Review Article

Developments in Bio-hydrogen Production from Algae: A Review

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Abstract: Diversification of biofuel sources has become an important energy issue. Bio-hydrogen production from microalgae has received much attention recently. However, commercial production of microalgae biofuels including bio-hydrogen is still not feasible due to the low biomass concentration and costly downstream processes. It has been reported that exposing some species of algae to environmental stress, e.g., by depriving the algae of sulfur in light, it is possible to produce significant amounts of hydrogen gas. However, this technology is still in its infancy and there is significant potential for technology development and improvement at every level. This review discusses the biological hydrogen production by microalgae (direct bio-photolysis, indirect bio-photolysis, photo fermentation and dark fermentation) and optimization of key parameters to enhance hydrogen production. The effects of different stress reactions on production of the valuable components are described. This knowledge can be used to evaluate the possibilities for producing hydrogen and high value products efficiently in the same process. Further studies of these topics may result in a sustainable process where solar energy can be converted into hydrogen in an integrated manner, where production efficiencies are sufficient for an economic exploitation of algal technology using algal stress reactions.

Keywords: Algal stress reactions, bio-hydrogen, hydrogenase, microalgae, microwave irradiation

INTRODUCTION

Our current energy consumption worldwide is in the proximity of 15 TW, while the energy consumption rate in 2050 has been estimated to be at least 27 TW (Lewis and Nocera, 2006). The majority of this energy is at the moment obtained from fossil fuels and any change requires improved technology for use of alternative energy sources. Fossil fuels are non-renewable energy source and also have seriously negative impacts on the environment. The use of fossil fuels cause excessive global climate change because emissions of greenhouse pollutants and the formation of compounds COx, NOx, SOx, CxHy, ash and other organic compounds that are released into the atmosphere as a result of combustion. The atmospheric concentration of carbon dioxide has been rising extensively since the Industrial Revolution and has now reached dangerous levels not seen in the last 3 million years (Le Quéré et al., 2012). Global warming is caused by the emission of greenhouse gases. 72% of the totally emitted greenhouse gases are Carbon Dioxide (CO₂), 18% Methane (CH₄) and 9% Nitrous Oxide (NOx). Carbon dioxide emissions therefore are the most important cause of global warming. CO₂ is inevitably created by burning fuels like fossil oil, natural gas, diesel and petrol. The increase in greenhouse gas emission will result in global warming, climate change, environmental degradation and health problems (Quadrelli and Peterson, 2007; Shaishav et al., 2013). The carbon dioxide is released to the atmosphere where it remains for 100 to 200 years. This leads to an increasing concentration of carbon dioxide in our atmosphere which in turn causes the average temperature on Earth to rise. Recent investigations have shown that inconceivable catastrophic changes in the environment will take place if the global temperatures increase by more than 2°C (3.6°F). A warming of 2°C (3.6°F) corresponds to a carbon dioxide (CO₂) concentration of about 450 ppm (parts per million) in the atmosphere (Momirlan and Veziroglu, 2005).

Reducing demand for energy intensive services, improving the efficiency of energy usage and development of renewable energy resources, must all combine to alleviate the crises of fossil fuel depletion, global warming and environmental degradation. It is important to develop an alternative energy sources that are clean, renewable and environmentally friendly for future world’s stability (Melis and Happe, 2001). There is no doubt that solar energy is the largest sources of
Table 1: Comparison of various hydrogen production processes—advantages and disadvantages (Karthic and Shiny, 2012; Das and Veziroglu, 2008)

