development, and hydrogen, the cleanest sustainable energy source, has enormous promise. Thus, employing biological creatures like bacteria, algae, or archaea to produce hydrogen gas is a sustainable method known as bio-hydrogen production. Hydrogen is a clean, environmentally beneficial biofuel with several excellent features. In the research study, consortia of *E. coli* and *E. aerogenes* were used for the anaerobic fermentation of bio-hydrogen production and the VFA's that were collected from the effluent were measured and used as a media for the photo-fermentation thereafter. Samples from Titagarh Sewage Treatment plants were collected and various parameters like pH, COD, TSS, TDS, VFA, ammonia, carbohydrate and ammoniacal nitrogen were calculated and based on these, fermentation were carried out thereafter. Also, nowadays VFA is an important platform chemical for biofuel, bioplastics, food and pharma industries. In our study, we have also recovered VFA from the wastewater effluent by adsorption on activated carbon and chemical precipitation by calcium salts and it was seen that adsorption has a better efficiency than precipitation in the recovery process. The recovered VFA can be used as a chemical in the future.

Keywords: Sustainability, Energy, Bio-hydrogen, Anaerobic Fermentation, *E. coli, E. aerogenes,* VFA, biofuel, bioplastics, adsorption, chemical precipitation.

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1.INTRODUCTION

Due to consumer demand, geopolitical unpredictability, cost of fossil fuels in the future, and the environmental concerns about the greenhouse effect makes biomass the most sustainable energy source and power providing billions with supply security while promoting agricultural livelihoods [1]. Since the beginning of the industrialized era, fossil fuel burning has accounted for around 80% of world energy consumption. Furthermore, the world's energy consumption has grown exponentially over the last ten years and will continue to do so throughout the next fifty years. This can be explained by a number of reasons such as a growing population, an economic lifestyle, society, modernization, and industrialization, and enhanced environmental requirements. Fossil fuels make for a large portion of our energy needs, with coal accounting for 27.2%, oil for 32.8%, and natural gas for 20.9% when it comes to transportation and heating. Even though they only make up just over one percent of the world's overall primary energy requirements, 5.8% of nuclear power, 2.3% of hydroelectric dams, and 10.2% of combustible biomass and waste contribute to the energy system after fossil fuel [2]. About 0.8% of the globe's power requirements can be supplied by geothermal, wind, and solar energy collectively. Although the conventional fossil-based fuels have a major share in the primary energy consumption, but they are considered unsustainable as they contribute to the formation of the greenhouse gases that has already above the 450 ppm CO_2 "dangerously high" level [4]. Among the various alternative energy sources, four stand out as strategically significant sustainable energy sources: synthesis gas (syn gas), natural gas, hydrogen, and biofuel. Because of its renewable nature, hydrogen gas produces low carbon dioxide when it is produced and burns a unit of weight and can be readily transformed into electricity using a fuel cell. It might make a great

alternative energy resource in the future. Compared to the existing chemical method of producing H_2, biological H_2 generation provides cleaner H_2 in a sustainable manner with simpler technology and more appealing potential. Even though the current industrial hydrogen production system relies on chemical processing units, the current biohydrogen research trend indicates that industrial production of biohydrogen could become a reality very soon. Also, 1, shows potential pricing for hydrogen and fossil fuels until the year 2015. The image below implies that although prices for fossil fuels are expected to rise, those for hydrogen are expected to fall. These prices will cross at the $10 kWh-1 range by 2018 [5]. However, by about 2015, the price of hydrogen will be competitive with that of fossil fuels due to its better utilization efficiency (η=1.35).

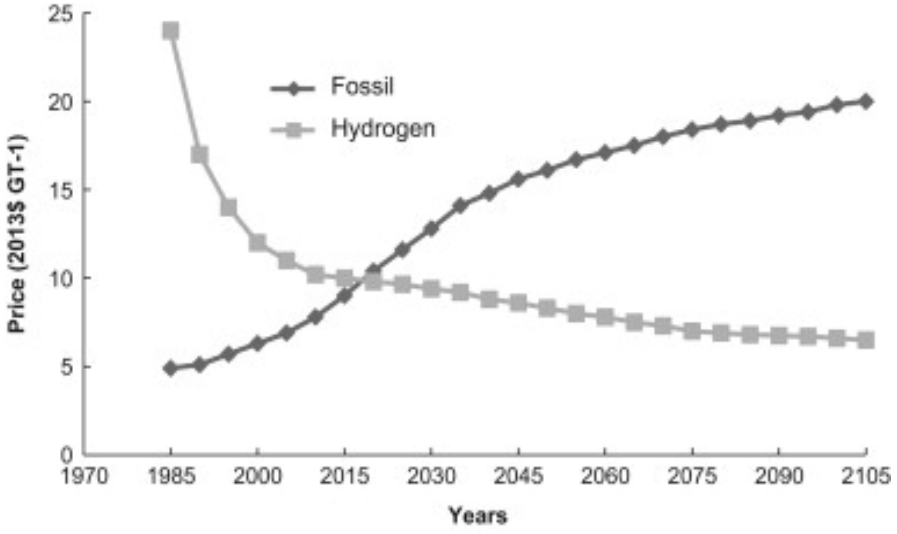

Figure 1: Fossil fuel and hydrogen price predictions vs. time (Hamad et al., 2014)

1.1 NEED FOR BIO-HYDROGEN

For global prosperity, energy is essential and hydrogen being the cleanest source of sustainable energy offers tremendous potential. The vital connection between sustainable energy services and renewable energy sources is made possible by hydrogen fuel cells and associated hydrogen technologies [3]. The energy density of hydrogen is higher and on combustion it yields water that do not contribute to the secondary pollutants. Therefore, biohydrogen production is a sustainable method of generating hydrogen gas using biological organisms such as bacteria, algae, or archaea. As an eco-friendly and clean biofuel, hydrogen offers many great qualities. Hydrogen, which has the chemical formula H_2, is a colorless, tasteless, odorless, and highly flammable gas at room temperature. Hydrogen possesses a boiling point of 22.28 K, a combustion energy of 120 MJ/kg, and a heat capacity of 14.4 kJ/kg K [6]. In the last 50 years, as the energy demand is high, bio-hydrogen gas production has constantly gained attention. As an alternative source of energy, the necessity of hydrogen has increased and is expected to contribute 8-10% in the energy market by the year 2025 [7]. It is anticipated that by 2025, hydrogen will account for around 11% of the overall 36% share of renewable energy, and that it may even reach 34% of the total renewable energy share of 69% of the total by 2050 [9, 10]. At present, natural gas produces 40% of H_2, heavy oils and naphtha produce 30%, coal produces 18%, electrolysis produces 4%, and biomass produces roughly 1% of H_2.[8]. Everything that is produced has an impact on society, the economy, and the environment, all of which determine its sustainability. The advantages of biohydrogen for society, economy, and environment are summed up in Fig 2:

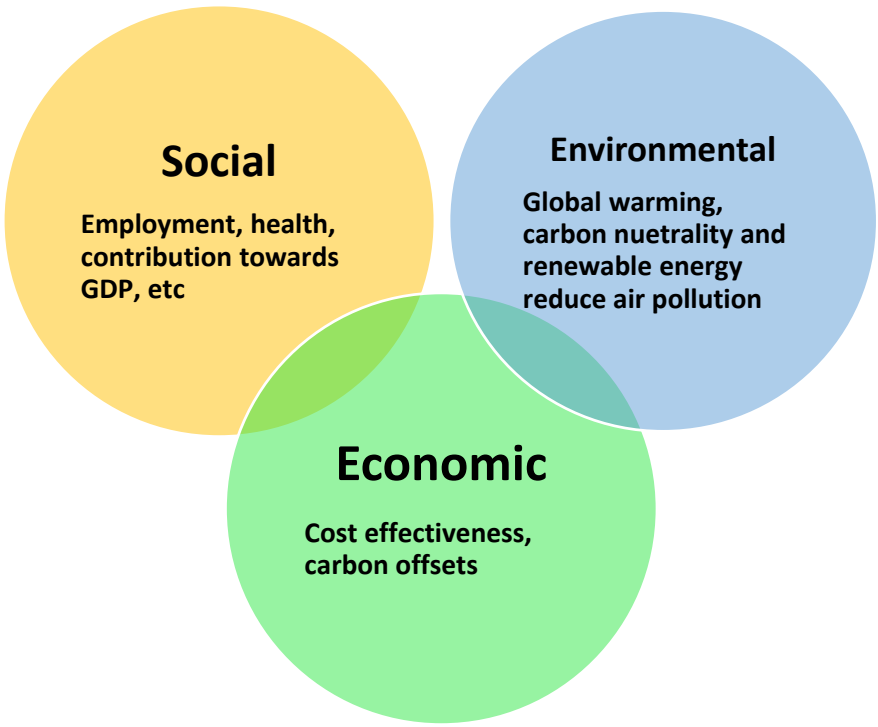

Figure 2: Benefits of Bio-hydrogen (Rathore et al; 2019)

Although there had been many researches since these years, a current possibility for the industrial production of hydrogen from the biological sources is a boon to this field. After H_2 has been used as a transportation fuel, improvement was seen in 2003. Some of these, like the International Journal of Hydrogen, Energy, Renewable and Sustainable Energy Reviews, are devoted exclusively to bioenergy. Since 1984, over 100 countries have conducted research on biohydrogen, and approximately 7834 papers have been published. China and the USA have contributed significantly to this research, with India coming in third [4,9]. The majority of the biohydrogen publications in SCOPUS, 2016, have been research articles (73%), followed by conference papers (11%) and review articles (10%). The absence of large-scale biogas plants in India prevents biogas from reaching its full potential. The generation and utilization of biogas are clearly far from the expected numbers based on these

tables. The conversion and utilization of garbage into biogas has enormous potential [10,11]. The industry has been able to realize energy savings for the adopted technologies, which has increased profitability.

1.3 DARK FERMENTATION – A COMMERCIALLY VIABLE PROCEDURE

Many new perspectives on the financial viability of dark fermentative biohydrogen production were exchanged. According to their findings, dark fermentation techniques promise to be more suitable for industrial use and sustainable production, and they also provide the possibility of producing biohydrogen [17]. In contrast to light fermentation, dark fermentation produces more hydrogen per unit area (HPR) and can use more complicated substrates, such as organic waste and wastewater and is also weather-independent. [18,19]. Although dark fermentation produces a lower yield than other processes, it has the distinct advantage of having a high production rate. Recently, stable compartments of mixed strains have been used to steadily boost production rate; additionally, sophisticated technologies have improved strain stability. Also, dark fermentation is advantageous over other techniques. Light energy is not directly needed for dark fermentation to occur. It can continuously produce hydrogen all day and all night long. When comparing dark fermentation to photo-fermentation and bio-photolysis, the hydrogen evolution rate—the quantity of hydrogen produced per unit of time—is higher [20-23]. The utilization of a broad range of potential substrates, such as organic waste materials and renewable biomass, can be achieved through dark fermentation, leading to comparatively cheaper costs. Complete conversion yields 12 mol hydrogen atoms from 1 mol of glucose:

$$12H_2 + 6CO_2 = C_6H_{12}O_6 + 6H_2O$$

With the process of dark fermentation, this yields 4 mol of hydrogen. Organic acids such as propionic, butyric, and acetic acids are the reaction's byproducts [32,33].

$C_6H_{12}O_6 + 6H_2O = 2CH_3COOH + 4\ H_2 + 2CO_2$

Through the utilization of an already-existing reactor and simple bioreactor design, dark fermentation could be simply scaled up [24]. Valuable products such as ethanol and VFA (acetic acid, butyric acid, lactic acid, etc.) can be recovered from dark fermentative effluent (DFE) and used as final products or as feedstock for other processes. Dark fermentative effluent, which uses starch as a substrate, primarily comprises butyrate and acetate. Numerous factors, including substrate kinds and concentrations, microorganism types, inoculum ages, pure or mixed bacterial cultures, pH levels, and temperature, significantly affect the generation of H_2 [25,26,27]. Depending on the final product that is produced, the fermentation of glucose yields varying amounts of H_2 and CO_2. As acetic acid and butyric acid are the end products, respectively, four moles and two mol of H_2 mol^{-1} glucose can be produced. The H_2 yield for the formation of mixed acid varied from 1 to 2.5 mol H_2 mol^{-1} glucose. H_2 is not produced during the synthesis of ethanol and lactic acid and the production of propionic acid consumes H_2 [26,28].

1.3.1 Selection of the microorganisms for hydrogen production from dark fermentation

Bacteria are the predominant microorganisms used in dark fermentation, a biological process for producing hydrogen. The viability of numerous rumen fungal strains for fermentation has not yet been investigated, despite reports that they can create hydrogen. Numerous fermentative hydrogen-producing bacteria have been identified and somewhat characterized thus far. These bacteria fall into three main categories: aerobes, facultative anaerobes, and strict anaerobes [24]. In the research study, we mainly worked with the genus Enterobacter which includes *Enterobacter aerogenes* and *Escherichia coli.* Four nickel-iron hydrogenases—hydrogenase-1, hydrogenase-2 (Hyd-2), hydrogenase-3 (Hyd-3), and hydrogenase-4 (Hyd-4)—are encoded in the *E. coli* genome. From these four hydrogenases, Hyd-3 is a component of the active anaerobic FHL complex and is encoded by the hycoperon Hyd-1 and Hyd-2 are known as uptake hydrogenases which catalyze hydrogen oxidation and are encoded by the hya and hyb operons respectively. Prior to Maeda and colleagues' latest findings, it was believed that Hyd-3 solely had hydrogen-producing activity. However, Hyd-3 also has hydrogen absorption activity [53]. As a result, Hyd-3 is a reversible hydrogenase that mostly catalyzes the synthesis reaction while both generating and using hydrogen. Hyd-4, which is encoded by the hyf operon, was initially suggested to have a second FHL complex due to its strong similarity with the hyc operon. Later research, however, revealed that Hyd-4 did not replace Hyd-3 in the production of hydrogen and that the hyf operon is not expressed in E. coli [54, 55]. Instead, it can be activated in the presence of effector-independent mutant proteins of FhlA (transcriptional activator of the FHL complex) or HyfR, an FhlA homologue of Hyd-4 [53-56].

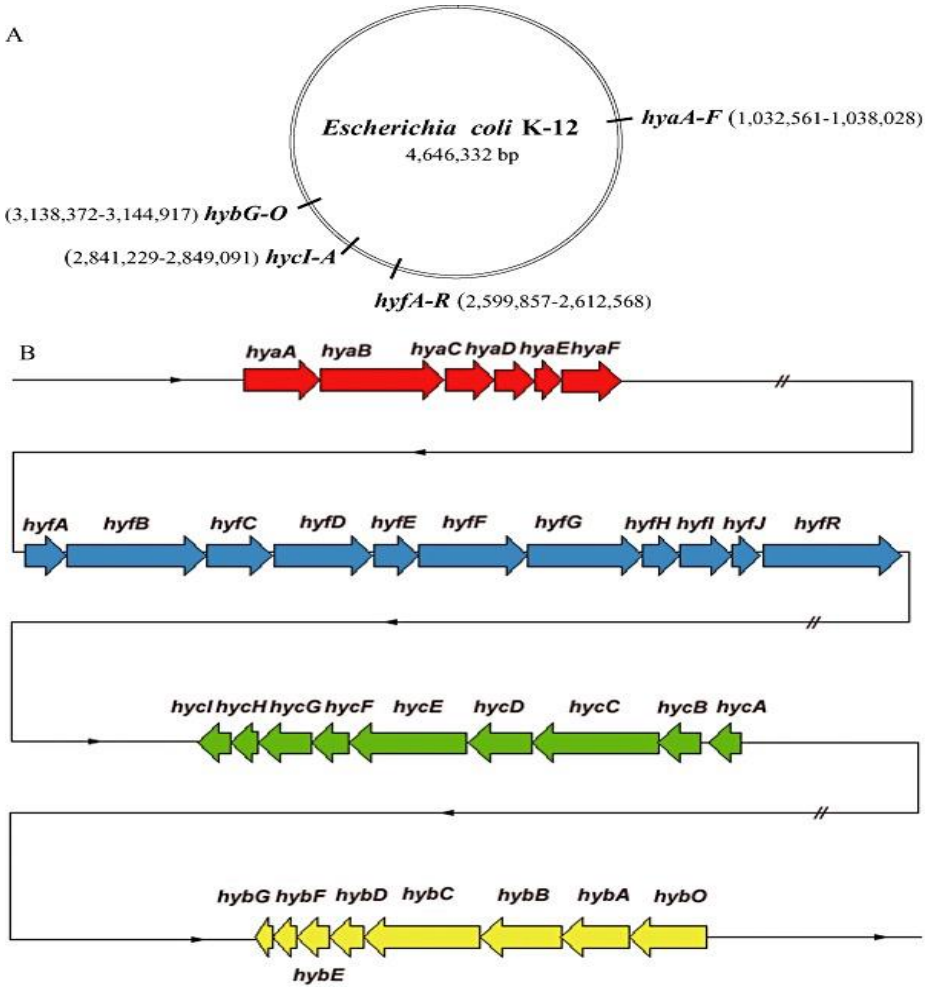

Figure 3: A. On the E. Coli K12 chromosome, the locations of the four structural hydrogenase operons.

B. The positions of the corresponding genes on the genome map are indicated by the values in brackets.

1.3.2 Metabolic pathways of hydrogen production in dark fermentation

The primary substrate for the metabolism of many anaerobic microbes is hydrogen. If energy-rich hydrogen molecules are accessible, these microbes can use them. They can also use the electrons from hydrogen oxidation to make energy. The organisms have an excess of electrons produced during metabolic activities due to the reduction of protons, which results in the production of hydrogen molecules, in the absence of external electron acceptors [29]. The primary enzymes controlling the metabolism of hydrogen are known as hydrogenases. The two fundamental hydrogenases are [Fe Fe]-hydrogenase and [Ni Fe]-hydrogenase. They differ in their evolutionary relationships and possess distinct active sites. Also, [Fe-Fe]-hydrogenases are more active in generating molecular hydrogen than [Ni-Fe]-hydrogenases, which are mainly involved in the oxidation of molecular hydrogen. It must be observed that [Fe-Fe]-hydrogenases are typically oxygen-sensitive. The reversible process is catalyzed by these enzymes: [Fe Fe]-hydrogenase and [Ni Fe]-hydrogenase. In any case, the core glycolytic process is typically activated by glucose or other carbon sources obtained from plant biomass or waste materials, producing pyruvate, ATP, and NADH. As of the present, three different kinds of critical metabolic sites are known to create hydrogen. The final byproduct of glycolysis, pyruvate, is broken down by pyruvate: formate lyase (PFL) into acetyl-CoA and formate. The formate: hydrogen lyase complex (FHL) then breaks formate down into H_2 and CO_2 [30]. The production of two H_2/glucose is the normal reaction for facultative anaerobes, such as Enterobacteria and *E. coli*. Glycolysis produces ATP and NADH, which can be utilized to make a range of reduced products in addition to biological hydrogen production. While some lactate is produced, facultative anaerobes primarily create ethanol as their final product. Various products, such as ethanol, butyrate, butanol, and acetone, can

be produced in stringent anaerobes based on the microorganisms and fermentation circumstances [24, 31]. Compared to other approaches, the dark fermentation process produces a larger amount and rate of biohydrogen. The low hydrogen concentration—roughly 40–60%—is the problem. For this reason, the fuel cells cannot use the fermentative effluent gas without a purification step [33,34].

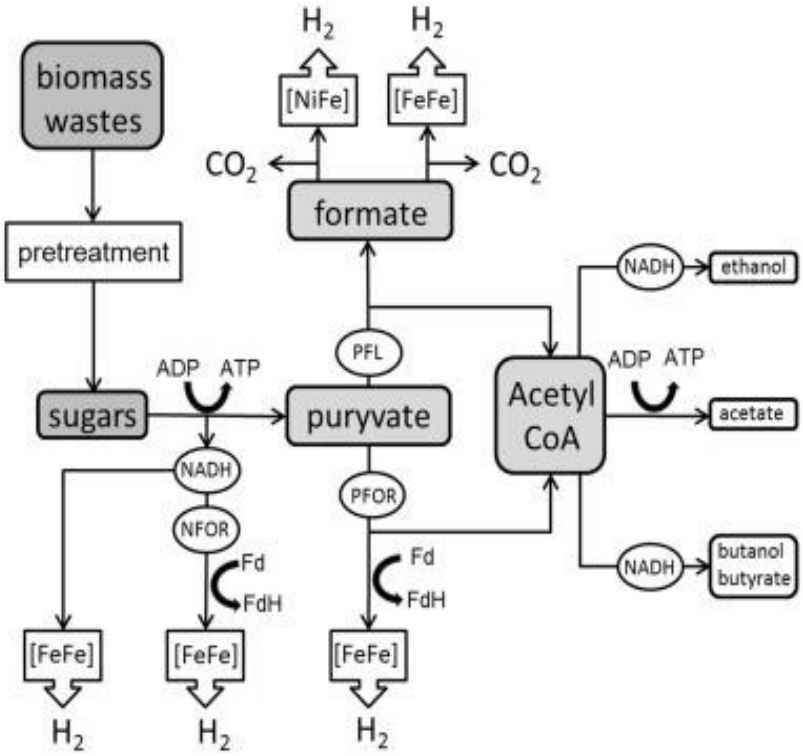

Figure 4: Metabolic pathways for the conversion of substrate during dark fermentation (Łukajtis et al; 2018)

1.4. ORGANIC ACIDS

The two aspects of the formation of organic acids are the metabolic pathways involved in biosynthesis and the industrial process strategy employed. Acids with a "long" biosynthetic pathway from glucose—which includes a large portion of the glycolytic pathway and the tricarboxylic acid cycle—and those with a "short pathway," which is essentially a glucose biotransformation, are classified into two categories. The pathways' regulation, upcoming advancements in metabolic control theory, and genetic modifications associated with them are all taken into consideration [35,36]. VFAs are organic acids with low molecular weight and significant hydrophilicity. It exists in the air, water, and soil, among other environmental compartments [40]. As they are vital to the metabolisms of higher plants, animals, and microbes and are the building blocks of many compounds like esters, ketones, aldehydes, alcohols, various organic acids like acetic acid, butyric acid, valeric acid, propionic acids and alkanes as well as biopolymers and biofuels. Acetic acid has a boiling point of 117 °C, while caprylic acid has a boiling point of 239 °C. As the number of carbon atoms per molecule increases, their solubility in water diminishes. For example, acetic acid is completely miscible in water, butyric acid is miscible, and caprylic acid has a solubility of 0.7 g dm^{-3}. As the number of carbon atoms per molecule increases, so does the density of VFAs. These acids can be distilled at atmospheric pressure and are typically made chemically [40,42]. Various catabolic pathways involved in the fermentation process are necessary for the generation of solvents and VFAs.It is possible to obtain VFA concentrations of 8–30 g/L using dark fermentation [35-39]. The synthesis of VFAs from FWs fermentation is significantly impacted by operational factors such as temperature, pH, hydraulic retention time (HRT), and organic loading rate (OLR), both in terms of yield and the relative distribution of the

various products. While most studies looked at each parameter's impact separately, they do have a synergistic effect on the microbial communities engaged in fermentation processes as well as on cellular metabolism [35].

