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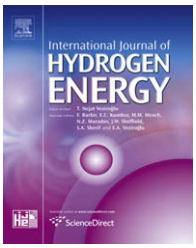

Metabolic pathway engineering for enhanced biohydrogen production

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ABSTRACT

Hydrogen is an ideal, clean, and potentially sustainable energy carrier for the future due to its abundance and non-polluting nature. Numerous bacteria, cyanobacteria, and algae are capable of producing hydrogen from water, solar energy, and a variety of organic substrates. Improvement of these diverse biochemical pathways is needed in order to make biohydrogen competitive with current production methods. This review summarizes some of the main biological pathways that produce hydrogen and their limiting factors. It also describes how metabolic engineering strategies are being used to overcome these limitations, increase yields, and broaden substrate utilization.

1. Introduction

In recent years there has been an increasing interest in alternative fuels due to rising petroleum costs, escalating diplomatic tensions with oil producing countries, and the rising levels of greenhouse gases in the atmosphere [1]. When used as a fuel, molecular hydrogen produces only pure water as a by-product, making it an attractive non-polluting energy carrier [2,3]. Hydrogen has the largest energy content per weight of any known fuel, and can be produced by various means [4,5]. Unfortunately, both of the main methods for hydrogen production, electrolysis of water and thermocatalytic reformation of hydrogen-rich compounds, usually require high-energy inputs obtained from non-renewable resources [5]. Biological production of hydrogen solves this problem by using microorganisms to convert biomass or solar energy into hydrogen gas [4,6].

There are several means of biohydrogen production that will be discussed in this review, including direct biophotolysis, indirect biophotolysis, photo-fermentation, and dark fermentation [5,7]. These diverse methods must be improved in order to make biohydrogen an economically viable alternative to other means of hydrogen production [4,5]. Metabolic engineering strategies provide a way to overcome the limitations that are part of the biochemical pathways used in hydrogen evolution. This review describes the main metabolic processes utilized by these different production methods and explains each of their limiting factors. Progress in the metabolic engineering of these pathways for enhanced production is summarized and substrate utilization prospects are discussed.

1.1. *Metabolic pathway engineering as a strategy*

The redirection of metabolic pathways for enhanced production of existing natural products, production of unnatural products, or degradation of unwanted molecules (e.g. environmental contaminants) is referred to as metabolic engineering. Metabolic engineering joins systematic and quantitative

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Table 1 – Nutrient manipulation and strategies for increasing substrate utilization in biohydrogen production.

Organism	Strategy	Reference
C. reinhardtii	HUP1, hexose uptake protein from *C. kessleri* was introduced to supply glucose.	Doebbe et al. [63]
A. variablilis	Supplement media with mannose. Examination of salinity and micronutrients on hydrogen production.	Shah et al. [65]
C. cellulolyticum	Express pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis* for greater cellulose-degrading capability.	Guedon et al. [98]
E. coli	Express invertase allowing for the utilization of sucrose.	Penfold et al. [117]
E. coli	Use added nutritional supplements to activate glycerol-fermenting pathway.	Murarka et al. [119]

analysis of pathways using molecular biology, modern analytical techniques, and genomic approaches. Since Bailey coined this definition for the emerging discipline of metabolic engineering, tremendous progress has been made in numerous fields [8–12]. Likewise, metabolic engineering could also hold the answer to biohydrogen production problems, by providing a way to eliminate bottle necks, increase carbon flow to the hydrogen-producing pathway, increase substrate utilization, and engineer more efficient and/or oxygen-resistant hydrogen-evolving enzymes (Tables 1 and 2) [13,14].

One of the most commonly used methods for performing metabolic engineering is the knockout of particular chromosomal genes [15–17]. Originally this was performed by creating mutations either by chemical methods or by ultraviolet radiation and then screening them [18]. This method however is very broad, results in many nonspecific mutations, and can be very time-consuming. Fortunately, with the advent of modern molecular microbiology and the availability of entire genome sequences, this method has been largely replaced by the directed disruption of chromosomal genes [17,19]. Furthermore, there has been tremendous progress in the development of computational tools and molecular biology techniques that are necessary for the effective implementation of metabolic engineering [11,20–23]. OptKnock, OptStrain, and OptReg are all computational frameworks that can be used to find the optimum modulation and deletion strategies for overproduction of fuels and chemicals [24–26].

Finally, studies on the effects of nutrient limitation and substrate utilization have revealed new regulatory mechanisms and alternative metabolic pathways in hydrogen-producing organisms [27,28]. A combination of systematic experimental design, gene knockouts, nutritional studies, heterologous expression, and artificial environments will be needed in order to achieve biohydrogen production levels that can compete with existing non-renewable production methods.

1.2. *Hydrogen-producing enzymes*

Each biohydrogen production method is dependent on the hydrogen-producing enzymes found inside the individual microorganism. These enzymes catalyze a very simple redox reaction: $2H^+ + 2e^- \leftrightarrow H_2$. In order to perform this reaction, most hydrogen-evolving enzymes have complex metallo-clusters as active sites and require special maturation proteins [4,29,30]. The three main hydrogen-evolving enzymes used by most biohydrogen evolving systems are nitrogenases, [NiFe]-hydrogenases, and [FeFe]-hydrogenases [31]. This review only briefly describes each enzyme, for a more detailed description of each of these enzymes and their maturation proteins please refer to reviews by Vignais and others [29–36].

Nitrogenases catalyze a reaction that fixes nitrogen into ammonia and is accompanied by the obligatory reduction of protons (H^+) to hydrogen [36]. The nitrogenase complex consists of two proteins encoded by three structural *nif* genes [35]. The first protein is the dinitrogenase or MoFe-protein, encoded by *nifD* and *nifK*, and the second protein is the dinitrogenase reductase or Fe-protein, encoded by *nifH* [35]. The reduction of nitrogen to ammonia with the accompanied production of hydrogen requires adenosine tri-phosphate (ATP) and proceeds at a slow turnover rate of about 6.4 s^{-1} [4].

In contrast, [NiFe]-hydrogenases work about 15 times better than nitrogenases at producing hydrogen and do not require ATP [4]. Not only can [NiFe]-hydrogenases produce hydrogen, but they can also work as uptake hydrogenases, utilizing the electrons from hydrogen and using them to reduce nicotinamide adenine dinucleotide phosphate

Table 2 – Strategies for overcoming the deleterious effects of oxygen on biohydrogen production.