<table>
<thead>
<tr>
<th>Process</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solar gasification</td>
<td>Good hydrogen yield</td>
<td>Effective solar collector plates are required.</td>
</tr>
<tr>
<td>Thermo-chemical gasification</td>
<td>Higher conversion can be achieved.</td>
<td>Gas conditioning and tar removal is to be done.</td>
</tr>
<tr>
<td>Pyrolysis</td>
<td>Gives carbonaceous material with bio-oil, chemicals and minerals</td>
<td>Catalyst deactivation will occur</td>
</tr>
<tr>
<td>Supercritical conversion</td>
<td>Sewage sludge can be used easily, difficult by gasification</td>
<td>Selection of supercritical medium</td>
</tr>
<tr>
<td>Direct bio-photolysis</td>
<td>Solar conversion energy increased by ten folds as compared to trees, crops.</td>
<td>Requires high intensity of light, low photochemical efficiency and O₂ is inhibitory</td>
</tr>
<tr>
<td>Photo-fermentation</td>
<td>A wide spectral energy can be used by photosynthetic bacteria.</td>
<td>O₂ is inhibitory on nitrogenase enzyme and light conversion efficiency is low.</td>
</tr>
<tr>
<td>Dark fermentation</td>
<td>It can produce H₂ without light.</td>
<td>Relatively lower H₂ yield. At higher H₂ yield, process becomes thermodynamically unfavorable.</td>
</tr>
<tr>
<td>Indirect bio-photolysis</td>
<td>Can produce relatively higher H₂ yield.</td>
<td>Product gas mixture contains CO₂ which has to be separated.</td>
</tr>
<tr>
<td></td>
<td>By-products (metabolites) can be efficiently converted to H₂.</td>
<td>Uptake hydrogenase enzymes are to be removed to stop degradation of H₂.</td>
</tr>
<tr>
<td></td>
<td>Has the ability to fix N₂ from atmosphere</td>
<td>30% O₂ present in gas mixture</td>
</tr>
</tbody>
</table>

renewable energy that we know of today. The different fields of technology for use of solar radiation include chemical/physical methods like photovoltaic, concentrating solar power, thermovoltaic, photochemical and thermochemical and use of biological approaches such as artificial photosynthesis and bio-photolysis (Rajeshwar et al., 2008). Practical use of solar energy requires conversion of the energy into an energy carrier and one of the promising candidates for alternative energy carriers is hydrogen. Hydrogen is seen by many as the fuel of the future because it has a very high energy density, three times that of petrol or diesel and because its use produces only water instead of greenhouse gases and other exhaust pollutants. Furthermore, using petrol and diesel in combustion engines waste at least two thirds of the energy in the fuel, whereas hydrogen can be used in fuel cells, which are about twice as efficient. Hydrogen is one of the most abundant elements in the world that accounts for 75% of the universe mass. It is a colorless, odorless, tasteless and a non-poison gas (Johnston et al., 2005). Currently, hydrogen is produced using non-renewable technologies such as steam reforming of natural gas (~50% of global H₂ supply), petroleum refining (~30%) or the gasification of coal (~20%). However, the viability of a future H₂ economy depends entirely upon the development of efficient, large-scale and sustainable H₂ production systems. The development of H₂ technologies has been given high priority in the European Union, the USA, Japan and China. This review intensely discusses the various approaches of photosynthetic hydrogen production from microorganisms particularly algae. It explores the potential for using various technologies for producing bio-hydrogen from solar energy using algae. Hydrogen produced through the action of living organisms is called bio-hydrogen.

Bio-hydrogen production: Hydrogen holds a promise as a potential clean, renewable and environmental friendly energy source. Currently 95 to 99% of hydrogen are produced from fossil fuel (Shaishav et al., 2013; Jo et al., 2006). The classical methods of producing hydrogen include steam reforming of natural gases, coal gasification and electrolysis of water (Jo et al., 2006). Conventional hydrogen gas production methods are energy intensive processes requiring high temperatures (>840°C) and not environmental friendly (Shaishav et al., 2013; Hsia and Chou, 2014). Electrolysis of water, although the cleanest technology for hydrogen gas production, can only be used in areas where electricity is cheap because electricity accounts for 80% of the operating cost of H₂ production (Karthic and Shiny, 2012). Recent reviews on hydrogen indicated that the worldwide need for hydrogen is increasing with a growth rate of nearly 12% per year for the time being and contribution of hydrogen to total energy market will be 8-10% by 2025 (Lemus and Duart, 2010). The advantages and disadvantages of various hydrogen production processes are outlined in Table 1 (Karthic and Shiny, 2012).

