Hydrogen Production from Wastewater Using Microorganisms - A Review

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Global energy consumption, powered primarily by fossil fuels, is expanding at an alarming rate. Biohydrogen is an exciting alternative fuel source which requires relatively little energy to produce, emits far lower levels of greenhouse gases, while using wastewater as a substrate. Biohydrogen can be produced by splitting water molecules photosynthetically, or by fermenting substrates in light and dark conditions. However, as these methods suffer from low efficiency and high production costs, biohydrogen is yet to see widespread commercial use. This paper summarizes present biohydrogen production technologies, as well as their current drawbacks and potential improvements. A complete analysis on biohydrogen produced from various sources of wastewater, as well as its economic potential and commercial viability, will also be covered. Wastewaters such as distillery wastewater, dairy wastewater and cassava wastewater have shown to be high-yielding wastewater substrates, giving up to 2.56 mol per mole substrate. Finally, the scope and viability of producing biohydrogen in India will be reviewed, along with a questionnaire used to interview two major wastewater generators and a potential biohydrogen user in India. While biohydrogen is currently in the laboratory stage in India, and is yet to achieve large scale commercial use, by adopting strategies to maximise yield and selection of the right inoculum and substrate, it has the potential to become a highly promising alternative to fossil-fuel based hydrogen for commercial usage.

Keywords: Biohydrogen, Wastewater, India, Commercial application

Introduction

The global energy demand has increased dramatically over the last few decades, growing from 66,429 TWh in 1970 to 178,899 TWh in 2022 (Ritchie et al., 2022)[1]. Fossil fuels are the dominant energy source as they made up approximately 82% of global energy consumption in 2022 (EI, 2023)[2]. Moreover, burning fossil fuels results in greenhouse gas emissions that heat our planet, depletes already limited resources, and negatively impacts health. For instance, 34.74 billion tons of CO_2 were emitted from burning fossil fuels (Ritchie et al., 2022)[1]. Also, burning fossil fuels causes air pollution, which resulted in eight million deaths in 2018 (Burrows, 2021)[3]. Therefore, in order to meet the world's energy needs while not damaging planetary and human health, we need to reduce the usage of fossil fuels and identify alternative environmentally-friendly fuels. In this regard, hydrogen (H_2) is promising because burning it produces only water, which does not pollute the air (Sivagurunathan and Lin, 2016)[4]. Hydrogen also has a high calorific value of 122 kJ per g, a number 2.7 times higher than that of fossil fuels. Therefore, hydrogen is becoming a promising candidate for using in fuel cells and electricity generation (Levin et al., 2004)[5]. However, 95% of commercial H_2 used today is produced from natural gas, coal, heavy oil, and naphtha in an energy-intensive manner, and is responsible for 830 million tonnes of CO_2 emissions every year (IEA, 2019)[6]. For hydrogen fuel to be successful, it needs to be more cost effective, less energy intensive and more environmentally friendly.

The biological production of H_2, or biohydrogen, is an exciting new technology that appears to have multiple advantages over fossil-fuel sourced hydrogen, such as being able to operate at ambient pressure and temperature and requiring less energy, the ability to use various organic wastes and wastewaters as substrates, resulting in a substantial 57-73% reduction in greenhouse emissions, and a high substrate conversion efficiency (Preethi et al. 2019; Arimi et al. 2015; Zhang et al. 2017)[7–9]. Biohydrogen can be produced electrochemically in a microbial fuel cell, by splitting of water molecules by microorganisms, or by fermenting organic materials using microorganisms in a reactor, which be discussed here. The organic materials that can be used for fermenting can include food wastes, agricultural wastes, animal dungs, and wastewaters, given their high degradability and high organic content. The diagrammatic representation for the same is given below in figure 1 (Banu et al., 2019)[10]:

This paper summarises the methods of producing biohydrogen and provides a detailed description of possible wastewater substrates that can be used for biohydrogen production, methods of increasing the H_2 yield, and the dark fermentation process.

Biohydrogen production is indeed hampered by a few issues, such as its high production cost - it costs between $10 to 20$ per Giga Joule (GJ) of energy produced from it compared to \$4.87 - \$5.62 per GJ for H_2 produced from fossil fuels - and its current applicability on at a small-scale level (Seddon, 2022; McFarland, 2022)[11,12]. I present the current picture of biohydrogen to identify the key bottlenecks to reduce cost and increase adoption. To further this aim, I supplement the research paper with insights from responses to a detailed questionnaire) administered to two major wastewater generators and one potential biohydrogen user and provide ideas to improve biohydrogen yield when using wastewater as a substrate.

Methods of Producing Biohydrogen

The production of biohydrogen can be broadly classified into two categories: light-dependent processes and light-independent processes. Light-dependent processes take place in the presence of sunlight, and can maybe further classified as hydrogen photosynthesis (splitting of water molecules to produce hydrogen gas using microorganisms) and photo fermentation. Light-independent methods do not require sunlight, and includes dark fermentation. Dark fermentation is especially advantageous as it utilizes fast growing bacteria and has a high hydrogen production rate (Ramprakash et al., 2022)[13]. Both light dependant and independent methods have great promise as they produce high yields of biohydrogen at low temperatures and at a low cost (Trchounian, 2015)[14]. Other techniques, such as genetic engineering and synthetic biology have also been used to improve the yield of biohydrogen (Ramprakash et al., 2022)[13]. The methods have been discussed in detail below:

Hydrogen Photosynthesis

Direct Photolysis: In this process, the microorganisms split the water molecules to produce hydrogen and oxygen. The process takes place in the presence of sunlight, and in the absence of oxygen. Microbial species such as Cyanobacteria have the potential to produce hydrogen by direct photolysis. The equation for the same is given below (Ramprakash et al., 2022)[13]:

$$2H_2O \rightarrow 2H_2 + O_2$$

This process is mediated by hydrogenase and nitrogenase enzymes, producing H_2 gas along with reducing N2 to ammonia (Ghiasian, 2019)[15].

This process does not require a carbon substrate as the reaction only uses light energy and water, and therefore has the potential to be highly energy efficient. It also possesses the advantage of using only CO_2 and water as input materials, making the entire process carbon-negative (Arimi et al. 2015)[8]. However, this method is affected by low conversion efficiency because of the inhibition of the hydrogenase enzymes by oxygen (Arimi et al. 2015, Ramprakash et al. 2022)[8,13]. The enzyme suffers irreversible inactivation by oxygen which greatly affects the efficiency of the reaction (Mohammadi and Vashisth, 2017)[16]. Nonetheless, many researchers have attempted to engineer the enzyme to make it more oxygen tolerant, and will be discussed in another section.