Method	Description	Reference
Sparging with inert gas	Keeps oxygen and hydrogen partial pressures low but adds cost.	Seibert et al. [51]
Sulfur deprivation in green algae	Partially inhibits PSII, resulting in less oxygen formation.	Kruse et al. [42]
Artificial thylakoid membrane proton channel expressed in green algae	Avoids an increase in the proton gradient, which redirects electrons from hydrogenase to reduce oxygen.	Lee et al. [53]
Expression of oxygen-tolerant [NiFe]-hydrogenase genes in cyanobacteria	Allows for hydrogen evolution during photosynthesis.	Xu et al. [55]
Temporal separation of photosynthesis and photophosphorylation in green algae and cyanobacteria	Fixes CO_2 in aerobic photosynthesis stage, followed by hydrogen production in anaerobic reactor.	Prince et al. [41]
Spatial separation using heterocysts in cyanobacteria	Heterocysts protect hydrogen-evolving enzymes from oxygen being produced in vegetative cells.	Tamagnini et al. [36]

(NAD(P)) [37–39]. The catalytic core of the [NiFe]-hydrogenases is composed of a heterodimeric protein [31]. The large subunit contains the Ni–Fe active site and the small subunit usually contains Fe–S clusters which serve as electron transfer points to the electron acceptor or donor [32].

The most efficient hydrogen-producing enzymes are [FeFe]-hydrogenases, which can have an activity 1000 times better than nitrogenases and about 10–100 times better than [NiFe]-hydrogenases [30]. These hydrogenases consist of one protein containing an Fe–Fe catalytic core and can have a variety of electron donors and acceptors [34]. [FeFe]-hydrogenases can either produce or consume hydrogen depending on their environment. All three of these enzymes are generally sensitive to oxygen and must be either spatially or temporally separated from it in order to produce hydrogen at optimal rates [29,34,36].

2. Photobiological biohydrogen production methods

Clearly, solar energy is the most abundant renewable resource available with 178,000 TW hitting the Earth per year [40]. This widely available resource has prompted many studies into the photobiological production of hydrogen. However, many recent reviews have dismissed this method of production due to low solar energy to hydrogen conversion efficiencies and expensive and complicated photo-bioreactors [4–6]. It is generally agreed that a stable and cost-effective solar conversion efficiency of at least 10% needs to be achieved so that this form of hydrogen production can compete with photoelectrical systems [4,41–43]. Fortunately, several advances in direct photolysis, indirect photolysis, and photo-fermentation have shown that metabolic engineering can be used to increase conversion efficiencies and improve productivity [42,44,45].

2.1. Direct photobiological production

Direct photolysis, the most extensively studied method of biohydrogen production, is carried out using photosynthetic microalgae or cyanobacteria. This method utilizes the [FeFe]-hydrogenases and [NiFe]-hydrogenases found in these photosynthetic organisms to convert solar energy and water into hydrogen [46,47]. However, the oxygen produced by photosynthesis greatly interferes with the efficiency of the hydrogenases and leads to low yields [5].

Both green algae and cyanobacteria depend on photosynthesis as the starting point for all subsequent hydrogen production. The conversion of solar energy to hydrogen starts with antenna pigments such as chlorophylls, carotenoids and phycobilisomes [41]. These pigments are part of complex antenna systems composed of many peptides and have the ability to capture photons and pass them to two photochemical reaction centers embedded in the thylakoid or cyanobacterial membranes [41]. Both green algae and cyanobacteria have evolved large light-harvesting antenna complexes that give them a survival advantage during low-light conditions [48]. Unfortunately, those large antenna systems decrease in efficiency as light levels increase due to self-shading and due to the fact that most of the absorbed photons are dissipated as fluorescence and heat [36].

The photons that do get passed on to the embedded photochemical reaction centers are used to drive electrons across the membrane, creating a membrane potential that can be used to drive other reactions (Fig. 1) [41]. Photosystem II (PSII) uses the potential to oxidize water and transfer the released electrons to cytochrome b_6f complex using plastoquinone (PQ) [40]. This complex then reduces plastocyanin (PC), which then reduces Photosystem I (PSI). PSI can then reduce a ferredoxin or flavodoxin, which in turn reduces ($NADP^+$) and produces NADPH. The protons that are released during this electron transfer generate a transmembrane electrochemical gradient of protons that is used by an ATPase to generate ATP. The ATP and NADPH produced are then used by the Calvin cycle to fix CO_2 to carbohydrate, which is then used in respiration and growth [41].

2.2. Strategies to improve direct photolysis in green algae

The rate of electron transfer from PSII to PSI is about 10 times lower than the rate of photon capture by the antenna pigments [4]. This means that about 90% of the photons being captured by the antenna systems are not being utilized [4]. In the green algae, *Chlamydomonas reinhardtii*, this efficiency can be increased by truncating the chlorophyll (Chl) antenna size of PSII rather than PSI [49]. A list of genes that confer a truncated antenna size in this green algae has been generated by random mutagenesis and is currently available [49]. RNA interference or RNAi has also been recently used to down regulate the entire family of light harvesting complexes (LHC) in *C. reinhardtii* [44]. This interruption resulted in less tightly stacked grana, 81% higher photosynthetic quantum yield, reduced sensitivity to photoinhibition, and an overall increased photosynthetic efficiency under high-light levels [44]. A study examining the detailed radiation characteristics of *C. reinhardtii* CC125 and its truncated chlorophyll antenna transformants *tla1*, *tlaX* and *tla1*-CW^+ was recently reported [50].