Bio-hydrogen is ideal as it can be operated at ambient temperature and pressure with minimal energy consumption and are more environmental friendly. Bio-hydrogen production methods can be broadly categorized into four primary groups (Fig. 1). Brief description of these processes is given in the later section.
Bio-hydrogen holds a promise as a potential clean, renewable and environmentally friendly energy source. There are three classes of biofuels: -First generation-made from food crops; Second generation-made from non-food crops or wastes; and Third generation-made using microbes. Third generation biofuels have several advantages over 1st and 2nd generation biofuels. Whereas first generation biofuels have caused increases in food prices, third generation biofuels would not. In comparison to second generation biofuels, third generation biofuels could capture sunlight energy 10 times more efficiently, meaning that smaller areas or land are needed to produce enough fuel (Shaishav et al., 2013; Momirlan and Veziroğlu, 2005). Many types of microbe can convert renewable energy sources into hydrogen. Bio-hydrogen is particularly attractive because of the excellent properties of hydrogen as a fuel and because bio-hydrogen is very easy to collect from the bioreactor (Rupprecht et al., 2006). Table 2 summarizes the different microorganisms that have been studied for bio-hydrogen production such as green algae, cyanobacteria (blue-green algae), photosynthetic bacteria and fermentative bacteria. Variety of organisms including the archaea, anaerobic and facultative aerobic bacteria, cyanobacteria and lower eukaryotes (i.e., green algae and protists) produce H₂ which may function singly or as a consortium of similar types or mixed cultures (Chandrasekhar et al., 2015).

The major biological processes for bio-hydrogen production are bio-photolysis of water by algae, dark fermentation, photo-fermentation of organic materials and the sequential dark and photo-fermentation processes (Das and Veziroğlu, 2001). Microorganisms are able to convert a diverse number of renewable resources into hydrogen (Levin et al., 2004). Microbial hydrogen production through the direct fermentation of organic wastes is one of the potential technologies for producing renewable hydrogen that couples the need for waste reduction and byproduct recovery, simultaneously (Show et al., 2012). The biological processes of hydrogen production are fundamentally dependent upon the presence of a hydrogen producing enzyme. These enzymes catalyze the chemical reaction $2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2$. Three enzymes carrying out this reaction are known; nitrogenase, Fe-hydrogenase and NiFe-hydrogenase (Hallenbeck and Benemann, 2002). Fe-hydrogenase enzyme is used in the bio-photolysis processes whereas photo-fermentation processes utilize nitrogenase. Among various hydrogen production processes, microbial/algal (biological) methods are known to be less energy intensive, for it can be carried...
out at ambient temperature and pressure. The type of light harvesting pigments, photosystems, source of reducing power and enzyme systems involved in various phototrophic hydrogen production by the organism’s are summarized in Table 3 (Dasgupta et al., 2010).

**Algae and microalgae:** Algae have received a great deal of attention as a novel biomass source for the generation of renewable energy. Algae are both unicellular and multi cellular autotrophic aquatic life forms. The unique feature of algae from the other entire microorganism is that they contain chlorophylls (chlorophyll a and b) which are usually found in higher plants. Chlorophyll is an important feature for photosynthesis which enables algae to absorb energy from light to fuel the manufacture of various biomasses. They are the most robust organism on earth as they are able to grow in a variety of habitats (Shaishav et al., 2013). Other components of algae are nucleus, cell wall, chloroplast containing accessory pigments, pyrenoid and adense region containing starch granules on its surface, stigma and flagella (Pelczar et al., 2008). Algae are generally divided into two groups, which are macroalgae and microalgae. Both groups of algae do not have roots, stems and leaves. Macroalgae, (or seaweeds) are photoautotrophic organisms that are able to produce and store organic carbons by utilizing CO$_2$ and HCO$_3$. Chloroxybacteria), eukaryotic microalgae (green algaeChlorophyta), red algae (Rhodophyta) and diatoms (Bacillariophyta) (Sambusiti et al., 2015). They are able to tolerate and adapt to a wide variety of environmental conditions (pH, temperature, light, etc.) and can be produced all year round (Uggetti et al., 2014). Moreover when cultured at optimal conditions, they are able to double in number within hours, thus permitting a short harvesting cycle (Razeghifard, 2013). Unlike macroalgae, microalgae are mainly composed of proteins (40-60%), carbohydrates (8-30%), lipids (5-60%) and other valuable components (pigments, antioxidants, fatty acids and vitamins) (Uggetti et al., 2014). Microalgae are the principal producers of oxygen in the world and exhibit enormous potential. Microalgae cultivation is an efficient option for the reduction of CO$_2$ from gaseous effluent and from the atmosphere (Chisti, 2007). The productivity per unit area of microalgae is high compared to conventional processes for the production of raw materials for biofuels and microalgae represent an important reserve of oil, carbohydrates, proteins and other cellular substances that can be technologically exploited (Chisti, 2007; Gressler et al., 2012).