Indirect Photolysis: Indirect photolysis occurs when carbon dioxide is used by bacteria in the presence of sunlight to produce carbohydrates, such as glucose. These carbohydrates are later broken down to produce energy, and hydrogen gas is released as a by-product. This process too is anaerobic, and is facilitated by the hydrogenase enzyme. *Cyanobacteria A variabilis* has been used as a promising microbe for this method; however, the production of hydrogen is inhibited due to sensitivity of the microorganisms to oxygen (Arimi et al., 2015)[8].

$$6CO_2 + 12H_2O \rightarrow C_6H_{12}O_6 + 6O_2 + 6H_2O$$

$$C_6H_{12}O_6 \xrightarrow{\text{Hydrogenase}} H_2$$

Photo-Fermentation

In photo-fermentation, volatile organic acids such as acetic acid and butyric acid are metabolised by microbes in the presence of light to produce hydrogen as a by-product. The reaction requires external energy to continue, which is supplied by light. The reaction proceeds as follows:

$$2CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2$$

Although the process can use both purple (such as *Chromatium sphaeroides* and *Rhotobacter sphaeroides*) and green bacteria (*Chlorobium sphaeroides*), the purple non-sulphur photosynthetic bacteria (*Rhotobacter sphaeroides*) are the most widely studied (Yin and Wang, 2022)[17]. Han et al. (2012)[18] achieved a biohydrogen yield of 794 mmol/mol-substrate (using volatile organic acids as a substrate with additions of 2.0 g/litre substrate of lactate and 2.0 g/litre substrate of succinate) using *Rhotobacter sphaeroides*.

The process has the advantage of high conversion rates, with a possible yield of 4 mol H_2 per mole substrate when using acetic acid as a substrate (Arimi et al. 2015)[8]. It also possesses low organic source requirement and high hydrogen content within the biogas generated (Yin and Wang, 2022)[17]. However, the use of expensive bioreactors can make this process expensive, and this process is hampered because only a limited number of substrates can be used (Arimi et al. 2015)[8]. Unlike dark fermentation, where any form of organic matter can be used, photo-fermentation can only use organic acids as substrates. The most important factors for improving production yields include bioreactor design and selection of biohydrogen substrate.

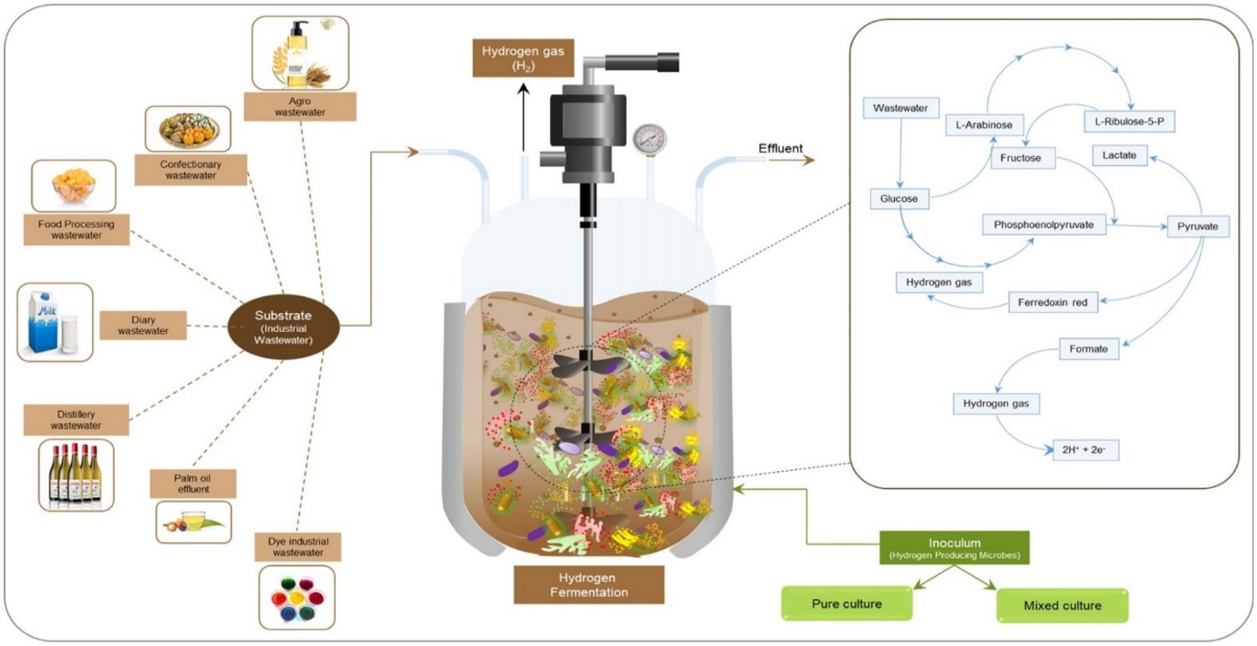

Fig. 1 A diagrammatic representation of the biohydrogen production process

This method of hydrogen production shows more promise when coupled with dark fermentation as will be covered in the section on photo-dark fermentation integration.

Dark Fermentation

In dark fermentation, the substrate is oxidised by anaerobic bacteria in the absence of light to generate electrons, which are used to produce hydrogen gas by reducing hydrogen ions. The entire process is facilitated by enzymes such as hydrogenases (Banu et al., 2019)[10]. The intermediate products that are produced include volatile fatty acids, as well as acetate, propionate and butyrate (Preethi et al., 2019, Arimi et al., 2015)[7,8]. The pathway that produces acetate is shown to be the optimal as it can produce up to a theoretical maximum of 4 molecules of hydrogen per molecule hexose as shown below:

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

The reaction does not require any additional energy source and is exothermic (Arimi et al., 2015)[8]. The possible substrates that can be used include sugars, amino-acids, glucose and wastewaters generated from sewage treatment plant, distilleries, sugarcane processing industries and dairy industries (Banu et al. 2019)[10]. Dark fermentation is the most promising method as it needs a lower external input energy to drive the reaction and the rate of hydrogen production is comparatively faster than other methods (Ramprakash et al. 2022)[13]. Treating wastewater is another advantage of dark fermentation (Yin and Wang, 2022)[17]. However, dark fermentation suffers from low H_2 yield, which is less than the biological theoretical maximum of 4 mole H_2 per mole glucose (Ramprakash et al. 2022)[13]. The hydrogen production from dark fermentation may be increased by pretreatment of the inoculum or substrate, addition of nanoparticles or by integrating the process with photo fermentation (Preethi et al. 2019)[7].