In order for direct biophotolysis to occur, the partial pressure of oxygen must be low enough to allow for the activation of the enzymes involved in hydrogen metabolism [5]. One of these enzymes is the reversible [FeFe]-hydrogenase, HydA1, which can be found in green algae [42]. This hydrogenase, which differs from most [FeFe]-hydrogenases by its lack of accessory Fe–S clusters at the N-terminus, uses the electrons from the reduced ferredoxin produced by PSI and combines it with the protons (H^+) in the medium to produce hydrogen gas (Fig. 1) [5]. However, the oxygen produced by PSII and the hydrogen produced by the [FeFe]-hydrogenase inhibits the constant evolution of hydrogen [40]. In fact, one of the main obstacles to hydrogen production using photobiological systems is that hydrogenases are extremely sensitive to inhibition by oxygen. Therefore many studies have sought to increase the oxygen tolerance of these enzymes (Table 2) [51]. Random and directed mutagenesis has been used to produce less oxygen-sensitive hydrogenases in *Chlamydomonas* and has succeeded in improving tolerance by about 10-fold [52]. Constant sparging of an inert gas can also be done in order to keep both gases at low partial pressures [4]. Unfortunately,

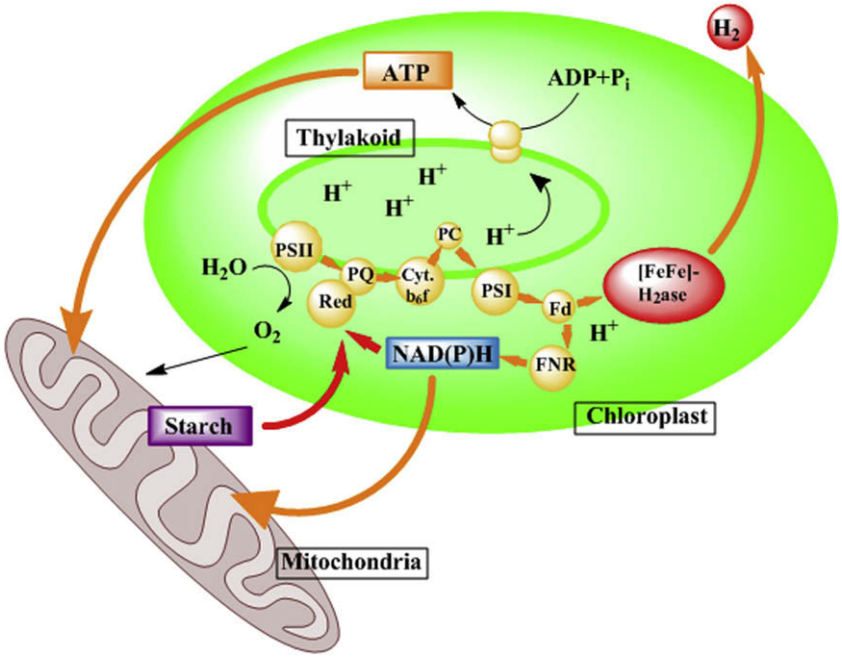

Fig. 1 – Photosynthesis and method of hydrogen production in green algae. Main electron transport during photosynthesis to the [FeFe]-hydrogenase is indicated by orange arrows. The red arrows show oxidation of reducing equivalents during respiration and Red indicates the NAD(P)H-plastoquinone oxidoreductase required for this reaction. Abbreviations: PS, photosystem; PQ, plastoquinone; Cyt. b_6f, cytochrome b_6f complex; PC, plastocyanin; Fd, ferredoxin; H_2ase, hydrogenase. Adapted from Happe 2002 [122].

sparging dilutes the gas stream and adds cost to the process. Therefore other more cost-effective methods must be found to reduce the deleterious effects of oxygen and hydrogen accumulation.

An alternative strategy for lowering the partial pressure of oxygen in green algae was implemented by Melis and colleagues in 2000 with *C. reinhardtii* [42]. This strategy uses sulfur deprivation to achieve low oxygen partial pressures and induce the hydrogen-evolving enzymes. Sulfur deprivation inhibits formation of the sulfur containing D1 polypeptide chain, which is the reaction center of PSII and results in the rapid decline of O_2 synthesis and CO_2 fixation [5]. Although impaired, the PSII system continues to function, producing the electrons necessary to evolve hydrogen and releasing a small amount of oxygen that is consumed by respiration [41].

Finally, a newly discovered oxygen sensitivity in green algae also exists that competes with hydrogenase-catalyzed hydrogen production by redirecting electrons to another transport pathway which reduces oxygen [53]. Lee has proposed that this oxygen sensitivity, which is a proton-gradient related problem, can be avoided by the genetic insertion of a hydrogenase promoter-programmed polypeptide proton channel into the algal thylakoid membranes [53]. This approach could also solve the additional problems associated with an increase in the proton-gradient, which include the restriction of hydrogen production, the competitive inhibition of hydrogen evolution by CO_2, and the requirement for bicarbonate binding at PSII for efficient photosynthesis [53,54].

2.3. *Strategies to improve direct photolysis in cyanobacteria*

Cyanobacteria can also perform direct biophotolysis, but do so primarily through nitrogenases and low activity bidirectional [Ni-Fe]-hydrogenases (Fig. 2) [33]. These enzymes do not produce hydrogen at the high rates of [FeFe]-hydrogenases. The nitrogenases in cyanobacteria are coupled to uptake [NiFe]-hydrogenases and are thought to provide a way for the cell to reutilize and regain the hydrogen formed by the nitrogenases [33]. The bidirectional hydrogenase, which is present in both nitrogen and non-nitrogen fixing strains, can evolve hydrogen under anaerobic conditions but only at low hydrogen partial pressures [36]. Therefore, utilizing this bidirectional hydrogenase also requires constant sparging of an inert gas. In order to combat this problem, The Venter Institute and NREL have recently expressed the bidirectional, oxygen-tolerant [NiFe]-hydrogenase genes, *hydS* and *hydL* from *Thiocapsa roseopersicina* in the cyanobacteria *Synechococcus* PCC7942 [55].

The bidirectional hydrogenase from cyanobacteria also doesn't require ATP to function and can suffer from a build up of ATP which then inhibits electron flow [36]. Fortunately, a variety of genetic tools exist to metabolically engineer cyanobacteria [21]. Improvement of cyanobacterial hydrogen production can be achieved by expressing a clostridial [FeFe]-hydrogenase in the cyanobacteria *Synechococcus elongatus* [56,57]. It must be noted, however, that the mechanism by which cyanobacteria are able to express an active [FeFe]-hydrogenase, without the co-expression of the appropriate

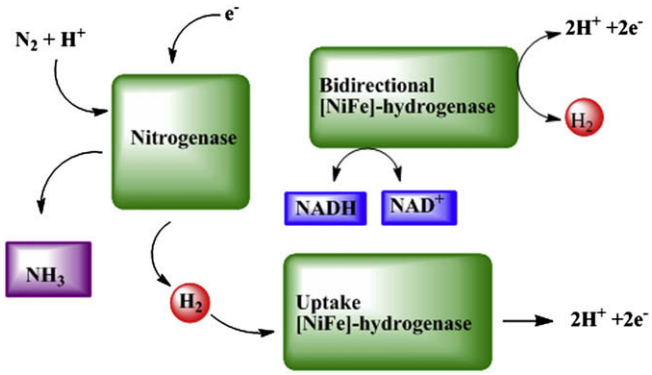

Fig. 2 – Methods of hydrogen production in cyanobacteria. The three main hydrogen-evolving enzymes of cyanobacteria are shown. Ammonia production by the nitrogenase results in hydrogen formation, some of which is consumed by the uptake hydrogenase. The bidirectional hydrogenase catalyzes the formation of hydrogen, but must be protected from oxygen inactivation. Adapted from Tamagnini 2002 [35].

maturation proteins, is still unknown [57,58]. The uptake of hydrogen by the [Ni-Fe]-hydrogenase in cyanobacteria can also be eliminated to increase hydrogen yield [59]. A recent review by Tamagnini highlighting the detailed regulation of hydrogenase expression and regulation in cyanobacteria can be referred to for further information [36].

