"PHOTO-BIOLOGICAL HYDROGEN PRODUCTION AND RECOVERY OF VOLATILE FATTY ACIDS FROM WASTE ACTIVATED SLUDGE"

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MASTER OF TECHNOLOGY

IN

ENVIRONMENTAL BIOTECHNOLOGY

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FACULTY OF INTERDISCIPLINARY STUDIES, LAW AND MANAGEMENT

(F.I.S.L.M)

JADAVPUR UNIVERSITY

KOLKATA

"PHOTO-BIOLOGICAL HYDROGEN PRODUCTION AND RECOVERY OF VOLATILE FATTY ACIDS FROM WASTE ACTIVATED SLUDGE"

A thesis

Submitted in partial fulfilment of the requirements for the award of degree of

M. Tech in Environmental Biotechnology

Jadavpur University

By

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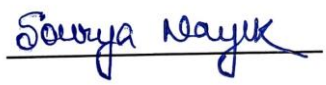
DECLARATION

I hereby declared that the work presented in this thesis report title **"Photo-biological hydrogen production and recovery of volatile fatty acids from waste activated sludge"** 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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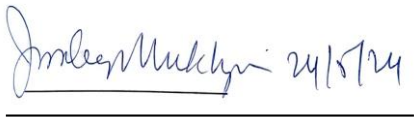

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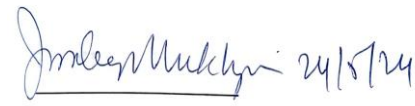

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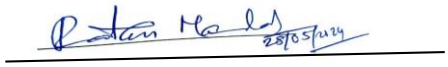

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We certify that Sourya Nayek thesis, **"Photo-biological hydrogen production and recovery of volatile fatty acids from waste activated sludge"**, is an authentic documentation of his work completed between September 2022 and May 2024, partially fulfilling the requirements for the Master of Engineering in Environmental Biotechnology degree from the Department of Environmental Studies (Registration Number 163792 of 2022–2023; Class Roll No. 002230904011 Examination Roll No. M4EBT24006). It is acknowledged that the undersigned only approves the thesis for the purpose for which it has been presented, and that by granting this approval, they do not necessarily support or approve any statements made, opinions stated, or conclusions drawn within.

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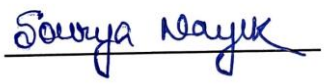

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ABSTRACT

The demand for energy is rising primarily because of technological advances. Fossil fuels are unable to generate energy with such massive amounts. Renewably generated clean energy can address this problem. Hydrogen is an environmental friendly and clean fuel that may be utilised in combustion machines or fuel cells for generating power. Starch wastes from various industrial food processing wastes are easily accessible, which makes them a viable source for the production of hydrogen (H_2). The production of H_2 during the fermentation process is based upon various external bioprocess parameters, including pH, temperature, and light intensity; substrate type and concentration (simple sugar or complex carbohydrate), mixed or pure microorganism strain; mode of operation (batch, fed batch, or continuous), fermentation technique (dark or photo) and the design and configuration of the bioreactor. Among various processes, photo fermentation way of Bio-H_2 production is thought to be one of the easy and cost-effective technique. Photo-fermentation is a potentially dynamic method that can utilise a range of feedstocks, such as the residues of dark fermentations, which can result in different two-stage system configurations, or different industrial and agricultural waste streams that are high in organic acids or sugars. This system's metabolic and enzymatic characteristic are described, and potential waste streams for practical use are explored.

The substrate in photo-fermentation technique was chosen as waste activated sludge which is produced in large amount in many water treatment plants around the country. The WAS is found to be reach in all type of nutrients needed for a bacterium to grow and able to produce gases. Purple non-sulphur bacteria were chosen to carry out the photo-fermentation process under proper illumination of light under anaerobic conditions. Different parameters were checked to enhanced the proper production of Bio-H_2 in lab scale. We have followed simple water displacement method to collect the produced gas in a 250-ml measuring cylinder. At the end of 96 hrs we could find 250 ml of water to get displaced and assume that that much amount of gases was produced. When we tried to execute the process by using WAS as substrate then at the same time interval there was gas collected. Different microorganism presents in the WAS obstructed ***R. sphaeroides*** to ferment the substrate. Next step will be pre-treatment of WAS and run the same process to overcome the challenges and to able to produce Bio-H_2 at an easy step.

Keywords: Sustainability, Energy, Bio-hydrogen, Anaerobic Fermentation *Rhodopseudomonas sphaeroides*, VFA, biofuel, bioplastics, adsorption, chemical precipitation.

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The massive energy content of hydrogen (H_2) makes it a potential fuel in the future [1]. Because hydrogen has a higher combustion value and is more sustainable as well as environmentally friendly than other fuels, some scientists claim that the modern era will be a hydrogen-driven society [2]. Hydrogen gas is not freely available, it can be prepared by both biological and chemical processes [3]. As hydrogen flames smoothly, emitting only water instead of CO_2 or other dangerous pollutants, it is a clean energy source. Using hydrogen as fuel, it satisfies the current global effort to achieve zero emissions.

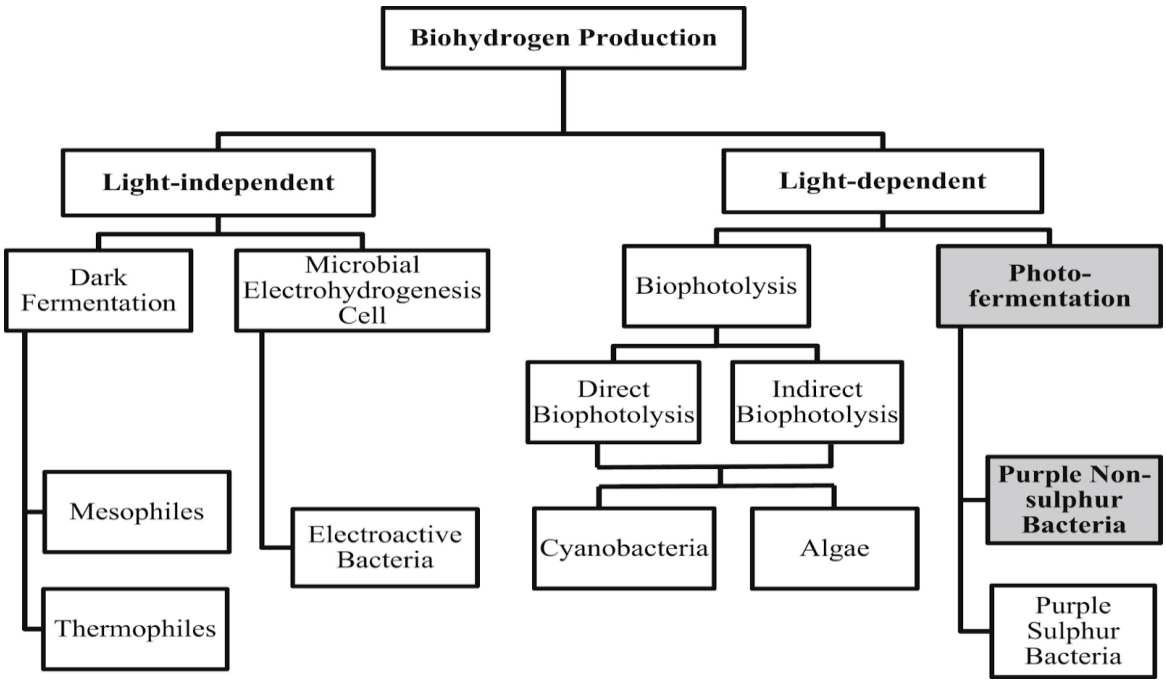


Figure 1 A schematic representation of various bio-hydrogen generation methods influenced by microorganisms [50]

The production of hydrogen from waste and wastewater, biomass, along with other renewable resources has drawn greater attention [15]. As per global statistics 8% of global hydrogen

demand can be produced by chemical processes such as steam reforming [4], photo-catalysis [5], gasification, biomass pyrolysis and electrolysis [6]. Biological process is easier and more advantageous over chemical process as it requires less input energy, can be operative at atmospheric pressure and room temperature in biological processes, fermentation is the best way for generating hydrogen [7]. Microorganisms, using simple sugar, produce CO_2 and H_2 in the fermentation process. The photo-fermentation process may be carried out with artificial or solar lighting, using different sources of carbon and nitrogen, including wastewater and dark fermentation effluents under a range of process, for example in batch or continuous mode [8] [9]. Since hydrogen provides an energy output of 122 kJ/g, equivalent to 2.75 times that of fossil fuels, it is an especially interesting energy carrier [15]. Furthermore, 1 g of hydrogen has approximately 142 kJ of energy, which makes hydrogen even more suitable to be utilised as fuel [17]. With plenty of advantages, such as the ability to use a variety of raw materials, including waste materials, environmental safety, a higher substrate to hydrogen conversion efficiency and the capacity to scale up for enormous scale hydrogen production at normal pressure and temperature, photo-fermentation is becoming increasingly important everyday [10]. Under anoxic or anaerobic conditions, photosynthetic bacteria convert organic substrates by fermentation into smaller molecules, H2, and carbon dioxide. This process is known as photo-fermentation [120]. Purple non-sulphur bacteria have been stated to be prospective for photo-fermentative hydrogen production due to their high substrate-to-hydrogen conversion rate as well as potential to decompose waste streams [11] [12] [13]. The process of photo-fermentation, which takes place in anoxic or anaerobic conditions, involves photosynthetic bacteria which ferments organic substrates to generate smaller molecules, like H_2 and CO_2 [14][8]. The production of BioH_2 by purple non-sulphur bacteria like *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* are becoming efficient day by day. Photo fermentative H_2 production is a membrane bound electron transfer process that occurs under a limited amount

of nitrogen (high C/N ratio), in anaerobic conditions, mediated by the nitrogenase enzyme complex in a wide range of absorption spectra [31]. Higher H_2 gas production and the utilisation of waste materials in the production process are benefits of the later approach [16]. In the upcoming decades, hydrogen is predicted to be employed as an alternative energy source and fuel because it has a tremendous future, that has carbon-free, clean, and a positive environmental image, it can replace fossil fuels [18]. In particular, photo-fermentation is effective for producing hydrogen from wastes, containing organic acids. It also serves as a desirable alternative to extract a greater amount of hydrogen from the sludge [19]. Different parameters, such as temperature, pH, inoculum amount, and microorganisms on photo-fermentative bio-hydrogen production have been studied [20]. An obvious advantage for photo-fermentation in comparison with dark fermentation is that, the PNS microorganisms are able to absorb and utilise a wide spectrum of light (522-860 nm) [29], and can also use organic substrates derived from various wastes for bio-hydrogen production [21].

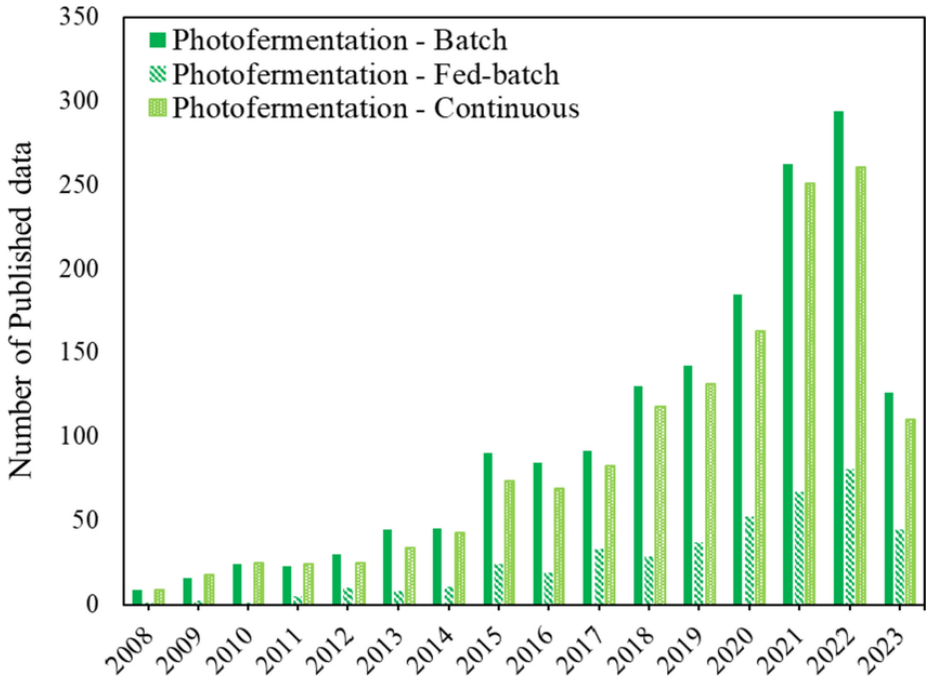
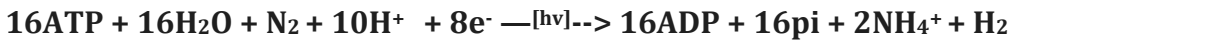


Figure 2: Graphical representation of Bio-H2 production [70]

1.1PHOTO-FERMENTATION:

Fermentation processes which involve light as a source of energy for photosynthesis are referred to as photo-fermentation. In comparison to dark fermentation, photo-fermentation has the advantage that light can replace carbohydrates as a source of energy. The following chemical equation depicts hydrogen production via photo-fermentation [21].



$$16ATP + 16H_2O + N_2 + 10H^+ + 8e^- \xrightarrow{[hv]} 16ADP + 16pi + 2NH_4^+ + H_2$$

Photosynthetic bacteria such as *Rhodopseudomonas sphaeroides* and *Rhodospirillum sp* are commonly applied and use small molecules of organic acids as carbon sources [22]. Purple non-sulphur (PNS) bacteria are highly promising in producing hydrogen via anaerobic photosynthesis and photo-fermentation [23]. The evolution of $BioH_2$ under the condition is catalysed by nitrogenase, which normally functions to catalyse the reduction of di-nitrogen to ammonia with the release of H_2 from reduced N_2. In the absence of other reducible substrates, nitrogenase continues to transform protons into $BioH_2$ [25]. The maximum light conversion efficiency is about 10% which has been reported by using PNS bacteria. Organic compounds can be completely converted into bio-hydrogen in the photo fermentation process, so this method can achieve larger bio-hydrogen yields and higher substrate efficiency compared to the dark fermentation process. Moreover, dark fermentation effluents rich in VFAs can be further used by photo-fermentative bacteria in photo fermentation for producing biohydrogen [24]. Ferredoxin acts as an electron carrier in the presence of an enzyme called nitrogenase in the cell membrane of photo fermentative bacteria. Nitrogenase enzyme controls the membrane bound electron

transfer mechanism which plays an important role in H_2 production [26]. Hydrogenase activity should be limited for enhancement of hydrogen gas production [27]. One of the primary conditions for efficient photo-fermentative production of hydrogen is an adequate amount of ATP. Purple non-sulphur bacteria can transform organic acid into hydrogen by breaking the thermodynamic barrier by means of light energy [28]. It has been found that iron is one of the important cofactors for the nitrogenase enzyme which boost the activity, which results in a faster rate of Bio-H_2 production [31].