Biohydrogen production by dark fermentation is reported to be technologically closest to commercial application (Yin and Wang, 2022)[17]. Studies have achieved a maximum yield of 4.84 mol H_2/mol lactose (Cardoso et al., 2014).

Photo Fermentation-Dark Fermentation iIntegration

One of the strategies to increase the yield of biohydrogen is integrating photo fermentation with dark hydrogen fermentation (Banu et al., 2019)[10]. Dark fermentation of hydrogen faces multiple drawbacks, such as low efficiency and low hydrogen yields. Only a part of the substrate is converted to hydrogen, and it is mostly converted into volatile fatty acids (VFAs) such as acetic acid, butyric acid and propionic acid, which is normally left unconverted and discharged off as effluents (Mishra et al., 2019; Banu et al., 2019)[10,19]. These volatile fatty acid substrates are readily metabolised by purple non-sulphur (PNS) bacterium, which produce hydrogen gas. This integrated dark-photo fermentation results in enhanced biohydrogen production, with it being theoretically possible to extract all hydrogen atoms from hexose as a gas (Arimi et al., 2015)[8]. High hydrogen yields in the range of 3.8 to 10 mole H_2 per mole substrate has been reported, with a theoretical maximum of 12 mole H_2 per mole substrate being possible. Mishra et al. (2019)[19] investigated the production of biohydrogen from palm oil effluent using two stage sequential dark and photo fermentation. They reported a hydrogen yield of 0.784 ml H_2 per ml substrate using dark fermentation, which increased to 3.064 ml H_2 per ml substrate using integrated dark and photo fermentation. The process also increases the degradation rate of the substrate (Yin and Wang, 2022)[17]. The overall reaction is as follows (Arimi et al. 2015)[8]:

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2$$

The process may be done in a two-stage process, using different bioreactors and inoculum for each stage, or a single-stage process, taking place in the same reactor and using a co-culture of anaerobes. The two-stage process has greater efficiency than the single-stage process, and higher yields have been reported too (Mishra et al., 2019)[19]. However, the process suffers from oxygen inhibition and long operation times, which affect the yield of hydrogen. Additionally, separate bioreactors and inoculum need to be used for each process, and the process parameters (pH, temperature etc.) must be externally controlled, which increases the cost of production and can prove challenging if the process is applied at a large scale. The single-stage process addresses these issues, and it is a more cost-effective manner to produce higher yields of biohydrogen and is better suited for practical applications, although hydrogen yields are not as high as those of the two-stage process. Additionally, the PNS bacterium take a long time to adapt from fermenting sugar substrates to fermenting VFAs, resulting in the accumulation of VFAs, inhibiting the process.

Table 1 summarises the advantages, disadvantages and ways to improve the various methods to produce biohydrogen (Arimi et al., 2015; Yin and Wang, 2022)[8,17].

Wastewater As a Source of Biohydrogen

When selecting the substrate for producing biohydrogen, the availability of the substrate, the amount of carbohydrate present in it, cost and hydrogen yield and production must be taken into account (Ramprakash et al., 2022; Arimi et al., 2015)[8,13]. Some ideal substrates for producing biohydrogen include glucose, xylose and lactose; however, they are expensive to get in pure form (Ntaikou, 2021)[24]. Therefore, using waste such as molasses, fruit peels and bagasse, and wastewater to produce biohydrogen has merit.

Every year, 359 billion cubic meters of wastewater is produced globally (Utrecht University, 2021)[25] Left un-or undertreated, this wastewater is responsible for diseases such as diarrhoea, cholera, dysentery and Hepatitis A, as well as growth of algal bloom (Ashworth, 2021). In the USA, 292 million tonnes of municipal solid waste (MSW) were generated in 2019, an 84 million tonne increase from the 208 million tonnes of MSW generated in 1990 (EPA, 2022)[2]. In this regard, industrial wastewater offers an organic-rich, carbon-neutral, renewable and inexpensive source for biohydrogen production, which additionally addresses water scarcity and water pollution (Sivagurunathan et al., 2017)[26].

Industrial wastewater contains highly degradable organic substances, which are ideal substrates to be used in biohydrogen as it maintains the balance between energy that is supplied and the energy that is extracted (Banu et al., 2019, Preethi et al., 2019)[7,10]. Many researchers have used wastewater from various sources such as sugar industry, food processing industry, distillery, chemical, palm oil industry, beverage industry (Sivagurunathan and Lin, 2016)[4], cheese whey and dairy, and each of them observed various different yields and production rates in the process (Banu et al., 2019)[10]. However, distillery wastewater was noted to have the greatest potential to produce biohydrogen, owing to its high sugar content (Arimi et al., 2015)[8]. Currently, distillery wastewater is the wastewater source that can provide the highest hydrogen yield (2.76 mol/mol glucose).

Other than the type of wastewater being used, various process parameters are important factors that influence the biohydrogen yield. For example, the HRT (hydraulic retention time, the average time the soluble compound remains in the bioreactor) is a key influencing factor for biological H_2 production. It is affected by factors such as the rate of reaction, biomass concentration and temperature (Arimi et al., 2015)[8]. On average, the lower HRT was found to have the best hydrogen production rate as it increases the Organic Loading Rate (The amount of organic matter being broken down per unit reactor volume, per unit time), whereas studies have found that hydrogen yield is highest with the lowest HRT. The HRT can be optimised by controlling the concentration of the substrate and sludge, temperature and nature of the microbes, with a high substrate to sludge ratio causing an increase in the optimum HRT, and a higher temperature decreasing the optimum HRT (Preethi et al., 2019; Arimi et al., 2015)[7,8]. Amorim et al. (2014)[27] investigated the effect of HRT on biohydrogen production from cassava wastewater. They found that the hydrogen yield increased from 0.13 mol H_2 to 1.91 mol H_2 per mole glucose as the HRT was decreased from 8 h to 2 h, and the hydrogen production rate increased from 0.20 to 2.04 L/L/h when the HRT was decreased from 8 to 1 h.