2.4. *Indirect photobiological production*

Indirect biophotolysis gets around the problems of direct biophotolysis through the temporal or spatial separation of photosynthesis from hydrogen evolution [4,41]. There are many different ways that indirect biophotolysis is done, but in most strategies, the first step involves growing the photosynthetic organism in large quantities to obtain biomass rich in carbohydrates. The second step varies, but usually involves using that biomass in a hydrogen-producing fermentation [4,5,47]. Some green algae, such as *C. reinhardtii*, have the ability to use stored carbohydrate under anaerobic conditions when mitochondrial oxidative phosphorylation is largely inhibited [42]. This alternative metabolic process produces ATP for the cell by rerouting the energy stored in the carbohydrate to a chloroplast [FeFe]-hydrogenase, using an NAD(P)H-plastoquinone oxidoreductase to facilitate ATP production via photophosphorylation (Fig. 1) [40]. Reduced ferredoxin from PSI is used to deliver the electrons from the break down of the carbohydrate directly to the active site of the [FeFe]-hydrogenase [60]. All green algae [FeFe]-hydrogenases are located in the chloroplast and are thus inhibited by the oxygen generated there during photosynthesis [42]. Therefore, temporal separation of hydrogen evolution and photosynthesis must be achieved by first growing the algae in large ponds and then placing them in an anaerobic reactor [41]. The first step fixes CO_2, providing biomass and carbohydrate stores, and the second step produces hydrogen from those stores. However, this process is not as energetically efficient as direct biophotolysis [41].

Cyanobacteria can use either a temporal or spatial separation of photosynthesis from hydrogen evolution in order to perform indirect biophotolysis [33]. The same two-stage method used for green algae can be applied to cyanobacteria in order to achieve the temporal separation of photosynthesis and hydrogen evolution. Both the bidirectional [NiFe]-hydrogenases and nitrogenases can be used to produce hydrogen gas in this two-stage process [41]. However, the bidirectional [NiFe]-hydrogenases require low hydrogen partial pressures and large amounts of reducing equivalents to operate efficiently [36]. In contrast, the nitrogenases of cyanobacteria require ATP to function and can produce hydrogen even under high H_2 partial pressures [35].

In addition to temporal separation, cyanobacteria can achieve spatial separation of photosynthesis and hydrogen production by the use of specialized cells called heterocysts. The heterocysts have reduced O_2 pressure and can evolve hydrogen through the use of nitrogenases even while photosynthesis is occurring inside other vegetative cells [35,36]. However, heterocyst formation is only achieved by 5–10% of the vegetative cells [36]. Therefore, an increase in heterocyst formation could result in significantly more hydrogen formation [36].

2.5. *Strategies to improve indirect photolysis in green algae*

Increasing the availability of electrons and protons for hydrogen evolution during anaerobic conditions has been achieved in *C. reinhardtii* by random gene insertion [42]. The *Stm6* strain that was isolated in that study had a modified respiratory metabolism, which provided it with large starch reserves and low dissolved oxygen concentration, both of which contributed to hydrogen production rates 5–13 times that of the wildtype [42].

Nutrient limitation and optimization can also be used to drastically change the metabolism of green algae [61,62]. Antal and Lindblad were able to show that sulfur deprivation in *Synechocystis* under a methane atmosphere, results in a 4-fold increase in hydrogen production due to accumulated glycogen stores [62]. A recent study by Burrows and colleagues examined the effect of nutrient optimization on hydrogen production in *Synechocystis* sp. PCC 6803 and revealed a 44-fold increase in accumulated glycogen resulting in a 150-fold increase in hydrogen production [61].

Substrate utilization can also be broadened in order to increase hydrogen yields from microalgae (Table 1). Recently the HUP1 (hexose uptake protein) from *Chlorella kessleri* was introduced into *C. reinhardtii* in order to supply glucose for heterotrophic growth in the dark [63]. This external glucose supply (1 mM) was shown to increase the hydrogen production capacity to about 150% of the high-hydrogen-producing strain, *Stm6* [63].

A new metabolic engineering strategy that incorporates silica sol–gel as a way to encapsulate *Synechocystis* sp. PCC 6803 has shown that this method is capable of altering metabolism and has the potential to increase hydrogen production [45]. However, this technique is still being optimized and more work must be done to determine the effect of encapsulation on short-term hydrogen production [45]. This area of research is relatively new and deserves more study.

2.6. Strategies to improve indirect photolysis in cyanobacteria

Recent studies have shown that heterocyst frequency can be increased by over-expression of *hetR* or by inactivation of *patS* or *hetN*, but these studies do not show the effect on hydrogen production [36]. The incorporation of a heterologous [FeFe]-hydrogenase into the heterocysts of cyanobacteria could also potentially provide a way to increase hydrogen production in this organism [41,57]. More studies must be done in order to determine the maturation genes in cyanobacteria that are involved in expressing an active heterologous [FeFe]-hydrogenase. The effect that growth media has on the heterocystous cyanobacteria *Anabaena variabilis* ATCC 29413 was explored, and it was found that hydrogen production could be increased 5.5-fold with proper nutritional requirements (Table 1) [64]. This study particularly highlighted the importance of systematically exploring nutritional requirements in conjunction with production designs [64]. In addition, supplementation of media with mannose has been shown to increase hydrogen production in *A. variabilis* SPU 003 by about 78% (Table 1) [65]. This same study also showed the importance of salinity and micronutrients on hydrogen production by this strain [65].