The microalgae biomass can produce biodiesel, bioethanol, biogas, bio-hydrogen and bio-oils (Fig. 2). Microalgae, although having simple structure, have a high photosynthetic efficiency with a growth doubling time as short as 24 h. Moreover, microalgae can be produced all year round. The species abundance and biodiversity of microalgae over a broad spectrum of climates and geographic regions make seasonal and geographical restrictions much less of a concern compared with other lipid feedstocks. The limitations of H$_2$ production by microalgae are mainly the absence of large scale method, low yield and energy conversion efficiency and inhibition of hydrogenase by the oxygen, by-product of photolysis. Sulfur deprivation is a key to avoid hydrogenase inhibition by oxygen. Under this condition, oxygen evolution is declined below respiration level and an anaerobic atmosphere is formed and hydrogenase may be kept active (Zhu et al., 2014; Zhang et al., 2014). In depth and important research has been carried out in the field of bio-hydrogen production since the mechanism of hydrogen production by sulfur

<table>
<thead>
<tr>
<th>Micro-Organism</th>
<th>Light–Harvesting Pigments</th>
<th>Photo system</th>
<th>Source of reducing power</th>
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<tbody>
<tr>
<td>Green Algae</td>
<td>Chlorophyll a, b Carotenoids</td>
<td>PSI and PSII</td>
<td>H$_2$O and/or organic substrate</td>
</tr>
<tr>
<td>Blue-green algae</td>
<td>Chlorophyll a Carotenoids Phycobilisome</td>
<td>PSI and PSII</td>
<td>H$_2$S, S$^0$, S$_2$O$_3$$^-$$^2$</td>
</tr>
<tr>
<td>Purple sulfur bacteria</td>
<td>Bacterio-chlorophyll a/b Carotenoids</td>
<td>Single photosystem similar to PSI</td>
<td>Organic acids</td>
</tr>
<tr>
<td>Purple non-sulfur bacteria</td>
<td>Bacterio-chlorophyll a/b Carotenoids</td>
<td>Single photosystem similar to PSI</td>
<td>H$_2$S, S$^0$, S$_2$O$_3$$^-$$^2$</td>
</tr>
<tr>
<td>Green sulfur bacteria</td>
<td>Chlorosomes, that containBacterio-chlorophyll a and either Bacterio-chlorophyll c, d, or e.</td>
<td>Single photosystem similar to PSI</td>
<td>-</td>
</tr>
<tr>
<td>Green gliding bacteria</td>
<td>Chlorosomes with Bacterio-chlorophyll c/d + Bacterio-chlorophylla.</td>
<td>Single photosystem similar to PSI</td>
<td>-</td>
</tr>
</tbody>
</table>
starvation, was discovered (Ghirardi et al., 2006; Melis, 2007). The majority of this research has focused on the model organism *C. reinhardtii*, which is where the process was initially detected.

**Hydrogenases in algae:** Hydrogen production in green algae is catalyzed by FeFe hydrogenases, which are small, bidirectional enzymes with high activities and very high sensitivity against oxygen (Vignais, 2008). FeFe hydrogenases can be found in both bacteria and algae and parts of the enzyme are very similar between the two groups. While the so-called H-cluster part of the enzymes where the active site is located is very similar, the major difference is the presence of an F-cluster part of the enzyme in bacterial FeFe hydrogenases. This F-cluster is the electron donor to the active site, while in hydrogenases which do not contain the F-cluster, the active site receives electrons directly from ferredoxin. Most algal hydrogenases do not have an F-cluster.