1.1.2. Photo-fermentative microorganisms:

Members of the α-proteobacteria, which are purple non-sulphur photosynthetic bacteria, are particularly adapted for photoheterotrophic application. They use their independent photosystem to produce ATP through the process of cyclic photophosphorylation in order to photosynthetically build the energy needed for their growth and survival in the presence of light. They receive the electrons required for their metabolic processes from hydrogen, inorganic ions (Fe^{2+}). These PNS bacteria undergo C3 cycle and use ATP and NADPH to fix CO2 and produce glucose from it, which allows them to grow photo-auto-lithotrophically on iron or hydrogen [19]. Purple non-sulphur bacteria has photosynthetic bacterio-chlorophyll, which uses light to generate the energy needed for photo-fermentation, which produces CO_2 and bio-hydrogen [30].

1.1.3. Metabolism of Bio-H_2 production by photo-fermentation:

The overall biochemical pathways for the photo fermentation process can be expressed as follows:

Ferredoxin (Fd) acts as electron carrier in presence of nitrogenase in the cell membrane [32]

$(CH_2O)_n$ —> Ferredoxin —> Nitrogenase —> H_2

^ ^

| | (1)

ATP ATP

The term "non-sulfur" originated from the belief that PNS bacteria did not grow photoautotrophically while using hydrogen sulphide as an electron source. PNS bacteria, instead of sulphur bacteria, are able to use sulphide as an electron donor, but not at large concentrations [32]. Nitrogenase is the main enzyme in the process of producing biohydrogen, among hydrogenases and nitrogenases. The nitrogenase catalyses the reduction of protons to produce hydrogen by using ATP in the absence of a nitrogen availability [33]. The production of bio-hydrogen by photo fermentation is restricted by the high ammonium contents in certain waste-waters, which inhibit the nitrogenase activity [34]. Theoretically, if acetic acid is the primary VFA in the fermentation medium then 1 mol of acetic acid can produce 4 mol of H_2 in the presence of light in anaerobic conditions.

$CH_3COOH + 2\ H_2O \rightarrow 4\ H_2 + 2\ CO_2$ ΔG_0 = +104 kJ (2) [35].

It was reported that the optimum pH and temperature range are 6.8 – 7.5 and 31–36 °C, respectively [36]. It has been reported that the lack of favourable carbon sources, uneven dispersion of light in the fermentation broth, and the metabolic H_2 generation from VFA synthesis are the main issues with photo-fermentation [37] [38].

1.2 ENZYMES RESPONSIBLE FOR BIO-H2 PRODUCTION:

Hydrogenases and nitrogenases are two distinct types of enzymes, facilitating the reduction of protons (H^+) to produce molecular hydrogen (H_2). These two enzymes are classified as metalloproteins as their active sites include metals [39].

1.2.1. Hydrogenase:

Using photosynthetic bacteria, it comes to light that the hydrogenase enzyme is in charge of the initial intake and evolution of bio-hydrogen during photo-fermentation. A simple redox reaction may be employed to illustrate this reversible reaction, as demonstrated in (3) [40]. The redox potential of the molecules that can interact with hydrogenase regulates the path of this reaction [41].

Hydrogenase: $H_2 \longleftrightarrow 2H^+ + 2e^-$ (3)

Most hydrogenases found in purple bacteria are [NiFe]-hydrogenase, which, based on the subunits of the enzyme, can produce or consume biohydrogen. The heterodimer of α and β makes up the core of the [NiFe]-hydrogenase enzyme [41]. A quick increase in the production of biohydrogen is triggered by [NiFe]-hydrogenase during photofermentation, and this is usually followed by a biohydrogen assimilation. Ni-containing hydrogenases have been found to be less sensitive to CO_2 and O_2 inhibition than [FeFe]-hydrogenases [43]. During photosynthesis, the only photosystem (PS) in bacteriochlorophylls uses light energy to generate electrons from the electron donors, which are organic acids and compounds. The electrons then flow through an H+-pumping electron transport chain and oxidised ferredoxin (Fd). Adenosine triphosphate (ATP) is synthesised using the proton motive force, whereas electrons can be stimulated repeatedly and recycled through the pathway of electron transfer or contributed to

NADP+ to create NADPH for biosynthesis by reverse electron transfer [42]. However, methanogenic bacteria have been found reported to possess [Fe]-hydrogenase, which promotes the reduction of carbonate with biohydrogen to methane [41].

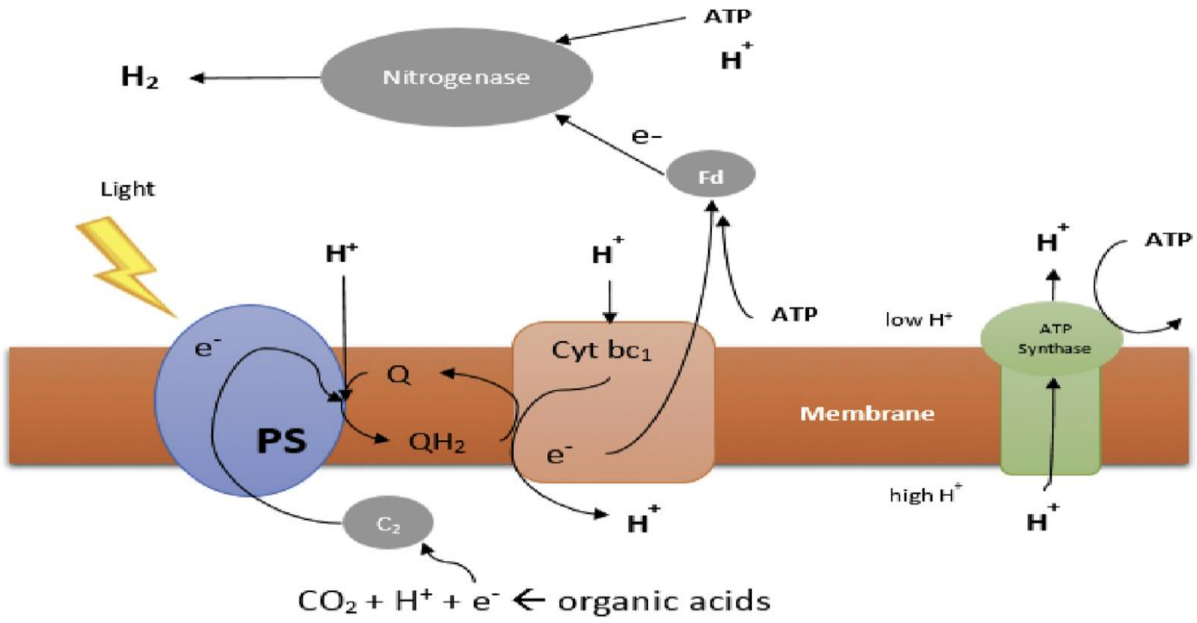


Figure 3 Hydrogen production via photo-fermentation by PNSB. [50].

1.2.2. Nitrogenase:

The nitrogen cycle on Earth depends on the enzyme nitrogenase, which catalyses N_2 fixation. It has been discovered that a broad spectrum of microbes, including bacteria and archaea, have this enzyme [44]. Nitrogenase is also able to produce H_2 during N_2 fixation. At the positions of nitrogen binding and reducing, distinct metal centres are found in the forms of nitrogenase that are found in molybdenum, vanadium, and iron. Dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein) are the two proteins that make up mo-nitrogenase, which has been studied the most [45].

Mo-nitrogenase:

$N_2 + 8H^+ + 8e^- + 16\ ATP \longrightarrow 2NH_3 + H_2 + 16\ ATP$ (4) [46]

Nitrogenase behaves as an ATP-powered hydrogenase in the absence of N_2, by simply producing H_2.

Absence of N2:

$8H^+ + 8e^- + 4\ ATP \longrightarrow 4H_2 + 4\ ADP$ (5) [47]

It has been found that severe nitrogen starvation conditions are associated with alternate nitrogenase expression [48]. Nitrogenase activity is disturbed by the presence of O_2 , N_2, and NH_4^+ [49]. Nitrogenase produces hydrogen through an irreversible process that is energy inefficient while four ATP are required to produce each mole of H_2. Two pathways exist for photosynthetic reduction in the hydrogenase system under anaerobic conditions: H_2 generation and CO_2 fixation [32].

1.3 PHOTO FERMENTATION: A BOON OR DISASTER TO THE INDUSTRY?

1.3.1 Advantages of Photo fermentation: -

- Using reduced chemicals and light energy, purple non-sulfur bacteria produce molecular H_2 in nitrogen-deficient circumstances, catalysed by nitrogenase [130].
- It has been found that *Rhodopseudomonas spheroides* can produce H_2 at rates varying from 80 to 100 ml H_2/l of culture [131] [132].
- The potential of these photoheterotrophic bacteria to convert light energy into H_2 has been studied using waste organic compounds as substrate [133], [134]. These investigations have taken place in batch processes [135], continuous cultures [136], [137], or cultures of bacteria immobilised in agar gel [138], carrageenan [139], porous glass, activated glass [140], or polyurethane foam [141].
- When acetic acid is the only VFA in the fermentation medium, potentially 4 mol of H_2 can be produced from 1 mol of acetic acid.

1.3.2 Disadvantages of photo fermentation: -

- High COD elimination rate and a high potential hydrogen yield have been associated with photo-fermentation; still the activity of the hydrogenase enzyme and light intensity hinder the commercial feasibility of hydrogen production [54].
- The light intensity is one of those factors influencing photo-fermentation activity. It has a negative impact on light conversion efficiency, increasing light intensity has a stimulatory effect on hydrogen yield and production rate [55] [56].

- Some limitations like low Bio-H2 yield, light limitation, cell washout, high cell concentration and ineffective against toxic/recalcitrant compounds present in IWWs, rpm speed and temperature hamper the production [66].
- The production also demands for a larger water content, greater capital costs, and a lower biomass concentration [67] [68].
- Ineffective against toxic compounds that already exist in wastewaters as well as an unpredictable yield of Bio-H_2 while running continuously [69].
- Lack of preferred C-sources, such as malate and lactate, inconsistent distribution of light across fermentation broth, and metabolic shift from production of hydrogen to PHB synthesis have all been identified as major issues with photo-fermentation.

1.4. ORGANIC ACIDS:

Organic acids having six or fewer carbon atoms are known as volatile fatty acids (VFAs). Examples of these acids are acetic acid, propionic acid, butyric acid, iso-butyric acid, and iso-valeric acid [71]. The commercial synthesis of volatile fatty acids (VFAs) demands the use of non-renewable sources as raw material. However, the use of non-renewable resources in this synthesis is becoming increasingly difficult because of climate change and the depletion of fossil fuel [74].

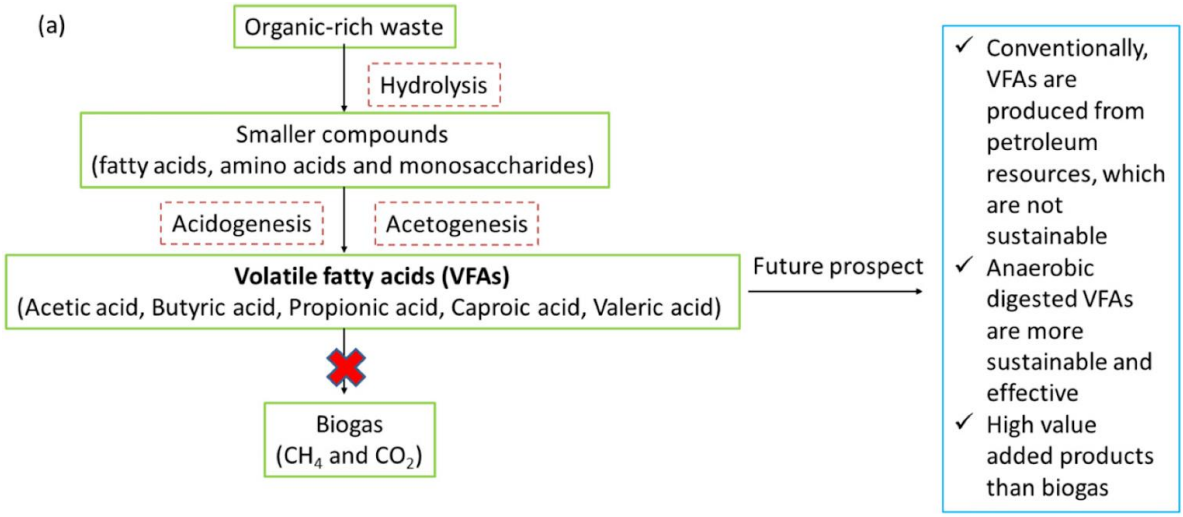


Figure 4: (a) Production pathway of VFAs [88].