The pretreatment of the substrate with other substances also has significant effects on the final hydrogen yield. By pretreating the substrates, the substrates are broken down into more readily fermentable products, and competing microbes are eliminated. Some methods of pretreatment include physical, mechanical, chemical, and biological (Preethi et al., 2019)[7]. Among the following, physical pretreatment methods using microwaves and thermal energy show the most promise for commercial

Table 1 Comparison of Different Methods of Hydrogen Production

Method	Advantages	Disadvantages	Substrate	Energy Source	Bacteria	Cost ($ per kg)	Strategies	Max Yield
Photolysis	No greenhouse gas emissions, Water substrate, Oxygen byproduct	Low biohydrogen yield, Oxygen inhibition, Expensive bioreactors	Water	Solar energy	Cyanobacteria	$2.13 to $7.24 (Direct) *, $1.24 (Indirect) **	Engineering of hydrogenase enzymes	490 ml/L substrate (Oncel et al., 2014)[20]
Photo-fermentation	High organic acid conversion, Higher yield than photolysis	Complex, expensive bioreactors, Limited substrates, Oxygen inhibition	Volatile organic acids	Organic substances, solar energy	R. capsulatus, R. Sphaeroides	$7.61	Coupling with dark fermentation, Advanced technology	3.53 mol H_2/mol acetate (Elkahlout et al. 2019)[21]
Dark fermentation	High hydrogen production rate, Easy operation, Waste treatment and energy production	Low H_2 yield, Low COD elimination	Sugars, amino acids, glucose, wastewaters	Organic substances	C. Sphaeroides, E. Sphaeroides	$7.52	Coupling with photo-fermentation, Pretreatment, Integration with MFC	4.84 mol H_2/mol lactose(Cardoso et al., 2014)[22]
Photo-dark Fermentation	High H_2 yields, High COD removal, Less organic acids in effluent	High production cost, Dual bioreactors	Sugars, amino acids, glucose, wastewaters	Organic substances	E. Cloacae, R. Sphaeroides	$7.80-$7.90 ***	Parameter optimization	22 mmol H_2/g COD(Mishra et al., 2019)[19]

*Capital cost is assumed to be $50 per m2 (Nikolaidis and Poullikas,
**Capital cost is assumed to be $135 per m2 (Nikolaidis and Poullikas, 2016)[23]
***estimated

use, owing to its ability to increase hydrogen yields and ease of implementation (Preethi et al., 2019; Arimi et al., 2015)[7,8]. Mohammedawi et al. (2019)[28] investigated the yield of biohydrogen from brewery wastewater after pre-treating the substrate with banana peels. They reported a production yield of 408.33 H_2 per L wastewater when using 50% BWW pretreated with 1 g/L banana peels for 2 hrs. They reported that the production yield was a 2.7-fold increase in the production yield over using raw BWW as a substrate. They reported that when pretreating the brewery wastewater, the ammonium concentration of brewery wastewater decreased and C/N ratio increased which resulted in the yield to increase.

Fermentation pH is another key factor in the production of biohydrogen that has a significant effect on the overall yield of biohydrogen. The optimal range of pH for biohydrogen production is 4.5 to 9, and a pH lower than 5 affects the hydrogenase enzyme and reduces hydrogen production (Stavropoulos et al., 2016; Liu et al., 2008)[29,30]. The pH depends on the microalgal species used, and decreases as the reaction proceeds due to build-up of volatile fatty acids. In a large-scale reactor, pH control is achieved by adding alkali‘s to neutralise the extra acidic products, and is optimised for maximum energy recovery; however, this can become expensive as large amounts of alkali are required to control the buildup of organic acids and CO_2 in the reactor (Mota et al., 2017, Arimi et al., 2015)[8,31]. Venkata Mohan et al. (2007)[32] investigated the effect of pH and substrate composition in the production of biohydrogen from chemical wastewater by selectively enriched anaerobic mixed consortia. They noted that a fermentation pH 6 had a higher H_2 production (1.25 mmol H_2 per g COD), which was more than the production at pH 5 (0.71 mmol H_2 per g COD) and pH 7(0.27 mmol H_2 per g COD). They also noted that the yield of H_2 using chemical wastewater as feed with glucose and sewage wastewater as co-substrates had a higher H_2 yield than only using glucose as a primary feed.

Organic Loading rate (OLR) has a major influence on biohydrogen production as the total conversion of carbohydrate is inversely proportional to OLR in the reactor (Preethi et al., 2019)[7]. Conditions The optimum HRT depends on the sludge loading rate, the pH conditions, the substrate type and concentration, the temperature and the reactor type (Arimi et al., 2015)[8]. A high OLR is difficult to obtain, as the pH decrease caused in the reactor reduces the substrate conversion rate (Arimi et al., 2015)[8]. As a high OLR increases the percentage of hydrogen in the biogas formed, optimising the reactor to increase the pH should lead to a higher OLR (Arimi et al., 2015)[8]. Azbar et al. (2009)[33] studied H_2 production from cheese processing wastewater via dark anaerobic fermentation was conducted using mixed microbial communities under thermophilic. They observed an average hydrogen production rate of 2.5 L H_2 /L/day and observed a maximum H_2 yield of 22 mmol H_2 per g COD at an HRT of 3.5 days (The COD was constant at 40 g/L). They observed hydrogen yields to be 3, 9 and 6 mmol/g COD, for OLR values of 47, 35 and 21 g COD/l/day (HRT was constant at 1 day).

Table 2 summarises the wastewater sources used for biohydrogen production, along with their respective hydrogen yields, production rates, HRT and inoculum.

Limitations and Commercial Viability of Biohydrogen

Hydrogen production has many advantages over hydrogen produced from fossil fuels such as lower carbon emissions and lower input energy, but it is limited by factors such as low substrate conversion efficiency and inhibition due to by-products from the fermentation processes (Venkata Mohan, 2010)[39]. Although a theoretical yield of 4 mole H_2 per mole glucose is possible using known pathways of producing biohydrogen, only yields in the range of 1-2 mole H_2 have been produced due to factors such as inhibition by by-products and oxygen. As discussed earlier, the by-products from dark fermentation of wastewater are in the form of volatile fatty acids, and their buildup can affect the hydrogen production and yield (Preethi et al., 2019)[7]. This problem can be tackled by integrating dark fermentation with photo fermentation, where the acidic by products can be fermented to produce biohydrogen. The single-stage process is better suited for use at a larger scale, as it does not require external control of process parameters and can be done in the same reactor (Mishra et al., 2019)[19].