Exposure to oxygen leads to a complete and irreversible inactivation of algal FeFe hydrogenase by destruction of the (4Fe-4S) domain of the active site H-cluster (Erbes et al., 1979; Stripp and Happe, 2009). This sensitivity against oxygen represents a challenge when the goal is to produce hydrogen from solar energy using the photosynthetic apparatus. Oxygen sensitivity of algal hydrogenases is an important topic which is being explored from many angles. The sulfur deprivation approach, which is a common method employed to enhance hydrogen production leads to anaerobic conditions in the culture by a partial inactivation of the oxygen producing PSII, thereby providing an environment for efficient hydrogen production. While promising for the production of clean and sustainable bio-hydrogen, these processes require improvement to be economically viable.

**Processes of bio-hydrogen production:** Table 4 gives a brief description of these processes. Although there are striking advantages, the low production rates, low substrate conversion efficiencies and accumulation of acid-rich intermediate metabolites from the acidogenic process are practical hindrances that must be overcome for the successful biological production of H₂. To overcome these limitations, many research projects on the biological production of H₂ are in progress and numerous novel approaches are being studied to address some of the existing problems and to overcome these problems by increasing the efficiency of the process.

**Strategies to enhance the bio-hydrogen production:** Molecular hydrogen has the potential to become the fuel of the future, but only if it is produced by a sustainable process. Hydrogen production from water photolysis under sunlight would be the cleanest energy conversion process; however, this process is hindered by low hydrogen productivity. New knowledge and technical innovations in hydrogen enzymes, electron carriers, biomaterials and nanotechnology may be able to overcome the intrinsic incompatibility of simultaneous hydrogen and oxygen evolution and splits water into separated gas streams. For a technically feasible hydrogen production with the help of algae, its efficiency must be increased by a factor of about 70 compared to the natural process.

Using green algae as a means of producing bio-hydrogen is a very good alternative as an attractive future energy carrier due to its conversion to energy yielding only pure water and it has the capability of eliminating all the problems that fossil fuels create (Show et al., 2012). However, the hydrogen yielded by biological processes is far too low compared to hydrogen produced by current chemical systems (Srirangan et al., 2011). Even though substantial progress is continuously being made, there are still many unknown aspects regarding hydrogen production mechanisms and how the efficiency can be improved. A fundamental understanding of this topic at every level is still needed in order to obtain a sustainable system in
Table 4: Summary of different processes of bio-hydrogen production