Microbial fermentation is another method that can be used to produce VFAs because these acids are the outcomes of different fermentation processes. Biological approaches are also becoming more common for producing VFAs because they are capable of employing renewable carbon sources as raw materials, which are becoming eco-friendlier [76]. A higher rate of productivity with fewer by products is achieved by employing pure sugars, such as glucose, xylose, etc., in the majority of VFA manufacturing processes [77] [78]. Nowadays, waste sludge is being used by researchers to create VFAs,

and a lot of study has been invested towards evaluating various waste [79]. A simpler and cheaper method of producing VFA and a potential alternative for waste treatment is waste sludge fermentation [80]. Because of the enormous quantities of wastes produced by the widespread usage of biological wastewater treatment, primary sludge (PS) and waste activated sludge (WAS) produced by municipal wastewater treatment plants are frequently studied for VFA synthesis [81]. Waste sludge-produced VFAs, which can be utilised as precursors for the synthesis of biogas, poly-hydroxyalkonate (PHA), and electricity [82]. There are different applications of volatile fatty acids.

1.4.1. Volatile Fatty Acids:

As a platform chemical for further bio-based post-stream processes, waste-derived VFA production offers a promising potential [83]. VFAs are widely used in the food, cosmetics, textile, bioenergy, and pharmaceutical industries [72] [73]. Microbial community composition is altered by low pH, causing a short-term variation to the VFA production system, although it was able to recover back shortly. The other two main acids were butyric and acetic acid [83]. The main platforms which are researched are sugar, thermochemical, biogas and carbon-rich chains. Highest yield, easy application to wastes and marine biomass, no additional enzymes, no sterilisation are required for VFA to convert them into useful chemicals [84]. The VFAs can be converted into useful chemicals like ketones, esters, 1-alcohols and 2-alcohols and polymers using different routes. One of the PHAs, polyhydroxybutyrate, can be converted into ethyl 3-ethoxybutyrate, which has been recently discovered as an exciting fuel providing oxygen with low pollutant emissions and high cetane values [85]. Moreover, VFAs can also be thermally converted into syngas or hydrogen [86].

1.4.2. Recovery of VFA:

The solutions are mixed with calcium-based salts to neutralise the organic acids; the resulting calcium carboxylate solutions are then typically dried by evaporation. The next step is to either crystallise or do further separation [87]. So far, there are several methods such as adsorption [89] [90], distillation and evaporation [91], extraction [92], electrodialysis [93] and pressure-driven membrane processes [94] have been implemented for VFAs recovery. Among the membrane-based purification processes microfiltration, ultrafiltration, nanofiltration, reverse osmosis and forward osmosis are regularly used to recover, purify and VFA concentration from mixed solutions[95]. The conventional approach of precipitation can be utilised to separate components from a mixture.

Adsorption by chemical precipitation on calcium hydroxide:

One of the most popular methods for recovery of volatile fatty acids (VFAs) is the calcium-based precipitation process. The pH adjustment is very important, the pH range is between 9 - 10 with adding NaOH or HCl. Addition of suitable calcium salts like Ca $(OH)_2$ or $CaCO_3$ the calcium ion reacts with the VFA to form insoluble calcium salts, which precipitate out of the solution, thorough mixing is needed to facilitate the reaction between calcium ions and VFAs. Allow the precipitate to settle to the bottom of the conical flask. Washing of precipitate with water or organic solvent to remove excess calcium ions or impurities. This process involves four steps to produce the final product: (1) The filtered fermentation liquid is first mixed with Ca $(OH)_2$ or $CaCO_3$, then (2) the calcium salts of the VFAs are filtered out of the aqueous solution, then (3) calcium salt is treated with sulfuric acid in order to remove the required VFA, and finally, the pure VFA is obtained by further purification [96] [97]. In the environmental related companies,

chemical precipitation is a bit cost effective as there is a high amount of H_2SO_4 and lime needed to carry out this process [98].

For the recovery of acetic acid, the desorbed solution was prepared on the following day by making three different conc. of NaOH i.e. 1, 0.1, 0.01 M in 95% of 100 ml of ethanol. The desorption solution was mixed thoroughly at 150 rpm for 2 hours and from prepared 100 ml desorbed sample 25 - 30 ml of the desorbed solution was transferred to the wastewater sample containing VFA bound to activated charcoal and left overnight so the whole VFA gets desorbed and floated on the surface of the charcoal. The next day the sample was vacuum filtered to extract the VFA. To extract the correct amount of VFA the desorbed solution was mixed with organic solvent called dichloromethane and put to a separating funnel for extracting the aqueous phase. The aqueous phase was heated at 40 -50°C to evaporate the solvent, gradually the concentration of the acetic acid increased and reached its glacial point, where it solidified into clear, crystalline mass. After the recovery of acetic acid, it was also quantified in HPLC for cross checking.

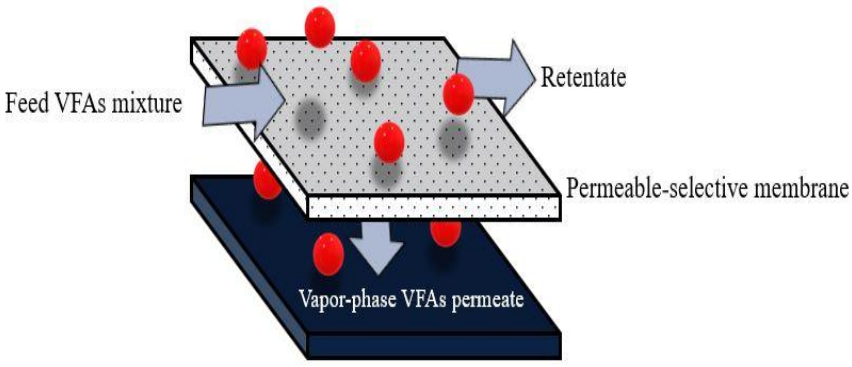


Figure 5: Precipitation of VFA using calcium salts

Adsorption on activated charcoal:

Another simple method is by chemical adsorption using activated charcoal as adsorbent. Multiple materials, including neutral polymeric resins, crosslinked poly (4-vinyl pyridine), zeolite molecular sieves, titanium dioxide (TiO2), activated carbon, and iron oxide nanoparticles, have been recommended for the adsorption of VFAs [127]. Affinity separation methods including adsorption and liquid-liquid extraction have also been studied with the goal of recovering VFA. Because it functions as a conductive material with a notably particular surface area, granular activated carbon (GAC) provides microorganisms with more areas to adhere. This enhances mass transfer and shortens the distance between syntrophic partners, which boosts biofilm formation and target product yield. By promoting electron transfer, altering metabolic pathways, and allowing the formation of an electron transfer chain, GAC encourages bacterial contacts and electron sharing.

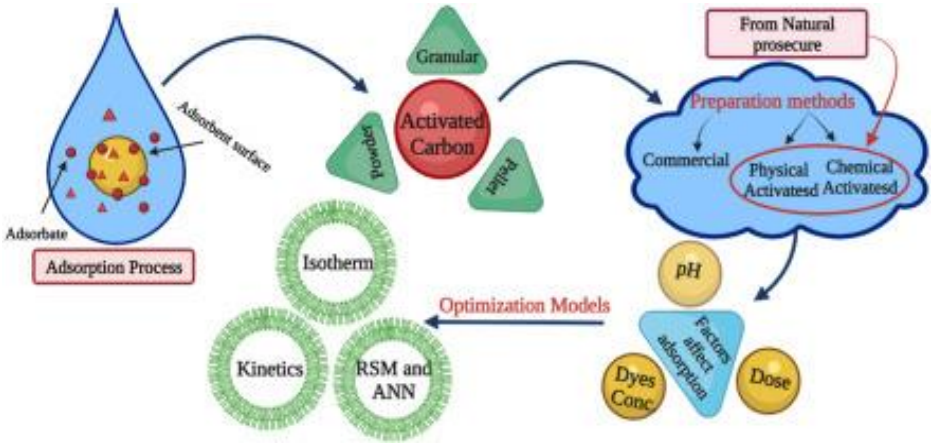


Figure 6: Adsorption of VFA on activated carbon [129]

2. LITERATURE REVIEW:

Biological production of hydrogen presents an attractive replacement within the overall framework of environmentally conscious and renewable power sources [99]. Compared to the present commercial manufacturing techniques Bio-H_2 production process is more safe, harmless and eco-friendly [100]. Wastewater treatment plants can get involved in Bio-H_2 production as different bacteria can survive in highly contaminated environments and produce bio-hydrogen [50]. By the combination of reduced molecules and light energy, photosynthetic bacteria use nitrogenase to catalyse the synthesis of molecular hydrogen in nitrogen-deficient environments (organic acids) [101]. Purple non-sulphur photosynthetic bacteria are believed to be potential H2 producers [106],[107]. These purple non-sulphur bacteria can still utilise simple organic acids like acetic acid as electron donors when thriving in an anaerobic environment. Ferredoxin uses the energy from ATP to move the charged particles to the nitrogenase enzyme This nitrogenase enzyme may use additional energy in the form of ATP to decrease proton back into hydrogen gas when nitrogen is not present. It has been observed that carbon dioxide (CO_2) and hydrogen gas (H_2) are produced in this manner from the main component of organic acids [102]. Although fermentation-based H_2 production is very productive and has the potential to utilise renewable biomass sources [103], its commercial use is restricted by the high chemical oxygen demand (COD) [104] of the effluent and the poor theoretical conversion efficiency of thermal value (4 mol H_2/mol glucose). A beneficial aspect of photosynthetic production of H_2 is that it may use a range of carbon sources, including waste from industry [105]. It has been noted that selected carbon sources are important for microbial growth along with efficient H_2 generation [108]. Although only 20 μM of ammonia has been shown to interfere with the nitrogenase enzyme responsible

for H_2 production, using nitrogen sources sparingly is very important [117]. Research focus has recently shifted to the application of integrated organic sources (organic acids and sugars). Even fewer studies are available on the impact of mixed carbon sources on photo-fermentative H_2 synthesis [109], regardless of the fact that there have been few reports of employing mixed carbon sources metabolism for efficient and cheap H2 production, the mechanisms of the process still remain unexplored [110]. Different parameters such as C:N ratio, carbon and nitrogen source, light efficiency, temperature and nutrient medium are very important for the production of Bio-H2 [111],[112],[113][114]. Among all of these factors which influence the electron donor, carbon sources are the most essential [115]. Considering photo fermentation, has the potential to be used with an extensive range of raw materials, is highly effective, ecologically friendly, and has the ability to produce staggering quantities of hydrogen at room

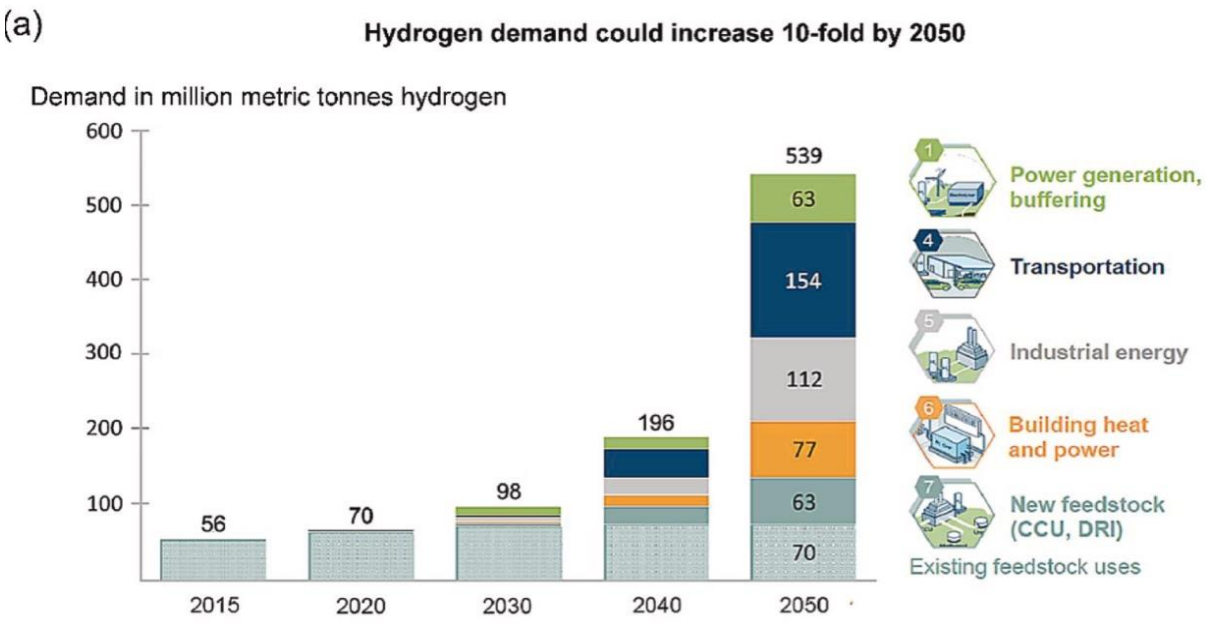


Figure 7: Demand for hydrogen in next 30 years [121].

temperature and pressure, it has grown popular [118]. The drawback is that the process's real hydrogen yields are considerably lower than its theoretical maximum value of 12 mol H_2/mol glucose [104]. This technique further makes it accessible to utilise a variety of organic materials, like lignocellulosic wastes, sewage, and industrial effluents, as a substrate for microbial growth. This process produces biohydrogen and offers an approach to remediate waste [119]. Industrial wastewater has been used and treated with anoxygenic photosynthetic microorganisms [122] to produce hydrogen and clean water to be used for further domestic purposes.