As mentioned earlier, the sensitivity of the hydrogenase enzyme to oxygen is a problem that must be addressed. Many researchers have made attempts to engineer hydrogenase enzymes to cope with oxygen. Mohammadi and Vashisth (2017)[16] investigated the pathways and diffusion channels of O2 in the hydrogenase enzyme. Through this, they were able to identify several areas that could be potential candidates for engineering to increase the oxygen tolerance of hydrogenase enzymes. Additionally, Bringham et al. (2012)[40] experimented with the hydrogenase enzymes found in C. pasteurianum, and achieved a large decline in the enzyme's oxygen sensitivity. Researchers have tried to engineer the enzyme to be oxygen resistant by modifying specific gas channels in the reactors, but since all attempts have led to a reduction in biohydrogen production, it has been recommended that engineered hydrogenase enzymes be used in fuel cell technologies rather than producing biohydrogen. (Ramprakash et al., 2022).[13]

Biohydrogen production is also inhibited by the presence of methanogens. Methanogenic bacterium harvest hydrogen produced during fermentation and use it to produce methane from carbon dioxide, which reduces the productivity of the process (Banu et al., 2019)[10]. Yang et al. (2007)[41] reported that the number of methanogens could be higher if the HRT of the system was longer, when using cheese whey as a substrate and mixed microbial communities (untreated) as inoculum. The activity of methanogens can be suppressed primarily by pre-treating the inoculum by methods such as heat-shock pretreatment (Banu et al., 2019)[10].

The production of hydrogen by microorganisms has gained considerable attention in the few decades due to the pollution less nature, recyclability and high efficiency of conversion of substrate (Preethi et al., 2019)[7]. In fact, a "Biohydrogen economy" has been postulated by the scientific community as a solution to the current global energy crisis (Ramprakash et al., 2022)[13]. The returns from producing biohydrogen from various different organic substances and wastewaters depend on costs levied on raw material, equipment, and transportation as well as technological development (Ramprakash et al., 2022)[13]. However, the commercialisation of biohydrogen is yet to commence as it is facing issues of high production cost and low yield of hydrogen and the lack of knowledge of biochemical reaction as well as involved enzymes. (Preethi et al., 2019; Goswami et al., 2021)[7,42].

The cost of biohydrogen is affected by several parameters, including bioreactor operation, resources for collecting biohydrogen, control systems, storage of hydrogen gas and material and nutrient costs (Ahmed et al., 2021)[43]. Currently, biohydrogen costs \$2.13 to \$7.24 per kg via direct bio photolysis, \$1.24 via indirect bio-photolysis and \$7.54 to \$7.63 via fermentative methods (Nikolaidis and Poullikas, 2016, Dincer and Acar, 2015)[23,44]. This is assuming the operating cost are kept minimum and parameters such as gas separation and handling was not considered (Nikolaidis and Poullikas, 2016)[23]. Nevertheless, biohydrogen has been shown to be profitable. Gholkar et al. (2021)[45] performed an economic evaluation of a biohydrogen plant in India. The total capital investment was \$144.6 million, and assuming a market value of \$10 per kg hydrogen, the payback period was 3.78 years with a return rate of 22%. Resnick (2004)[46] calculated a biohydrogen production cost of \$30.7 per GJ using photo-fermentation. The operating cost was taken to be \$193 million, which included power costs (\$2.5 million), water costs (\$0.01 million), labour costs (\$23 million), supplies (\$3.5 million), culture production costs (\$2.7 million), gas separation and handling costs (\$0.13 million), and a subtotal operating cost (¿\$175 million), resulting in a capital cost of \$1.41 per GJ per year. Dark fermentation had a lower capital cost of \$0.64 per GJ per year, owing to lower cost of the bioreactor. All studies attempting to estimate the costs for producing biohydrogen have concluded that biohydrogen is more expensive than conventional hydrogen fuels. Additionally, many of the studies failed to account for important costs in the production process, such as gas separation costs and labour costs. Additionally, the cost of producing hydrogen from natural gas can be as low as 0.7\$ per kg (IEA, n.d)[6], which is close to half the estimated cost of the biohydrogen. Therefore, further research must focus on accurately modelling all the factors in the operating cost, to obtain an accurate price figure for hydrogen production. Additionally,

Table 2 Comparison of Hydrogen Production from Different Wastewater Sources

Wastewater Source	Hydrogen Production Rate (L/L/D)	Hydrogen Yield	Type of Reactor	Reactor Conditions (pH, Temperature)	Inoculum	Source
Beverage	37.5	1.62 mol/mol hexose	Continuously Stirred Tank	pH 5.7 to 6.4; 37 °C	Enriched Microflora	(Sivagurunathan and Lin, 2016)[4]
Cheese Whey	2.5	22 mmol H_2 per g COD	Up-flow Anaerobic Sludge Blanket	pH 5.5; 35-37 °C	Anaerobic seed sludge from anaerobic digester	(Azbar et al., 2009)[33]
Dairy	19.2	2.56 mol H_2 / mol carbohydrate	Anaerobic Fluidised-bed	pH 3.7 to 4.3; 24-30 °C	Biomass from fermentation	(Silva et al., 2019)[34]
Brewery	12	1.5 mol H_2 per mole carbohydrate	Anaerobic batch	pH 3.5; 37 °C	Anaerobic bacteria consortium	(Pachiega et al., 2018)[35]
Palm oil effluent	43.2-45.6	18 mmol /g-COD	Anaerobic batch	pH 5.5; -	Clostridium butyricum, Rhod pseudomonas palustris	(Mishra et al., 2019)[19]
Chemical	0.24	1.25 mmol/g-COD	Anaerobic batch	pH 6; 27°C - 31°C	Selectively Enriched Anaerobic mixed consortia	(Venkata Mohan et al., 2007)[32]
Food processing	5.04	5.71 mmol /g-COD	Anaerobic batch	pH 6.4; 23 °C	Treated soil inoculum	(Van Ginkel et al., 2004)[36]
Sugar processing	2.18	1.48 mol H_2 /mol sucrose	Anaerobic Sequencing batch	pH 4.5; 31°C	Seed sludge	(Won et al., 2013)[37]
Cassava Wastewater	49	1.91 mol/mol glucose	Anaerobic Fluidised bed reactor	pH 5; 26-30 °C	Sludge from swine wastewater treatment	(Amorim et al., 2014)[27]
Distillery	5.15	2.76 mol/mol glucose	100 m3 scale bioreactor	pH 5.2-7; 37 °C	Defined bacterial co-cultures	(Vatsala et al., 2008)[38]

Distillery wastewater has the highest yield of hydrogen (2.56 mol/mol glucose), followed by dairy wastewater and cassava wastewater. However, the hydrogen production rate of distillery wastewater is comparatively lower (5.15 L/L/D) (Table 2). Therefore, the wastewater with the best balance of high hydrogen yield and production rate is Cassava wastewater, owing to its high energy content of 40 g carbohydrate per litre (Amorim et al., 2014).[27]

biohydrogen produced must be produced in a large scale, must be priced competitively and must utilize various strategies to attempt to reduce the cost, and increase productivity (which will be discussed later).