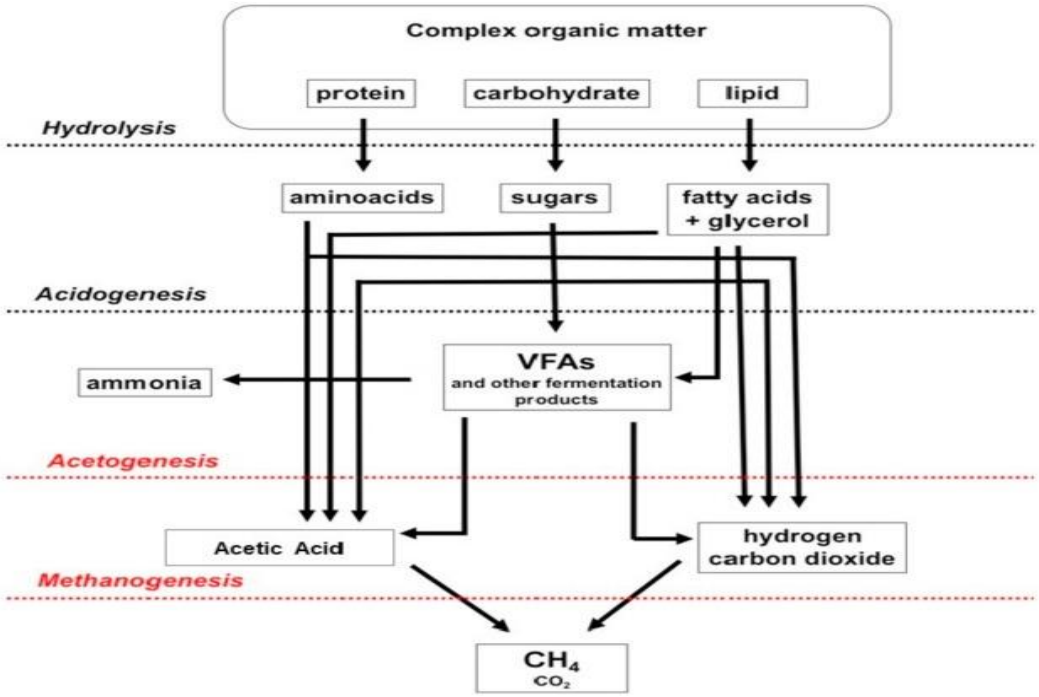

Figure 5: The anaerobic digestion process cascade. To obtain maximum VFAs, the phase separation is indicated by the red line. (Strazzera, et al; 2018)

1.4.1 Importance of volatile fatty acids in industries

VFAs are the main source of energy for ruminants, meeting around 80% of their maintenance energy needs, and a vital source of carbon and energy for the microorganisms engaged in the metabolism of the organic carbon cycle on Earth [41]. During wastewater treatment, sludge is an organic waste that is unavoidable. Sludge comprises a variety of microorganisms in addition to 10% (w/w) carbohydrates, 50% protein, 10% fat, and 30% miscellaneous materials, such as fibre and ribonucleic acid (RNA). The components of sludge—protein, fat, carbs, and RNA—have been effectively transformed into biodiesel, biogas, bioplastic, biosurfactants, bio-flocculants, biopesticides, ethanol, and other products in recent years [40, 43]. The importance of the various volatile fatty acids can be listed in the table below: -

TYPE OF VOLATILE FATTY ACIDS	**APPLICATIONS IN INDUSTRY**	**REFERENCES**
Acetic acid	Vinyl acetate-derived polymers are used in the manufacture of various derived molecules and can be found in wood panels, paper bags, cardboard boxes, labels, adhesives, latex paints, premium paper coating, textiles, and cement additives. In addition, it is utilized as a precursor for cellulose acetate, alcohol acetates, halogenated acetic acid, acetic anhydride, citrate esters, diketene, methyl acetoacetate,	Bastidas-Oyanedel et al; 2015

	acetoacetamides, and acetoacetylated polymers. These precursor molecules have numerous uses in the manufacturing of agrochemicals (fungicides, insecticides), pharmaceuticals (aspirin, vitamin E, beta-lactam and oxacillin antibiotics, antiepileptic drugs), and dye, colorant, and polymers.	
Butyric acid	It is widely recognized for its anticancer effects and is used in the chemical and pharmaceutical industries. It works by inducing morphological and biochemical differentiation in a range of cells, which simultaneously suppresses neoplastic features. Butyric acid derivatives have been used to treat malignancies, hemoglobinopathies (such as leukemia and sickle cell anaemia; SCA), and to shield hair follicles from alopecia brought on by chemotherapy and radiation therapy. The primary use of butyric acid in the chemical industry is in the production of polymers made of cellulose acetate butyrate. The addition of a butyryl group to cellulose acetate polymers	Dwidar et al; 2012

	results in a polymer with improved flexibility, increased hydrophobicity, and resistance to light and cold that is more soluble in organic solvents.	
Propionic acid	In addition to effectively preventing the formation of mould on surfaces, propionate salts can also stop *Listeria monocytogenes* from growing when mixed with lactic and acetic acids. Within the plastics sector, it finds application in the production of cellulose-based plastics, which include air filters, textiles, reverse osmosis membranes, lacquer formulations, and moulding polymers. Pharmaceutical companies mainly employ sodium propionate in animal therapy to treat wound infections and as a component of anti-arthritic and conjunctivitis medications. Propionate salts and butyl rubber are used as scent bases in the cosmetics sector to enhance the consistency and longevity of goods. Propionate salts and butyl rubber are used as scent bases in the cosmetics sector to enhance the consistency and longevity of goods.	Gonzalez-Garcia et al; 2017

Caproic acid	As an emerging platform chemical, capric acid has immediate applications as an antibacterial, feed additive, and plant growth stimulator. Additionally, it can serve as a precursor for a variety of products, such as paint additives, lubricants, scents, and medications.	Chen et al; 2017

Table 1: Applications of various volatile fatty acids

1.4.2 Recovery of volatile fatty acids

1. Recovery by adsorption on activated carbon

As the VFA concentrations in mineral-rich streams is low, recovery of the organic acids from the fermented wastewaters is challenging. VFAs must be separated from extremely diluted aqueous streams using an affinity agent. Effective VFA recovery by affinity separation requires careful consideration of the design and selection of an affinity agent. Although retrieving the VFAs from the affinity agent becomes difficult, a strong affinity may result in an efficient VFA separation. For the purpose of recovering VFA, affinity separation techniques such as adsorption and liquid-liquid extraction have also been investigated. Granular activated carbon (GAC) gives microorganisms more places to adhere by acting as a conductive substance with a noticeably specific surface area [57]. By reducing the distance between syntrophic partners and improving mass transfer, this raises the production of biofilms and increases the yield of the target products. GAC promotes electron sharing and

interactions among bacteria by facilitating electron transfer, modulating metabolic pathways, and enabling the creation of an electron transfer chain [58]. The method of producing acid by hydrolytic fermentation is further optimized by this technique. Pretreatment methods are currently the main focus of most research on the anaerobic fermentation of lignocellulosic biomass [59, 60]. Studies examining the effects of incorporating GAC on microorganisms and VFA generation in lignocellulosic biomass without the use of intricate pretreatments or enzymes have not been conducted. Substances can be separated from complex and diluted solutions using adsorption. The mass of adsorbate per kg of adsorbent is a common definition of capacity which is used to express the efficacy of adsorption. To finish the recovery procedure and renew the adsorbent, the adsorbate must be desorbed from the adsorbent once adsorption has taken place. High adsorbent selectivity for the target component is crucial when applying adsorption to complicated solutions like fermented wastewater [48]. Purolite A133S, a tertiary amine-functionalized resin, was found to give 35% greater adsorption yields than granular activated carbon (GAC) when single- and multicomponent mixes of volatile fat acids (VFAs) (acetic, propionic, and butyric) were adsorbed onto it [49]. Amberlyst A21, a tertiary amine, was shown to be an effective candidate for adsorption/desorption investigations after an effective VFA adsorption from a real grape pomace digestate. The adsorption yields for acetic acid and actual grape pomace digestate were approximately 61 and 11%, respectively [50,51]. They also discovered that adding NaOH to ethanol and water during desorption produced a yield of about 99%. Furthermore, the impact of the resins' amine functionalization type on a VFA mixture revealed that nonfunctionalized resin exhibited greater selectivity compared to functionalized resin (primary, secondary, and tertiary) [52].

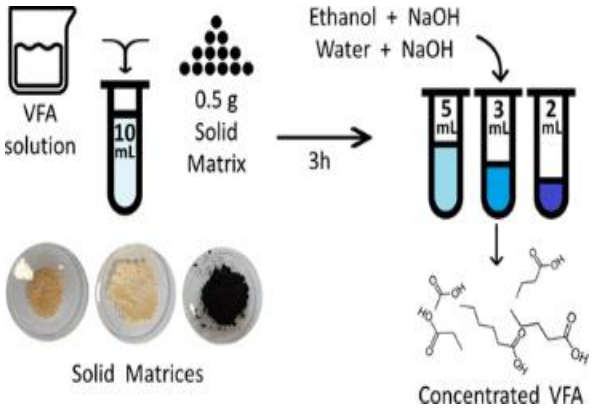

Figure 6: Recovery of volatile fatty acids by granular activated carbon adsorption (Rizzioli et al; 2021)

2. Recovery by chemical precipitation

By adding sulfuric acid, precipitation is a straightforward and effective technique for extracting calcium acetate, or acetic acid, from fermentation broth. The recovery step's primary operating parameters, the temperature at which the recovered acetic acid solution concentrated, and the kind and quantity of adsorbent utilized to remove pigment (color) were all optimized. Salts based on calcium are added to the solutions to neutralize the organic acids; the resulting calcium carboxylate solutions are then typically concentrated by evaporation. Calcium acetate was precipitated and more easily removed when the molar ratio of calcium acetate to sulfuric acid was 1:1 and the pH was raised to a level higher than the pKa (3.86) [110]. This made it possible to separate and recover the acetic acid supernatant more successfully.

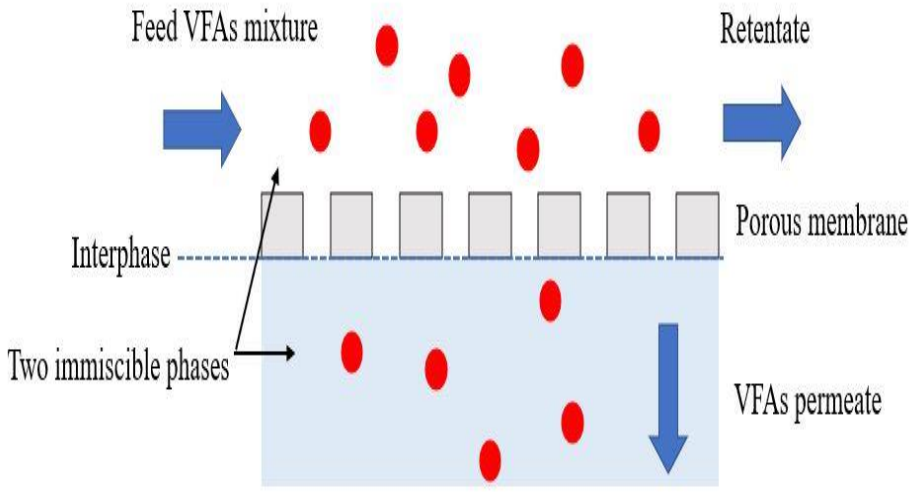

Figure 7: Recovery of volatile fatty acids by chemical precipitation of calcium salts (Sukphun, et al; 2021)

Calcium acetate solution may be added to aid with precipitation, which took more than 18 hours to complete and could be mixed at up to 220 rpm. After that, crystallization or more separation must be done [105, 108]. Another common technique is precipitation, which involves a series of processes depending on the type of precipitant to separate the VFAs. It was discovered that a key element in the precipitation-based acetic acid isolation process was the molar ratio of calcium acetate to sulfuric acid [106, 107, 108]. The separation and final purification steps of the calcium precipitation process, which generates a significant amount of solid waste and can cost up to 50% of the total production cost, have been used. While conventional calcium precipitation is straightforward and dependable, it is costly and hostile to the environment since it uses sulphuric acid and calcium oxide and generates a significant amount of solid waste in the form of calcium sulphate sludge [109].

2. REVIEW OF LITERATURE

2.1 HOW THE CONCEPT OF BIOHYDROGEN CAME INTO EXISTENCE?

Rapid urbanization and industrialization are largely dependent on energy. The Nobel Laureate Dr. Richard Smalley asserts that "energy is the single most critical challenge facing humanity" [61]. The primary energy sources used in today's systems are fossil fuels, which have finite supplies. The growing amounts of carbon dioxide (CO_2) in the atmosphere and worries about global warming pose a threat to the world's ability to continue using fossil fuels to supply the majority of its energy needs. The rate at which the global need for energy is increasing is concerning. Energy consumption increased from 82.2 to 86.7 MBD (million barrels per day) between 2004 and 2007, with an average annual growth rate of 1.8%. [61,62,63]. The supply of energy will not always be met by petroleum output. Over the following 15 years, the pace of oil production is anticipated to reach its peak. It will be necessary to investigate alternative fuel sources to meet the growing demand for energy. Thus, one of the most important and pressing issues of our day is energy security [63, 64]. The availability of fossil fuels, especially crude oil, is limited to a few regions of the world, and political, economic, and ecological variables determine how long supplies will last. They will grow more expensive when those reserves run out. The development of new technologies for electricity generation will become more crucial as global oil resources diminish. Reducing the use of fossil fuels and increasing the availability of alternative energy sources and use of fossil fuels and improving the availability of ecologically friendly energy sources, such as fuel cells and renewable sources are essential to mitigate the effects of global warming [63,65].

2.1.1 Hydrogen Economy- A progress towards climate and energy

Fuel cells and hydrogen, together known as the "hydrogen economy," hold immense potential for addressing our worries about climate change and supply security in a way that is very different from the norm. It's possible that the 21st century will be the fuel cell century, in contrast to the 19th century, which was the century of the steam engine and the 20th century, which was the century of the internal combustion engine. Therefore, the creation and commercialization of cutting-edge technology for the production, storage, and use of hydrogen will expedite the shift to a hydrogen economy [63].

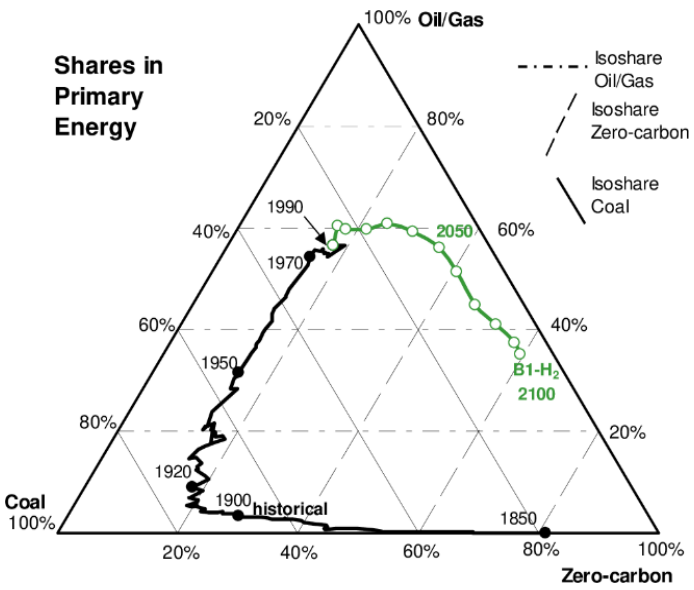

Figure 8: A sustainable development scenario for the hydrogen economy in the 21st century (Barreto et al; 2003)

As per the "18S approach", which focuses on the aspects and components of innovation in the hydrogen system whose assessment ought to motivate public and private stakeholders to assess, comprehend, and put into practice policies and plans in order to accomplish a seamless shift to a hydrogen economy [87]. The analysis focuses on the critical role that corporate actors play in any transition, particularly disruptive ones like the one towards a hydrogen economy [88, 89]. It also examines the role of visions in the transition towards a sustainable chemical industry, which, among other things, depends on CO2 as a feedstock and hydrogen as a reducing agent [90, 91]]. In order to prevent sunk costs, it is crucial for stakeholders to invest in long-lived, low-carbon capital stocks that the policy's execution "appears certain and credible".

Figure 9: The 18S method for the hydrogen transition (Falcone et al; 2021)

A list of indicators is provided by prospective investors to consider in order to determine whether or not a hydrogen economy is taking shape. An emphasize that the shift is a worldwide challenge that requires acceptability in industry and the general public, pointing

out that "without market and hydrogen acceptance in the rest of the world, investments will be wasted [89, 90, 91]."

2.2 SUSTAINABILIY ASSESSMENT OF BIO-HYDROGEN

Recent life cycle assessments have indicated that the development and use of some bioenergy fuels may have deteriorated environmental performances (LCA) [12, 13]. Given that they are produced from low-cost organic wastes rather than intentionally grown plants, some bioenergy systems are thought to pose less of a risk to sustainability. The adoption of biohydrogen technology is contingent upon the state of the infrastructure in a given location. Therefore, to facilitate the application of biohydrogen technology, infrastructure development must be specified and built [13]. In addition to technological evaluations, sustainability results should look into the variables influencing fuel and energy use throughout different regions of the world. Several researchers have employed diverse gasification techniques, a range of gasification agents, and examination of the impacts of various operational factors, including temperature, the proportion of gasification agents to biomass, biomass feed compositions, moisture contents equivalency ratio, on the production of syngas and the syngas's calorific values [13-15]. An energy study of a hybrid biomass system for producing biohydrogen entails assessing the system's energy consumption and conversion efficiency. To maximize the system's energy use and pinpoint areas for development is the aim of energy analysis. To optimize a hybrid biomass system for the production of hydrogen, energy analysis is essential. In order to maximize efficiency, minimize expenses, and lessen environmental effect, the energy analysis's findings can help with decision-making and system optimization [14]. So, for proper sustainability assessment, it is also necessary to know the advantages and disadvantages of the various

bio-hydrogen techniques, Hydrogen can be produced from a variety of sources, but each source has certain advantages and disadvantages that can be outlined in the below Fig 3.

Process	Advantages	Disadvantages
Solar gasification	Good hydrogen yield	Effective solar collector plates are required
Thermo-chemical gasification	Higher conversion can be achieved	Gas conditioning and tar removal is to be done
Pyrolysis	Gives carbonaceous material with oil, chemicals and minerals	Catalyst deactivation will occur
Supercritical conversion	Sewage sludge can be used easily, difficult by gasification	Selection of supercritical medium
Direct bio-photolysis	H_2 can be produced directly from water and sunlight	Requires high intensity of light, low photochemical efficiency and O_2 is inhibitory.
Indirect bio-photolysis	Blue green algae can produce hydrogen from water. It has the ability to fix N_2 from atmosphere	Uptake hydrogenates are to be removed.
Photo-fermentation	A wide spectral energy can be used by photosynthetic bacteria.	O_2 is inhibitory on nitrogenase enzyme and light conversion efficiency is low.
Dark fermentation	It can produce H_2 without light. No oxygen limitations and can produce several metabolites as by-products. Various substrates can be used in this anaerobic process.	Relatively lower H_2 yield. At higher H_2 yield, process becomes thermodynamically unfavorable.
Two-stage fermentation	Can produce relatively higher H_2 yield. By-products (metabolites) can be efficiently converted to H_2.	Requires continuous light source which is difficult for large scale processes.