2.1. HOW DID BIO-HYDROGEN COME INTO EXISTENCE?

The hydrogen produced biologically by the use of bacteria, algae, archaea, or their consortia from renewable resources is known as bio-H_2 [60]. As it flames smoothly and only emits water when burnt, it causes no hazard to public health or causes contamination of the air, water, or land. It also doesn't add to particulate matter, greenhouse gases, or oxides of C, S, N, or H [61]. Many nations are now doing comprehensive studies on the production of Bio-H2 in order to safeguard the environment and public health. India's National Green Hydrogen Mission estimates that the country would achieve Net Zero by 2070 and energy independence by 2047. The cost of green hydrogen (6 USD/kg), was calculated by the Hydrogen Council (HC). Bio-H_2 finds widespread application in fuel cells and reciprocating combustion engines [62]. Bio-hydrogen has many advantages as they are eco-friendly, clean, renewable and have higher energy content (142 MJ/kg) than other fuels [63].

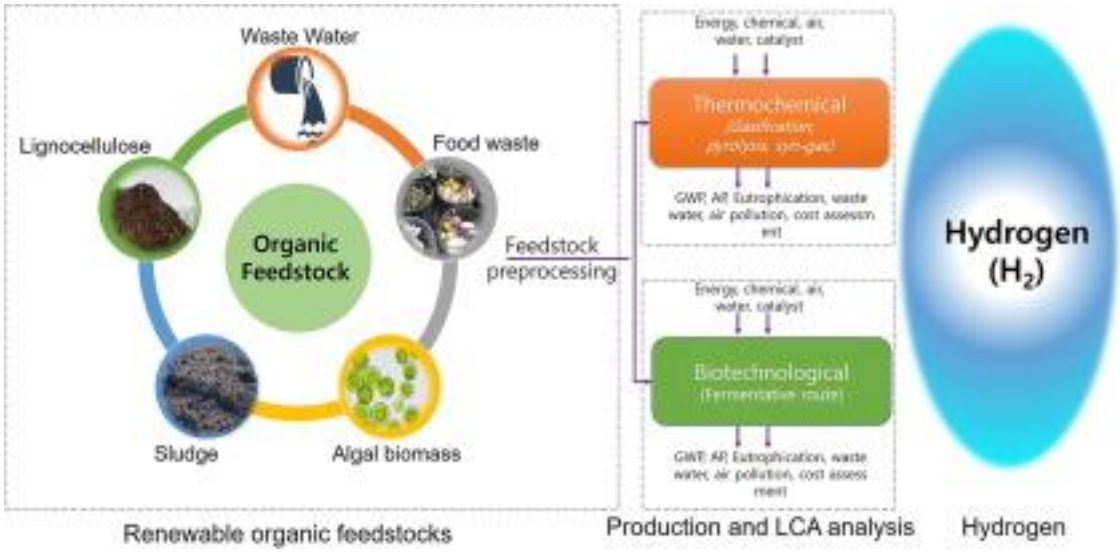


Figure 8: How bio-hydrogen evolved?

Bio-H_2 has the ability to stop the use of fossil fuel and zero emission of greenhouse gases [64]. Some of the drawbacks include low ignition energy and a limited flammability range. Bio-H2 has no colour and no smell, making it impossible to detect leaks. It also spreads readily through the atmosphere, causing jet fires, delayed ignition, and explosions [65]. Storage and transport are also a major challenging issue with bio-hydrogen.

2.2 LIFE CYCLE ASSESSMENT AND BIO-HYDROGEN PRODUCTION

According to recent life cycle assessments, the development and use of some bioenergy fuels may have worsened environmental performances (LCA). However, some bioenergy systems are believed to pose less of a risk to sustainability because they are derived from inexpensive organic wastes rather than purposefully grown plants. The infrastructure of a particular place has an impact on the uptake of bio-hydrogen technology. Recent life cycle assessments suggest that environmental performances (LCA) may have gotten worse as a result of the development and usage of various bioenergy fuels [150, 151]. But because certain bioenergy systems are made from cheap organic wastes instead of intentionally produced plants, it's thought that they provide less of a threat to sustainability. The adoption of bio-hydrogen technology is dependent on the infrastructure of a given location [154]. According to recent life cycle evaluations, the development and use of different bioenergy fuels may have worsened environmental performances (LCA).

However, certain bioenergy systems are considered to pose less of a danger to sustainability since they are based on inexpensive organic wastes rather than purposefully grown plants. The infrastructure of a particular place affects the uptake of bio-hydrogen technology. In order to produce bio-hydrogen, a hybrid biomass system's energy research must evaluate the system's energy conversion efficiency and consumption [152]. Energy analysis aims to optimise the system's energy use and identify potential areas for improvement. Energy analysis is necessary to maximise the generation of hydrogen from a hybrid biomass system. The results of the energy analysis can assist in decision-making and system optimisation in order to maximise efficiency, minimise costs, and diminish environmental damage [153].

2.3 BIOREACTOR CONFIGURATIONS –

PHOTOFERMENTATION BIOREACTOR

Rhodopseudomonas sphaeroides (MTCC 4066) the photo fermentative facultative anaerobe pre-culture was grown under proper conditions. Transparent 650 ml borosilicate reaction vessel (CCF) of working volume 500 ml was used as a photo fermentative batch reactor. The batch reactor was maintained at room temperature and under proper luminance and the lights were positioned on the side of the stirrers.

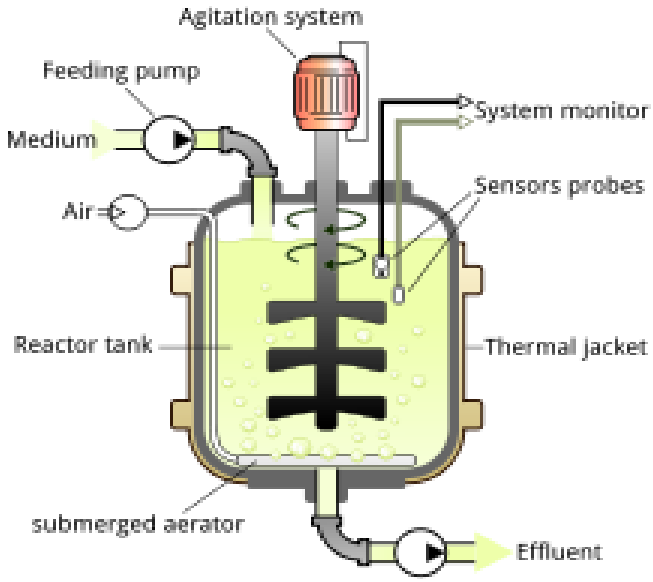


Figure 9: Photo bioreactor configurations

Cap of the reactor had only one outlet to collect the mixture of produced gases into the carbon dioxide trapper filled with KOH solution of 40% of total volume of the trapper. The reaction vessel was sealed with parafilm and flushed with nitrogen gas to ensure anaerobic conditions and eliminate oxygen (O_2) from the whole reaction vessel. The inoculum was centrifuged at 8000 rpm at 28°C to obtain the active cell mass and then the active cell mass was added in the hydrogen producing media at room temp in the LAF cabinet and put under a bright light source.

2.4 HOW VFA CAN BECOME A PLATFORM CHEMICAL FOR THE INDUSTRY?

VFAs are essential compounds with uses in the food, pharmaceutical, textile, and chemical industries, among others. They can be polymerized to create plastics and polymers like polyvinyl acetate or cellulose acetate propionate, or they can be chemically transformed to become esters. A further use involves biological conversion leading to the production of fertilisers, medium-chain fatty acids, or biopolymers such as polyhydroxyalkanoates [142] Biological conversion can also result in the production of bioenergy from VFAs, such as hydrogen and biogas, and power via fuel cells [143].

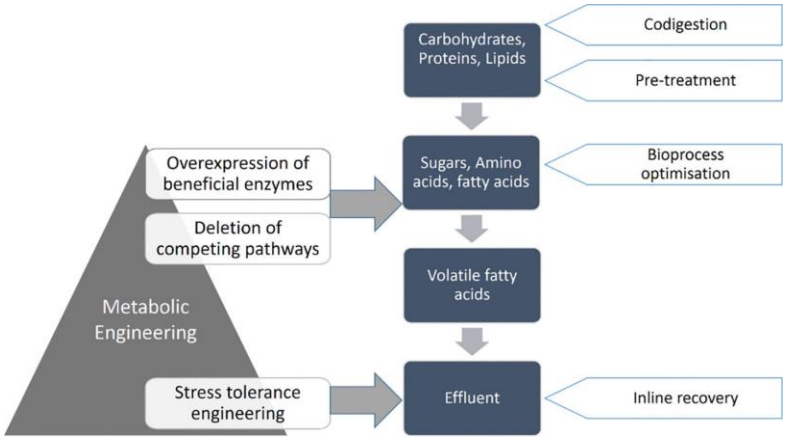


Figure 10: How VFA is produced from waste?

Petroleum-based chemical synthesis is the primary method used to meet the increasing demand for volatile floral acids (VFAs). The following are some of these techniques: (i) ethylene oxidation, methanol carbonylation for the synthesis of acetic acid; (ii) oxidation of propionaldehyde, ethylene hydro-carboxylation for the synthesis of propionic acid; and (iii) oxidation of butyraldehyde for the synthesis of butyric acid [144].

2.4.1 Importance of VFA

Given the importance of volatile fatty acids (VFAs) as platform chemicals in a variety of applications, the benefits derived from producing them through microbial fermentation exceed the expenses related to microbial production. The significance of key factors influencing the VFA yield is thoroughly discussed, along with the benefits and drawbacks that go along with them. These include: (i) the operational parameters that influence the fermentative VFA production efficiency; (ii) pre-treating different substrates to enhance their microbial activity; (iii) co-fermentation to balance the nutrient levels of diverse substrates and improve their digestion potential; and (iv) innovative reactor configurations that are known to increase VFA production [145]. VFAs serve as essential building blocks for the synthesis of biopolymers, biofuels, and other compounds such alcohols, alkanes, ketones, and esters [146]. One of the most appealing uses of volatile fatty acids (VFAs) derived from acidogenic fermentation is the synthesis of poly-hydroxyalkanoates (PHAs). PHAs are really biodegradable compounds that resemble plastic and can be used in place of polymers made from petroleum [147]. The biological removal of nutrients (nitrogen and phosphorus) from wastewaters is another widespread application for VFAs. Because wastewaters have a low carbon content, adding carbon sources is necessary to carry out a full denitrification phase. Typically, acetate, methanol, and ethanol are utilised to boost denitrification efficiency [148].

3. AIM AND OBJECTIVE OF THE STUDY:

3.1. AIM OF THE STUDY:

To utilize the waste activated sludge and use PNS bacteria to produce hydrogen under light condition and recover the rich organic acid content from waste activated sludge.

3.2. OBJECTIVE OF THE STUDY:

The primary objective of this study is to determine whether using fermentative bacteria in conjunction with waste activated sludge to make bio-hydrogen and then recover the rich organic acids from WAS is feasible and effective. The goal of the research is to determine whether this technique has the potential to be a sustainable and effective way to produce bioenergy and treat wastewater, which will help create environmentally friendly waste management and renewable energy solutions.

4. MATERIALS AND METHODS

4.1. SUBSTRATES AND REAGENTS

All the chemicals used in the experiment were present in the lab of School of Environmental Studies, Jadavpur University, Kolkata. The waste activated sludge which was used as the substrate in the experiment was collected from Sewage treatment plant, Titagarh, West Bengal. The activated sludge was stored at 4°C to keep the bacteria activated and also for further use.

4.2. MICROORGANISM AND CULTURE CONDITIONS: -

Rhodopseudomonas sphaeroides MTCC 4066 the photo fermentative facultative anaerobe was purchased from MTCC (Microbial Type Culture Collection and Gene Bank, CSIR Institute of Microbial Technology, Chandigarh, India) was used as photo fermentative H_2 production bacteria. The bacterial strain was cultivated in modified Biebl and Pfennig's media with an addition of CH_3COONa. Sodium pyruvate ($C_3H_3Na_3$) was used as a carbon source as mentioned in modified Biebl and Pfennig's media so there was no extra addition of any carbon sources. The composition of Biebl and Pfennig's media in g/L was KH_2PO_4 0.5; NaCl 4; NH_4Cl 0.7; $CaCl_2.2H_2O$ 0.2; $MgSO_4.7H_2O$ 2; $C_3H_3Na_3$ 3; Yeast 0.3; Ferric citrate (0.1% (w/v)) 5 mL/L; and trace element solution 1 mL/L. The composition of trace element solution in mg/L was $ZnCl_2$ 70; $MnCl_2.4H_2O$ 100; H_3BO_4 60; $CoCl_2.6H_2O$ 200; $CuCl_2.2H_2O$ 20; $NiCl_2.6H_2O$ 20; $NaMoO_4.2H_2O$ 40; HCl (25% v/v) 1 ml/L. The media was prepared and pH was adjusted to 6.8 - 7 [116] using 1N NaOH before putting it into the autoclave (121 °C, 15 psi, 15 min). After autoclaving the media was cooled in room temp and sodium pyruvate was added inside the laminar flow cabinet. The photosynthetic bacteria *R. sphaeroides* was allowed to grow under proper light for 10 days and their

growth was observed by measuring the colour density of their growing media at 600 nm using a visible light spectrophotometer.