Pilot scale projects conducted are important ways to predict realistic costs of biohydrogen. Li et al. (2012)[47] conducted an economic evaluation of beverage wastewater. They reported that when using a scale of 100 m3, an annual revenue of $184000 would be obtained, with an annual profit of $81000. The study noted that local costs in Hong Kong, the place of the study, was lower than those predicted by their software model, which was based on prices in the USA. This shows that the profitability of biohydrogen also varies with the region, and the cost of equipment there will determine its overall cost. Nevertheless, the study proves that, if done at the right scale and using the right methods, biohydrogen can be profitable. However, there are challenges associated when scaling up the process. Firstly, the overall efficiency of the process decreases the efficiency, and there is a loss of energy (Bhagchandanii et al., 2020)[48]. Additionally, capital costs are much higher, and there is difficulty in maintaining and controlling various process parameters (Li et al., 2012; Mota et al., 2017)[31,47].

There are many strategies that exist today that seek to maximise the yield of biohydrogen and reduce the overall cost. Some of them include integrating dark fermentation and electrofermentation. In this strategy, the volatile fatty acids produced by dark fermentation are used in a microbial fuel cell, in which organisms consume organic matter and transport the electrons produced in the reaction to the electrodes, producing electricity. Del Campo et al. (2012)[49] investigated the production of biohydrogen and electricity in a fuel cell. They achieved a yield of 1.4 mol H_2 per mole hexose along with a power generation of along with 550 mW/m2 power density that was produced from the MFC. The process results in lower production cost and less waste generated, but the process will face challenges when scaled up, as it has a low conversion efficiency, low productivity and energy wastage (Bhagchandanii et al., 2020)[48].

Another strategy that is showing promise is the use of nano-catalysts. Adding these nano-catalysts into fermentation media, which transfers photogenerated electrons to the fermentative bacteria, which increases the breakdown rate of the substrate, and enhances the production of hydrogen (Ramprakash and Incharoensakdi, 2020)[50]. Zhang and Shen (2007)[51] investigated the effect of gold nanoparticles on the production of biohydrogen from artificial wastewater. They achieved a maximum yield of 4.48 mol H_2 per mol sucrose, which represents the conversion efficiency of sucrose to hydrogen reached 56%. The addition of the nano-catalysts increases the light absorbing efficiency of the process, and aids the transporting of electrons in the process. Using nano-catalysts, including ion-based catalysts such as ferrous oxide, can also improve the cost-effectiveness of the biohydrogen production process, making it more suitable for commercial use. However, the usage of certain nano-catalysts could make the process environmentally unfriendly, and would necessitate restrictive operational parameters (Wu et al., 2023).[52]

Genetic engineering is also a well-known technique to increase the biohydrogen yield. By improving the expression of hydrogenases, knocking out competitive pathways and activating new pathways to increase the breakdown rate of the substrate to hydrogen, the production of biohydrogen can be increased (Ramprakash et al., 2022)[13]. E-coli is an ideal candidate for applying this technique, given its short doubling time, well-known metabolism and ease of genetic manipulation. Nyberg et al. (2015)[53] investigated biohydrogen production in a photobioreactor using genetically engineered cyanobacteria, and reported that the hydrogen yields were noticeably higher than those reported from the unmodified cyanobacteria. However, before achieving sustainable large-scale use, a more efficient growth and production system for the microorganisms should be developed, along with further improving the strain of the microorganism for commercial use.

To summarize, wastewater sourced biohydrogen may suffer many limitations, such as competition from methanogens, low efficiency resulting in high production costs and inhibition of the enzymes involved by oxygen. However, with pre-treating the substrate and incorporation of various strategies that include nano-catalysts, microbial fuel cells and enzyme engineering, biohydrogen appears to be a promising alternative to fossil fuel sourced hydrogen.

Biohydrogen Production from Wastewater in India

India, the 3rd largest GHG emitter in the world and with the world's highest population, is one of the world's major wastewater generators. India's energy sector emitted 2.5 billion tonnes of CO_2 in 2021, and its urban centres generates 26.41 km3 of sewage every day, of which only 28% is treated (Ritchie et al, 2020; Minhas et al., 2022; IEA,2021)[1,6,54]. India is also a major importer of coal as a primary energy source, importing 200 million tonnes of coal in 2020 (Oskarsson et al., 2021)[55]. Coal is a highly polluting energy source, and is non-renewable. Therefore, there is a need to find an energy source in India that has the potential to reduce CO_2 emissions and wastewater contamination, along with meeting India's energy demand.

India is estimated to use around 5 million tonnes of hydrogen annually for various purposes such as petroleum refining, fertilisers and metal refining (MNRE, 2023)[56]. However, most of this hydrogen is sourced from steam reforming of natural gas, called grey hydrogen., which is highly polluting as it releases greenhouse gases. India's hydrogen market is growing, and Morgan Stanley predicts that it will reach $19 billion in 2030, with the main drivers being green and blue hydrogen (Bhutra, 2023)[57]. The main industries expected to drive this are the fertilising, refining and steel industries, which will account for 88% of hydrogen sales. Reliance Industries Limited (RIL) recently announced plans to start green hydrogen production in 2025, with claims that the cost of said green hydrogen will reduce from the current figure of $8 per kg to $1 per kg by 2030 (The Times of India., 2023)[56].

India is water scarce, with 600 million people in India facing extreme water stress, and therefore producing biohydrogen from wastewater is important (Nath and Parmar, 2022)[58]. Biohydrogen produced from wastewater holds a lot of promise in a country like India. It has the potential of integrating wastewater treatment and energy generation. However, the scope of biohydrogen in India is very limited as most of the research about biohydrogen is still in the developmental stages and mostly confined to small scale trials in the laboratory (Pathak et al., 2016)[59]. If biohydrogen is to be a major source of energy in India, development of new strategies to produce biohydrogen, optimising bioreactor design and selecting the optimal substrate will be key.