Figure 10: Advantages and disadvantages of various techniques of hydrogen production (Pandu and Joseph 2012)

Due to the poor light utilization efficiency and challenges in building reactors for hydrogen production, the conversion of solar energy into hydrogen by photosynthetic bacteria is not easily applied in reality [16]. On the other hand, fermentative hydrogen production (mainly dark) has the benefits of a quick hydrogen production rate and easy operation. Additionally, it can produce fermentative hydrogen using a various type of microorganisms that has been discussed earlier.

2.3 SYNERGYSTIC RELATIONSHIP BETWEEN DIFFERENT TYPES OF FERMENTATIVE BACTERIA

From the various research studies and following the physiological data, metabolic by-products, and hydrogen generation were analyzed. The results showed that acetone-butanol fermentation produced approximately 30% of the overall yield, while photosynthetic bacteria produced 70% of the hydrogen [81]. Because of their great metabolic diversity, microorganisms can ferment a variety of substances, both within and between species or strains. An organic molecule serves as the final electron acceptor in fermentation, an anaerobic redox process in which partial substrate oxidation occurs (typically the same substrate itself or an intermediary from substrate oxidation). Microbial H2 metabolism is extremely diverse and pervasive at the taxonomic, community, and ecosystem levels, as several recent investigations have shown [84]. Sugars and amino acids are common fermentation substrates; glucose is typically thought of as the model substrate. H2 is not created as the only reduced product in any thermodynamically possible dark fermentation process carried out by recognized microbes; rather, it is produced in conjunction with volatile fatty acids (VFA) and/or alcohols [85,86]. Microbes do not benefit from H2 evolution in and of itself, but some components of the microbial community require it in order to get rid of extra electrons. During microbial fermentation, molecular hydrogen synthesis is a means to effectively release surplus reductant as a diffusible gas, mostly by the regeneration of NAD+ from NADH. There are two phases to the co-fermentation process. The first is the dark fermentation and cellulose breakdown stage. In this stage, anaerobic bacteria (Enterobacteriaceae) and cellulose-degrading bacteria (*Proteiniclasticum*) hydrolyze cellulose to carbohydrates in order to release hydrogen initially and produce organic acids

(mostly acetate and butyrate) [82]. The large amount of substrate promotes high HPR (a phosphocarrier protein that controls energy metabolism by forming a cooperative interaction with several enzymes in *E. coli*) during dark fermentation and quick replication of acetone-butanol fermentation. Nevertheless, the buildup of organic acids prevents bacteria from photosynthesis [82, 83]. Bacteria that break down cellulose and produce hydrogen also facilitated the transfer of carbon sources. Research has indicated that a significant build-up of organic acids can lower the pH of fermentation and prevent the formation of hydrogen. Thus, the transfer of carbon and the creation of hydrogen will both benefit from the presence of acid-degrading bacteria. Meanwhile, at the terminal phase of co-fermentation, certain non-hydrogen-producing bacteria, like *Sporolactobacillus*, occur primarily to reduce acidic stress by consuming butyrate and lactate, which act as acid neutralizers to promote stable hydrogen production and thereby extend the duration of hydrogen production [83].

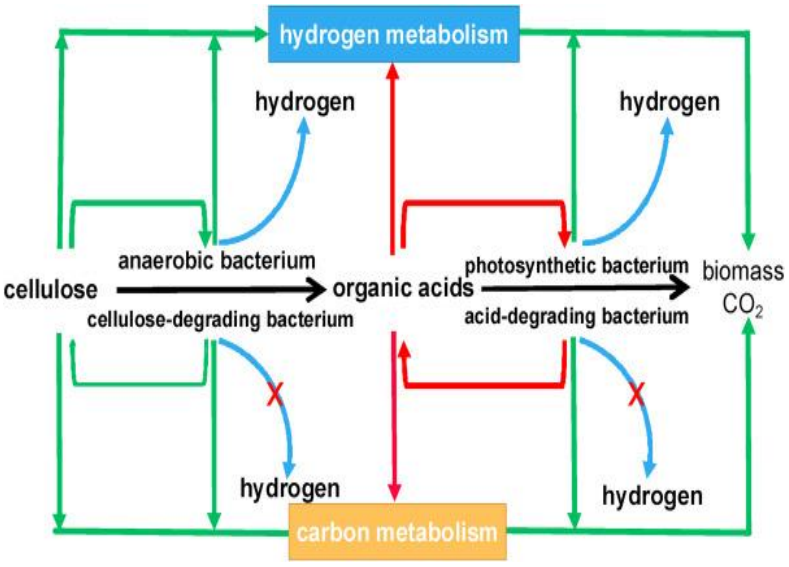

Figure 11: A syntrophic co-fermentation paradigm for the synthesis of biohydrogen. (Wang, et al; 2021)

2.4 RECENT TRENDS IN BIO-HYDROGEN PRODUCTION BY DARK FERMENTATION

Despite the fact that dark fermentation has many advantages, the majority of research have shown hydrogen yields of less than 2 mol/mol-glucose. Because of this, the majority of research conducted in the last few decades has been on how to increase the hydrogen production [69]. Since 2000, the production of biohydrogen has expanded using a number of methods and procedures, as the graph below illustrates. In the year 2020, there has been a maximum biohydrogen production prior to the previous years.

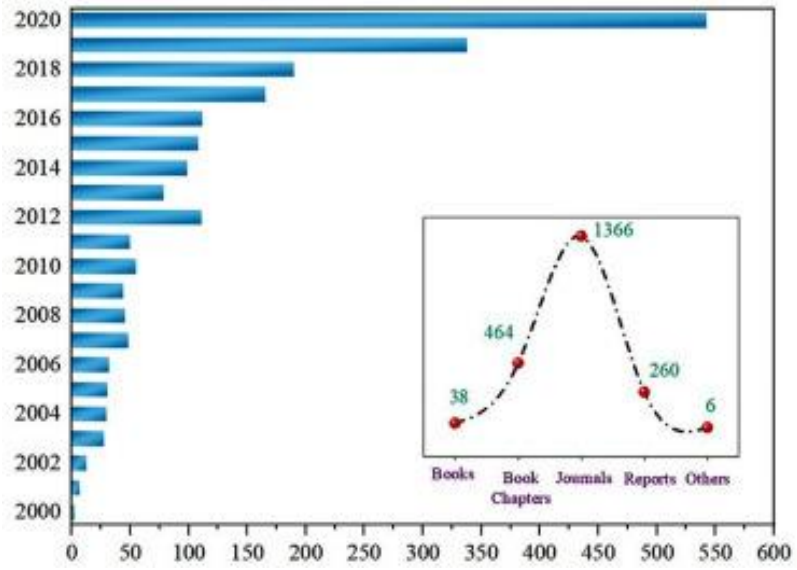

Figure 12: A perspective, cost-effectiveness, constraints, and future scope of trends in dark biohydrogen production strategies and their connections to the shift to a low-carbon economy (Sharmila et al; 2012)

A number of strategies have been devised to maximize the fermentation process's effectiveness. In order to increase the accessibility of complex organic compounds, some writers have investigated various pretreatment techniques (such as homogenizer, acid, alkali, ultrasonic, and biological pretreatments) in order to advance. Additionally, some researchers have improved the operating conditions (such as temperature and hydraulic retention time) in order to maximize the productivity of the bacteria that produce hydrogen.

In addition, a number of researchers co-fermented several feedstocks with complicated features in an effort to maximize their utilization throughout the dark fermentation process [70,71].

2.4.1 Transfer of Bio-H2 Production Technology

To address the current global energy dilemma, the scientific community has suggested a bio-H_2 economy. Bio-H_2 can be created from waste biomass using integrated technologies, which has the twin benefits of producing clean energy and using trash. The economic and environmental sustainability of bio-based technologies is evaluated by measuring their effectiveness in process design, resource utilization, and carbon footprint emissions [72]. Global food security, sustainable agriculture, bio-based energy, safe and healthy food, and industrial applications of renewable resources are the five key areas covered by **the National Research Strategy for Bioeconomy 2030** [73] presented by the Federal Ministry of Education and Research (Germany). Biological processes include acidogenesis, bioelectrogenesis, photosynthesis, methanogenesis, fermentation, and acetogenesis can efficiently handle food waste (both avoidable and inevitable) and can be incorporated into bio-based manufacturing [74,75]. As energy demand rises, the shift from a linear economy dependent on fossil fuels to a circular economy will quicken. It has been proposed that biogenic waste might be processed in biorefineries to create bio-based goods and used as a feedstock for the bioeconomy. Biochemicals (organic acids), biomaterials, bioelectricity, biofertilizers, bionanomaterials, animal feed, and biofuels (bio-H2, biodiesel, biohydane, and bio-CH4) can all be produced by biorefineries [76]. The majority of businesspeople and investors view biogas as an energy source. Notwithstanding these advantages, the project including biorefineries still necessitates a high-risk investment because of a number of other

aspects, such as resource availability, waste separation, product efficiency, operational problems, and economic feasibility considerations [77, 78, 79]]. Integrated bioprocessing is one of the bioprocessing technologies that incorporates decarbonization into a sustainable framework to produce bio-based goods that are on par with those made with fossil fuels. For instance, alcohols, bioplastics, microbial lipids, and bioelectricity can all be made using VFAs, which are created during acidogenesis. Additionally, CO2 can be captured and diverted to other necessary molecules [79,80].

Figure 13: Tech-economics of bioprocessing in conjunction with fermentation. (Ahmad et al; 2023)

2.5 MAJOR CHALLENGES THAT WERE SEEN WHILE PRODUCING BIO_HYDROGEN BY DARK FERMENTATION IN INDIA

From various research studies, the major challenges that the world is facing in the recent years and despite of several researches, the incorporation of biohydrogen into the commercial world was a total failure due to the several reasons. The various reasons are addressed in the below:

- **Feedback Inhibition: -** Many fermentations process deals with the issue of feedback or product inhibition, and Bio-hydrogen is not the only inhibitor in cases where H_2 and VFAs generated during the acidogenic process are the primary inhibitors. By changing the electron flow of biochemical pathways, elevated H_2 partial pressure in the system has been directly linked to influence the metabolic shift and varied production of fermentative metabolites (primarily acetate, butyrate, propionate, and ethanol) which in turn, affects the conversion efficiencies of the reactions and overall yield of Bio-hydrogen production [92].
- **Presence of Inhibitors: -** The bulk of inorganic inhibitors in dark fermentation are composed of heavy metals. While small levels aid in dark fermentation through the activation of several enzymes, heavy metals found in excessive concentrations in sludge are harmful [93]. The precise threshold at which dark fermentation is inhibited depends on the heavy metal, feedstock, and substrate types. According to Sharma et al.'s work on municipal solid waste utilizing *Enterobacter aerogenes* and *E. coli*, the inhibitory potential of heavy metals for hydrogen generation is Cu < Cr < Pb < Hg.

- **Partial Pressure: -** One of the delicate factors to take into account when investigating the production of biohydrogen is the partial pressure of hydrogen. If H2 accumulated in the headspace, the system's partial pressure of hydrogen would increase. Le Chatlier's principle states that an accumulation of hydrogen will significantly impede the subsequent reaction [94, 98]. Therefore, the higher the partial pressure of hydrogen in the reactor, the more detrimental an effect it has on hydrogen generation. Studies have also shown that a critical component of prolonged hydrogen production is the partial pressure of hydrogen.
- **Gaseous Environment: -** Oxygen-susceptible attribute of the enzymes impact on hydrogen generation, it is mandatory to sustain an anaerobic atmosphere in the system. Impact of gaseous environment on biohydrogen production has been reported by various authors [95]. Generation of hydrogen was found to be naturally influenced by the existence of varying concentrations of inert or anoxic gases like argon, CO2, and increased hydrogen production by Anabaena variabilis when CO2 was introduced repeatedly during the growth cycle, exposure to CO2 heightened the levels of reductants impacting enhanced hydrogen yield during the process.
- **Hydraulic Retention Time:** - Reactor volume and flow rate, typical fermentation time, design, energy needs, operating costs, and other aspects are all crucial to take into account. In the process of producing hydrogen, optimal HRT is crucial. The most important factor in the entire process is thought to be sourcing microbial cultures that have been vetted for their capacity to withstand the mechanical disruptions caused by the constant volumetric flow [96, 97, 98].

2.6 NEED FOR PRE-TREATMENT

The pre-treatment process helps to boost anaerobic digestion to increase H2 production and speeds up the hydrolysis stage, which lessens the effect of the rate-limiting step. To selectively enrich acidogenic H_2 generating inoculum, a range of mixed cultures were subjected to a number of pre-treatment techniques, including heat shock, chemical, acid, alkaline, oxygen-shock, load-shock, infrared, freezing, etc. [126].

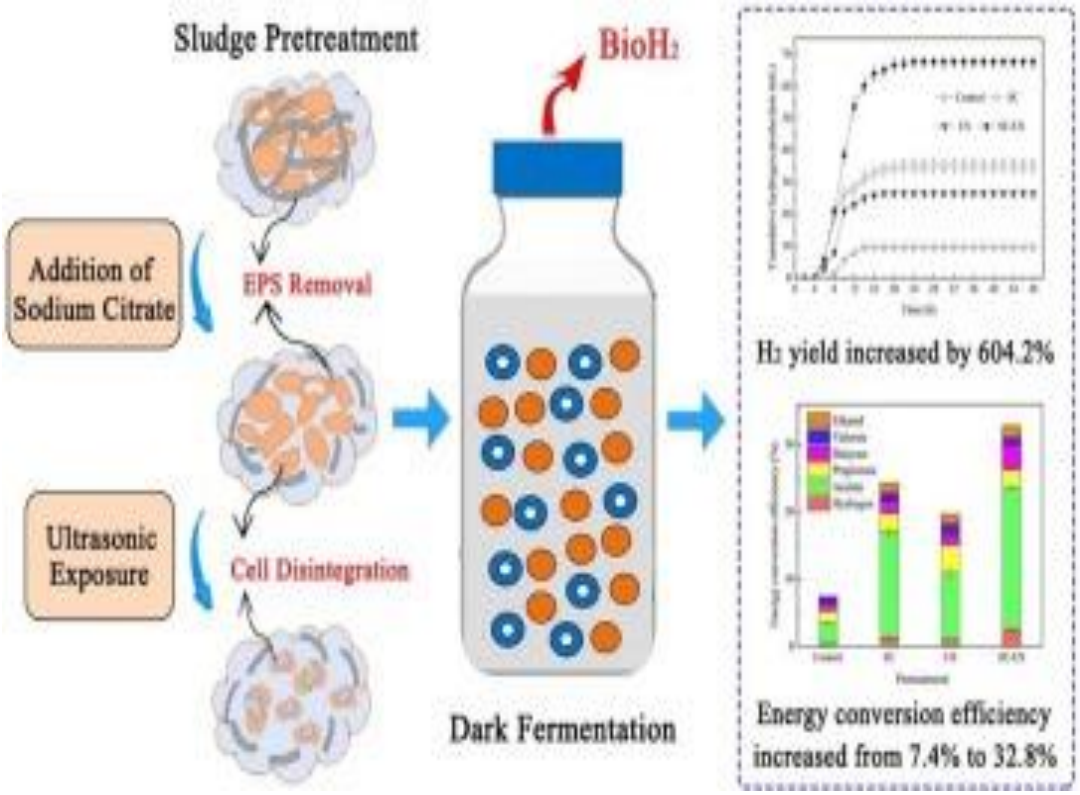

Figure 14: Production of bio-hydrogen from waste activated sludge treated with a combination of ultrasonic and sodium citrate: Microbial community and energy conversion. (Yang and Wang 2020)

The activity of the acidogenic group of bacteria was thought to be essential in controlling the organism's metabolic pathways, which were also significantly influenced by pH. According

to reports, the methanogenic bacteria's ideal pH range was between 6.0 and 7.5, but the acidogenic bacteria operated well below 6.0 pH 4-6. The key for preventing both methanogenesis and solventogenesis was thought to be in the pH range of 5.5–6.0.

Pretreatment techniques for the selective enrichment of anaerobic consortia that produce hydrogen have a significant impact on glucose's acid-forming pathways and H_2 yield [127, 128]. The fermentation pathways of glucose varied depending on the pretreatment techniques used. A drop in H_2 yield was seen in the fermentation route of glucose when it changed from ethanol and butyric types to propionate and butyric types, in conjunction with an increase in temperature or the use of alkaline or acid pretreatment. It was advised that the chemical pretreatment approach be used instead of heat shock and base treatment, despite the fact that certain data suggested the latter. Chemical additions changed the ambient and physiological parameters while increasing yield and productivity [129]. This shown that depending on the waste properties of specific inoculum or substrates, appropriate treatment is required. Based on these findings, it is believed that the potential cause of the discrepancy may be the varied approaches taken in their research regarding the kinds of substrates (corn stover and sugar-beet pulp) that were utilized for pretreatments, given that these substrates differ in composition and origin [130].

3. AIMS AND OBJECTIVES OF THE STUDY

3.1 AIM OF THE STUDY:

To utiliz**e** waste-activated slurry and fermentative bacteria to create bio-hydrogen while recovering rich organic acids from waste-water discharge.

3.2 OBJECTIVES OF THE STUDY:

The primary objective of this study is to investigate the feasibility and efficacy of utilizing waste-activated slurry in conjunction with fermentative bacteria to produce bio-hydrogen, while concurrently recovering rich organic acids from wastewater discharge. The study aims to assess the potential of this process as a sustainable and efficient method for both bioenergy production and wastewater treatment, contributing to the development of environmentally friendly solutions for waste management and renewable energy generation.

4. MATERIALS AND METHODS:

4.1 Microorganisms and growth culture medium:

Escherichia coli and *Enterobacter aerogenes,* the good hydrogen producers were used in the research study. It is a rod-shaped, facultatively anaerobic, Gram-negative bacterium [99, 101]. The strain was cultivated aerobically overnight at 37 °C in nutrient broth medium (Sarada Chemicals, India). By subculturing once a month, the strain was kept aerobically on nutrient agar medium at 37 °C. It was discovered that glucose (10 g L^{-1}), $(NH_4)2HPO_4$ (1.25 g L^{-1}), $FeSO_4 \cdot 7H_2O$ (210.5 mg L^{-1}), $MgSO_4 \cdot 6H_2O$ (215.2 mg L^{-1}), $NiCl_2 \cdot 6H_2O$ (3.2 mg L^{-1}), and a C/N/P ratio of 10:3:1 were the ideal dietary needs for this organism for high-rate hydrogen generation. It has previously been documented that this organism is efficient in producing hydrogen by using monosaccharides (like glucose, fructose, and xylose), disaccharides (like sucrose and cellobiose), and polysaccharides (like potato starch and C.M. cellulose). [101]

Using consortia of E. coli and E. aerogenes, a different medium known as the Minimal Nutrient media (M9) was utilized for the synthesis of biohydrogen. The M9 salts were composed of NH_4Cl (5 g L^{-1}), KH_2PO_4 (15 g L^{-1}), NaCl (2.5 g L^{-1}), and Na_2HPO_4 (64 g L^{-1}). In addition, additional nutrients such as 20% glucose, 1(M) $MgSO_4$, and 1(M) $CaCl_2$ were added to the broth and grew aerobically overnight at 37°C with a C/N/P ratio of 40:1:5 [102].

Each lab and pilot-scale hydrogen generation experiment's inoculum were created independently. The inoculum used in lab- and bench-scale bioreactors for hydrogen production was created by growing Enterobacter aerogenes in nutrient broth medium in

anaerobic conditions. Every instance employed an inoculum size of 7% v/v, and the inoculation process was carried out in an aseptic environment [100, 101]. The anaerobic conditions were maintained by flushing the reactor vessel with liquid nitrogen in the experimental setup. The experiments were carried out in 500ml (working volume) reactor vessel under anaerobic conditions.

4.2 Sample collection and analysis

Water samples were taken from different areas of the Titagarh Sewage Treatment Plant. Both the raw water and the water from the primary and secondary clarifiers were gathered and examined. After collection, various parameters like COD, Ammoniacal nitrogen, TSS, TDS, alkalinity, carbohydrate content, volatile fatty acids and ammonia were also evaluated and checked. The samples were stored in jerry cans in the refrigerator.

4.2.1 COD estimation

The term "chemical oxygen demand" (COD) refers to the milligrams per litre (mg/L) unit of measurement used to describe the amount of oxygen needed to chemically oxidize organic and inorganic materials in water. It is frequently employed as a gauge for the concentration of organic contaminants in water. In a COD test, a potent oxidizing agent is added to a water sample under particular circumstances, and the amount of oxygen used during the reaction is measured. Elevated COD levels are a sign of a high organic pollutant concentration, which can lower oxygen levels in water bodies, causing environmental damage and killing aquatic life.

For the research study, a refluxing flask was filled with 20 ml of the waste water, 0.4 gms of H_2SO_4, and three to four glass beads. The procedure involved adding 10 ml of 0.25(N)

potassium dichromate solution, 30 ml of conc H_2SO_4 and powdered mercuric sulphate, diluting the solution to 150 ml with distilled water, refluxing it for one to two hours until the color turned reddish brown at 70 to 80 degrees Celsius.

Figure 15: Reddish brown color of the water sample after refluxing

The reddish-brown solution is titrated against standardized 0.1(N) Mohr salt and two to three drops of ferroin indicator until a deep green color appears. The same process was also used to prepare a blank.