<table>
<thead>
<tr>
<th>Process</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct biophotolysis</td>
<td>Biophotolysis is the action of light on biological systems that result in dissociation of water into molecular hydrogen and oxygen; H₂O→H₂+½O₂.</td>
<td>Johnston et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>The solar energy is directly converted to hydrogen. 2H₂O+‘light energy’→2H₂+O₂.</td>
<td>Akkerman et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>Cyanobacteria or green microalgae, can use light to carry out photosynthesis (they possess chlorophyll a and the photosynthetic systems: PSI and PSII).</td>
<td>Ghiroardi et al. (2000, 2006)</td>
</tr>
<tr>
<td></td>
<td>The pigments in PSI (P680) absorb the photons with a wavelength shorter than 680 nm, generating a strong oxidant capable of splitting water into protons (H⁺), electrons (e⁻) and O₂.</td>
<td>Hallenbeck and Benemann (2002)</td>
</tr>
<tr>
<td></td>
<td>The electrons reduce the ferredoxin (Fd) and/or nicotinamide adenine dinucleotide phosphate (NADP⁺) into their reduced forms.</td>
<td>Azwar et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>Under special conditions, the reduced ferredoxin can also be used by hydrogenase or nitrogenase to reduce protons for evolution of molecular hydrogen (2H⁻→2Fd→H₂+2Fd).</td>
<td></td>
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<tr>
<td></td>
<td>Disadvantage: The enzyme hydrogenase is very sensitive to oxygen (O₂), hence when a certain amount of O₂ are present it will inhibit hydrogenase activity and stops it from producing hydrogen</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Advantage: Solar conversion in cyanobacteria or green microalgae is 10 fold more than compared with trees or crops.</td>
<td></td>
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<tr>
<td>Indirect biophotolysis</td>
<td>Indirect biophotolysis avoids the inhabitation of hydrogenase by separating the hydrogen production process from the oxygen production process into two stages.</td>
<td>Prince and Kheshgi (2005)</td>
</tr>
<tr>
<td></td>
<td>At first it involves the splitting of water molecules by sunlight to produce protons and oxygen and at the same time carbon dioxide fixation occurs to produce storage carbohydrate, followed by the production of hydrogen gas by hydrogenase: 12H₂O+6CO₂+‘light energy’→C₆H₁₂O₆+6O₂</td>
<td>Karthic and Shiny (2012)</td>
</tr>
<tr>
<td></td>
<td>C₆H₁₂O₆+12H₂O+‘light energy’→12H₂+6CO₂</td>
<td>Momirland and Veziroglu (2005)</td>
</tr>
<tr>
<td></td>
<td>Example: Blue-green algae (cynobacteria)</td>
<td>Mathews and Wang (2009)</td>
</tr>
<tr>
<td></td>
<td>Cyanobacteria that produces hydrogen can either be nitrogen fixing (ex: non-marine Anabaena sp) or non-nitrogen fixing organism (ex: Synechococcus)</td>
<td>Das and Veziroglu (2008)</td>
</tr>
<tr>
<td></td>
<td>Advantage: H₂ evolution is separated from O₂ evolution.</td>
<td>Lin and Jo (2003)</td>
</tr>
<tr>
<td></td>
<td>Disadvantage: Significant ATP requirement of nitrogenase</td>
<td>Das and Veziroglu (2008)</td>
</tr>
<tr>
<td>Dark fermentation</td>
<td>Involves the production of hydrogen in a dark environment without the presence of sunlight, water and oxygen.</td>
<td>Vardar-Schara et al. (2008)</td>
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<tr>
<td></td>
<td>Fermentative/hydrolytic microorganisms hydrolyzes complex organic polymers to monomers which are further converted to a mixture of lower molecular weight organic acids and alcohols by necessary H₂-producing acidothetic bacteria.</td>
<td>Nath and Das (2011)</td>
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<tr>
<td></td>
<td>Anaerobes utilizes glucose as substrate to produce pyruvate and NADH through glycolysis. Oxidation of NADH by Ferredoxin reduction and NADH-ferredoxin reductase are able to produce additional hydrogen. Pyruvate is then oxidized to acetyl-CoA which is then further converted to acetyl phosphate resulting in the production of ATP and excretion acetate from which hydrogen can be derived</td>
<td>Hallenbeck and Benemann (2002)</td>
</tr>
<tr>
<td></td>
<td>Advantages: Uses a variety of carbon sources. Can produce hydrogen without light. Produces valuable by-products eg. Butyric acid, lactic acid and acetic acid. There is no oxygen limitation problem.</td>
<td></td>
</tr>
<tr>
<td>Photo-fermentation</td>
<td>It is a fermentative conversion of organic substrates into hydrogen and carbon dioxide by using sunlight as the source of energy.</td>
<td>Manish and Banerjee (2008)</td>
</tr>
<tr>
<td></td>
<td>Under anaerobic conditions these bacteria are able to use simple organic acids as electrons donors which are transported to nitrogenase enzyme by ferredoxin using energy in the form of ATP. In the absence of nitrogen, nitrogenase enzyme reduces proton into hydrogen gas using extra energy in the form of ATP.</td>
<td>Akkerman et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>CH₃COOH+2H₂O+light→4H₂+2CO₂</td>
<td>Azwar et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>Using light as the energy source, the organic acid substrates is oxidized using the tricarboxylic acid cycle (TCA), producing electrons, protons and carbon dioxide. The produced electrons are then delivered to cytochrome c and are shuttled through a number of electron-transport-chain using NAD/NADH before being delivered to ferredoxin. At the same time protons are pumped through the membranes forming proton gradient, which then drives ATP production by ATP synthase. The ATP produced are used to drive the activity of nitrogenase enzyme to catalyze the production of hydrogen gas from protons.</td>
<td>Mathews and Wang (2009)</td>
</tr>
<tr>
<td></td>
<td>Example: Purple non sulfur bacteria (PNS)</td>
<td>Kim and Kim (2011)</td>
</tr>
<tr>
<td></td>
<td>Disadvantage: Need N₂ limit condition. Need pretreatment of industrial effluent as it may be toxic.</td>
<td></td>
</tr>
</tbody>
</table>