The Ministry of New and Renewable Energy (MNRE) put forward a National Green Hydrogen Mission in 2022, with the aim of making India a leading supplier and producer of green hydrogen in the world, along with meeting its goal of being net-zero by 2070. According to the mission, India should have a capacity of producing 5 million metric tonnes per annum (MMTPA) of green hydrogen (which includes biomass-based hydrogen, electrolysis of water and gas purification), and have an additional renewable energy capacity of 125 GW by 2030, along with restricting carbon emissions to 2 kg CO_2 for every kg of hydrogen produced (Rai, 2023)[60]. This will be done in a 2-phase approach which will be focussed on creating demand, initiatives for indigenisation and penetration of sectors. However, to meet its net zero goal, India must accelerate its green hydrogen production to 7 MMTPA in 2040 and 114 MMTPA in 2070. Currently, India produces only 6 MMTPA of hydrogen, with grey hydrogen being its main driver and green hydrogen making up a negligible part of this whole. If this target is to be met, India will need to make sure renewable power (especially solar power) is available, increasing demand for green hydrogen in industries such as steel, fertiliser and cement, developing infrastructure in the form of pipelines, transporting network, liquefaction plants and compressors, furthering research and development in the field and providing subsidies for green hydrogen over its fossil-fuel sourced counterpart. With this in place, Green Hydrogen has the potential to become one of India's main energy suppliers (Kaushal, 2022)[61]. The MNRE supports various research projects on producing biohydrogen, and storage of hydrogen gas as metal hydrides (Pathak et al., 2016)[59].

The MNRE also supports various pilot scale projects that are being undertaken in India to produce biohydrogen (Pathak et al., 2016)[59]. For example, a group of researchers at the Indian Institute of Technology in Kharagpur performed a pilot scale experiment to produce biohydrogen from various wastewater substrates, reporting yields of up to 1.5 moles of H_2 per mole hexose (Das, 2009)[62]. They later installed a biohydrogen plant with a capacity of 10 m3 using substrates such as distillery wastewater with the support of MNRE (Pandey, 2016)[63]. These initiatives are meant to accelerate research and development of biohydrogen. Furthermore, the project seeks to optimise the physio-chemical parameters for the highest possible hydrogen yield, build two additional 10 m3 plants in New Delhi and Ahmedabad with generation capacities of 30,000 to 50,000 litres every day and build a 1000 m3 bioreactor for commercial application (Das, n.d.)[64]. Researchers also developed a large-scale hydrogen bioreactor of 12.5 m3 capacity using organic wastewater effluents in Chennai (Pathak et al., 2016).[59]

Many pilot scale projects have been done in India with the aim of achieving the highest yield at the lowest cost. They serve as important indicators of how successful biohydrogen will be. For example, Vatsala et al. (2008)[38] investigated the hydrogen production from cane distillery wastewater using defined bacterial co-cultures at a scale of 100 m3, and achieved a yield of 2.76 mol per mole glucose and a production rate of 0.53 kg/100 m3/h. Also, India was set to get its first biomass-based hydrogen production plant in Khandwa district of Madhya Pradesh (Ramesh, 2022)[65]. It was to be run as a joint venture between Watomo Energies and Biezel green and it claimed to produce 1 tonne of biohydrogen from 30 tonnes of biomass. It was scheduled to open in July 2022.

The scope of producing biohydrogen in India is very high, but it faces challenges (Pathak et al., 2016)[59]. Firstly, Biohydrogen production in India follows a technology-push marketing model, that assumes that there will be demand for the biohydrogen. This model is flawed, as there is no communication between the end-users and producers, causing a greater likelihood that the technology will fail in India. Also, the production of biohydrogen does not involve the local community, who could be major users of this technology. Thirdly, biohydrogen loses favour to conventional fuels, as the policy frameworks gives subsidies to the conventional fuels, making them cheaper than biohydrogen. The technology faces a lack of financial aid from the government. Finally, the biohydrogen will most likely be transported by tankers rather than pipelines in India. Although pipelines require less maintenance and have very low energy consumption, their progress is limited in India due to problems in land acquisition (Reuters, 2019)[66]. Therefore, most of the hydrogen fuel would most likely be transported in tankers, which would limit the overall efficiency of the biohydrogen. This problem can be tackled, however, by storing the hydrogen in the form of metal hydrides (which will be discussed later), the transportational problems can be mitigated.

The National Green Hydrogen Mission, however, can address a few of the challenges mentioned, such as the limited efficiency, high production cost, and policy constraints and lack of demand for the biohydrogen in India. Firstly, the mission has planned for an outlay of 19744 crore INR ($2.38 billion), with over 1644 crore INR ($177 million) to support pilot scale projects and 400 crore INR ($48.3 million) for R&D (MNRE, 2023)[2]. The Mission also includes plans for public awareness, stakeholder outreach, and collaboration with the private sector. The collaboration between the MNRE, involved government departments and private sector industrialists will involve sharing of resources, a dedicated research fund and the development of a R&D program. The collaboration is of great importance, as it enables sharing of expertise between the concerned private sector industrialists and the government departments, speeds up the R&D process, and improves the efficiency and quality of the designed reactors (Rahman et al., 2014)[67]. In addition, the Mission facilitates advances in improving the process's efficiency, reducing biohydrogen's costs and improving awareness of the technology to the public.

For the better understanding of the region, I interviewed two major wastewater generators and one major hydrogen user. The questionnaire included details of the quantity and content of the wastewater generated by them, along with insights about biohydrogen production in the region along steps towards improvement and commercial usage (supporting information).