4.2.2 Ammoniacal nitrogen by Kjeldahl method

Ammoniacal nitrogen is the measure of nitrogen in the organic matter as ammonia, a toxic pollutant that poisons humans and disrupts the balance of the water ecology systems. In wastewater systems, where organic matter is more, a high concentration of ammoniacal nitrogen inhibits the natural nitrification creating water hypoxia and also depletes dissolved oxygen. The ammonia that can be recovered from the ammoniacal nitrogen can be further used for pretreating the waste activated sludge.

Place the homogeneous wastewater sample (1 g) in the digestion tube after precisely weighing it or pipetting the appropriate amount of protein fraction solution (20 ml myofibrillar or sarcoplasmic protein fraction or 40 ml non-proteinous nitrogen fraction). Add 20 ml of cone H2SO4, 3 to 5 anti-bumping granules, and 7 g of catalyst. As a blank, create a tube with the aforementioned substances and the fish sample removed. Place the exhaust manifold cover over the tube, insert it into the digestor that has been preheated, and digest for 15 minutes at a temperature of between 110 and 130°C (if the sample is not liquid, skip this step). Set the digestor's temperature to 420°C, which is the typical digestion temperature. Then, digest the sample for another 15 minutes or until the solution turns bright green—one hour for the wastewater sample. After removing the tube, let the sample stand until it cools. Add 60 ml of distilled water very slowly.

Turn on the distillation equipment and give it a 10-minute pre-wash. Fill a 250 ml conical flask with 25 ml of 4% boric acid, then set the flask underneath the condenser so that the condenser tip is submerged in the boric acid solution. Attach the distillation apparatus to the digestion tube containing the sample digest. Gently pour 60 ml of 40% NaOH into the material that has been digested. To start the distillation process, switch on the steam supply valve right away. Cook for 4 minutes, or until the boric acid has completely absorbed all of the ammonia. After lowering the

conical flask and making sure the condenser tip is not submerged in solution, heat the mixture for an additional minute. Clean the condenser's tip using distilled water. Conical flask with ammonia distillate should be placed on a magnetic stirrer. After adding 1 mL of the indicator, titrate the sample with conventional 0.1N sulfuric acid until the solution turns pinkish-green in color. Check the amount of acid used in the titration.

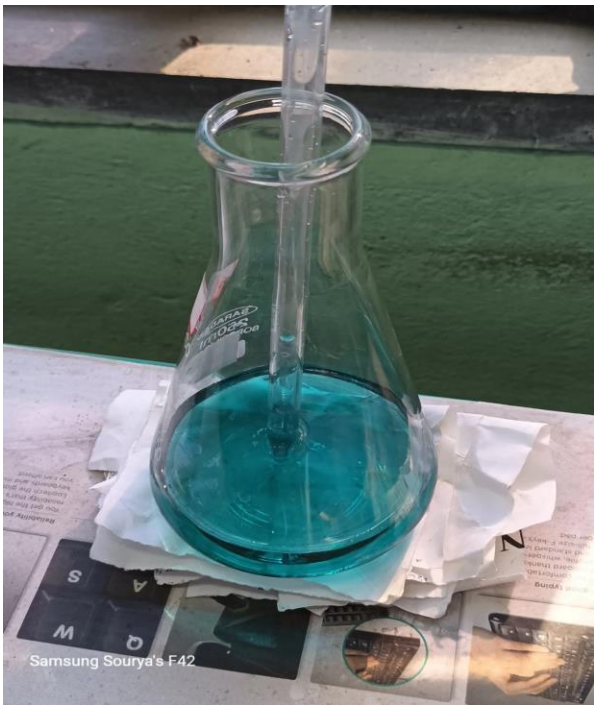

Figure 16: Blue color of the wastewater sample before digestion.

4.2.3 Total suspended solids and Total Dissolved solids estimation

Total Dissolved Solids (TDS) refers to the total concentration of dissolved inorganic and organic substances present in water. These substances can include salts, minerals, metals, ions, and some organic compounds that are dissolved in water rather than suspended as solids. TDS is typically expressed in milligrams per liter (mg/L) or parts per million (ppm).

To make sure that the treated wastewater satisfies regulatory requirements prior to disposal, TDS measurement is crucial. Wastewater with elevated TDS levels may include contaminants that need further treatment to get rid of. Additionally, monitoring total dissolved solids is crucial for determining the quality of the water, safeguarding public health and the environment, maximizing water use across industries, and guaranteeing adherence to legal requirements for water treatment and disposal.

Total Suspended Solids (TSS) refers to the measure of solid particles suspended in water or wastewater. These particles can include organic matter, inorganic matter, and microorganisms that are not dissolved in the water but are instead suspended and carried along by the flow. TSS is typically expressed in milligrams per liter (mg/L) or parts per million (ppm). Measuring total suspended solids (TSS) is crucial for assessing how well wastewater treatment systems work. Elevated total suspended solids (TSS) in wastewater may suggest that treatment processes need to be improved or adjusted in order to guarantee that discharge meets all applicable regulations. Total suspended solids measurement is, in general, an essential metric for wastewater treatment, environmental monitoring, and guaranteeing the safety and quality of water resources for aquatic ecosystems and human use.

25 mL of the waste water sample are metered out using a graduated cylinder and poured onto filtering paper. A filter flask is filled with the filtrate after suction is applied, along with a little amount of waste water. A waste water sample is put into a filtration device and allowed to pass through the filter. After filtering, the filter paper is dried in an oven at 103–

105 ° C for an hour, and its weight is measured once more. The suction is then maintained for three minutes.

4.2.4 Alkalinity of the water sample

The buffering capacity of a wastewater sample, or its resistance to pH variations, is measured by alkalinity. Variations in pH can have an impact on how well different wastewater treatment methods work. Operators can evaluate a sample's capacity to sustain a pH that is stable and ensure ideal circumstances for treatment processes by evaluating its alkalinity. Sustaining the proper alkalinity levels in wastewater systems and treatment plants can aid in preventing equipment and infrastructure corrosion. Acidic substances can come from a variety of sources, including natural organic matter or industrial processes, and are frequently found in wastewater. The ability of the sample to neutralize acids, or alkalinity, keeps the pH from falling to levels that can be hazardous to the environment or treatment procedures. For best results, various treatment techniques, including chemical precipitation or biological nutrient removal, may need a certain alkalinity level.

Operators can measure alkalinity and make necessary adjustments to treatment operations to guarantee effective removal of pollutants and adherence to regulatory criteria. The water sample is measured to 50 mL in a conical flask. It is mixed with five drops of methyl orange till the mixture turns yellow-orange in color. After then, it is titrated against the burette's $0.02(N)H_2SO_4$ until it reaches the end point, which is pink. The procedure is carried out three times, and the burette's $0.02(N)\ H_2SO_4$ levels are recorded.

4.2.5 Carbohydrate content of the water sample by Anthrone method

Organic substances called carbohydrates are frequently discovered in wastewater, which comes from a variety of sources including home sewage, food processing, and agricultural practices. Analyzing the carbohydrate content gives information about the wastewater's total organic load. High glucose concentrations may be a sign of organic pollutants that need to be treated to avoid harmful effects on the environment. Carbohydrates may ferment during the wastewater treatment procedures, resulting in the production of organic acids, gases, and other pollutants. Monitoring the amount of carbohydrates in a mixture aid in determining the likelihood of fermentation and how it might affect the efficiency of treatment, the creation of gas, and odors.

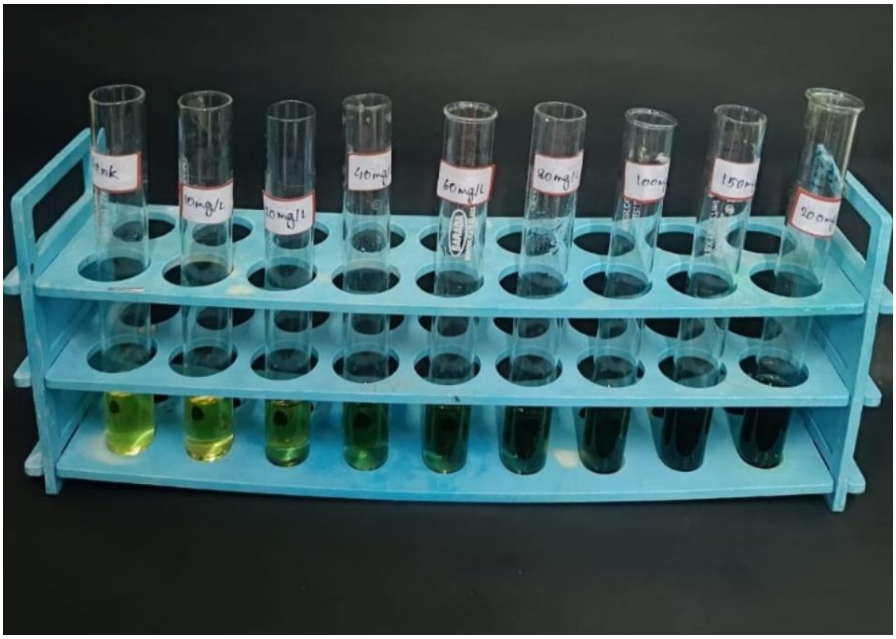

Figure 17: Dilutions of various concentrations for carbohydrate estimation

A 200 ug glucose per ml of distilled water stock solution is made. A series of glucose dilutions in varying amounts are made with distilled water. Using tubes 2 through 9 to generate a standard curve, use tube 1 as a blank. Add 5 ml of the Anthrone reagent to each tube and thoroughly mix by vertexing. Let the tubes cool. After covering the tubes with covers and heating them to boiling point for ten minutes, allow them to cool to room temperature before measuring the absorbance at 620 nm in comparison to a blank.

4.3 VFA ESTIMATION:

In the research study, VFA was determined using two methods: Spectrophotometric method and HPLC method. The two methods are discussed below:

4.3.1 VFA estimation by Montgomery method

The Montgomery method is a traditional VFA measurement procedure. Compared to the distillation approach, the methods for preparing the esters in this traditional colorimetric composite determination of the VFA were comparatively easy and accurate. However, compared to the spectrophotometric approach, the colorimetric method was more precise and less labor-intensive due to the colorimeter's involvement in measuring the optical density of the prepared samples/standards. In contrast to the Montgomery approach, the modified spectrophotometric method allows for the direct measurement of the sample or standard's absorbance using a single or double beam spectrophotometer [108].

Using a substantial excess of a non-volatile alcohol that was miscible with water in all quantities and sulphuric acid as a catalyst seemed to be the easiest method. After testing ethylene glycol, 2-methoxyethanol, and glycerol, ethylene glycol was ultimately chosen because it produced good ester yields and low blank values. Glycol generally has to be purified in order to use a beam spectrophotometer consistently. For simplicity, these conditions were set as the standard as significant increases in the quantities of sulfuric acid and glycol resulted in only marginal improvements in the ester production. The combination needs to be heated. The ester yield practically reaches its maximum after 2 minutes when 2.2 ml of the above-mentioned mixture is heated up in a boiling-water bath, and after 4

minutes a slow decline starts. Three minutes of heating produced the best effects, and it was also found to be helpful to quickly cool the mixture to room temperature.

The method that was ultimately chosen involved adding the hydroxamate solution to a significant amount of ferric chloride solution that contained enough sulfuric acid to provide a final pH value of 1.6 (before dilution). Effervescence is not dangerous, and in the examination of sewage sludge, an inaccuracy of less than 2% occurs when there is a 0.1 pH unit shift in the optical density. If appropriate attention is taken in the preparation and measurement of reagents, a pH value of 1.6 0.1 can be easily attained because sulfuric acid alone has significant buffering action at this level. The color that is created is reasonably stable, fading at a pace of 1.3% per hour over a period of 3 hours or 0.5% per hour over a period of 19 hours.

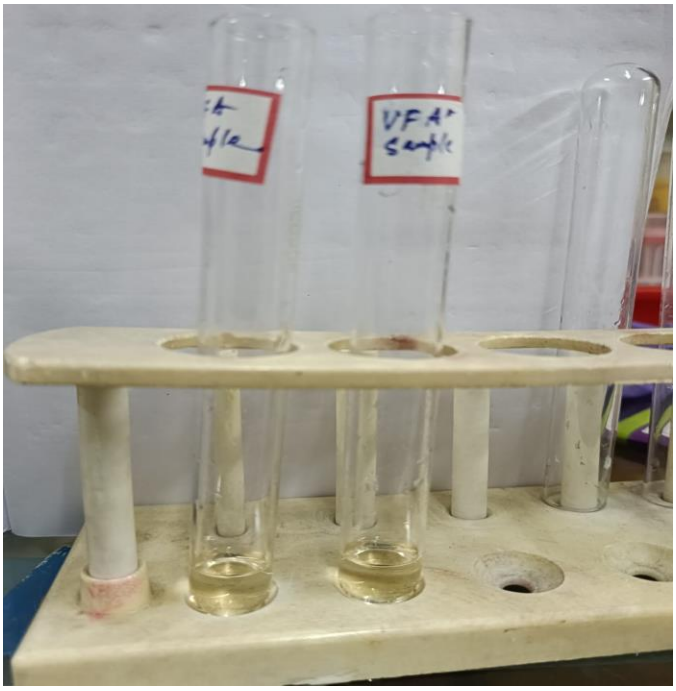

Figure 18: Color development after the addition of $FeCl_3$

Use a filter assist to help with the sample's filtering for clarity; in certain circumstances, centrifuging the sample will yield sufficient clarity. 0.5 mL of the alcohol is poured into a test tube that is dry (12.5 x 1.5 cm. A 1.7 ml of acidic ethylene glycol reagent from a burette is added and mix well. Avoid letting the test tube come into direct contact with the heating source by heating it in a boiling water bath for three minutes. Afterward, the test tube in cold water is cooled and a 2.0 ml of 4.5 N sodium hydroxide and 0.5 ml of hydroxylammonium sulphate solution is thereafter added. The mixture is put aside for one minute if the volatile acid content is predicted to be more than 5000 ppm.

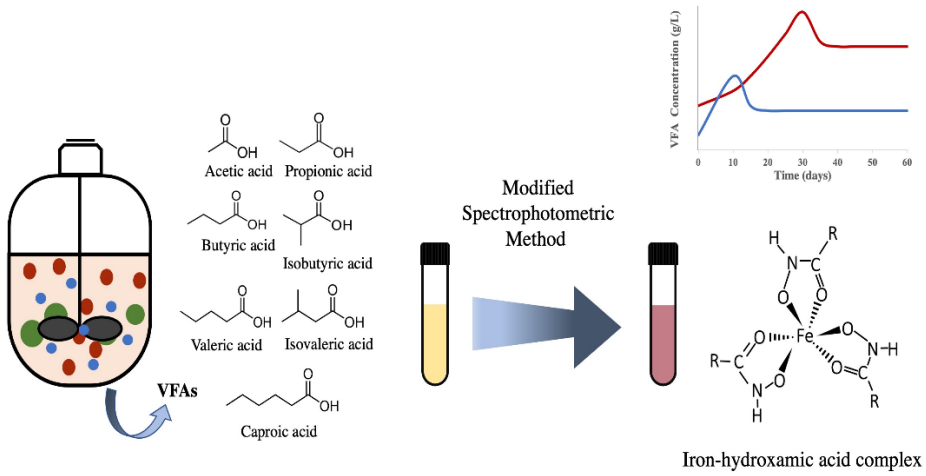

Figure 19: An Enhanced Spectrophotometric Approach for Quantifying High-Range Volatile Fatty Acids in Mixed Acid Fermentation of Organic Waste (Aramrueang et al; 2022)

The test-tube solution into a 25-ml calibrated flask is poured along with 10 ml of acidic ferric chloride reagent. Rinse away any remaining residue with water and water is added as required and given a good shake. To allow dissolved gases to escape, leave the stopper off for five minutes and the optical density is measured at 650nm, Precautions must be taken to

avoid gas bubbles to form in the optical cell. After one hour of color development, the reading is taken.

4.3.2 VFA estimation by HPLC method using C18 column

The HPLC studies were conducted in an isocratic environment at 55 degrees Celsius. H_2SO_4 at a concentration of 0.005 mol/L made up the mobile phase. Before being used, the solution was degassed by sonication for ten minutes after being filtered through a 0.45 mm Millipore membrane. The injection volume was 20 ml, and the flow rate was 1.0 ml/min. Refractive index detectors were used to evaluate carbohydrates (sucrose, glucose, and fructose) and UV-Vis detectors at 210 nm were used to analyze volatile fatty acids (acetic, propionic, iso-butyric, and butyric acids).

4.4 Estimation of ammonia by phenate method

The principle of phenate method is generation of an intensely blue compound, indophenol which is formed by the reaction of ammonia, hypochlorite and catalysis of phenol by sodium nitroprusside. The interference caused by the precipitation of calcium and magnesium ions at high pH levels is eliminated by complexing these ions with citrate. Other trivalent types of nitrogen do not cause any interference. filtering or distilling out turbidity that interferes. Remove hydrogen sulphide by acidifying samples to pH 3 with diluted HCL and forcefully aerating until the sulphurous odor is eliminated.

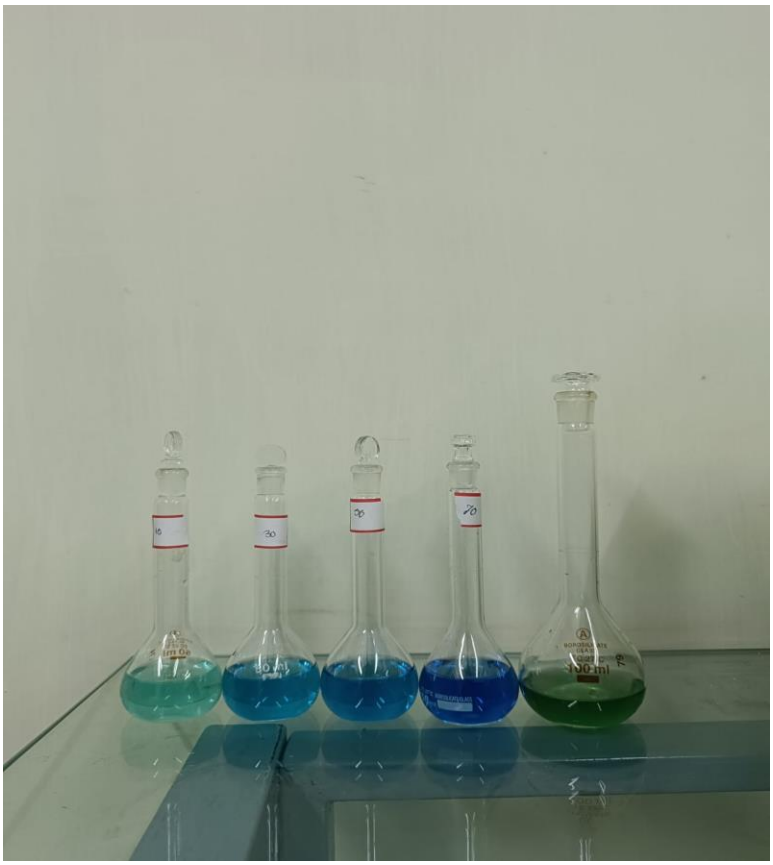

Figure 20: Standard solutions of ammonia of varying concentrations

To a 25 ml sample in a 50ml Erlenmeyer flask, add with proper mixing after addition, 1ml phenol solution, 1ml sodium nitroprusside solution, add 2.5ml oxidizing solution. Cover with plastic wrap or parafilm wrapper and let the color develop at room temperature for at least 1 hr. The color is consistent within 24hr and the absorbance is measured at 640 nm. A blank is been prepared with at least two standards. The standard of varying concentrations is prepared from stock ammonia solution to minimize the error. A standard curve is prepared for the standard solutions [103, 104].