Figure 11:R. sphaeroides grown in abundance of light and in growing media

4.3. SAMPLE COLLECTION AND ANALYSIS:

Waste water samples were collected from four different sites of Titagarh WWTP. Raw, primary and secondary wastewater was collected and examined. After collection, different parameters like TDS, TSS, COD, ammoniacal nitrogen, alkalinity, carbohydrate, VFA and ammonia were also evaluated and tested.

Figure 12: Sample container with wastewater sample from Titagarh

4.3.1 TSS and TDS estimation:

Total dissolved solid is a measure of the combined content of all inorganic and organic substances contained in a liquid in molecular, ionized or micro-granular suspended form. A well-mixed sample is filtered through a prepared glass fibre filter into a clean conical flask. The portion of the sample that is not retained by the filter paper is dissolved solid. The filter paper is dried to a constant weight at 180°C and the difference in the weight of the dish represents the dissolved solid concentration. Dissolved solids are those which pass through a water filter or filter paper. They include some organic materials, salts, inorganic nutrients and toxins. To make sure the treated wastewater fulfils the requirement preceding to disposal, measurement of TDS is important. It is measured in milligrams per litre(mg/L) or in parts per million(ppm). Moreover, the measurement of TDS is needed to determine the quality of water, ensure public health and environment.

TSS stands for total suspended solids that can be retained on a filter paper and are capable of settling out of the water column onto the stream bottom when stream velocities are low. They include silts, clay, planktons, organic wastes and inorganic precipitation such as those from acid mine drainage. It is also measured in milligrams per litre(mg/L) or in parts per million(ppm). TSS is a key indicator of water quality, high levels of TSS indicates pollution or contamination which gives a negative impact on aquatic and human environment. In aquatic ecosystems TSS can harm aquatic life by inhibiting proper light penetration, nutrient transport and pollutants. TSS measurements are used to evaluate the effectiveness of wastewater treatment processes. By measuring the TSS the pollution sources can also be identified and if possible corrective actions can also be taken.

For measuring the TDS, 25 ml of water sample was taken and filtered through filter paper. The filtered water was collected in a glass beaker. The wet filter paper was put into a hot air oven at 103-105°C to let it get dried. The dried weight of the filtered paper was noted. For measuring the TSS, the filtered water which was collected in the TDS experiment was used. 5 ml of that collected water was placed in a porcelain crucible and put in a hot air oven for 1-2 hours until no trace of water was left behind. The crucible was weighed immediately after cooling to avoid absorption of moisture due to hygroscopic nature and the weight was noted.

4.3.2. COD estimation:

The term COD (Chemical Oxygen demand) refers to the milligrams per litres (mg/l) unit of measurement used to describe the amount of oxygen needed to chemically oxidize the organic and inorganic matter in water. In a COD test an effective oxidizing agent is added to the water sample under particular conditions and the amount of oxygen used is measured during the reaction. An abundance of organic contaminants that harm the aquatic environment and the natural environment are detected by high COD levels.

For our research study, in a refluxing flask 20 ml of waste water sample was taken followed by 30 ml of conc. H_2SO_4. The procedure also includes addition of 10 ml of 0.25(N) $K_2Cr_2O_7$

and the total volume was made up to 250 ml. The solution was added with a pinch of both silver nitrate and mercuric sulphate. Then the solution was put for refluxing at 70-80°C for 2 hours. After two hours of refluxing the colour changed to reddish brown as the ferroin indicator was added to mark the colour change due to heating. After the refluxing was done, the sample was allowed to cool down at room temperature and was titrated

against 0.1(N) Mohr salt until the colour changed from reddish brown to deep green. Blank was prepared at the same time by following the same procedure.

Figure 13: Reddish brown colour appearance after refluxing

4.3.3 Alkalinity estimation:

The ability of a water sample to neutralise acids is referred to as its alkalinity. Calcium carbonate ($CaCO_3$) content is commonly expressed in milligrams per litre (mg/L) or in comparable units like millie equivalents per litre (meq/L). The buffering capacity of water is primarily dependent on its alkalinity, which is regulated by compounds such as hydroxides, carbonates, and bicarbonates. Bi-carbonate represent a major form of alkalinity, since it is formed in considerable amounts from action of CO_2 upon basic materials in the soil. Although many materials may contribute to alkalinity of water, the major portion of the causes in natural water are - hydroxide, carbonates and bicarbonates. The alkalinity of water is principally due to the action of weak acid and strong base and such substances act as a buffer to resist a drop in pH resulting from acid addition.

Process Control: The success rate of biological treatment procedures might be impacted by alkalinity. It assists in keeping pH levels in wastewater treatment facilities within the range that is best for microbial activity, which is essential for the decomposition of organic materials.

Acid Neutralisation: Acids from natural or industrial processes are frequently present in wastewater. By acting as a buffer, alkalinity helps to neutralise these acids and avoid abrupt pH fluctuations, which may be detrimental to aquatic life in receiving waters as well as biological treatment operations.

Regulatory Compliance: Alkalinity thresholds for wastewater disposal licences are frequently imposed by regulatory bodies.

Corrosion Control: Alkalinity has an impact on wastewater's corrosiveness as well. Acidic conditions brought on by low alkalinity can damage pipelines and equipment.

Pollution Indicator: Variations in alkalinity levels can reveal the presence of certain contaminants or modifications in the wastewater's composition, offering important insights for process improvement and troubleshooting.

For our research study, 25 ml of water sample was taken followed by the addition of a few drops of methyl orange till the water sample colour changes to yellowish orange, it was then titrated against 0.02(N) conc. H_2SO_4 till the colour changes to pink which marks its end point.

4.3.4. Ammoniacal nitrogen analysis by Kjeldahl method:

The measurements of the amounts of ammonia and organic nitrogen are combined into a single value by the Total Kjeldahl Nitrogen (TKN) test. Main objective of this method is to oxidise organic molecules by applying concentrated sulfuric acid. The Kjeldahl

technique is divided into three primary phases overall. The procedures consist of titration, distillation, and digesting.

Digestion: This technique involves heating the sample in the presence of sulfuric acid. By oxidation, the acid breaks down the organic material, releasing reduced nitrogen in the form of ammonium sulphate. Usually, potassium sulphate is added to raise the medium's boiling point. When we get a transparent, colourless solution, the sample has completely broken down. During Kjeldahl digestion, H_2SO_4 oxidizes organic matter to CO_2 and H_2O.

Distillation: The solution is now distilled, and to turn the ammonium salt into ammonia, a tiny amount of sodium hydroxide is added. Following distillation, the vapours are captured in a unique solution made of water and hydrochloric acid.

Titration: Then, using back titration, the level of ammonia or nitrogen contained in the sample is determined. A portion of the HCl is neutralised as the ammonia dissolves in the acid-trapping solution. It is possible to back titrate the remaining acid using a standard base solution, such as NaOH or another base.

For our study, 25 ml of wastewater sample was taken in a digestion flask and mixed with borate buffer to remove ammonia and NaOH was added to get an alkaline pH of around 9 - 9.8. After that 1 ml of $CuSO_4$, 5 gm ofK_2SO_4 , and 10 ml of conc. H_2SO_4 was added in the digestion process and made the volume to 50 ml. Glass beads were added and the kjeldahl flask was put for digestion for 30 mins. Boiling was done till the volume turned to half. As digestion continues, coloured or turbid samples will become transparent and colour changed to shades of yellow. 5 -10 ml of boric acid as an absorbent solution was added and the distillate colour changed to violet after reacting with boric acid and finally the distillate was titrated against H_2SO_4 to determine ammonia in the sample.

Figure 14: Blue colour after distillation

4.3.4. Carbohydrate analysis by Anthrone method

Specifically, for biological samples such as plant extracts or food products, the Anthrone method is a widely used technique for analysing carbohydrates. Furfural is created when carbohydrates are dehydrated using conc.H_2SO_4. The reagent's active form is anthranol, which is the enol tautomer of Anthrone. It interacts by condensing with the carbohydrate furfural derivative, giving solutions that are diluted a green colour and concentrated solutions a blue hue that is measured calorimetrically. The highest absorption is shown at 620 nm in the blue-green solution.

Reaction:

(i) Hydrolysis to monosaccharides

Disaccharide —->Monosaccharide

(ii) Dehydration---product is a furfural

Monosaccharide —-> Furfural

(iii) Reaction of furfural with Anthrone

Furfural + Anthrone —> reagent Blue green complex

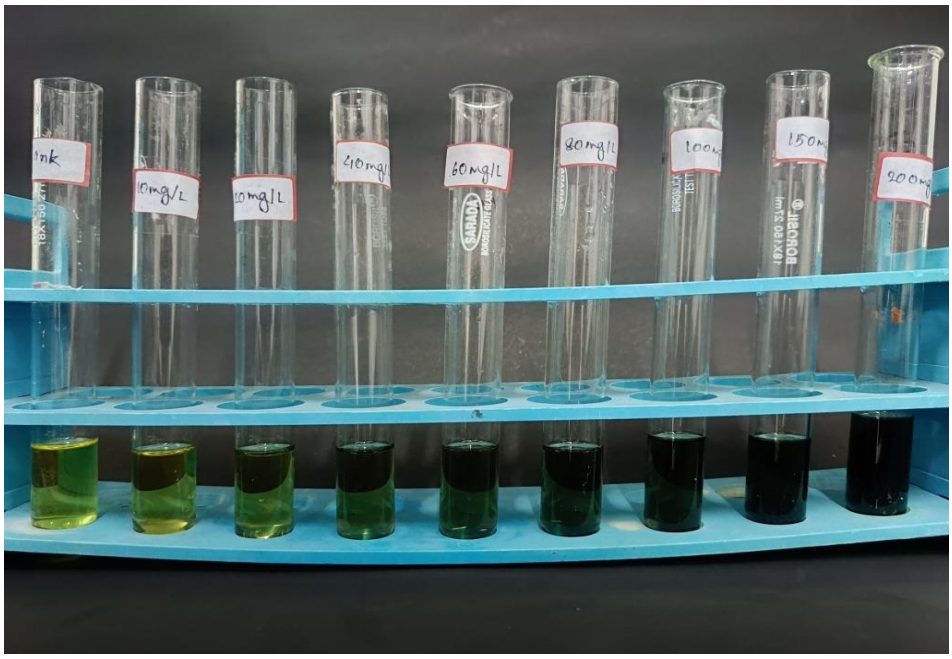


Figure 15: Carbohydrate estimation

Anthrone reagent preparation: Dissolve 2g of Anthrone in 1 litre of concentrated H_2SO_4. Use freshly prepared reagent for the assay

For our study, 300µg ,500µg and 1 ml of sample was taken in three separate test tubes to carry out the experiment. 5 mL of the prepared Anthrone reagent was added into each tube and mixed thoroughly by vortexing. The tubes were covered with aluminium foil or caps and was put in a boiling water bath for 10 minutes. Cool the test tubes at room temperature and measure the optical density at 620 nm against a blank.

4.4. VFA analysis

C2-C7 monocarboxylic aliphatic acids, or low-molecular-mass carboxylic acids, are crucial metabolites and intermediates in biological processes. These carbohydrates are referred to as short-chain fatty acids (SCFAs) or volatile fatty acids (VFAs) [123]. Organic matter breaks down anaerobically, producing volatile fatty acids. As a result, wastewater,

leachates from waste sites, and activated sludge all contain large amounts of them. VFAs often build up in a variety of environmental compartments, with streams containing a greater amount of these pollutants [124].

4.4.1. VFA analysis by Montgomery method:

The spectrophotometric process is based on the widely used Montgomery technique, a colorimetric ferric hydroxamate method for determining carboxylic esters [126]. In our study, both low and high concentration standards were prepared and measured at 513 nm. In case of measuring the VFA of samples different steps were followed:

Step 1. In a test tube 400 µl sample was pipetted followed by 400 µl of Ethylene glycol and 100µl of conc. H_2SO_4 was added. The test tube was put into a boiling water bath for 10 mins.

Step 2. After taking out the sample from the boiling water bath it was cool down to room temp.

Step 3. To the test tube containing the sample, 500µl of 1.8 gm of hydroxylamine hydrochloride solution (NH2OH.HCl), 500µl of diluted HCl, 2 ml of 3.5 gm ferric chloride solution and 500µl of 7.5 gm of NaOH were added.

Step 4. The absorbance was measured at 513 nm by UV-Visible spectrophotometer.

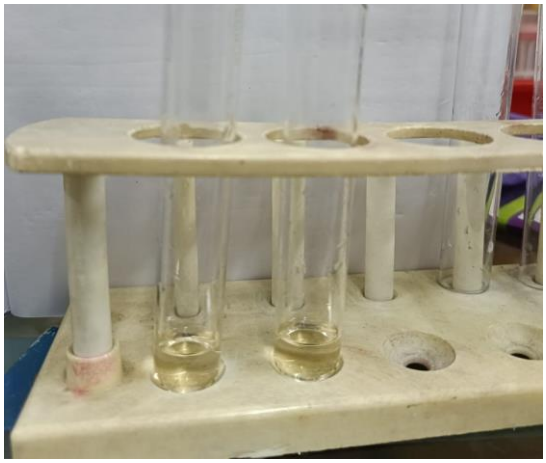


Figure 16: Colour generation after adding of FeCl3

4.4.2. Working of HPLC:

Pump: Delivers the mobile phase at a constant flow rate and high pressure.

Injector: Introduces the sample into the mobile phase stream.

Column: Contains the stationary phase where separation occurs.

Detector: Identifies and quantifies the separated components as they elute from the column.

Data System: Collects and analyses the detector signal to produce chromatograms.