The first wastewater generator was a sugar industry plant in Pugalur, a municipality in the state of Tamil Nadu, India. The plant generates an average of 4,00,000 litres every day, along with 50,000 litres of sewage every day. The wastewater that is generated in the industry is treated in an effluent treatment plant using an activated sludge process, and the treated wastewater is used in process and equipment cooling purposes. Although not currently using hydrogen fuel, the generator was also willing to assess the technology and the suitability of the effluent (Sugar industry wastewater), and based on that start a pilot trial and, later, a commercial plant. The generator was also willing to support labs and startups working to produce biohydrogen sustainably. Sugar industry wastewater is a highly promising wastewater substrate, and high yields and production rates have been observed when using the same (Refer to table 2 for more information). The generator explained that the cost and environmental impact of biohydrogen would be the main drivers of the technology's success. His views are not entirely correct, however, as there will be many more factors that determine biohydrogen's success, including its ease of transport and storage, support from the government and efficiency of production (Pathak et al., 2016).[59]

It was highlighted that the choice of substrate would play a key role in the production of biohydrogen, where the substrate should be abundant and cost effective, as well as environmentally friendly. There are many wastewater substrates available in

India, and considering the large capacity of waste produced by Indian urban centres, there should be many readily available and at very low cost (Minhas et al., 2022)[54]. Additionally, it was noted that the major bottlenecks in the biohydrogen production process is the storage of said biohydrogen. Storing hydrogen is a major factor of biohydrogen production, and is directly correlated with cost (Ahmed et al., 2021)[43]. Hydrogen gas can be stored the form of metal hydrides, especially magnesium hydrides, seems to be a highly promising method to store hydrogen in large quantities at low cost. Magnesium hydrides are also possessing good heat resistance, reversibility and recyclability, aiding their suitability for storing biohydrogen (Sakintuna et al., 2007)[68]. Therefore, as it can potentially store large amounts of hydrogen at low costs, storing biohydrogen appears to be an easily solvable bottleneck.

For further insights, I conducted an interview with a director of a well-known power systems company. The director was also willing to support labs and startups trying to produce biohydrogen sustainably. It was noted that the transportation of the biohydrogen produced would be a major bottleneck to look into, considering most of the fuel would be transported be tankers rather than pipelines in India. A key assumption here is that the hydrogen fuel would be transported as either a compressed gas or liquid hydrogen. However, with the advent of metal hydride storage, the high costs and safety risks involved with transporting hydrogen as a compressed gas or liquid would be significantly reduced (Minhas et al., 2022)[54]. With adequate investment, metal hydride storage will make hydrogen storage cheaper and more convenient.

The director also argued that biohydrogen would not be successful, citing the low efficiency and yield of the biohydrogen, and therefore high costs to produce and sell. Studies have proven that using wastewater as a substrate for biohydrogen can be economically feasible (Li et al., 2012)[47]. Moreover, there are multiple strategies in place that seek to boost the efficiency of the process, such as sequential fermentation, nano-catalysts, genetic engineering and microbial fuel cells (Ramprakash et al., 2022)[13]. The National Green Hydrogen Mission supports various research efforts and pilot scale projects in India, with the aim of making biohydrogen an alternative to fossil fuels (Pathak et al., 2016)[59]. Finally, the technology of biohydrogen is still in its early stages, and it would be unfair to judge its success before it develops towards large-scale commercialisation. Biohydrogen is indeed a highly compelling technology, and it appears necessary to further spread awareness of this technology to major wastewater generators and potential users.

Lastly, I interviewed the director of a textile company as well as an investor in climate-tech startups, the second wastewater generator. She indicated that this process is more useful for companies that use hydrogen in their production process, or the storage and transport of hydrogen would make the process unviable. However, as mentioned earlier, metal hydride storage makes hydrogen storage cheaper and more convenient, therefore it does not appear unviable to store and transport hydrogen. However, the utility of the produced biohydrogen depends on the industry that would utilise it. Unless the factory utilises hydrogen gas, producing biohydrogen would be unprofitable, as the costs of running the plant (assumed to be 50 m3) would amount to $93 million per annum, and the cost of energy per GJ would be more that that of conventional electricity ($10 per GJ), making it a loss (Li et al., 2012; Seddon, 2022)[11,47]. Instead, a wiser strategy would be to sell the produced biohydrogen, and if strategies are implemented to reduce costs and improve the yield, the biohydrogen sold could be profitable.

Biohydrogen production in India seems highly promising, especially since it utilises wastewater and requires low energy inputs and can be performed in ambient pressure and temperature conditions. However, if it is to reach large scale utilization, the process needs to be scaled up to a sufficient size (¿100 m3), the amount of investment must be increased and various strategies must be incorporated to make this process profitable in India. Interviews conducted with major wastewater generators and the power systems company proved that biohydrogen is still not widely known in India, but has the scope of becoming a major energy source in India provided costs of production are low and the biohydrogen is used at the site of generation.

Conclusion and Outlook

The world's energy demand is increasing at alarming rates, and fossil fuels are the primary source for said energy. Hydrogen fuel is considered as an alternative fuel source, primarily as it is clean and has a higher energy density than fossil fuels. However, most of the hydrogen produced today is via highly polluting methods such as steam reforming of naphtha and natural gas. Biohydrogen can be considered as an eco-friendly and sustainable way of producing hydrogen as it can use organic waste products (including wastewater) as substrates and does not require large amounts of energy. This review summarizes the current methods of biohydrogen production, the scope of using wastewater as a substrate for biohydrogen, the limitations faced by biohydrogen from wastewater and its commercial viability and the current picture of biohydrogen in India, with a few ideas on the way forward. Wastewater is a highly promising as a substrate, primarily as it integrates wastewater treatment and energy generation and it has a high organic content. Exploitation of said wastewater results in favourable yields. For reaching sustainable commercial use, biohydrogen production must overcome its low efficiency and high production cost. The low efficiency of biohydrogen can primarily be attributed to inhibition of oxygen and build-up of inhibitory by-products, and further research must focus on developing oxygen-tolerant hydrogenase enzymes and new pathways to metabolize the by-products. Additionally, finding robust and high yielding strains of microorganisms, and optimising the reactor's design will help increase the process's efficiency. Breakthroughs in locating a suitable strain and engineering the hydrogenase enzyme will streamline biohydrogen's transition towards large scale commercial use. Currently, biohydrogen is more expensive to produce than fossil-fuel derived hydrogen. However, pilot scale projects have found that biohydrogen can be produced cheaply and profitably, by producing at a large scale using commonly-available, high yielding wastewater substrates. Additionally, optimising parameters in the reactor such as pH, temperature, and HRT, incentivizing biohydrogen as an environmentally friendly alternative fuel, breakthrough research, and competitive pricing will aid biohydrogen's rapid transition to large scale use. Wastewater generated biohydrogen holds considerable promise in India, primarily as it can use the large quantities of wastewater generated by its cities. Many pilot scale projects have been undertaken in India with the aim of maximising biohydrogen yield and achieving commercial viability. The MNRE in its National Green Hydrogen Mission mentioned that India would have a production capacity of 5 million tonnes of green hydrogen by 2030. With the right investment, implementation of initiatives based on improving the yield and cost and subsidies to reduce biohydrogen cost, wastewater-sourced biohydrogen could be a major part of India's energy demand.

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