4.5 Thermodynamics of Bio-hydrogen production processes

4.5.1 Growth curve of *E. coli* and *E. aerogenes*

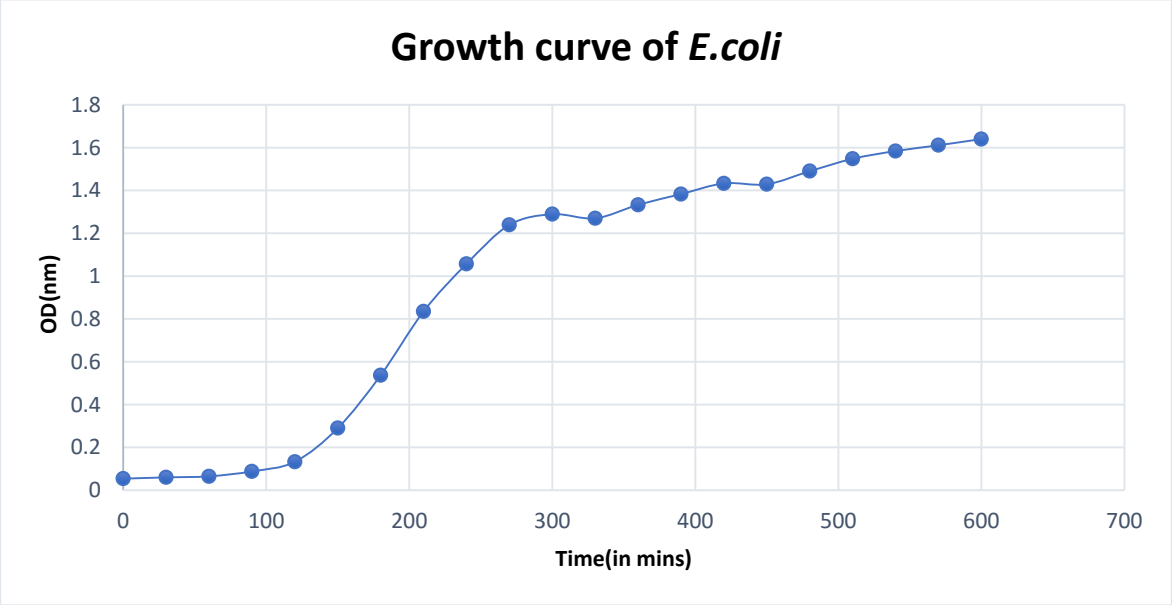

Figure 21: Growth curve of E. coli

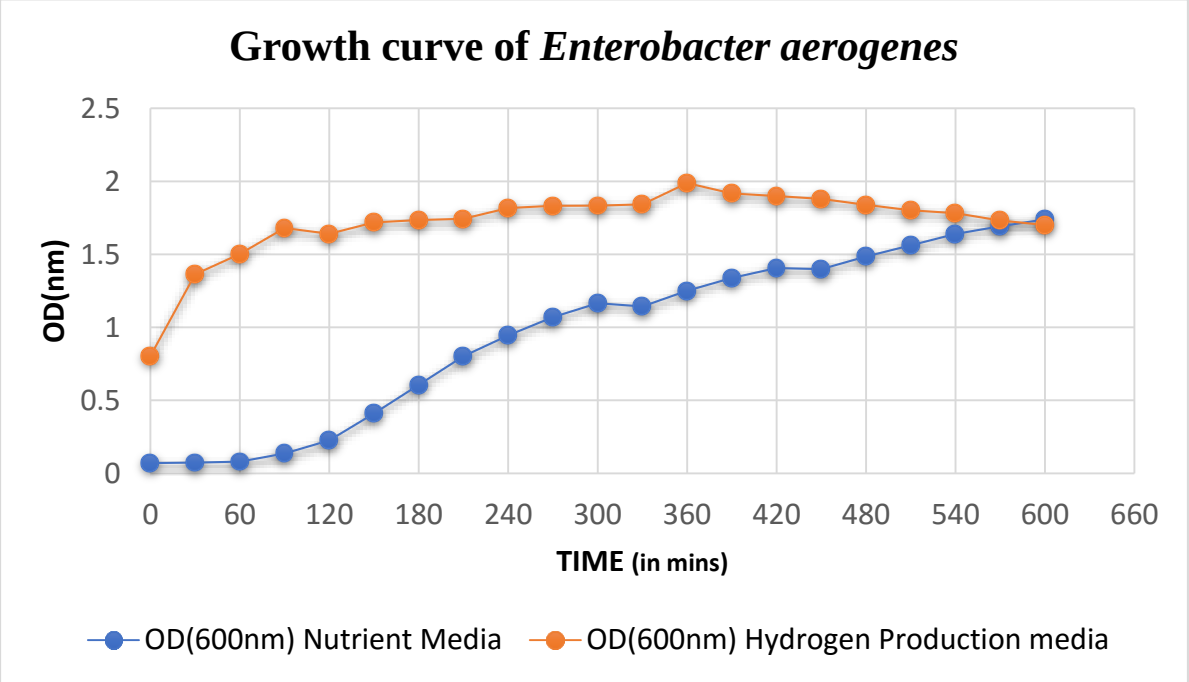

Figure 22: Growth curve of Enterobacter aerogenes

4.5.2 Thermodynamics of Bio-hydrogen production processes

The process's thermodynamics determines the hydrogen yield. Four mol H_2/mol glucose is the greatest hydrogen yield that can be produced from glucose. Studies reveal that the majority of mesophiles have hydrogen yields that are just 2 mol H_2/mol glucose. This is explained by the fact that the complete oxidation of glucose to CO_2 and H_2 has a positive Gibbs energy change and will not occur without the input of additional energy under typical conditions (1 M concentrations of the reactants, 25C, pH 7). Several tests have demonstrated that the synthesis of acetate from glucose promotes the synthesis of hydrogen. Only four mol of hydrogen, the theoretical maximum, can be created. When glucose is converted to acetate while simultaneously producing CO2 and hydrogen [94, 114]. But the process of producing acetate is not a redox reaction. Consequently, in theory, this response is feasible only in the event that hydrogen receives all of the reducing equivalents. In actuality, though, the reaction will only be advantageous if the hydrogen pressure is maintained low—either by hydrogen-consuming microbes or by manually draining the reactor of hydrogen gas.

Fermentative reactions involved in pathway	ΔG° (KJ/mol)	Comments
$NADH + H^+ + pyruvate^- \rightarrow NAD^+ + lactate^-$	−25.0	Highest positive Gibbs free energy; hence this reaction is most unfavorable
$2NADH + 2H^+ + acetyl\text{-}CoA \rightarrow 2NAD^+ + ethanol + CoA$	−27.5	
$NADH + H^+ + pyruvate^- + NH_4^+ \rightarrow NAD^+ + alanine + H_2O$	−36.7	
$NADH + H^+ \rightarrow NAD^+ + H_2$	+81.1	Highest negative Gibbs free energy; hence this reaction is most favorable
$2\ Ferredoxin\ (red) + 2H^+ \rightarrow 2\ ferredoxin(ox) + H_2$	+3.1	

Figure 23: Values of Gibbs Free Energy for Various Fermentative Reactions (Balachandar et al; 2013)

The two primary reducing equivalents used in the fermentation of glucose are NADH and Fd. The midway redox potentials of NAD/NADH and oxidized ferredoxin/reduced ferredoxin are 398 mV and 320 mV, respectively. To maintain the process, recyclable reducing equivalents are employed [115]. These electron carriers can be recycled by a variety of mechanisms, including the one in which NADH reduces pyruvate to create ethanol. The reduction of protons with NADH to produce molecular hydrogen has a redox potential of about 414 mV under normal circumstances. The potential of this pair is greater than that of NADP/NADH. As a result, the oxidation of NADH to produce molecular hydrogen is limited thermodynamically [94]. In contrast, it is preferable for ferredoxin to be oxidized in order to form molecular hydrogen. But the production of other reduced products, including lactate and ethanol, is more likely than hydrogen. However, yields of up to 4 mol H2/mol glucose have been reported in thermophiles. The greater temperature at which hydrogen synthesis takes place could be the cause of the thermophiles' increased yields. This is predicated on the equation that links the process's enthalpy (H), temperature (T), and entropy (S) to Gibb's free energy.

$$\Delta G_0 = \Delta H - T\Delta S_0$$

4.6 Design Parameters

With proper mathematical calculations and modelling, the reactor vessel and the CO_2 trapper were designed and used to ensure accuracy of the process. Initially, a setup was established with proper research and study, but later on it failed to displace gas due to negative pressure, improper headspace and maximum height of the CO_2 trapper. Therefore, according to the design parameters, each glassware was designed which can be better described in the below fig 23.

Reactor Vessel **CO_2 trapper** **Gas column**

Figure 24: Design of the various glass wares used in bio-hydrogen production for dark fermentation

4.7 Experimental setup:

4.7.1 Hydrogen Production in Batch Process

1. **Old setup**

Figure 25: Experimental setup for bio-hydrogen production

There was a problem in the old setup, due to the lack of designing and configuration. The CO_2 trapper was too large as compared to the reactor vessel. Also, there was not enough space on the headspace that created too much negative pressure on the column. Due to excessive methane formation, large bubbles were seen in the trapper and this in turn would tear the parafilm on the reactor vessel. Droplets of water vapor were seen in the reactor vessel indicating gas formation.

2. New setup

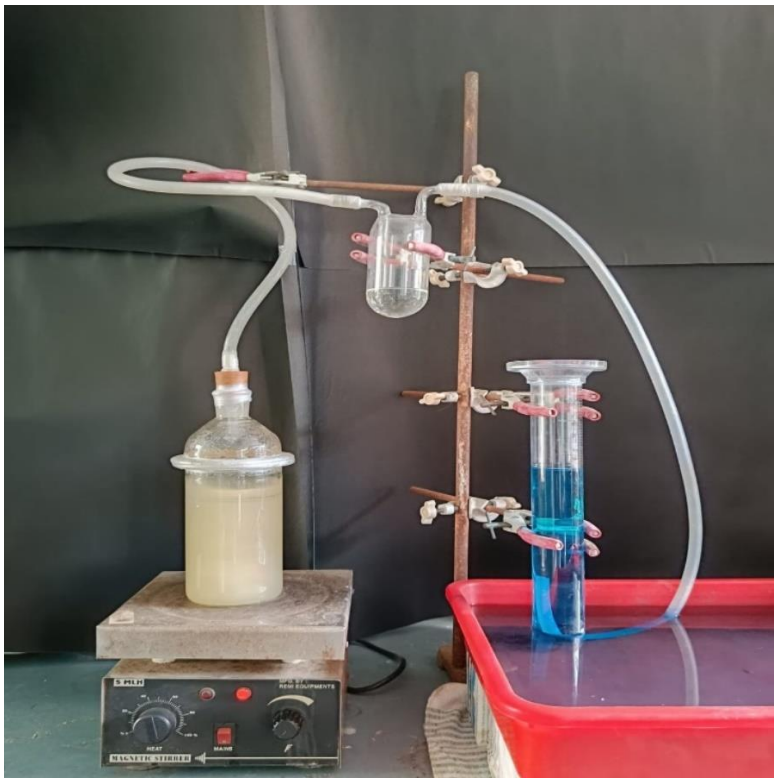

Figure 26: Experimental setup for bio-hydrogen production

The new setup was designed following the design paradigm of IIT Kharagpur. In the new setup, a simple measuring cylinder of 250ml was used and methylene blue dye was used inside for better displacement of gas. However, this system is not viable for collection as there are no outlet. Therefore, in future, a new setup would be designed with proper collection and estimation measures.

5. RESULTS AND DISCUSSIONS

5.1 REAL TIME ESTIMATION OF DOUBLING TIME OF BOTH THE ORGANISMS

5.1.1 Dry cell weight vs OD graph

For *E. coli,* the linear relationship was used to determine the dry cell weight of the fermentations performed in the research work.

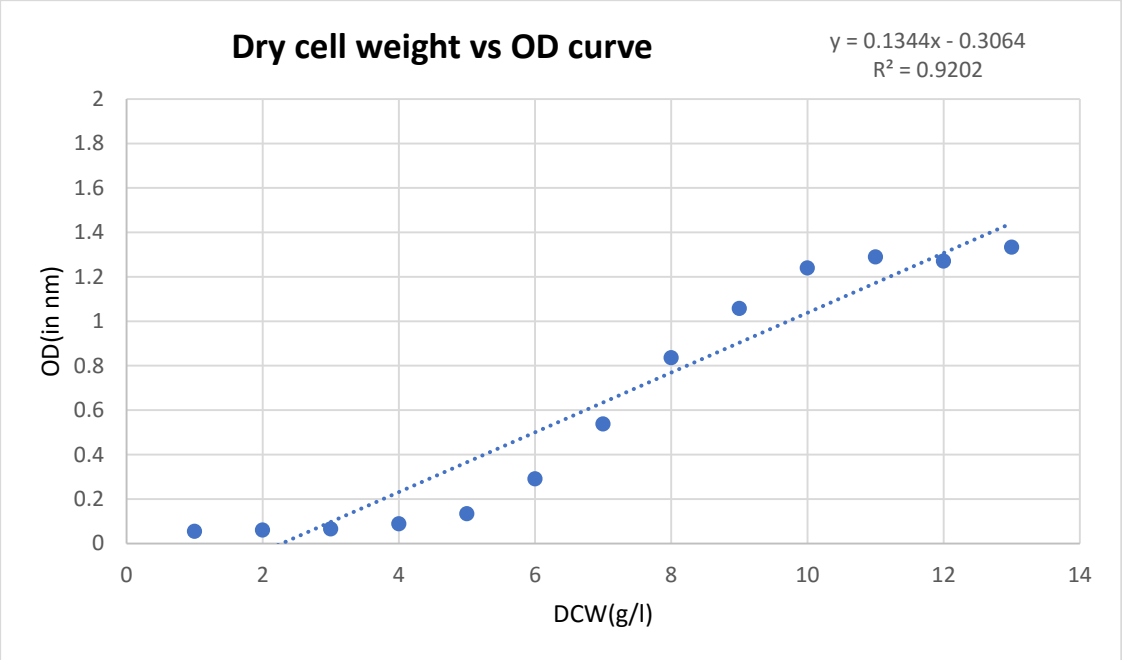

Figure 27: Dry cell weight Vs OD curve

For *E. aerogenes,* the linear relationship was used to determine the dry cell weight of the fermentations performed in the research work.

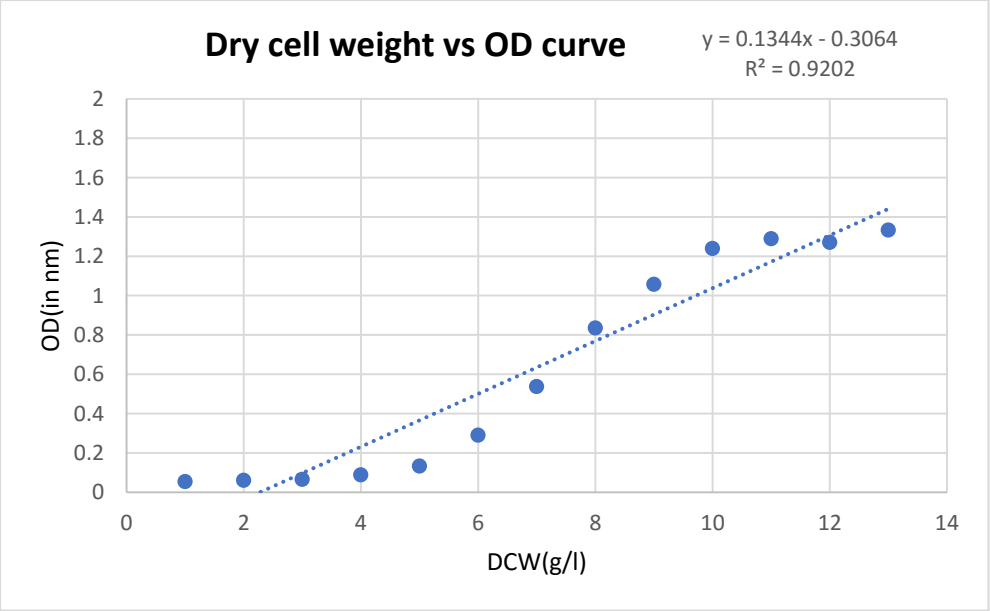

Figure 28: Dry cell weight Vs OD curve

5.1.2 Cell biomass concentration of the organism

From the graph y=mx+c, x is calculated for each organism. For *E. coli* the initial biomass concentration was 2.402mg/l and the final biomass concentration was 2.568mg/l. For *E. Aerogenes*, the initial biomass concentration was 1.568 mg/l and the final biomass concentration was 2.302 mg/l.

5.1.3 Specific growth rate of the organism

The specific growth rate is calculated by the following equation:

$$\mu = \ln(X) - \ln(X_0) / t_2 - t_1$$

where, μ= Specific growth rate (h^{-1})

X= Final Biomass Concentration

X_0= Initial Biomass Concentration

t_2 = Final Time

t_1= Initial Time

The specific growth rate of *E. coli* is 0.0134 h^{-1} and that of E. *Aerogenes* is 0.123 h^{-1}.

5.1.4 Doubling time estimation of the organism

The doubling time estimation is very important for the fermentation processes. Each organism has different doubling time for which it can replicate more and more. The doubling time is calculated by the formula:

$$Td = \ln 2 / \mu$$

The doubling time of *E. coli* is 5 hours and that of E. *Aerogenes* is 5.6hours. This signifies that both the organisms have a faster growing rate and is commercially feasible for use.

5.1.5 Calculation of the total gas pressure

The total gas pressure in the vessel is calculated by:

$$P = \rho g h$$

where, P= pressure=density, g =specific gravity and h = height

The total gas pressure is 2934.7 kg/m^3.

The gas pressure of H_2 is calculated by:

$$PV = nRT$$

The H_2 gas pressure is 2.92 kPa.

5.2 CULTURE AND INOCULATION

After examining the doubling time of the organism, the organisms were incubated at 37 ° C and for both the organisms (E*. coli and E. aerogenes*), the mid log phase was 3hours. Although, in the nutrient media, the growth of E. aerogenes was good, but it failed to produce bio-hydrogen even in the mid log phase. Therefore, for *E. aerogenes*, certain supplements were added keeping in mind about the C: N: P ratio. As for dark fermentation, in the mid log phase the hydrogen production was maximum, so in the research study, after 3 hours of incubation in the incubator, the cultures were mixed and a total of 7% of the culture medium was added in the hydrogen production media in the reactor vessel for bio-hydrogen production.

Figure 29: Experimental setup for bio-hydrogen production

5.3 BIOHYDROGEN GAS PRODUCTION

5.3.1 Using salt media

A simple salt media was chosen keeping in mind about the carbon, nitrogen and phosphorus sources that would initiate both growth and gas production. It was seen that after 10 hours, only 950 ml of total $_{gas}$ was displaced

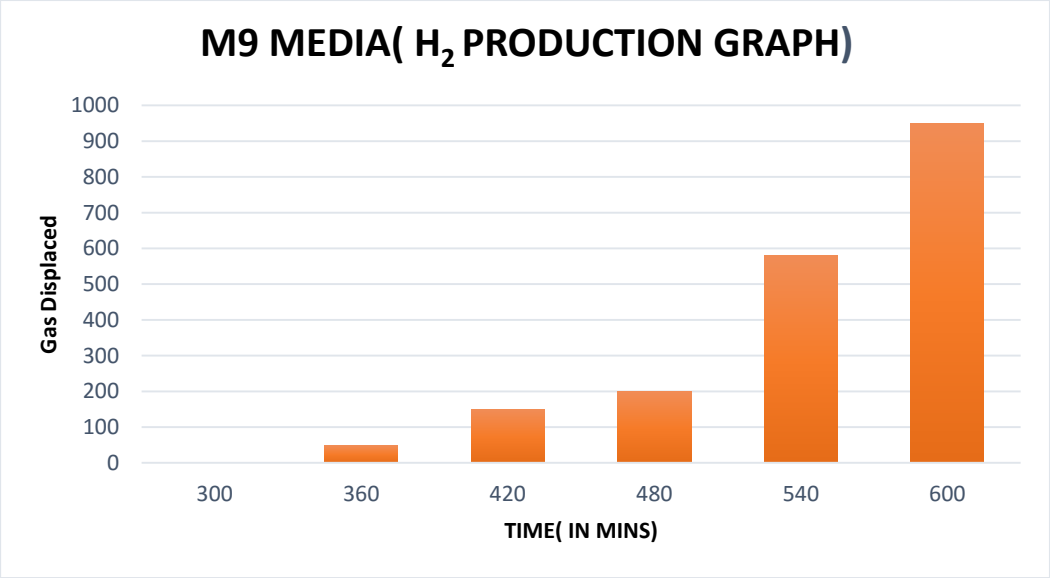

***Figure 30*: Displacement of gas using M9 media**

5.3.2 Using hydrogen production media

A hydrogen production media was chosen keeping in mind about the carbon, nitrogen and phosphorus sources that would initiate both growth and gas production. It was seen that after 10 hours, only 1950 ml of total gas was displaced.

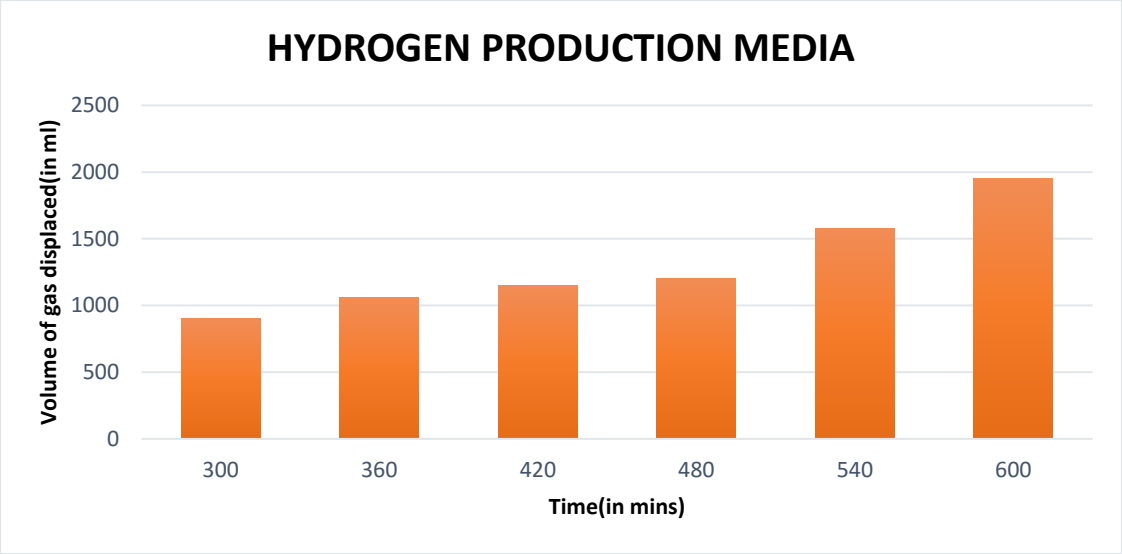

Figure 31: Displacement of gas using Hydrogen Producing media

5.3.3 Using wastewater effluent

After the wastewater was collected and chosen keeping in mind about the carbon, nitrogen and phosphorus sources that would initiate both growth and gas production. It was seen that after 10 hours, only 5760 ml of total gas was displaced.