High Performance Liquid Chromatography (HPLC) (LC-10 AT, VP, Shimadzu, Japan) with an ultra violet detector was implemented to analyse VFA. A C18 column was employed in mobile phase conditions with 0.05 N of 90% H_2SO4. The column's temperature and flow rate were maintained at 30°C and 0.6 mL/min, respectively [126]. For our study, as we had only two standard samples called Acetic and Valeric acid we prepared them as standard against sample we got the peak accordingly. The sample, mobile phase, wash solution was first vacuum filtered and degassed using a gas sonicator for 10 mins to remove all the present gas bubbles in the solutions. The VFA were analysed at 210 nm.

4.5 VFA RECOVERY

4.5.1 ADSORPTION BY CHEMICAL PRECIPITATION

One of the most popular methods for recovery of volatile fatty acids (VFAs) is the calcium-based precipitation process. The pH adjustment is very important, the pH range is between 9 - 10 with adding NaOH or HCl. Addition of suitable calcium salts like $Ca(OH)_2$ or $CaCO_3$ the calcium ion reacts with the VFA to form insoluble calcium salts, which precipitate out of the solution, thorough mixing is needed to facilitate the reaction between calcium ions and VFAs. Allow the precipitate to settle to the bottom of the conical flask. Washing of precipitate with water or organic solvent to remove excess calcium ions or impurities. This process involves four steps to produce the final product: (1) The filtered fermentation liquid is first mixed with $Ca(OH)_2$ or $CaCO_3$, then (2) the calcium salts of the VFAs are filtered out of the aqueous solution, then (3) calcium salt is treated with sulfuric acid in order to remove the required VFA, and finally, the pure VFA is obtained by further purification [96] [97]. In the environmental related companies, chemical precipitation is a bit cost effective as there is a high amount of H_2SO_4 and lime needed to carry out this process [98].

For the recovery of acetic acid, the desorbed solution was prepared on the following day by making three different conc. of NaOH i.e. 1, 0.1, 0.01 M in 95% of 100 ml of ethanol. The desorption solution was mixed thoroughly at 150 rpm for 2 hours and from prepared 100 ml desorbed sample 25 - 30 ml of the desorbed solution was transferred to the wastewater sample containing VFA bound to activated charcoal and left overnight so the whole VFA gets desorbed and floated on the surface of the charcoal. The next day the sample was vacuum filtered to extract the VFA. To extract the correct amount of VFA the desorbed solution was mixed with organic solvent called dichloromethane and put to a separating funnel for extracting the aqueous phase. The aqueous phase was heated at 40 -50°C to evaporate the solvent, gradually

the concentration of the acetic acid increased and reached its glacial point, where it solidified into clear, crystalline mass. After the recovery of acetic acid, it was also quantified in HPLC for cross checking.

Figure 17: Precipitate of VFA along with calcium salt

4.6. AMMONIA ESTIMATION BY PHENATE METHOD:

Alkaline phenol and hypochlorite react with ammonia to form indophenol blue that is proportional to the ammonium concentration. The phenate method is based on the Berthelot colour reaction caused by sodium nitroprusside. For our study we have prepared the reagents as follows:

Phenol solution: Mix 11.1 ml of liquid phenol with 95% of ethyl alcohol to a final volume of 100 ml.

Sodium nitroprusside: 0.5% w/v: dissolve 0.5 gm of sodium nitroprusside in 100 ml of deionized water. Can be stored up to 1 month in an amber bottle.

Alkaline citrate: dissolve 200 gm trisodium citrate and 10 gm sodium hydroxide in deionized water. Dilute up to 1000 ml.

Oxidizing solution: Mix 100 ml alkaline citrate solution with 25 ml sodium hypochlorite.

Standard ammonium solution: use stock ammonium solution and water to prepare a calibration curve in a range appropriate for the concentration of the sample.

For our study, 25 ml of sample was taken in 50 ml of Erlenmeyer flask followed by 1 ml of phenol solution, 1 ml of sodium nitroprusside solution and 2.5 ml of oxidizing solution. The Erlenmeyer flask was covered by aluminium foil and left for an hour to generate colour. After that the absorbance was measured at 640 nm using the UV-Visible spectrophotometer. Blank was also prepared separately.

Figure 18: Sample and standard for ammonia estimation by Phenate method

4.7. DESIGN PARAMETERS:

Figure 19: CO2 Trapper, Reaction vessel and Gas column

The diameter of the reaction vessel is 7.6 cm, the gas column has a diameter of 4 cm and the trapper is made with a diameter of 8 cm.

4.8. EXPERIMENTAL SETUP:

BIO-HYDROGEN IN BATCH PROCESS:

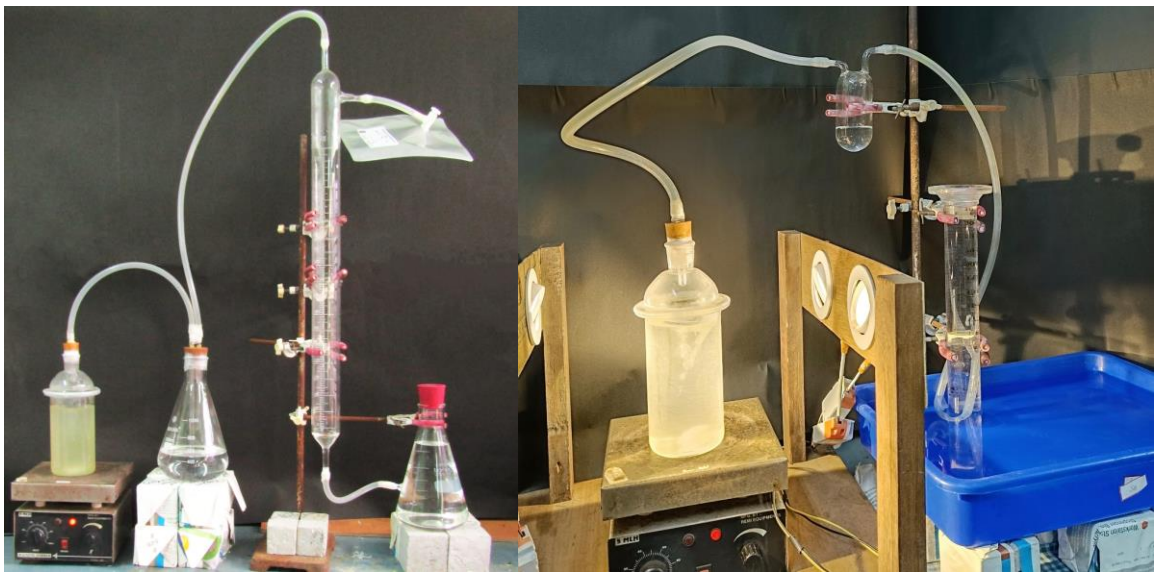


Figure 20: Old setup: **New Setup:**

The challenges faced in continuing with the old setup was, the CO_2 trapper and the gas collecting column. The CO_2 trapper being too large in volume as compared to the gas column resulted in the displacement of whole water present in the gas column followed by overflowing of the reservoir so there was no collection of gas in the column. So, the necessary changes made in the new setup was quite simple. We have followed a simple water displacement method of collecting gas using a 250ml measuring cylinder. The CO_2 trapper was designed with a volume of 100ml and filled with 40% of KOH solution. We could see a larger number of gas bubbles in the trapper.

5. RESULT AND DISCUSSION:

5.1 Growth curve for photo fermentative bacteria:

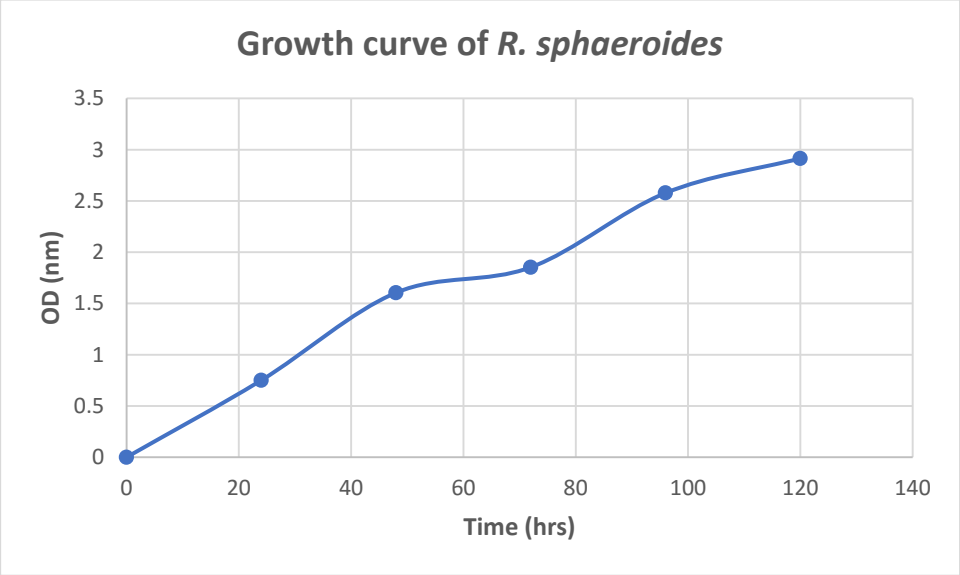


Figure 21: Growth curve of *Rhodopsuedomonas sphaeroides.*

5.2. BIO-HYDROGEN PRODUCTION:

5.2.1. Using Biebl pfennig's media:

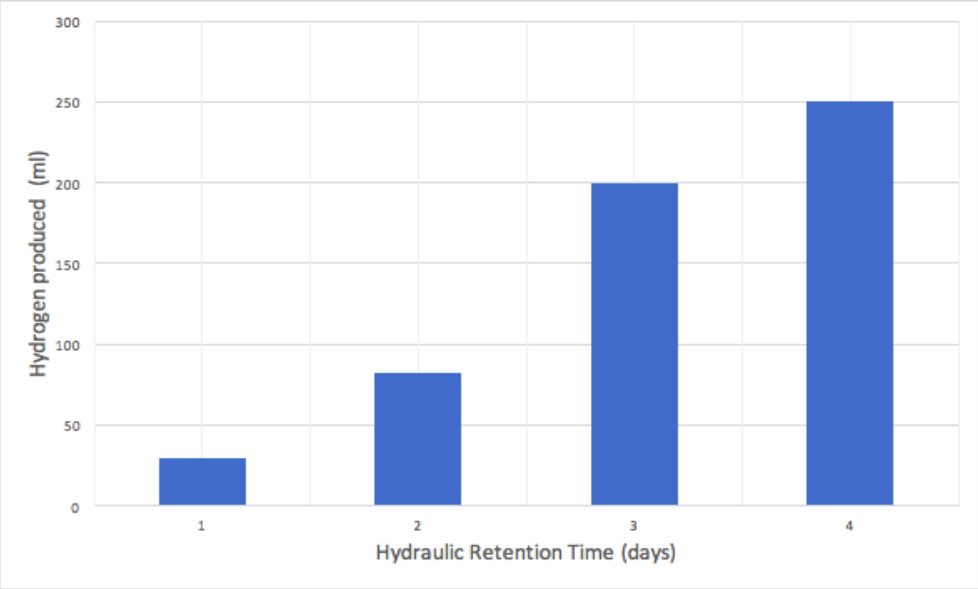


Figure 22: Production of gas in following four days using Biebl Pfennig's media

Using Biebl Pfennig's media 250ml of water was displaced from the gas collecting column so we can say there was 250 ml of gases produces in 96 hours in the proper source of light and substrates.

5.2.2. Using Waste Activated Sludge:

In our study when we put the waste activated sludge in the reaction vessel, the WAS got fermented as there was a change in colour from black to green with enormous growth of bacterial cell. The WAS along with the PNS bacteria was measured spectrophotometrically at 600nm we found change in OD as compared to the initial value. But when the WAS were added with extra carbohydrates in the reaction vessel, only the bacterial growth was enhanced and production of gas also occurred, but due to presence of different obstacle in the WAS the gas pressure didn't build up in the vessel so that it could displace the water in the column and get collected. So, in the photo fermentation process, fermentation of WAS only occurred but we were unable to collect the produced gases. So, there was no water displacement seen from the column.

5.3. DETERMINATION OF BIO-HYDROGEN PRODUCTION:

Due to unavailability of gas chromatography, the exact amount of hydrogen produced remains unknown. In our research study, we have used the simple water displacement method to collect the produced gases which included hydrogen and methane. The CO_2 already got trapped in the KOH solution.

5.4. ACETIC ACID ESTIMATION:

5.4.1. Acetic acid estimation by Montgomery method:

5.4.1.1. Using Biebl Pfennig's media:

A standard curve was prepared for Biebl pfennig's media is shown below:

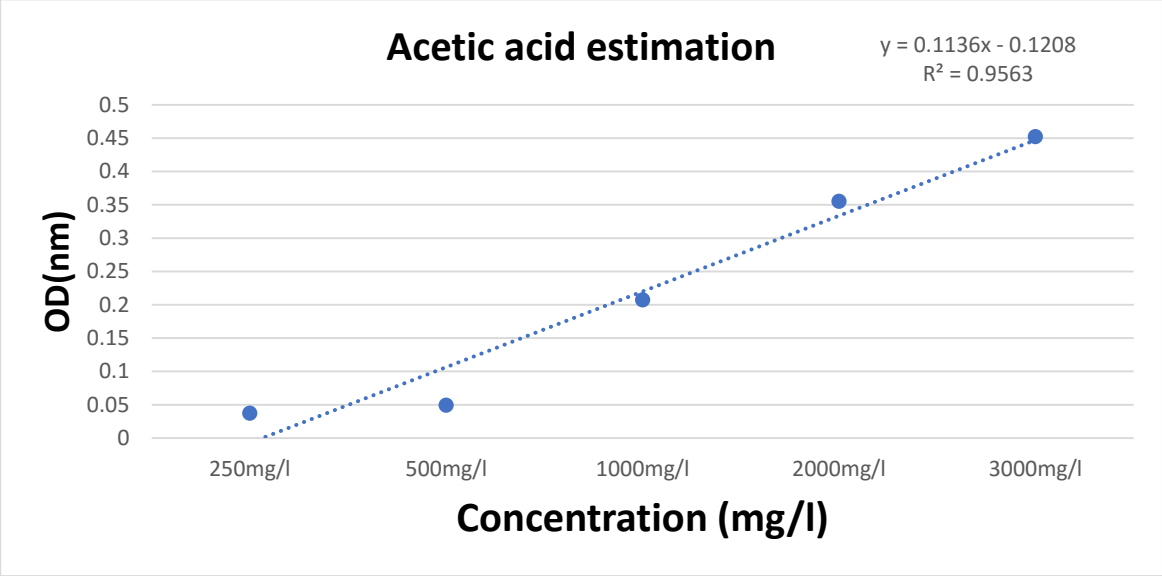


Figure 23:Acetic acid estimation using Biebl Pfennig's media

As the acetic acid concentration was lower to 750mg/l as estimated by Montgomery method, so an additional amount of acetate was added to enhance the process in photo-fermentation.