2.7. Photo-fermentation

One of the other main sunlight-dependant hydrogen production methods is called photo-fermentation [5]. This fermentation is carried out by nonoxygenic photosynthetic bacteria that use sunlight and biomass to produce hydrogen [47]. Purple non-sulfur (PNS) and green sulfur (GS) bacteria such as *Rhodobacter spheroids* and *Chlorobium vibrioforme*, respectively, are capable of producing hydrogen gas by using solar energy and reduced compounds [40]. Their photosynthetic systems differ from oxygenic photosynthesis due to their requirement for reduced substrates and their inability to oxidize water [40]. Nitrogenases are the main enzymes utilized by these bacteria and require nitrogen-deficient conditions in order to produce hydrogen [5]. The PNS bacteria are capable of using a range of organic acids, while the GS bacteria prefer H_2S, S, or thiosulfate as a substrate [40]. The substrate is oxidized using the tricarboxylic acid cycle (TCA) and the produced electrons are shuttled through an electron transport chain that uses NAD/NADH and ferredoxin before a nitrogenase combines the derived electrons with a proton to produce hydrogen gas and restore redox balance to the cell [40,66]. This production method has high conversion efficiencies from organic substrates but is still plagued by the reliance on ATP-consuming nitrogenases, expensive photo-bioreactors and inefficient light harvesting antenna [4,40].

2.8. Strategies to improve photo-fermentation in PNS bacteria

The PNS bacteria, *Rhodobacter capsulatus*, was recently improved for hydrogen production by eliminating poly-hydroxyalkanoate (PHA) synthesis and knocking out the uptake hydrogenase [67]. Accumulation of PHA storage compounds such as poly-3-hydroxybutyrate (PHB) competes for electrons used in hydrogen formation and the membrane-bound uptake hydrogenase lowers yields [67].

Another improvement strategy used in PNS bacteria involved the genetic modification of the electron transfer chains in *Rhodobacter capsulatus* [68]. This study showed that the elimination of the cyt *cbb3* oxidase, which serves as a redox signal to the RegB/RegA regulatory system, which in turn increases nitrogenase expression, increases hydrogen production by 2-fold [68]. The uptake hydrogenase of *R. capsulatus* was also eliminated in this study [68]. Recently Kim and colleagues reported that two PII-like proteins, GlnB and GlnK, were knocked out in order to interrupt the repression of nitrogenase by ammonium ions in *Rhodobacter sphaeroides* [69]. This study resulted in a strain of *R. sphaeroides* that could produce hydrogen with an ammonium ion concentration of 2 mM, although some repression of nitrogenase activity was still present [69]. This same study was able to clone the gene encoding the [FeFe]-hydrogenase, *hydA* from *Clostridium acetobutylicum* into *Rhodospirillum rubrum*, and also over-expressed the native *R. rubrum* [FeFe]-hydrogenase, *hydC* [69]. It was found that both [FeFe]-hydrogenases required pyruvate as an electron donor and that pyruvate formate lyase and formate hydrogen lyase are active during fermentative and photoheterotrophic growth [69].

3. Dark fermentation

The theoretical yields of hydrogen from dark fermentations depend largely on the type of anaerobic organisms that are used in the fermentation. Although glucose can theoretically provide 12 mol of hydrogen per mol glucose, there are no metabolic pathways existing in nature that would allow this, since cell growth would not be possible [70]. Facultative anaerobes evolve 2 mol of hydrogen from each mole of glucose consumed, whereas strict anaerobes evolve 4 mol [71]. However, these theoretical yields are based on known metabolism and can be increased by engineering the metabolic pathways that convert glucose into hydrogen [13,47,72,73].

Dark fermentation seems to hold the best promise for biohydrogen production due its low cost, a relatively high production efficiency, and stable hydrogen evolving enzymes [4,5,47]. [NiFe]-hydrogenases and [FeFe]-hydrogenases can be utilized in dark fermentations by using pure cultures or a mixture of anaerobic microorganisms [74]. Since no oxygen is produced or consumed in these reactions, both types of hydrogenases are less likely to be inactivated by oxygen [47]. Organic wastes from agriculture or sewage can be fed into large anaerobic bioreactors, achieving the dual goals of waste management and hydrogen production [75,76]. Dark fermentations also solve the problem of expensive photo-bioreactors which are necessary for direct biophotolysis and photo-fermentations [47]. The bioreactors required for these dark fermentations can be kept simple and reduce production cost by lowering the amount of maintenance and area required to operate [4,5]. However, several reviews have identified obstacles such as hydrogen consumption by uptake hydrogenases, substrate utilization, and overall low production yields due to inefficient metabolic pathways that limit the

viable macroscale production of hydrogen from dark fermentation [4,6,46].

3.1. *Strict anaerobe dark fermentation*

Strict anaerobes utilize dark fermentations to break down glucose into pyruvate and NADH (Fig. 1) [4]. Pyruvate is then converted to acetyl-CoA and CO_2 by way of pyruvate ferredoxin oxidoreductase [4]. The acetyl-CoA is then converted to acetyl phosphate and results in the formation of ATP and the excretion of acetate [77] The oxidation of pyruvate into acetyl-CoA requires the reduction of a ferredoxin (Fd) by pyruvate-ferredoxin oxidoreductase, which is then oxidized by a hydrogenase that regenerates oxidized Fd and hydrogen gas [77]. Additional hydrogen can be produced from the NADH that is generated during glycolysis [32]. The NADH is oxidized by Fd reduction and an NADH-ferredoxin reductase, but only at very low partial pressures of hydrogen (<60 Pa) [32,78]. The highest theoretical yields of hydrogen, 4 mol hydrogen per mole of glucose, are produced when acetate is the fermentation end-product [5]. Butyrate formation yields 2 mol of hydrogen per mole of glucose and even lower yields are associated with propionate and reduced end-products such as alcohols and lactic acid [5,32].

The obligate anaerobe, *C. acetobutylicum*, has long been of great interest due to its ability to ferment various substrates into valuable end-products such as acetone, butanol, and ethanol (Fig. 3) [79]. Fermentation by this organism has two distinct phases [80]. The first phase is characterized by rapid growth and high hydrogen production along with acetic and butyric acid production [80]. The second phase is characterized by slower growth, low hydrogen production, and solvent production [80]. These phases are known as the acidogenic and solventogenic phases, respectively [79]. Determination of whether clostridium utilizes a solvent or acidic producing pathway is dependant on ATP and NADH levels [80].