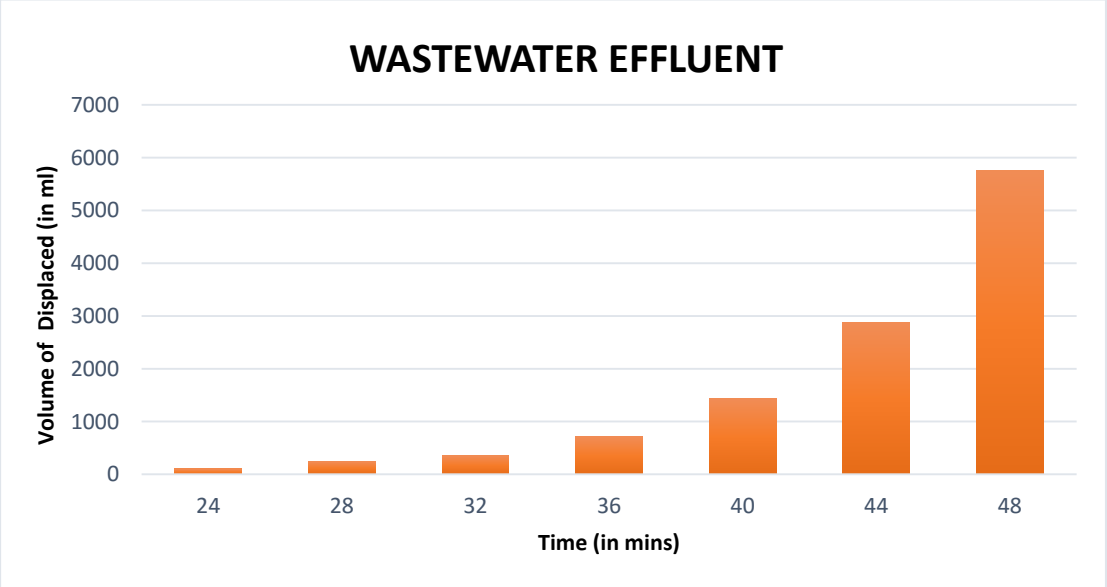

Figure 32: Displacement of gas using Wastewater effluent

5.4 DETERMINATION OF BIO-HYDROGEN GAS PRODUCTION

Due to the unavailability of Gas chromatography, the exact ml of hydrogen is unknown. In the research study, we have used water displacement method to displace gases. We have got a number of gases which includes methane, hydrogen, Hydrogen sulfide, ammonia and very small traces of carbon dioxide as a CO_2 trapper was used.

5.5 ACETIC ACID ESTIMATION

5.1 Acetic acid estimation by Montgomery method

5.1.1 Using nutrient media

A standard curve was prepared for the nutrient media shown below:

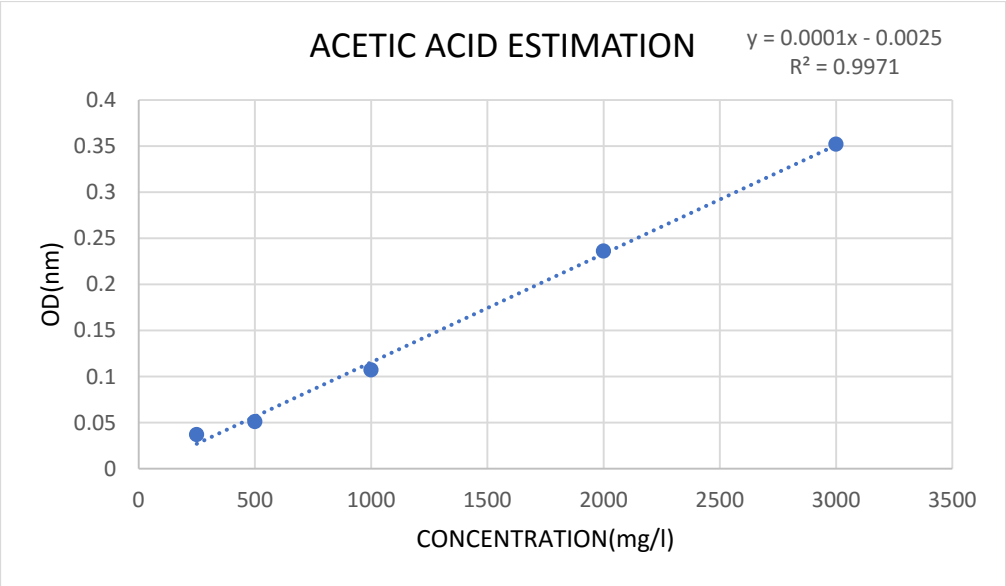

Figure 33: Acetic acid estimation using nutrient media

For the nutrient media, after dark fermentation, the acetic acid concentration was 750mg/l at 513nm. So, an additional acetate salt was added for the photo-fermentation procedure.

5.1.2 Using Hydrogen production media

A standard curve was prepared for the Hydrogen production media shown below

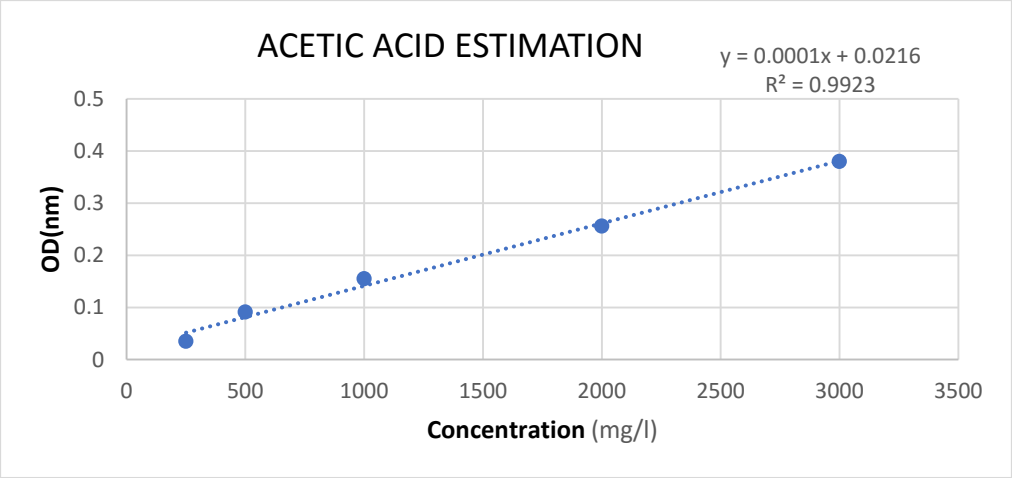

Figure 34: Acetic acid estimation using Hydrogen production media

For the Hydrogen production media, after dark fermentation, the acetic acid concentration was 950mg/l. at 513nm. So, an additional acetate salt was added for the photo-fermentation procedure.

5.1.3 Using Wastewater effluent

A standard curve was prepared for the Hydrogen production media shown below

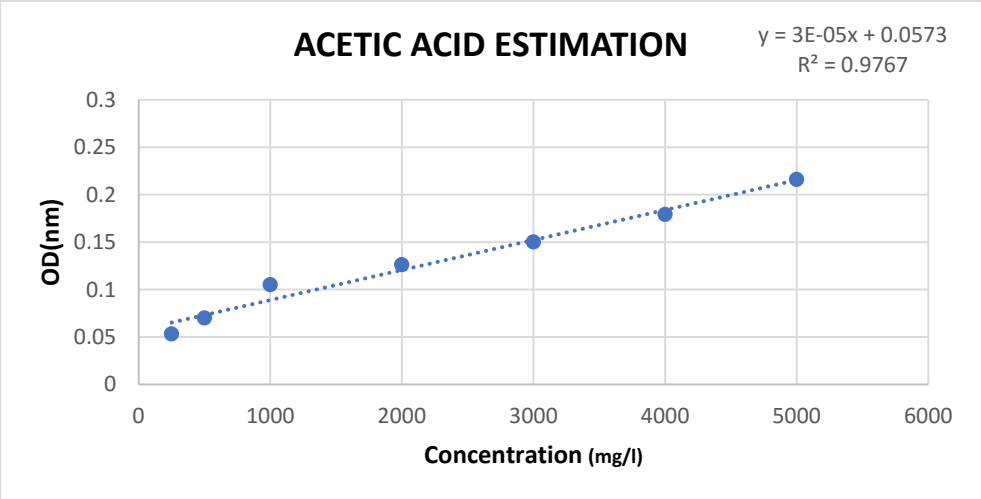

Figure 35: Acetic acid estimation using wastewater effluent

As there was no pre-treatment, the initial VFA concentration was measured at 513nm and it was 4345 mg/l. As the Acetic acid concentration was too high for the dark fermentation effluent, it was diluted and the decision of VFA recovery came into place.

5.6 AMMONIA ESTIMATION

Phenate method was chosen to determine ammonia.

5.1 Using nutrient media

A standard curve was prepared for the nutrient media shown below:

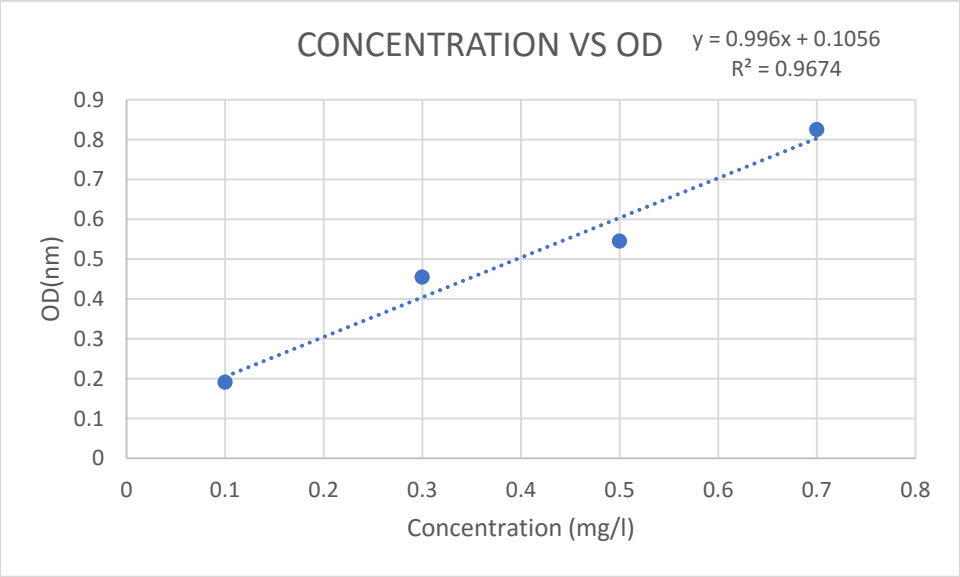

Figure 36: Ammonia estimation using M9 media

For the M9 media, after dark fermentation, the ammonia concentration was 0.6 mg/ml at 640 nm.

5.7 CARBOHYDRATE ESTIMATION FOR WASTEWATER EFFLUENT

Anthrone method was chosen to determine sugar content of the wastewater sample.

A standard curve was prepared for the wastewater effluent shown below:

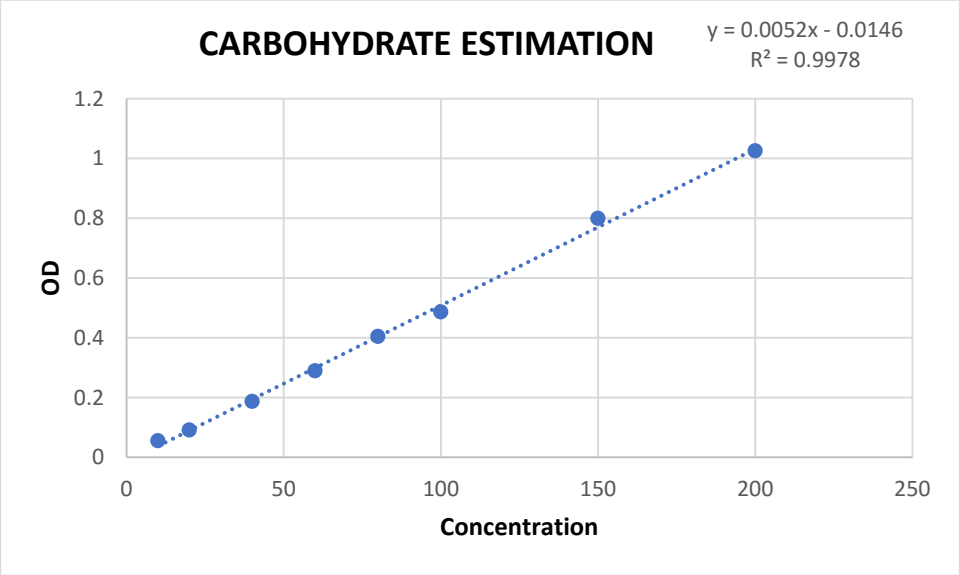

Figure 37: Carbohydrate estimation using Wastewater effluent

For the wastewater effluent, the carbohydrate concentration of the wastewater was 46 mg/ml at 620 nm. As the concentration of glucose was very low to initiate the dark fermentation, an additional 9.8g/l of glucose was added. Although the process is not commercially viable, it was used to test the accuracy and rightness of the process.

5.8 CHARACTERIZATION OF THE VARIOUS PARAMETERS IN THE WASTEWATER EFFLUENT

Number of parameters	Values
pH	6.8
COD (in ppm)	1700
Volatile fatty acid (in ppm)	4345
Carbohydrate content (in ppm)	46
Total suspended solids (in ppm)	860
Total Dissolved Solids (in ppm)	266
Alkalinity (in ppm)	1380
Ammoniacal nitrogen (in ppm)	140

Table 2: Characterization of the various parameters in the wastewater effluent

The COD was beyond the permissible limit which indicates low DO levels that will promote the growth of both anaerobic and facultative anaerobes which is a plus for the research study. The growth of anaerobic microbes will help in fermentation thereafter. As the sugar content was much less than it was expected, initially glucose was added to initiate the process. The high TSS and TDS values will interfere in the fermentation process, therefore for avoiding interferences, the wastewater effluent was filtered using a vacuum pump.

5.9 MASS BALANCE IN THE ADSORPTION-DESORPTION EXPERIMENTS BY ACTIVATED CARBON

5.9.1 Before recovery of VFA

Samples	Initial amount of acetic acid (in mg/l)
Secondary effluent (First visit)	2090
Secondary effluent (Second visit)	100
Lagoon	2020
Refrigerated effluent	1500
Raw water	500
Primary effluent	250

Table 3: Acetic acid concentration before recovery

5.9.2 After recovery of VFA

Samples	Initial amount of acetic acid (in mg/l)	Amount of VFA adsorbed on the activated carbon	Amount of VFA desorbed
Secondary effluent (First visit)	2090	150	1200
Secondary effluent (Second visit)	107	0	156
Lagoon	2020	120	2000
Refrigerated effluent	1500	50	2500

Raw water	500	0	400
Primary effluent	250	0	300

Table 4: Acetic acid concentration after recovery

5.9.3 HPLC generated peak and area under the curve for various samples at 210 nm

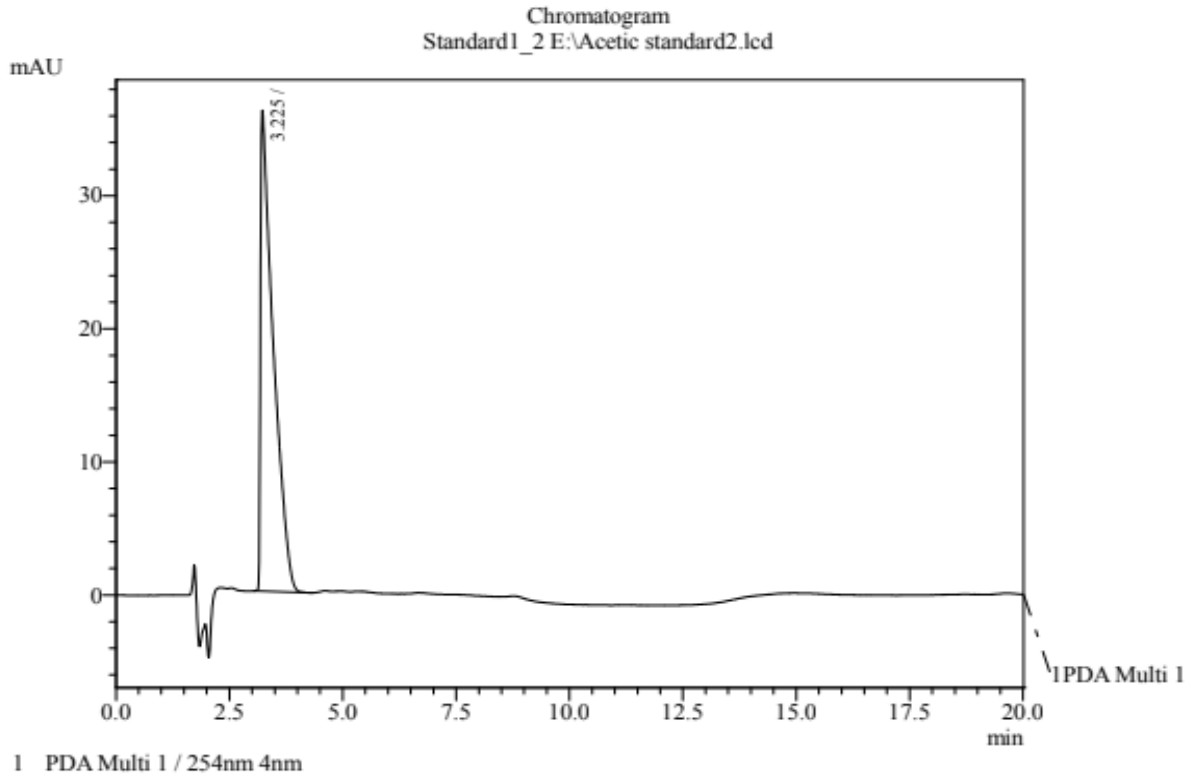

Figure 38: Acetic acid standard of 100 ppm

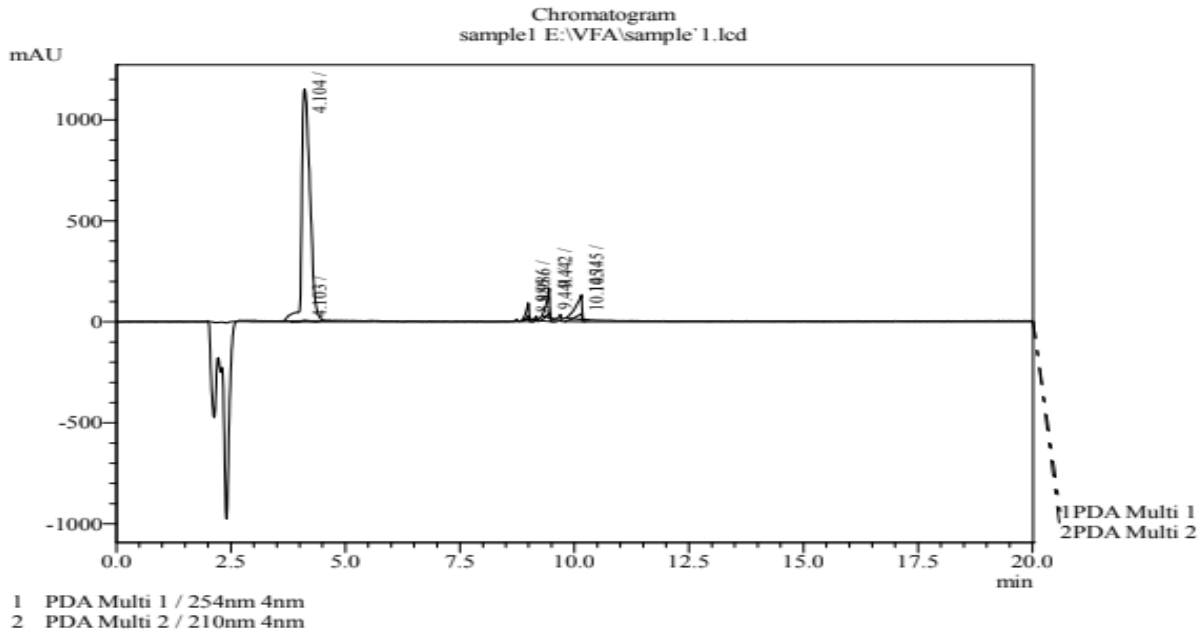

Figure 39: Adsorbed acetic acid of the secondary effluent

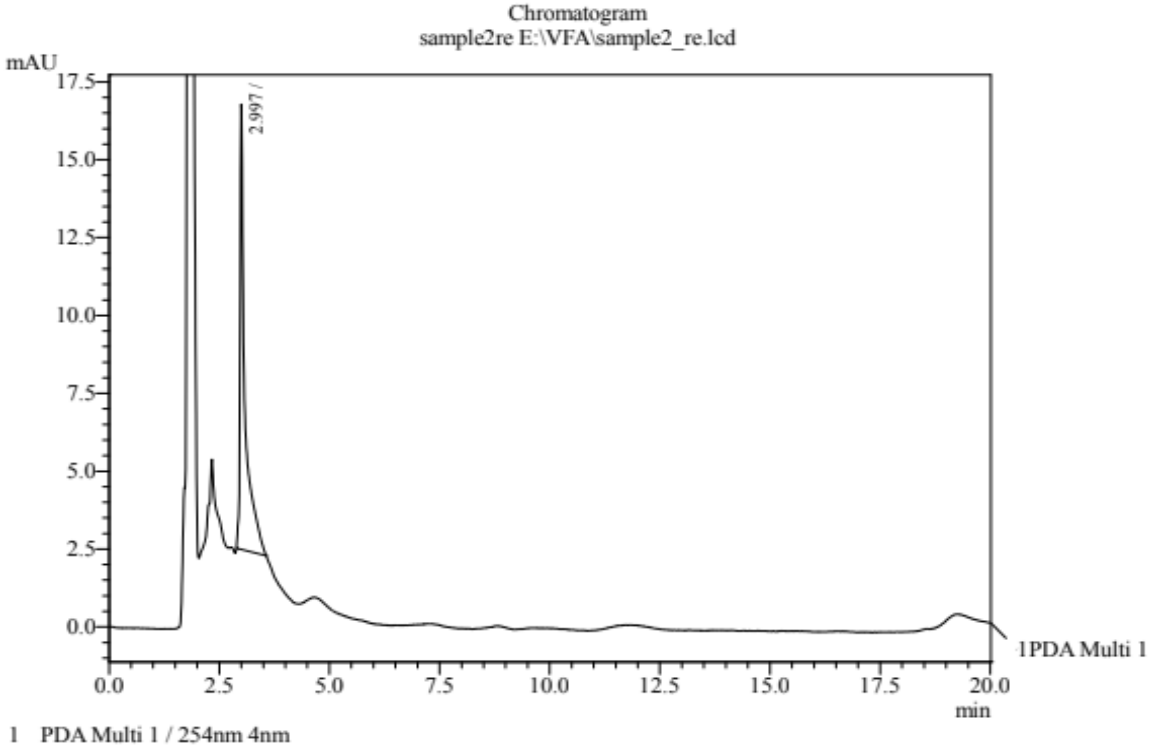

Figure 40: Desorbed acetic acid of the secondary effluent

5.10 MASS BALANCE IN THE ADSORPTION-DESORPTION EXPERIMENTS BY CHEMICAL PRECIPITATION

5.10.1 After recovery of VFA

Samples	Initial amount of acetic acid concentration	Amount of VFA desorbed
Secondary effluent	130	22.9
Primary effluent	100	10