5.4.1.2. Using Waste water effluent:

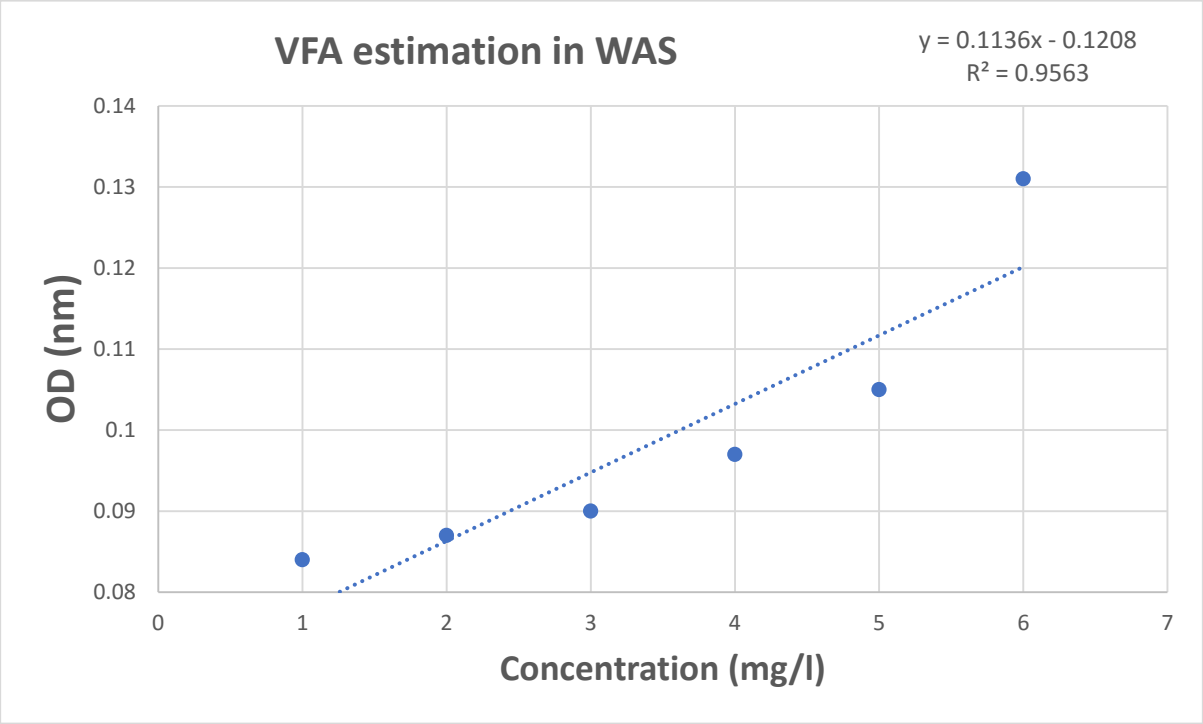


Figure 24:Acetic acid estimation in waste activated sludge.

As there was no pre-treatment, the initial VFA concentration was measured at 513nm and it was 4345 mg/l. The photo fermentation process went good with it but there was random uptake of VFA by the present bacteria in the WAS. Later, the WAS was autoclaved and prepared for photo-fermentation without pre-treatment.

5.5. AMMONIA ESTIMATION:

We have followed Phenate method to estimated ammonia.

5.5.1. In Biebl Pfennig's media:

A standard curve was plotted for Biebl Pfennig's media

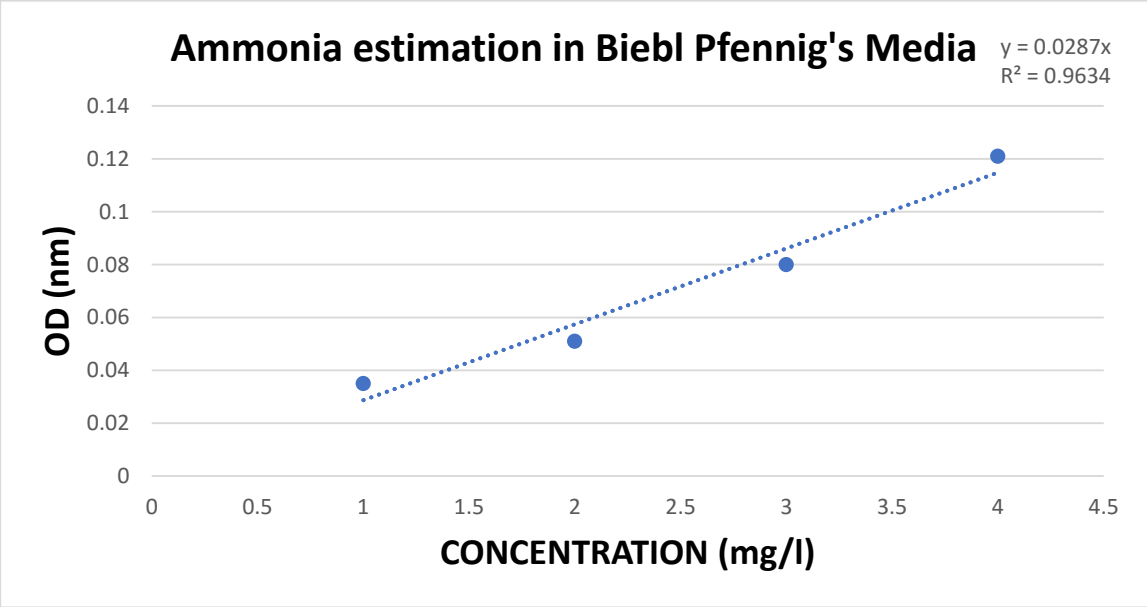


Figure 25:Ammonia estimation in Biebl Pfennig's Media

For Biebl pfennig's media the ammonia concentration was found 0.6mg/ml at 640 nm.

5.6. CARBOHYDRATE ESTIMATION FOR WASTEWATER EFFLUENT:

Anthrone method was chosen for our study.

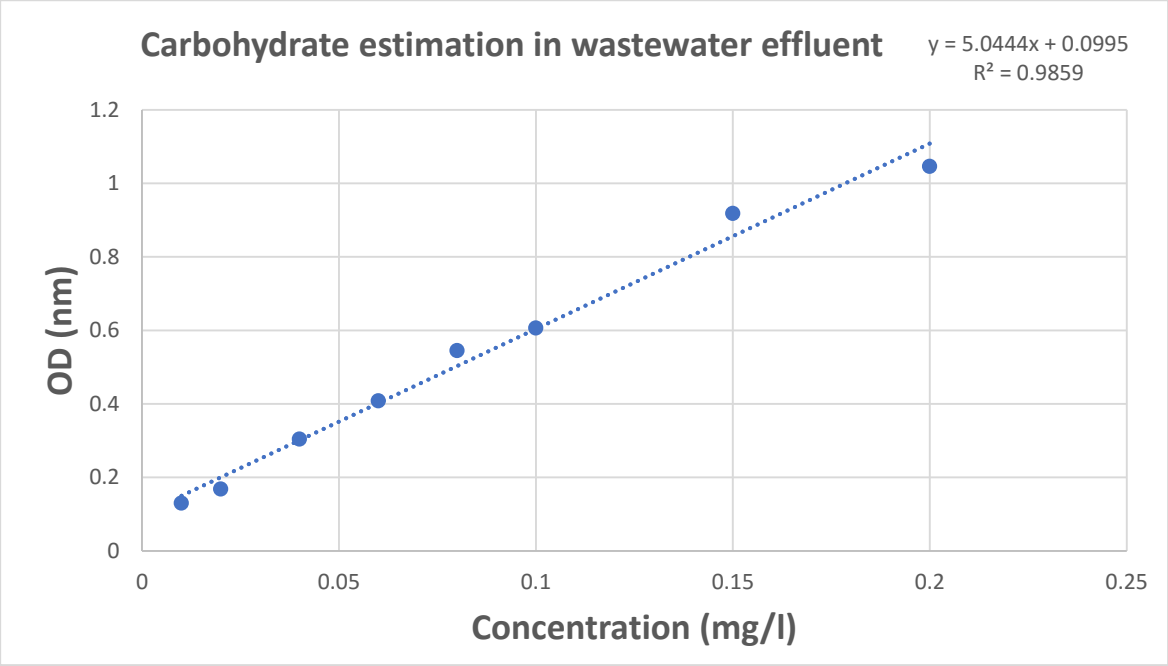


Figure 26: Carbohydrate estimation using Wastewater effluent.

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 photo fermentation, an additional 20 g/l of glucose was added. Although the process is not commercially viable, it was used to test the accuracy and rightness of the process.

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5.7. CHARACTERIZATION OF VARIOUS PARAMETRES IN THE WASTE WATER EFFLUENT

PARAMETRES	VALUES
pH	6.8
COD (Chemical Oxygen Demand in ppm)	1700
Volatile Fatty Acid (VFA in ppm)	4345
Carbohydrate (in ppm)	46
TSS (Total Suspended Solids in ppm)	860
TDS (Total Dissolved Solids in ppm)	266.07
Alkalinity (in ppm)	1380
Ammonical Nitrogen (in ppm)	140

Table 1: Characterization of Various Parametres In The Waste Water Effluent

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

5.7.1 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 2: Acetic acid concentration before recovery

5.7.2 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 3:Acetic acid concentration after recovery