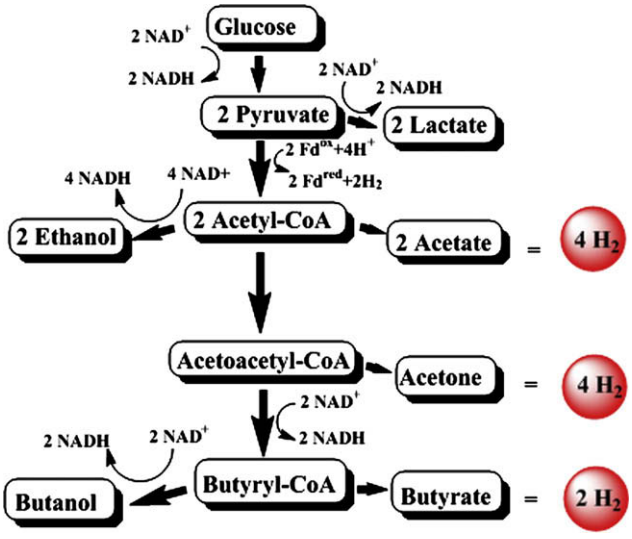

Fig. 3 – Anaerobic fermentation in clostridium. The simplified metabolic pathways for the break down of glucose and production of various metabolites in *C. acetobutylicum* are shown. Each arrow indicates one or several reactions in series along the pathways. The amount of hydrogen produced by the [FeFe]-hydrogenase during the formation of each metabolite is also shown. Abbreviations: Fd, ferredoxin; ox, oxidized; red, reduced. Adapted from Chin 2003 [123].

Recently, *C. acetobutylicum* has been completely sequenced, and both ann [FeFe]-hydrogenase and a [NiFe]-hydrogenase have been found in its genome [81]. However, the [FeFe]-hydrogenase is 10-fold more active, suggesting that it is the major producer of hydrogen gas [58,82]. The mechanism of the [FeFe]-hydrogenase is closely linked to Fd and the enzyme NADH-ferredoxin reductase [82]. During acidogenic metabolism, reduced Fd is oxidized by the [FeFe]-hydrogenase to produce hydrogen gas, while the resulting oxidized Fd is recycled back to the reduced form by an NADH-ferredoxin reductase that oxidizes NADH into NAD^+ in the process [80,83]. This ferredoxin-redox system is one of the ways that *C. acetobutylicum* is able to regenerate the NAD^+ needed to drive glycolysis [80].

The *hydA* gene from *C. acetobutylicum* encodes for the [FeFe]-hydrogenase [84]. However, this enzyme needs accessory proteins in order for an active hydrogenase to be expressed. The *hydE*, *hydF*, and *hydG* genes from *C. acetobutylicum* encode for the necessary accessory proteins and their heterologous expression along with *hydA* have been shown to produce an active [FeFe]-hydrogenase in *E. coli* [58,85]. Although active, the expressed [FeFe]-hydrogenase in *E. coli* still requires its native ferredoxin counterpart for *in vivo* activity [85]. There may be two enzymes with two modes of regulation that serve as an NADH-ferredoxin reductase and a ferredoxin-NAD reductase but neither has been identified in the *C. acetobutylicum* annotated sequence [81,83]. Recently, the native Fd counterpart for HydA was identified as CAC0303 and was cloned and expressed in *E. coli* [86].

C. acetobutylicum has also been shown to be the fastest reported microorganism for hydrogen production from hexose, with a rate of 2.4 $LH_2\,L^{-1}\,h^{-1}$ [82,87]. However, a recent study showed that *C. beijerinckii* L9 attained the highest hydrogen yield from glucose (2.81 mol/mol-glucose) when compared to *C. acetobutylicum* [88].

3.1.1. *Strategies to improve hydrogen production in Clostridium*

C. acetobutylicum is an ideal candidate for metabolic engineering due to the availability of genetic tools for gene knockout and gene over-expression [89–91]. Many studies have been done on *C. acetobutylicum* to determine the conditions that need to be met for solventogenesis to occur and therefore much is known about its metabolism [80,83,92]. Antisense RNA has been used to downregulate the gene expression in *Clostridium* and provides another method that can be used to increase hydrogen production in this organism [93,94]. One of the main problems encountered with dark fermentation is the inhibition of hydrogen production by the accumulation of fermentation end-products [95]. The production and accumulation of acetic and butyric acids resulted in lower hydrogen yields and a total undissociated acid concentration of 19 mM initiated solventogenesis [95]. Different strains of *Clostridium* produce different ratios of end-products thus affecting their hydrogen-producing potential

[87]. The elimination of butyric acid formation and the increased production of acetic acid would provide for increased hydrogen yield from glucose [87]. Although acetate production would increase hydrogen yield to 4 mol of hydrogen per mole of glucose, this is still not enough for the process to be an economically viable alternative to existing hydrogen production methods [4]. In order to increase yields to 8–9 mol of hydrogen per mole glucose, the acetate that is produced as a by-product could be converted to hydrogen using a newly developed microbial fuel cell-based process which only has an energy cost equivalent to 1.2 mol of hydrogen per mole glucose.[96]. Recently, increased hydrogen production in *C. paraputrificum* M-21 has been achieved by over-expression of the *hydA* gene encoding the [FeFe]-hydrogenase [97]. This 1.7-fold increase in hydrogen production over wildtype resulted in the increase of acetic acid production due to over-oxidation of NADH [97].

Some *Clostridium* species are capable of breaking down cellulose into hydrogen, thus making them extremely attractive for biohydrogen production (Table 1) [87]. Recently, Ren and associates showed that *C. cellulolyticum* and *C. populeti* produce the highest hydrogen yields from cellulose [87]. However, this same study found that *C. acetobutylicum* achieved the highest hydrogen yield (2.3 mol/mol hexose) from cellobiose [87]. It was speculated that the cellulose-degrading pathway from *C. populeti* or *C. cellulolyticum* could be expressed in *C. acetobutylicum*, yielding a strain capable of high hydrogen production from cellulose [87]. The cellulose-degrading capabilities of *C. cellulolyticum* were recently improved by the heterologous expression of pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis* [98]. This metabolic engineering approach lowered the excess pyruvate generated from cellulose break down and effectively reduced the toxic effect of pyruvate build up [98].