Table 5: Acetic acid concentration after recovery

5.10.2 HPLC generated peak and area under the curve for various samples at 210 nm

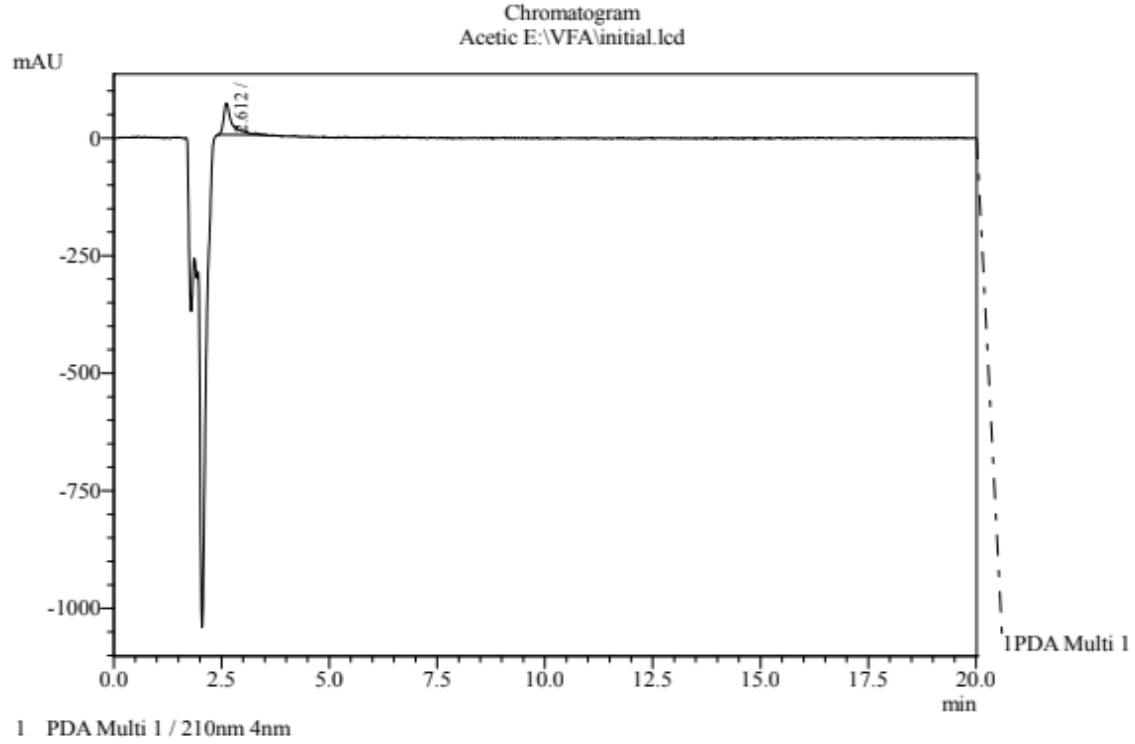

Figure 41: Initial amount of acetic acid concentration of the secondary effluent

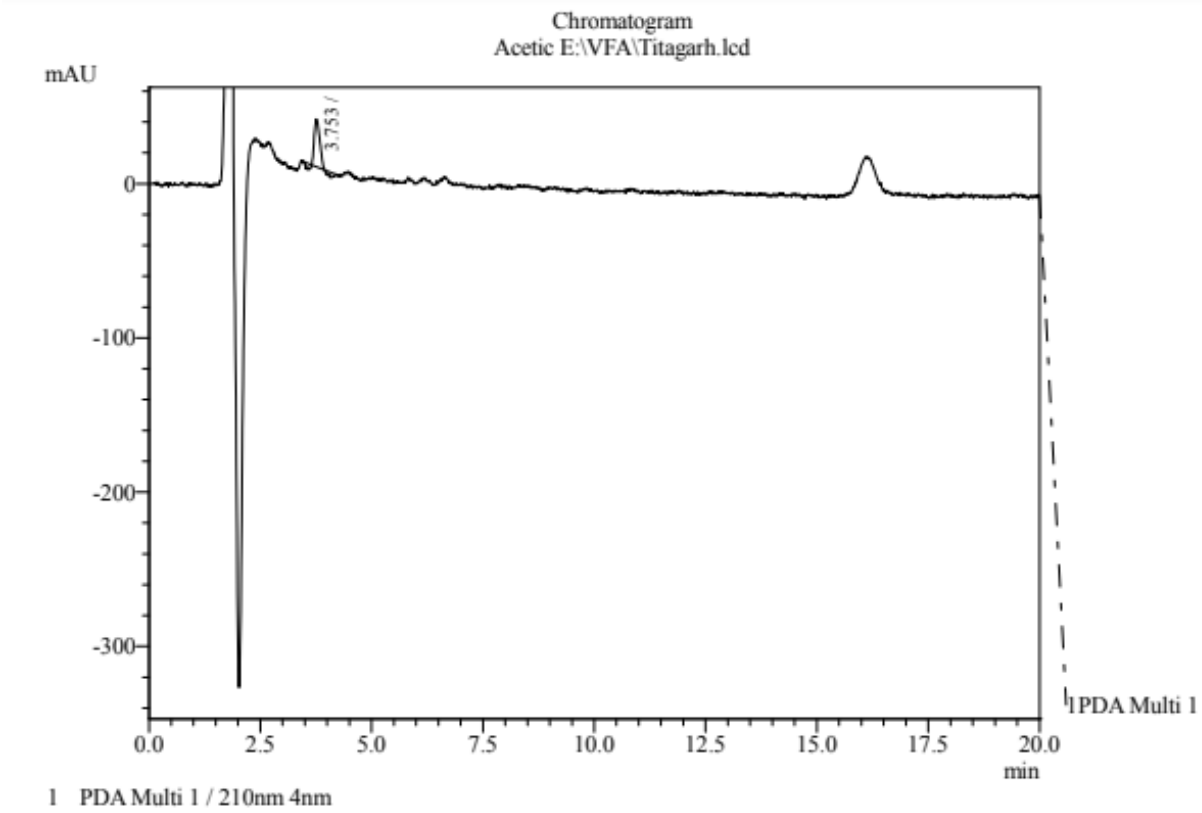

Figure 42: Desorbed acetic acid concentration of the secondary effluent

6. FUTURE SCOPE

6.1 Future perspectives of Bio-hydrogen production

An increasingly popular approach to addressing the primary economic challenge is the shift to integrated H_2 manufacturing techniques that can increase H_2 yields. In addition, the H_2 generation strategies are supported by the use of two-stage integration, or combined dark and photo fermentative processes. An integrated dark-photo-fermentative process using sugar beetroot thick juice for growth and H_2 production has been successfully demonstrated, and it can improve energy recovery by 90.3% when compared to a single stage hydrogen production [116]. This needs to be thoroughly investigated. Furthermore, practically whole conversion of various carbohydrates into "green" hydrogen with close-to-theoretic stochiometric conversion yields is made possible by the application of synthetic enzymatic pathways using a collection of recombinant enzymes. While rates of conversion and total prices still require improvement, cost estimates indicate that an ecologically benign and sustainable strategy can eventually compete with thermochemically produced H_2. The potential for H_2 generation in the years to come is contingent upon both scientific and economic developments, including the development of bioreactors, genetically modified microbes, and efficient manufacture of thermostable enzymes [4].

Furthermore, practically whole conversion of various carbohydrates into "green" hydrogen with close-to-theoretic stochiometric conversion yields is made possible by the application of synthetic enzymatic pathways using a collection of recombinant enzymes [117, 118]. While rates of conversion and total prices still require improvement, cost estimates indicate that an ecologically benign and sustainable strategy can eventually compete with thermochemically produced H_2. The potential for H_2 generation in the years to come is

contingent upon both scientific and economic developments, including the development of bioreactors, genetically modified microbes, and efficient manufacture of thermostable enzymes. The utilization of less expensive lignocellulosic substrates and the invention of suitable and effective bioreactor types are essential if hydrogen fermentation is to meet the world's demand for renewable energy [116, 4]. Before large-scale bio-hydrogen production can be applied effectively, there are still many obstacles to overcome in the design, building, operation, and management of suitable bioreactors. The production of bio-hydrogen can be guided and scale-up issues can be addressed by applying CFD (Computational Fluid Dynamics) to dark-fermentation. Furthermore, by combining dark-fermentation with photo-fermentation, scientists and practitioners will be able to maximize the amount of hydrogen produced overall in addition to solving issues with substrate feedback inhibition during dark-fermentation [117, 119]. All things considered, producing hydrogen by dark fermentation is a viable method for the long-term development of renewable hydrogen for commercial and industrial purposes.

7. CONCLUSION

Technologies based on biohydrogen are still in their infancy. Current methods have potential for real-world use, but in order for biohydrogen systems to become economically viable, they need to be able to produce enough H_2 to run fuel cells big enough for real-world use. Biohydrogen systems have promising futures if bioreactor designs are optimized, gases are quickly removed and purified, and enzyme pathways that compete with systems that produce hydrogen are genetically modified [120]. The size of the bioreactor would be drastically decreased even if certain dark-fermentation methods were to improve the rate of H_2 synthesis by ten times. This would make it far easier to overcome the scale-up engineering hurdles and produce new prospects for real-world implementations. Numerous outcomes have demonstrated increased hydrogen yield from various substrates and inoculum when paired with an efficient and suitable pretreatment method. Also, it was shown that, when compared to alternative techniques for producing bio-hydrogen from wastes high in carbohydrates, the sequential or combined bioprocesses of dark and photo-fermentations appear to be the most appealing strategy [123, 124]. Two essential factors require optimization: an appropriate substrate and a perfect microbial culture capable of effectively converting the substrate into hydrogen. Scientific breakthroughs include organism genetic modification, hydrogenase enzyme engineering, the use of better bioreactors, and hybrid processes can all help to improve hydrogen generation [124, 125]. The future of biological H2 production is dependent on a number of factors, including social acceptance, economic factors (such as the cost of fossil fuels), and technological advancements in the form of hydrogen energy systems and genetically engineered microorganisms that enhance efficiency.

REFERENCES

1. Rathi, B.S., Kumar, P.S., Rangasamy, G. and Rajendran, S., 2024. A critical review on Biohydrogen generation from biomass. International Journal of Hydrogen Energy, 52, pp.115-138.
2. Boodhun, B.S.F., Mudhoo, A., Kumar, G., Kim, S.H. and Lin, C.Y., 2017. Research perspectives on constraints, prospects and opportunities in biohydrogen production. International Journal of Hydrogen Energy, 42(45), pp.27471-27481.
3. Levin, D.B., Pitt, L. and Love, M., 2004. Biohydrogen production: prospects and limitations to practical application. International journal of hydrogen energy, 29(2), pp.173-185.
4. Singh, A. and Rathore, D., 2017. Biohydrogen: next generation fuel. Biohydrogen production: sustainability of current technology and future perspective, pp.1-10.
5. Hamad, T.A., Agll, A.A., Hamad, Y.M. and Sheffield, J.W., 2014. Solid waste as renewable source of energy: current and future possibility in Libya. Case studies in thermal Engineering, 4, pp.144-152.
6. Boodhun, B.S.F., Mudhoo, A., Kumar, G., Kim, S.H. and Lin, C.Y., 2017. Research perspectives on constraints, prospects and opportunities in biohydrogen production. International Journal of Hydrogen Energy, 42(45), pp.27471-27481.
7. Kumar Gupta, S., Kumari, S., Reddy, K. and Bux, F., 2013. Trends in biohydrogen production: major challenges and state-of-the-art developments. Environmental technology, 34(13-14), pp.1653-1670.

8. Rathore, D., Singh, A., Dahiya, D. and Nigam, P.S.N., 2019. Sustainability of biohydrogen as fuel: Present scenario and future perspective. AIMS Energy, 7(1), pp.1-19.
9. Singh, J. and Gu, S., 2010. Biomass conversion to energy in India—A critique. Renewable and sustainable energy reviews, 14(5), pp.1367-1378.
10. Nahar, G., Mote, D. and Dupont, V., 2017. Hydrogen production from reforming of biogas: Review of technological advances and an Indian perspective. Renewable and Sustainable Energy Reviews, 76, pp.1032-1052.
11. Melamu, R., 2008. Hydrogen as an energy product from agriculturally produced sugars and starches in South Africa.
12. Masilela, P. and Pradhan, A., 2021. A life cycle sustainability assessment of biomethane versus biohydrogen–for application in electricity or vehicle fuel? Case studies for African context. Journal of Cleaner Production, 328, p.129567.
13. Diallo, A.D.D., Alkhatib, M.F.R., Alam, M.Z. and Mel, M., 2021, November. Simulation of biomass and municipal solid waste pellet gasification using Aspen Plus. In IOP Conference Series: Materials Science and Engineering (Vol. 1192, No. 1, p. 012023). IOP Publishing.
14. Diallo, A.D.D., Alkhatib, M.F.R., Alam, M.Z. and Mel, M., 2021, November. Simulation of biomass and municipal solid waste pellet gasification using Aspen Plus. In IOP Conference Series: Materials Science and Engineering (Vol. 1192, No. 1, p. 012023). IOP Publishing.
15. Rizvi, S.M.M., Taqvi, S.A.A., Khan, M.M., Zabiri, H., Qadir, D., Ouladsmane, M. and Metwally, A.S.M., 2023. Techno-economic sustainability assessment for bio-hydrogen

production based on hybrid blend of biomass: A simulation study. Fuel, 347, p.128458.

16. Pandu, K. and Joseph, S., 2012. Comparisons and limitations of biohydrogen production processes: a review. International Journal of Advances in Engineering & Technology, 2(1), p.342.
17. Masilela, P. and Pradhan, A., 2022. Systematic literature review of the sustainability and environmental performance of dark fermentative biohydrogen production. Journal of Cleaner Production, 372, p.133541.
18. Zhang, T., Jiang, D., Zhang, H., Jing, Y., Tahir, N., Zhang, Y. and Zhang, Q., 2020. Comparative study on bio-hydrogen production from corn stover: photo-fermentation, dark-fermentation and dark-photo co-fermentation. International Journal of Hydrogen Energy, 45(6), pp.3807-3814.
19. Park, J.H., Chandrasekhar, K., Jeon, B.H., Jang, M., Liu, Y. and Kim, S.H., 2021. State-of-the-art technologies for continuous high-rate biohydrogen production. Bioresource technology, 320, p.124304.
20. Rittmann, S. and Herwig, C., 2012. A comprehensive and quantitative review of dark fermentative biohydrogen production. Microbial cell factories, 11, pp.1-18.
21. Lee, H.S., Vermaas, W.F. and Rittmann, B.E., 2010. Biological hydrogen production: prospects and challenges. Trends in biotechnology, 28(5), pp.262-271.
22. Kapdan, I.K. and Kargi, F., 2006. Bio-hydrogen production from waste materials. Enzyme and microbial technology, 38(5), pp.569-582.

23. Ren, N., Guo, W., Liu, B., Cao, G. and Ding, J., 2011. Biological hydrogen production by dark fermentation: challenges and prospects towards scaled-up production. Current opinion in biotechnology, 22(3), pp.365-370.
24. Cao, Y., Liu, H., Liu, W., Guo, J. and Xian, M., 2022. Debottlenecking the biological hydrogen production pathway of dark fermentation: insight into the impact of strain improvement.Microbial Cell Factories, 21(1), p.166.
25. Das, S.R. and Basak, N., 2021. Molecular biohydrogen production by dark and photo fermentation from wastes containing starch: recent advancement and future perspective. Bioprocess and biosystems engineering, 44, pp.1-25.
26. Reddy, C.V., Reddy, K.R., Harish, V.A., Shim, J., Shankar, M.V., Shetti, N.P. and Aminabhavi, T.M., 2020. Metal-organic frameworks (MOFs)-based efficient heterogeneous photocatalysts: synthesis, properties and its applications in photocatalytic hydrogen generation, CO2 reduction and photodegradation of organic dyes. International Journal of Hydrogen Energy, 45(13), pp.7656-7679.
27. Mohanraj, S., Anbalagan, K., Rajaguru, P. and Pugalenthi, V., 2016. Effects of phytogenic copper nanoparticles on fermentative hydrogen production by Enterobacter cloacae and Clostridium acetobutylicum. International Journal of Hydrogen Energy, 41(25), pp.10639-10645.
28. Abubackar, H.N., Keskin, T., Yazgin, O., Gunay, B., Arslan, K. and Azbar, N., 2019. Biohydrogen production from autoclaved fruit and vegetable wastes by dry fermentation under thermophilic condition. International Journal of Hydrogen Energy, 44(34), pp.18776-18784.

29. Łukajtis, R., Hołowacz, I., Kucharska, K., Glinka, M., Rybarczyk, P., Przyjazny, A. and Kamiński, M., 2018. Hydrogen production from biomass using dark fermentation. Renewable and Sustainable Energy Reviews, 91, pp.665-694.
30. Hallenbeck, P.C., 2005. Fundamentals of the fermentative production of hydrogen. Water Science and Technology, 52(1-2), pp.21-29.
31. Hallenbeck, P.C., Ghosh, D., Skonieczny, M.T. and Yargeau, V., 2009. Microbiological and engineering aspects of biohydrogen production. Indian journal of microbiology, 49, pp.48-59.
32. Banu, J.R., Kavitha, S., Kannah, R.Y., Bhosale, R.R. and Kumar, G., 2020. Industrial wastewater to biohydrogen: possibilities towards successful biorefinery route. Bioresource technology, 298, p.122378.
33. Ahmed, S.F., Rafa, N., Mofijur, M., Badruddin, I.A., Inayat, A., Ali, M.S., Farrok, O. and Yunus Khan, T.M., 2021. Biohydrogen production from biomass sources: metabolic pathways and economic analysis. Frontiers in Energy Research, 9, p.753878.
34. Mona, S., Kumar, S.S., Kumar, V., Parveen, K., Saini, N., Deepak, B. and Pugazhendhi, A., 2020. Green technology for sustainable biohydrogen production (waste to energy): a review. Science of the Total Environment, 728, p.138481.
35. Strazzera, G., Battista, F., Garcia, N.H., Frison, N. and Bolzonella, D., 2018. Volatile fatty acids production from food wastes for biorefinery platforms: A review. Journal of environmental management, 226, pp.278-288.
36. Kosse, P., Lübken, M. and Wichern, M., 2016. Selective inhibition of methanogenic archaea in leach bed systems by sodium 2-bromoethanesulfonate. Environmental Technology & Innovation, 5, pp.199-207.