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

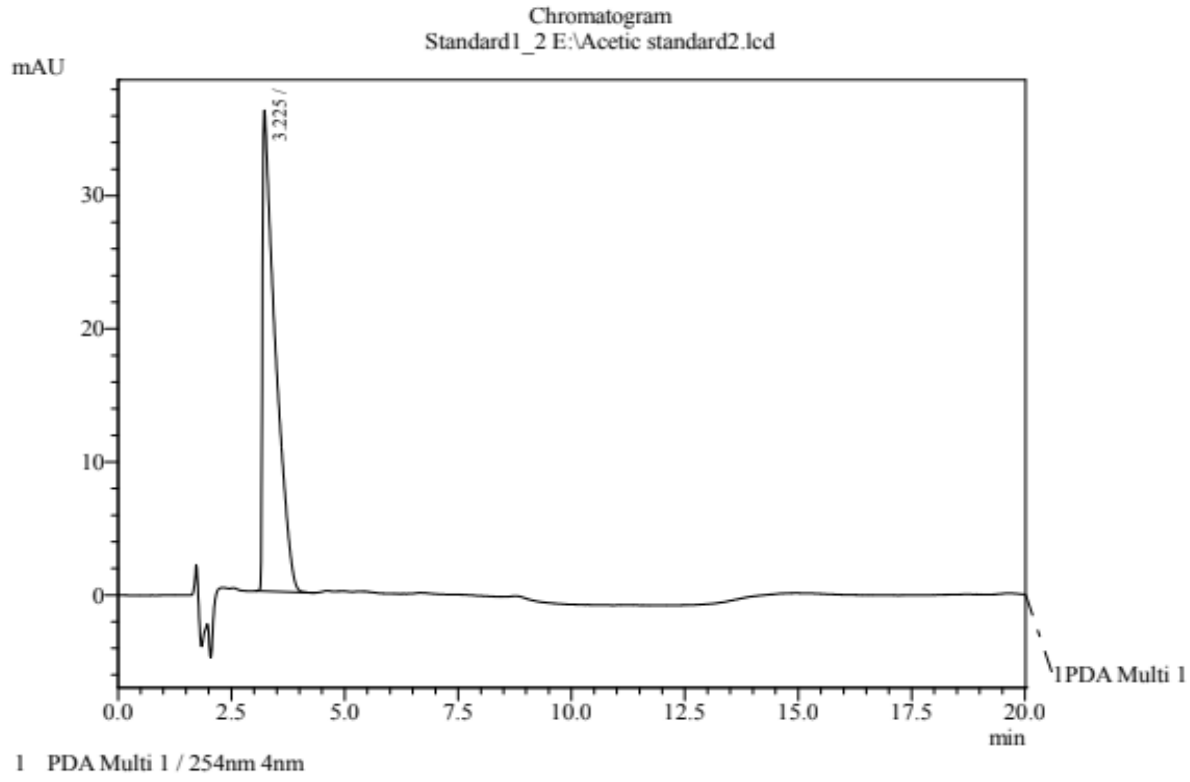

Figure 27:Acetic acid standard of 100 ppm

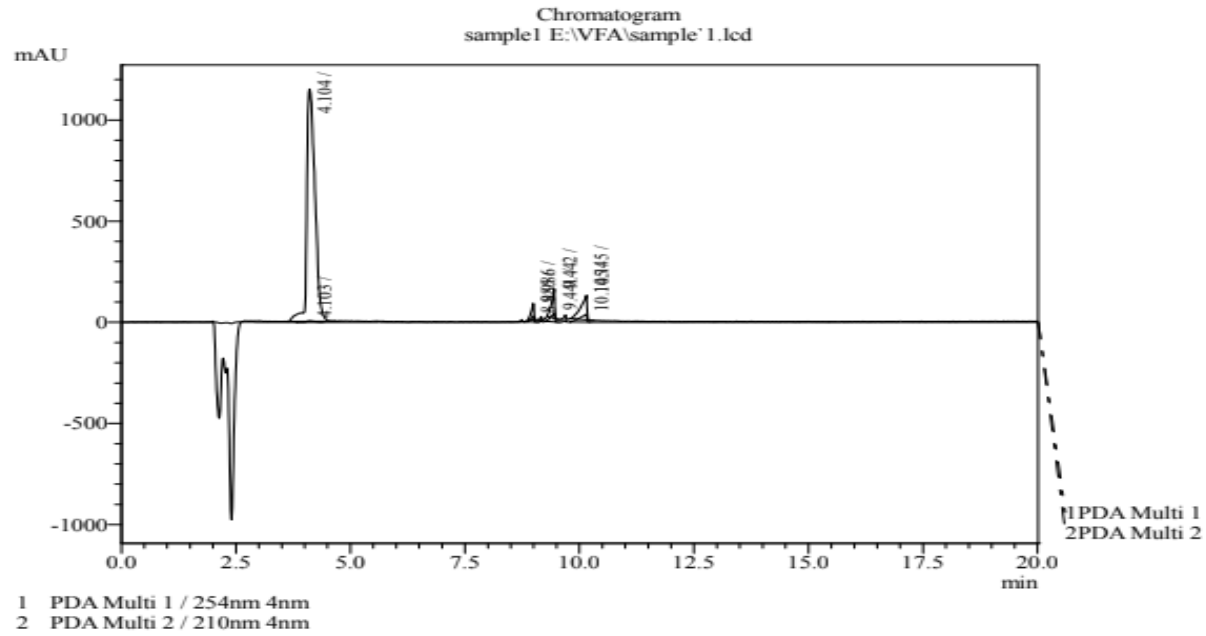

Figure 28: Adsorbed acetic acid of the secondary effluent

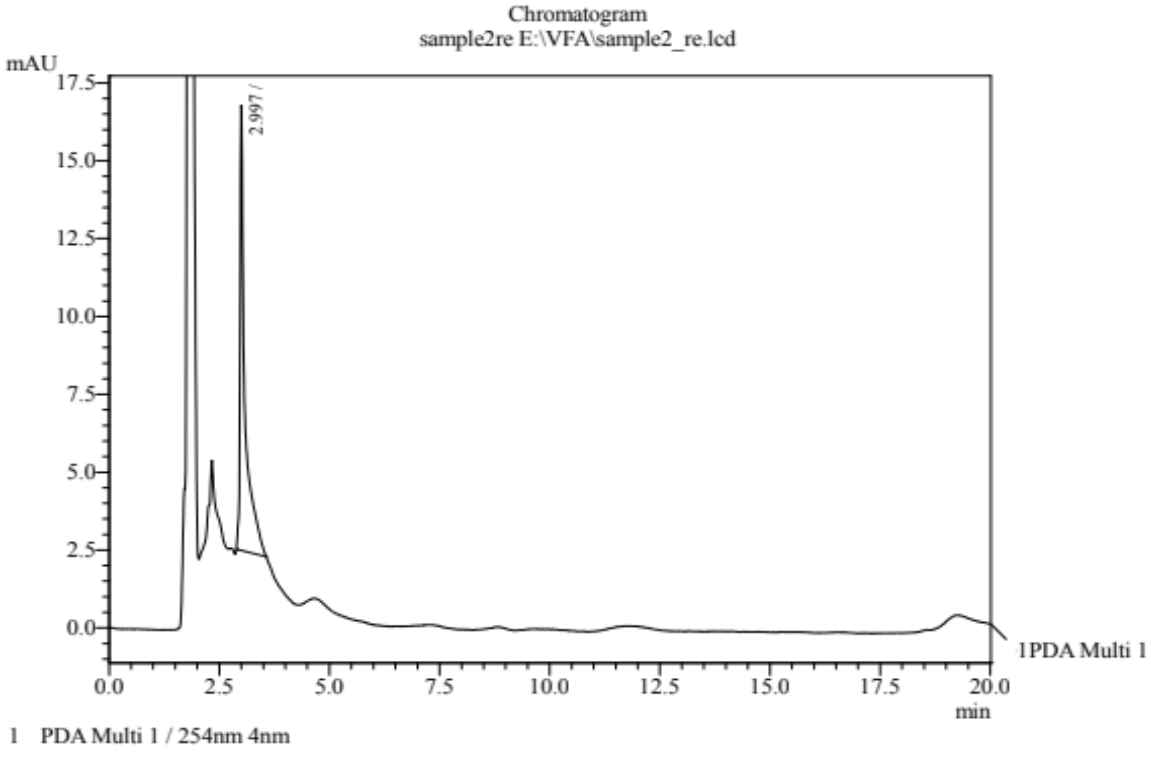

Figure 29: Desorbed acetic acid of the secondary effluent

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

5.8.1 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 4: Acetic acid concentration after recovery

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

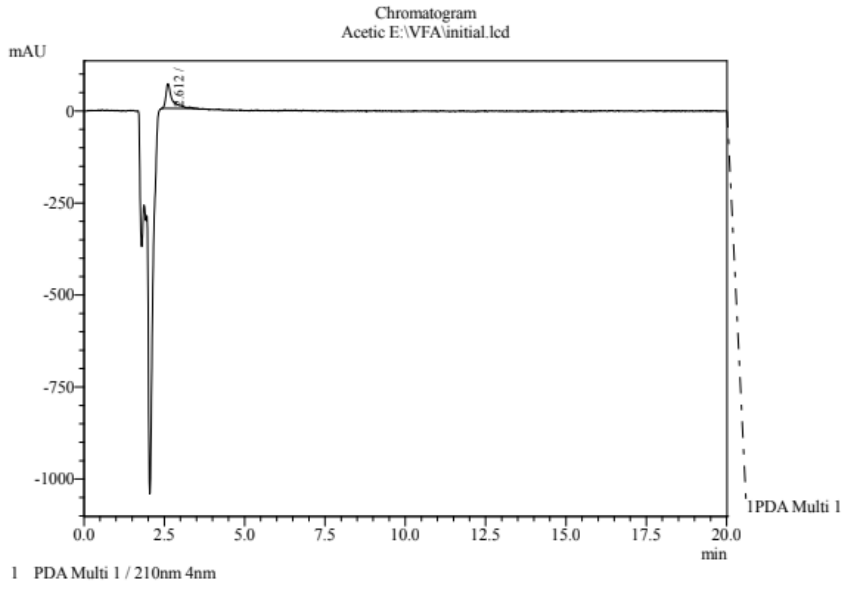

Figure 30: Initial amount of acetic acid concentration of the secondary effluent

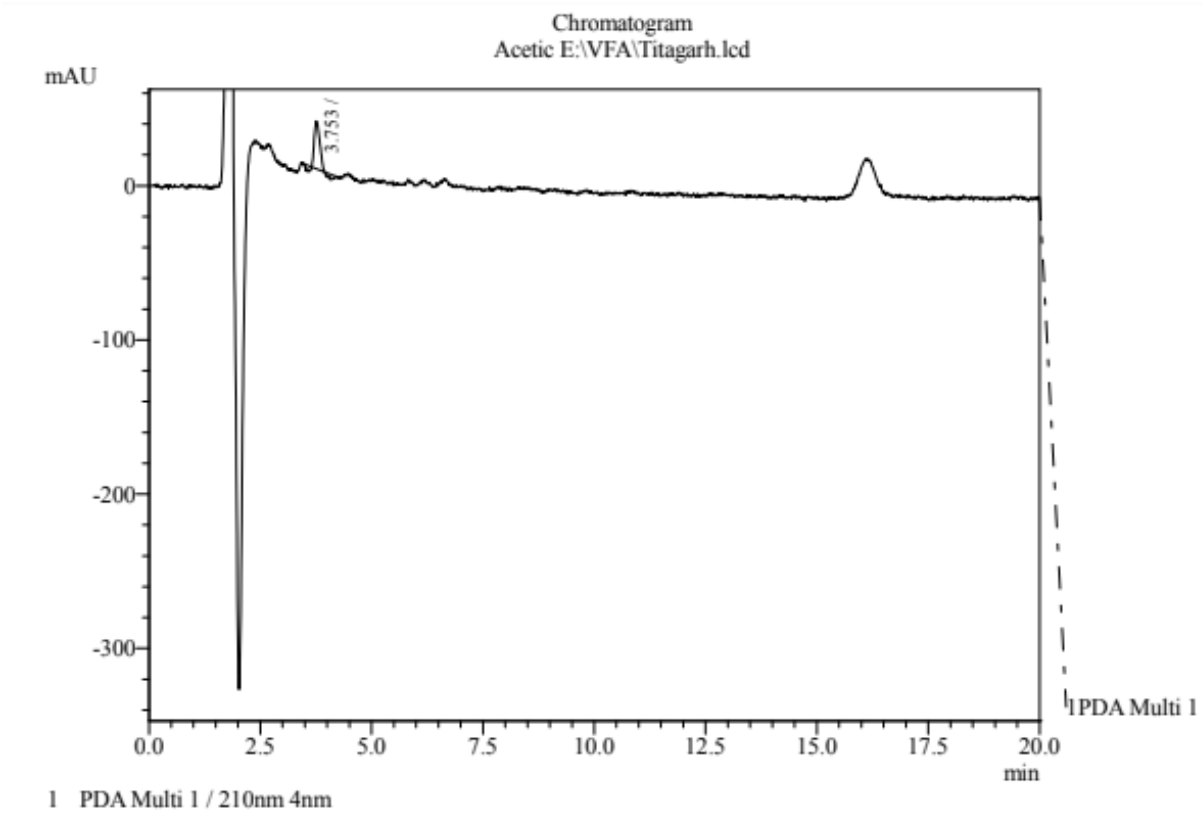

Figure 31 : Desorbed acetic acid concentration of the secondary effluent

6. FUTURE SCOPE

6.1 FUTURE PERSPECTIVES OF BIO-HYDROGEN PRODUCTION

- Numerous critically significant variables are required for photo fermentative H2 generation. The cumulative H2 generation in the photo-bioreactor is improved by the axial and uniform application of light across a larger surface area of illumination [128]. Uses a wide range of light, utilises organic wastes as substrate and also can use dark-fermentation effluents as substrate.
- The light efficiency denotes the ratio between generated H2 energy to the light energy supplied [53]. Furthermore, there is no production of oxygen and the process is able to utilise a wide range of light intensity (520–860 nm) [57].
- Dark-fermentation effluents consisting of VFAs such as acetic, butyric, propionic, lactic, malic acid were also used as substrates in photo-fermentation by PNS bacteria for bio-hydrogen production [51]. It also helps in bioremediation. Photo-fermentation utilises a wide range of substrates, such as effluent from dark-fermentation and has high substrate conversion efficacy, it is one of the processes that has the potential to generate huge quantities of hydrogen [52].
- The stoichiometric bioconversion of substrate to hydrogen can be made possible through the photo-fermentative production of hydrogen technique. Along with Bio-H_2 production, PNS bacteria also helps in synthesising various biochemicals which include lipids, polyhydroxybutyrate (PHB), and biodegradable thermopolymer. These biochemicals exhibit distinct competing metabolic processes [58] [59].
- Although solar energy is a free and pure energy source, it has certain drawbacks, such as heating issues and fluctuating intensity. It is important to create technologies that can

be used for large-scale fermentation that utilise both artificial and solar light. While there may be an initial cost increase, there may be long-term cost savings.

- The procedure of fermentation is significantly impacted by a number of external process variables, including agitation, light intensity (in the case of photo-fermentation), temperature, and pH. For effective H_2 generation, those factors must be optimised, and fermentation must be carried out under ideal conditions. This calls for extensive study and advancements in optimisation methods.
- Multi-strain and multi-substrate systems have been found to boost H_2 subsequent generations; when using complex carbohydrates as a substrate, using several strains might enhance substrate conversion efficiency. This suggests that the pre-treatment step may be removed, which would reduce the cost of fermentation; thus, a full study of this system if necessary.
- As the enzyme plays a crucial role in H_2 generation and it contains metal cofactors like iron (Fe) and nickel (Ni), the addition of these metals in the form of a nanoparticle is important to boost H_2 production. Much investigation is needed for improving the utility and screening of different nanoparticles that play a function in H_2 generation.
- One of the main approaches used for enhancing bio-hydrogen production in the future by changing PF's metabolic pathways is through genetic engineering. But as this technology is still in its early stages, additional investigation is needed to fully understand its potential [149].

7. CONCLUSION:

Photo Fermentative H_2 production depends on many simultaneous critical parameters. Higher surface area of illumination with axial and uniform light distribution enhances the total production of hydrogen in the photo-bioreactor. It was also observed that the presence of high levels of COD, VFA, Carbohydrate and pH helped in producing high amount of hydrogen in the lab scale. In our study, the rapid consumption of carbohydrate also showed a negative impact on gas production. When the synthetic media was change to wastewater as hydrogen production media for PNS bacteria the we found that the externally added glucose source help in more amount of hydrogen production as compared to the presence of carbohydrate in the wastewater.

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