3.2. *Facultative anaerobe dark fermentation*

E. coli is an excellent example of a facultative anaerobe, which means that it is able to grow in anaerobic and aerobic conditions. When growing in aerobic conditions, pyruvate generated from the break down of sugars is mainly used by pyruvate dehydrogenase (PDH) and then further converted through the TCA cycle or into acetate [99]. In contrast, most pyruvate generated during anaerobic conditions is converted by pyruvate formate lyase (PFL) and produces formate and acetyl coenzyme A (AcCoA) (Fig. 4). The AcCoA is further broken down into acetate and ethanol and a portion of the pyruvate is converted into lactate depending on conditions. In addition, the cell produces succinate. This anaerobic production of organic acids is referred to as mixed acid fermentation [100].

Absence of exogenous electron acceptors, such as oxygen and nitrate, requires an alternative way to regenerate NAD^+ in *E. coli* cells. In anaerobic conditions, the excreted products from the fermentation are in reduced form and help cells maintain a constant supply of NAD^+ for glycolysis [101]. The formation of lactate, ethanol, and succinate generates NAD^+, while the production of acetate yields ATP from substrate phosphorylation. The resulting ATP is essential because an incomplete TCA cycle under anaerobic conditions does not generate ATP [99].

Formate is produced in anaerobic conditions in order to get rid of extra reducing equivalents that would have been lost through the reduction of NAD^+ under aerobic conditions [102]. In addition, formate can be further broken down into hydrogen gas and carbon dioxide during acidic conditions to maintain pH of the fermentation broth and to lower the concentration of formate in the cell [39,102].

E. coli utilizes two types of hydrogen metabolism [103]. The first involves respiratory hydrogen oxidation (uptake) that is combined with quinone reduction [103,104]. The second is hydrogen evolution during mixed acid fermentation [103]. Four hydrogenase isoenzymes have been identified in the *E. coli* genome [105]. Two hydrogenases (hydrogenase 1 and 2) are involved in periplasmic hydrogen uptake, while the others (hydrogenases 3 and 4) are part of cytoplasmically oriented formate hydrogenase complexes [31,102,103,105]. The uptake hydrogenases 1 and 2 are multi-subunit, membrane-bound, nickel-containing Fe/S proteins encoded by the *hya* and *hyb* operons, respectively [31]. Hydrogenase 3, located on the *hyc* operon, produces hydrogen from formate as a part of the formate hydrogen lyase complex (FHL-1), which is active during mixed-acid fermentation at slightly acidic pH. Hydrogenase 4, located on the *hyf* operon, has been shown to produce hydrogen as part of the FHL-2 complex at slightly alkaline pH [106,107]. However expression of hydrogenase 4 is not significant in the wild-type strain [105].

The FHL complex of *E. coli* is a multi-enzyme complex that catalyzes the reversible formation of equimolar amounts of hydrogen and CO_2 from the oxidation of formate as a response to the acidic conditions under anaerobic fermentations [73]. Formate dehydrogenase (FDH-H), which is encoded by *fdhF*, is the only protein in the FHL complex that is not encoded by the *hyc* operon. This gene is regulated by the presence of hydrogenase 3 (Hyd-3), and four polypeptides which are all encoded on the *hyc* operon [105]. Hydrogenase 3 (Hyd-3) is composed of a cytoplasmically oriented large subunit, encoded by *hycE* and a small subunit encoded by *hycG*. The remaining four polypeptides along with the product of *hycG* are membrane integral electron transfer components [102].

Transcription of the FHL complex is activated by the gene product of *fhlA*, called the FHL activator protein (FHLA) [105]. The FHL repressor protein (HycA) is encoded by *hycA*, which is found on the same operon as Hyd-3 [73]. This repressor binds to FHLA and stops FHL transcription [108].

3.2.1. *Metabolic engineering strategies for in E. coli*

The model organism and facultative anaerobe, *E. coli*, has undergone extensive engineering for increased hydrogen production due to its well known metabolic pathways, its relative flexibility for genetic manipulation, and the availability of an established genetic engineering tool kit [17,18,47,109].

Methods for introducing specific gene knockouts into *E. coli* are readily available, including a recent method that allows for gene disruption without leaving a scar [17,110]. The Keio collection contains in-frame, single-gene knockout mutants of *E. coli* that can be used to quickly create strains with multiple gene disruptions using P1 mediated transduction [111,112]. Likewise, the ASKA library contains a complete set of *E. coli* ORF clones and provides a valuable resource for metabolic engineering studies [113].

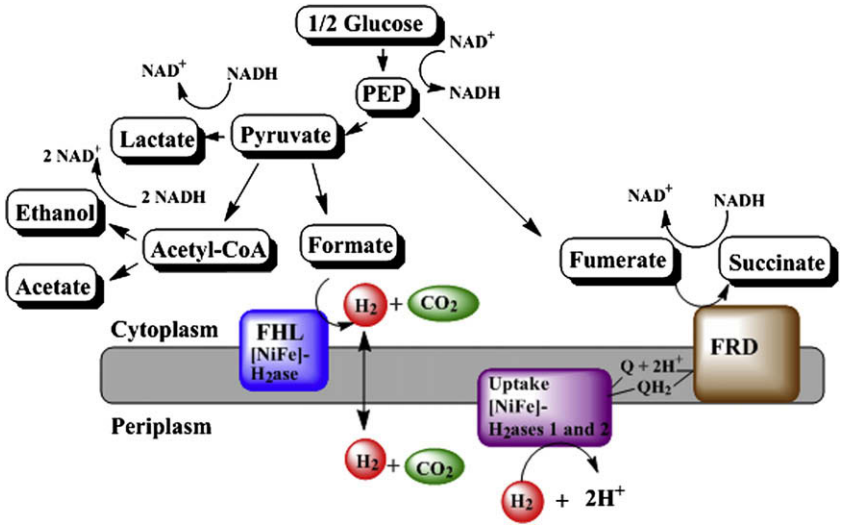

Fig. 4 – Mixed acid fermentation for biohydrogen production in *E. coli*. The metabolic pathways for the break down of glucose are shown with arrows indicting one or more reactions in series. The orientation of the hydrogen-evolving formate–hydrogen lyase (FHL) complex in the membrane is shown along with the uptake hydrogenases and fumarate reductase (FRD). Abbreviations: PEP, phosphoenol-pyruvate; Q, quinine pool; QH_2, quinol. Adapted from Murarka 2008 [119].