37. Infantes, D., Del Campo, A.G., Villaseñor, J. and Fernández, F.J., 2011. Influence of pH, temperature and volatile fatty acids on hydrogen production by acidogenic fermentation. International Journal of Hydrogen Energy, 36(24), pp.15595-15601.
38. Hassan, G.K., Massanet-Nicolau, J., Dinsdale, R., Jones, R.J., Abo-Aly, M.M., El-Gohary, F.A. and Guwy, A., 2019. A novel method for increasing biohydrogen production from food waste using electrodialysis. International Journal of Hydrogen Energy, 44(29), pp.14715-14720.
39. Weide, T., Brügging, E., Wetter, C., Ierardi, A. and Wichern, M., 2019. Use of organic waste for biohydrogen production and volatile fatty acids via dark fermentation and further processing to methane. International journal of hydrogen energy, 44(44), pp.24110-24125.
40. Pandey, A.K., Pilli, S., Bhunia, P., Tyagi, R.D., Surampalli, R.Y., Zhang, T.C., Kim, S.H. and Pandey, A., 2022. Dark fermentation: Production and utilization of volatile fatty acid from different wastes-A review. Chemosphere, 288, p.132444.
41. Aluwong, T., Kobo, P.I. and Abdullahi, A., 2010. Volatile fatty acids production in ruminants and the role of monocarboxylate transporters: a review. African Journal of Biotechnology, 9(38), pp.6229-6232.
42. Lee, W.S., Chua, A.S.M., Yeoh, H.K. and Ngoh, G.C., 2014. A review of the production and applications of waste-derived volatile fatty acids. Chemical Engineering Journal, 235, pp.83-99.
43. Jhariya, U., Dafale, N.A., Srivastava, S., Bhende, R.S., Kapley, A. and Purohit, H.J., 2021. Understanding ethanol tolerance mechanism in Saccharomyces cerevisiae to enhance

the bioethanol production: Current and future prospects. BioEnergy Research, 14, pp.670-688.

44. Bastidas-Oyanedel, J.R., Bonk, F., Thomsen, M.H. and Schmidt, J.E., 2015. Dark fermentation biorefinery in the present and future (bio) chemical industry. Reviews in Environmental Science and Bio/Technology, 14, pp.473-498.
45. Dwidar, M., Park, J.Y., Mitchell, R.J. and Sang, B.I., 2012. The future of butyric acid in industry. The Scientific World Journal, 2012.
46. Gonzalez-Garcia, R.A., McCubbin, T., Navone, L., Stowers, C., Nielsen, L.K. and Marcellin, E., 2017. Microbial propionic acid production. Fermentation, 3(2), p.21.
47. Chen, W.S., Strik, D.P., Buisman, C.J. and Kroeze, C., 2017. Production of caproic acid from mixed organic waste: an environmental life cycle perspective. Environmental Science & Technology, 51(12), pp.7159-7168.
48. Reyhanitash, E., Kersten, S.R. and Schuur, B., 2017. Recovery of volatile fatty acids from fermented wastewater by adsorption. ACS sustainable chemistry & engineering, 5(10), pp.9176-9184.
49. da Silva, A.H. and Miranda, E.A., 2013. Adsorption/desorption of organic acids onto different adsorbents for their recovery from fermentation broths. Journal of Chemical & engineering data, 58(6), pp.1454-1463.
50. Rebecchi, S., Pinelli, D., Bertin, L., Zama, F., Fava, F. and Frascari, D., 2016. Volatile fatty acids recovery from the effluent of an acidogenic digestion process fed with grape pomace by adsorption on ion exchange resins. Chemical Engineering Journal, 306, pp.629-639.

51. Rizzioli, F., Battista, F., Bolzonella, D. and Frison, N., 2021. Volatile fatty acid recovery from anaerobic fermentate: focusing on adsorption and desorption performances. Industrial & Engineering Chemistry Research, 60(37), pp.13701-13709.
52. Reyhanitash, E., Kersten, S.R. and Schuur, B., 2017. Recovery of volatile fatty acids from fermented wastewater by adsorption. ACS sustainable chemistry & engineering, 5(10), pp.9176-9184.
53. Maeda, T., Sanchez-Torres, V. and Wood, T.K., 2007. Escherichia coli hydrogenase 3 is a reversible enzyme possessing hydrogen uptake and synthesis activities. Applied microbiology and biotechnology, 76, pp.1035-1042.
54. Self, W.T., Hasona, A. and Shanmugam, K.T., 2004. Expression and regulation of a silent operon, hyf, coding for hydrogenase 4 isoenzyme in Escherichia coli. Journal of bacteriology, 186(2), pp.580-587.
55. Self, W.T. and Shanmugam, K.T., 2000. Isolation and characterization of mutated FhlA proteins which activate transcription of the hyc operon (formate hydrogenlyase) of Escherichia coli in the absence of molybdate. FEMS microbiology letters, 184(1), pp.47-52.
56. Vardar-Schara, G., Maeda, T. and Wood, T.K., 2008. Metabolically engineered bacteria for producing hydrogen via fermentation. Microbial Biotechnology, 1(2), pp.107-125.
57. Li, Q., Gao, X., Liu, Y., Wang, G., Li, Y.Y., Sano, D., Wang, X. and Chen, R., 2021. Biochar and GAC intensify anaerobic phenol degradation via distinctive adsorption and conductive properties. Journal of Hazardous Materials, 405, p.124183.

58. Ma, J., Wang, Z., Li, L., Shi, Z., Ke, S. and He, Q., 2022. Granular activated carbon stimulated caproate production through chain elongation in fluidized cathode electro-fermentation systems. Journal of Cleaner Production, 365, p.132757.
59. Kumar, R., Kim, T.H., Basak, B., Patil, S.M., Kim, H.H., Ahn, Y., Yadav, K.K., Cabral-Pinto, M.M. and Jeon, B.H., 2022. Emerging approaches in lignocellulosic biomass pretreatment and anaerobic bioprocesses for sustainable biofuels production. Journal of Cleaner Production, 333, p.130180.
60. Chen, W., Zeng, Y., Liu, H., Sun, D., Liu, X., Xu, H., Wu, H., Qiu, B. and Dang, Y., 2023. Granular activated carbon enhances volatile fatty acid production in the anaerobic fermentation of garden wastes. Frontiers in Bioengineering and Biotechnology, 11.
61. Ritter, S.K., 2003. Green solutions to global problems. Chemical & Engineering News, 81(39), pp.31-31.
62. Baxter, L.W. and Calandri, K., 1992. Global warming and electricity demand: A study of California. Energy Policy, 20(3), pp.233-244.
63. Nayak, B.K., Pandit, S. and Das, D., 2013. Biohydrogen. Air Pollution Prevention and Control: Bioreactors and Bioenergy, pp.345-381.
64. Arent, D.J., Wise, A. and Gelman, R., 2011. The status and prospects of renewable energy for combating global warming. Energy Economics, 33(4), pp.584-593.
65. P. Boisen and M. Lage. Fact Sheet: NG/biomethane used as vehicle fuel. NGVA Europe (2009)
66. Barreto, L., Makihira, A. and Riahi, K., 2003. The hydrogen economy in the 21st century: a sustainable development scenario. International Journal of Hydrogen Energy, 28(3), pp.267-284.

67. Zhong, J.J., 2011. 2.14—Bioreactor Engineering. Comprehensive Biotechnology, 2nd ed.; Moo-Young, M., Ed, pp.165-177.
68. Garcia-Aguirre, M., Saenz-Alvaro, V.A., Rodriguez-Soto, M.A., Vicente-Magueyal, F.J., Botello-Alvarez, E., Jimenez-Islas, H., Cardenas-Manriquez, M., Rico-Martinez, R. and Navarrete-Bolanos, J.L., 2009. Strategy for biotechnological process design applied to the enzymatic hydrolysis of agave fructo-oligosaccharides to obtain fructose-rich syrups. Journal of Agricultural and Food Chemistry, 57(21), pp.10205-10210.
69. Kim, M.S., Cha, J. and Kim, D.H., 2013. Fermentative biohydrogen production from solid wastes. In Biohydrogen (pp. 259-283). Elsevier.
70. Monlau, F., Trably, E., Barakat, A., Hamelin, J., Steyer, J.P. and Carrere, H., 2013. Two-stage alkaline–enzymatic pretreatments to enhance biohydrogen production from sunflower stalks. Environmental science & technology, 47(21), pp.12591-12599.
71. Sharmila, V.G., Tamilarasan, K., Kumar, M.D., Kumar, G., Varjani, S., Kumar, S.A. and Banu, J.R., 2022. Trends in dark biohydrogen production strategy and linkages with transition towards low carbon economy: An outlook, cost-effectiveness, bottlenecks and future scope. International Journal of Hydrogen Energy, 47(34), pp.15309-15332.
72. Katakojwala, R. and Mohan, S.V., 2021. A critical view on the environmental sustainability of biorefinery systems. Current Opinion in Green and Sustainable Chemistry, 27, p.100392.
73. Federal Ministry of Education and Research (BMBF), 2011. National Research Strategy BioEconomy 2030—Our Route Towards a Bio Based Economy.

74. Imbert, E., Ladu, L., Morone, P. and Quitzow, R., 2017. Comparing policy strategies for a transition to a bioeconomy in Europe: The case of Italy and Germany. Energy Research & Social Science, 33, pp.70-81.
75. Wohlfahrt, J., Ferchaud, F., Gabrielle, B., Godard, C., Kurek, B., Loyce, C. and Therond, O., 2019. Characteristics of bioeconomy systems and sustainability issues at the territorial scale. A review. Journal of Cleaner Production, 232, pp.898-909.
76. Ramprakash, B., Lindblad, P., Eaton-Rye, J.J. and Incharoensakdi, A., 2022. Current strategies and future perspectives in biological hydrogen production: A review. Renewable and Sustainable Energy Reviews, 168, p.112773.
77. Ncube, A., Fiorentino, G., Colella, M. and Ulgiati, S., 2021. Upgrading wineries to biorefineries within a Circular Economy perspective: An Italian case study. Science of the Total Environment, 775, p.145809.
78. Mohan, S.V., Nikhil, G.N., Chiranjeevi, P., Reddy, C.N., Rohit, M.V., Kumar, A.N. and Sarkar, O., 2016. Waste biorefinery models towards sustainable circular bioeconomy: critical review and future perspectives. Bioresource technology, 215, pp.2-12.
79. Poggi-Varaldo, H.M., Munoz-Paez, K.M., Escamilla-Alvarado, C., Robledo-Narváez, P.N., Ponce-Noyola, M.T., Calva-Calva, G., Ríos-Leal, E., Galíndez-Mayer, J., Estrada-Vázquez, C., Ortega-Clemente, A. and Rinderknecht-Seijas, N.F., 2014. Biohydrogen, biomethane and bioelectricity as crucial components of biorefinery of organic wastes: a review. Waste management & research, 32(5), pp.353-365.
80. Ahmad, A., Rambabu, K., Hasan, S.W., Show, P.L. and Banat, F., 2023. Biohydrogen production through dark fermentation: Recent trends and advances in transition to a circular bioeconomy. International Journal of Hydrogen Energy.

81. Banu, J.R., Kannah, R.Y., Kumar, M.D., Gunasekaran, M., Sivagurunathan, P., Park, J.H. and Kumar, G., 2018. Recent advances on biogranules formation in dark hydrogen fermentation system: mechanism of formation and microbial characteristics. Bioresource technology, 268, pp.787-796.
82. Sargsyan, H., Trchounian, K., Gabrielyan, L. and Trchounian, A., 2016. Novel approach of ethanol waste utilization: biohydrogen production by mixed cultures of dark-and photo-fermentative bacteria using distillers' grains. international journal of hydrogen energy, 41(4), pp.2377-2382.
83. Wang, Y., Jing, Y., Lu, C., Kongjan, P., Wang, J., Awasthi, M.K., Tahir, N. and Zhang, Q., 2021. A syntrophic co-fermentation model for bio-hydrogen production. Journal of Cleaner Production, 317, p.128288.
84. Saady, N.M.C., Chaganti, S.R., Lalman, J.A., Veeravalli, S.S., Shanmugam, S.R. and Heath, D.D., 2012. Effects of linoleic acid and its degradation by-products on mesophilic hydrogen production using flocculated and granular mixed anaerobic cultures. International journal of hydrogen energy, 37(24), pp.18747-18760.
85. Greening, C., Biswas, A., Carere, C.R., Jackson, C.J., Taylor, M.C., Stott, M.B., Cook, G.M. and Morales, S.E., 2016. Genomic and metagenomic surveys of hydrogenase distribution indicate H2 is a widely utilised energy source for microbial growth and survival. The ISME journal, 10(3), pp.761-777.
86. Cabrol, L., Marone, A., Tapia-Venegas, E., Steyer, J.P., Ruiz-Filippi, G. and Trably, E., 2017. Microbial ecology of fermentative hydrogen producing bioprocesses: useful insights for driving the ecosystem function. FEMS Microbiology Reviews, 41(2), pp.158-181.

87. Dincer, I. and Acar, C., 2017. Innovation in hydrogen production. International Journal of Hydrogen Energy, 42(22), pp.14843-14864.
88. Falcone, P.M. and Hiete, M., 2019. Exploring green and sustainable chemistry in the context of sustainability transition: The role of visions and policy. Current Opinion in Green and Sustainable Chemistry, 19, pp.66-75.
89. Fisch-Romito, V., Guivarch, C., Creutzig, F., Minx, J.C. and Callaghan, M.W., 2021. Systematic map of the literature on carbon lock-in induced by long-lived capital. Environmental Research Letters, 16(5), p.053004.
90. Kovač, A., Paranos, M. and Marciuš, D., 2021. Hydrogen in energy transition: A review. International Journal of Hydrogen Energy, 46(16), pp.10016-10035.
91. Falcone, P.M., Hiete, M. and Sapio, A., 2021. Hydrogen economy and sustainable development goals: Review and policy insights. Current opinion in green and sustainable chemistry, 31, p.100506.
92. Dahiya, S., Chatterjee, S., Sarkar, O. and Mohan, S.V., 2021. Renewable hydrogen production by dark-fermentation: Current status, challenges and perspectives. Bioresource technology, 321, p.124354.
93. Sohale, A.P., Janardanan, S., Yadav, D., Dash, B. and Yadav, M.D., 2023. Dark Fermentative Biohydrogen Production: Recent Advances and Challenges. Industrial & Engineering Chemistry Research, 62(37), pp.14755-14771.
94. Balachandar, G., Khanna, N. and Das, D., 2013. Biohydrogen production from organic wastes by dark fermentation. In Biohydrogen (pp. 103-144). Elsevier.

95. Nagarajan, D., Lee, D.J. and Chang, J.S., 2019. Recent insights into consolidated bioprocessing for lignocellulosic biohydrogen production. International Journal of Hydrogen Energy, 44(28), pp.14362-14379.

96. Silva-Illanes, F., Tapia-Venegas, E., Schiappacasse, M.C., Trably, E. and Ruiz-Filippi, G., 2017. Impact of hydraulic retention time (HRT) and pH on dark fermentative hydrogen production from glycerol. Energy, 141, pp.358-367.

97. Lu, C., Wang, Y., Lee, D.J., Zhang, Q., Zhang, H., Tahir, N., Jing, Y., Liu, H. and Zhang, K., 2019. Biohydrogen production in pilot-scale fermenter: effects of hydraulic retention time and substrate concentration. Journal of cleaner production, 229, pp.751-760.

98. Kamaraj, M., Ramachandran, K.K. and Aravind, J., 2020. Biohydrogen production from waste materials: benefits and challenges. International journal of environmental science and technology, 17(1), pp.559-576.

99. Kumar, N. and Das, D., 2000. Enhancement of hydrogen production by Enterobacter cloacae IIT-BT 08. Process Biochemistry, 35(6), pp.589-593.

100. Mishra, P., Balachandar, G. and Das, D., 2017. Improvement in biohythane production using organic solid waste and distillery effluent. Waste Management, 66, pp.70-78.

101. Balachandar, G., Varanasi, J.L., Singh, V., Singh, H. and Das, D., 2020. Biological hydrogen production via dark fermentation: A holistic approach from lab-scale to pilot-scale. International Journal of Hydrogen Energy, 45(8), pp.5202-5215.

102. Yari, K., Fatemi, S.S.A. and Tavallaei, M., 2012. High level expression of recombinant BoNT/A-Hc by high cell density cultivation of Escherichia coli. Bioprocess and biosystems engineering, 35, pp.407-414.

103. Solorzano, L., 1969. DETERMINATION OF AMMONIA IN NATURAL WATERS BY THE PHENOLHYPOCHLORITE METHOD 1 1 This research was fully supported by US Atomic Energy Commission Contract No. ATS (11-1) GEN 10, PA 20. Limnology and oceanography, 14(5), pp.799-801.

104. Pason, T.R., Maita, Y. and Lalli, C.M., 1984. A Manual of Chemical and Biological Methods for Seawater Analysis.

105. Lee, S.C. and Kim, H.C., 2011. Batch and continuous separation of acetic acid from succinic acid in a feed solution with high concentrations of carboxylic acids by emulsion liquid membranes. Journal of membrane science, 367(1-2), pp.190-196.

106. Huang, H.J., Ramaswamy, S., Tschirner, U.W. and Ramarao, B.V., 2008. A review of separation technologies in current and future biorefineries. Separation and purification technology, 62(1), pp.1-21.

107. Tao, B., Passanha, P., Kumi, P., Wilson, V., Jones, D. and Esteves, S., 2016. Recovery and concentration of thermally hydrolysed waste activated sludge derived volatile fatty acids and nutrients by microfiltration, electrodialysis and struvite precipitation for polyhydroxyalkanoates production. Chemical Engineering Journal, 295, pp.11-19.

108. Aktij, S.A., Zirehpour, A., Mollahosseini, A., Taherzadeh, M.J., Tiraferri, A. and Rahimpour, A., 2020. Feasibility of membrane processes for the recovery and purification

of bio-based volatile fatty acids: A comprehensive review. Journal of Industrial and Engineering Chemistry, 81, pp.24-40.

109. Zacharof, M.P. and Lovitt, R.W., 2013. Complex effluent streams as a potential source of volatile fatty acids. Waste and biomass valorization, 4, pp.557-581.

110. Min, D.J., Choi, K.H., Chang, Y.K. and Kim, J.H., 2011. Effect of operating parameters on precipitation for recovery of lactic acid from calcium lactate fermentation broth. Korean Journal of Chemical Engineering, 28, pp.1969-1974.

111. Sukphun, P., Sittijunda, S. and Reungsang, A., 2021. Volatile fatty acid production from organic waste with the emphasis on membrane-based recovery. Fermentation, 7(3), p.159.

112. Montgomery, H.A.C., Dymock, J.F. and Thom, N.S., 1962. The rapid colorimetric determination of organic acids and their salts in sewage-sludge liquor. Analyst, 87(1041), pp.949-955.

113. Aramrueang, N., Lomwongsopon, P., Boonsong, S. and Kingklao, P., 2022. Improved Spectrophotometric Method for Determination of High-Range Volatile Fatty Acids in Mixed Acid Fermentation of Organic Residues. Fermentation, 8(5), p.202.

114. Thauer, R.K., Jungermann, K. and Decker, K., 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriological reviews, 41(1), pp.100-180.

115. Amend, J.P. and Shock, E.L., 2001. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. FEMS microbiology reviews, 25(2), pp.175-243.

116. Prabakar, D., Manimudi, V.T., Sampath, S., Mahapatra, D.M., Rajendran, K. and Pugazhendhi, A., 2018. Advanced biohydrogen production using pretreated industrial waste: outlook and prospects. Renewable and Sustainable Energy Reviews, 96, pp.306-324.

117. Ren, N., Guo, W., Liu, B., Cao, G. and Ding, J., 2011. Biological hydrogen production by dark fermentation: challenges and prospects towards scaled-up production. Current opinion in biotechnology, 22(3), pp.365-370.

118. Kim, J.E., Kim, E.J., Chen, H., Wu, C.H., Adams, M.W. and Zhang, Y.H.P., 2017. Advanced water splitting for green hydrogen gas production through complete oxidation of starch by in vitro metabolic engineering. Metabolic engineering, 44, pp.246-252.

119. Brar, K.K., Cortez, A.A., Pellegrini, V.O., Amulya, K., Polikarpov, I., Magdouli, S., Kumar, M., Yang, Y.H., Bhatia, S.K. and Brar, S.K., 2022. An overview on progress, advances, and future outlook for biohydrogen production technology. International Journal of Hydrogen Energy, 47(88), pp.37264-37281.

120. Kotay, S.M. and Das, D., 2010. Microbial hydrogen production from sewage sludge bioaugmented with a constructed microbial consortium. International journal of hydrogen energy, 35(19), pp.10653-10659.

121. Kotay, S.M. and Das, D., 2007. Microbial hydrogen production with Bacillus coagulans IIT-BT S1 isolated from anaerobic sewage sludge. Bioresource technology, 98(6), pp.1183-1190.

122. Kraemer, J.T. and Bagley, D.M., 2007. Improving the yield from fermentative hydrogen production. Biotechnology letters, 29, pp.685-695.

123. Liu, C.Z. and Cheng, X.Y., 2010. Improved hydrogen production via thermophilic fermentation of corn stover by microwave-assisted acid pretreatment. International Journal of Hydrogen Energy, 35(17), pp.8945-8952.

124. Karthic, P. and Joseph, S., 2012. Comparison and limitations of biohydrogen production processes. Res J Biotechnol, 7(2), pp.59-71.

125. Yang, G. and Wang, J., 2020. Biohydrogen production from waste activated sludge pretreated by combining sodium citrate with ultrasonic: Energy conversion and microbial community. Energy conversion and management, 225, p.113436.