The engineering and regulation of *E. coli* hydrogenases has the potential to lead to an *E. coli* strain that is capable of producing large quantities of hydrogen [47]. This potential has led to multiple studies which have tried to optimize hydrogen production through metabolic engineering and has been reviewed recently by Vardar-Schara and colleagues [32]. Several ways of improving hydrogen production in *E. coli* have been described (Table 3). Some of these methods involve the disruption of lactate production, succinate production, or both. These studies show that hydrogen production is increased by directing carbon flow into making formate. The hydrogen yield was increased from 1.08 mol/mol glucose to 1.82 mol/mol glucose in the SR15 strain lacking lactate and succinate production [71]. Yoshida also showed that the elimination of lactate and succinate formation allowed for faster FHL induction by aerobically grown cells once they were added to an anaerobic system [114].

Another metabolic engineering target for greater hydrogen production is the regulation of the FHL complex. The over-expression of this complex can be achieved by either eliminating HycA, the repressor, or truncating the N-terminus of FhlA so that it is no longer dependant of formate to activate the *hyc* operon [115]. Yoshida et al. created an FHL over-expressing strain, SR13, that contained an *hycA* deletion and a plasmid over-expressing *fhlA* [116]. The combination of *hycA* inactivation with the over-expression of the FHL activator *fhlA* managed to achieve a 2.8-fold increase in hydrogen production yield compared to the wild-type when formate was used as a substrate [73]. Penfold and colleagues were also successful in creating an *hycA* null strain called HD701, which doubled the rate of hydrogen evolution over the wild-type strain (MC4100) at 100 mM glucose [73]. Lower concentrations of glucose gave up to a 14-fold increase in hydrogen production over wildtype [73]. Furthermore, in order to address the uptake hydrogenases, double deletions of hydrogenase 1 and hydrogenase 2 from *E. coli* have been reported along with the disruption of the twin arginine transport (Tat) protein system which is responsible for translocation of the uptake hydrogenases to their membrane sites [32]. In addition, substrate

Table 3 – Strategies for increased hydrogen production in *E. coli*.

Strain	Strategy	References
E. coli SR15 (*ΔldhA ΔfrdBC*)	Eliminate competing metabolites. Allows for faster FHL induction.	Yoshida et al. [114]
E. coli SR13 (*ΔhycA fhlA*$^+$)	Knockout repressor and over-express the inducer.	Yoshida et al. [116]
E. coli HD701 (*ΔhycA*)	Repressor knockout repressor and lower glucose concentrations to increase yields.	Penfold et al. [73]
E. coli BW25113 (*ΔhyaB ΔhybC ΔhycA ΔfdoG ΔldhA ΔfrdC ΔaceE*)	Eliminate uptake hydrogenases, competing metabolites, knock out repressor, over-express inducer, decrease competing formate consumption, and prevent formate export.	Maeda et al. [118]
E. coli BL-21	Express [FeFe]-hydrogenase from *E. cloacae*.	Chittibabu et al. [76]
E. coli TG1/pBS(Kan)Synhox	Express bidirectional [NiFe]-hydrogenase from *Synechocystis* sp. PCC 6803, which eliminates hydrogen uptake activity.	Maeda et al. [32]

utilization was also increased by transforming strains of *E. coli* enhanced for hydrogen production with the pUR400 plasmid encoding invertase activity, allowing the utilization of sucrose and resulting in a 3.2 μmol (mg protein)$^{-1}$ h^{-1} rate of hydrogen evolution (Table 2) [117].

Recently, Maeda and colleagues showed that extensive combinatorial strategies could increase hydrogen production significantly [32]. They eliminated competing pathways, deleted the uptake hydrogenases and *hycA*, over-expressed *fhlA*, decreased formate consumption by other metabolic pathways, and prevented formate exportation. This strategy resulted in a strain capable of a 4.6-fold higher hydrogen production rate from glucose than wildtype and doubled the hydrogen yield from glucose to 1.3 mol/mol glucose [32]. A recombinant strain of *E. coli* was also engineered that contained the [FeFe]-hydrogenase gene from *Enterobacter cloacae* IIT BT-08 [76]. The resulting strain had a hydrogen yield of 3.12 mol/mol glucose, which was much higher than either *E. cloacae* or *E. coli* wildtype [76]. This study in particular shows that much higher hydrogen yields can be obtained with recombinant organisms. Maeda and colleagues also recently engineered a recombinant *E. coli* strain expressing the bidirectional [NiFe]-hydrogenase from *Synechocystis* sp. PCC 6803 [118]. This strain was found to have a 41-fold increase in hydrogen yield over wildtype, which could be attributed to an inhibition of native hydrogen uptake hydrogenases [118].

Recent work by Dharmadi and Murarka shows that glycerol can be utilized in dark fermentations by *E. coli*, with hydrogen as a major product [28, 119]. It is interesting to note however that the ability of *E. coli* to ferment glycerol is dependant on the presence of added nutrient supplements [119]. The availability of glycerol as a waste by-product of biodiesel production and its highly reduced state make it an attractive substrate for future biohydrogen production from dark fermentations.

4. Conclusion

Tremendous progress is being made in the field of metabolic pathway engineering, in regard to increasing biohydrogen production in microorganisms. Studies utilizing strategies that alter native metabolisms and combine the heterologous expression of genes necessary to keep toxicity and redox levels stable need further exploration [32,66]. The speed at which new genomes are being sequenced guarantees that there will be a large library of diverse proteins to fulfill many metabolic engineering requirements. For example, Nagy and colleagues were recently able to apply gene shuffling strategies to express recombinant [FeFe]-hydrogenases from *C. acetobutylicum* and *Clostridium saccharobutylicum* in *E. coli* [120]. King has also used *E. coli* to generate recombinant [FeFe]-hydrogenases from both *Clostridium* and green algae [58]. The integration of these fast hydrogen-producing enzymes into non-native hosts could provide the way to increase hydrogen yields to a commercially competitive level.

Engineering various substrate utilization pathways into hydrogen-producing organisms could also increase the economic viability of biohydrogen production. Recently a review by Yazdani suggested that glycerol utilization could be a path for the economic viability of the biofuels industry [121].

Optimization of biohydrogen-producing methods should also focus heavily on nutrient levels, since it has been shown that nutritional factors can drastically alter metabolism and hydrogen production capabilities. More studies addressing the effects of nutrients and micronutrients on hydrogen production should be done in order to shed more light on the complex regulatory networks that exist in hydrogen-producing systems. Engineered microorganisms optimized to produce high-yields of hydrogen at competitive rates and able to utilize broader substrate ranges will surely increase the viable macroscale utilization and production of hydrogen from renewable resources.

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