the future. Therefore optimization of the physiological assay and growth parameters are required in order to enhance the hydrogen production from biological processes. Table 5 gives an account of some of the parameters along with the details of each of the process involve in optimizing the hydrogen production. The bio-hydrogen market: Research and development of biological hydrogen production have expanded significantly in the past decade. The International Energy Agency has commented that bio-hydrogen provides a high market potential in the future (Maniatis, 2003). Although no commercial scale renewable bio-
Table 5: Details of various strategies to enhance hydrogen production from microalgae

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two stage photosynthesis: Separation of oxygen and hydrogen production process through Sulphur deprivation</td>
<td>• In direct bio-photolysis oxygen and hydrogen are co-evolved together. The hydrogenase enzyme is extremely sensitive to oxygen and a slightest amount of oxygen present will completely inhibit the activity of hydrogenase (Srirangan et al., 2011). To circumvent this problem a two stage bio-photolysis process was develop to allow the temporal separation of oxygenc photosynthesis and photo-biological hydrogen production (Melis et al., 2000). • The two-stage bio-photolysis is done by sulphur deprivation. In the first stage the algal cells are grown in sulphur rich medium leading to vigorous cell growth and high rate of photosynthesis. In the presence of sulphur, green algae is able to reduce sulphur to sulphide and incorporate it into cysteine which is the central intermediate form of most sulphur compound, hence sulphur plays a key role in the growth of the algae cells (Ghirardi et al., 2000; Jo et al., 2006). Once sufficient amount of growth is obtained the algal cells are then transferred to a medium deprived of sulphur. Upon sulphur deprivation oxygen production notably declined due to defective Photosystem II repair cycle. This is because the biosynthesis of D1(reaction centre protein) which is an essential protein in the Photosystem II reaction centre, was damaged due to the inability of chloroplast to synthesize pertinent amount of sulphurous amino acids, cysteine and methionine that needs to be frequently replaced (Srirangan et al., 2011; Kothari, 2013). This results in anaerobiosis and with illumination by light, hydrogenase enzyme is activated leading to active production of hydrogen gas for several days (Srirangan et al., 2011; Melis and Happe, 2001; Zhang et al., 2002; White and Melis, 2006; Ghirardi et al., 2000; Melis, 2002; Melis et al., 2000). • Thus, in the presence of sulphur the green algae undergoes normal photosynthesis of water oxidation, oxygen evolution and biomass accumulation in order for the cells to grow. In the absence of sulphur the green algae turns into hydrogen production mode. This process is reversible hence is enables the cells to cycle between oxygen production and hydrogen production mode.</td>
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<tr>
<td>Solar Conversion Efficiency Avoids wastage of photon absorption by truncating the light harvesting chlorophyll (Chl) antenna size of Photo-system II and I</td>
<td>• Light utilization efficiency by algae is one of the most important factors in the hydrogen production, however, the solar conversion efficiency of algae cells currently is below 1% (Hallenbeck and Benemann, 2002; Allakhverdiev et al., 2010), which is not high enough to compete with the current petrochemical methods (Mussunguz et al., 2007). • The low solar conversion energy by green algae is due to their genetic tendency to assemble large arrays of light absorbing chlorophyll (Chl) antenna in their photosystems (Melis, 2002). At high solar intensities the rate of photon absorptions by the chlorophyll antenna in the chloroplast far exceeds the rate of photosynthesis which eventually results in dissipation and loss of excess photons via non-photochemical photons by fluorescence or heat (Melis, 2002). Hence 95% of the absorbed photons will be wasted resulting in low solar conversion efficiency and cellular productivity. Moreover, the algae cells at the surface of the mass culture are subjected to severe photo inhibition because of over absorption by the algae cells at the surface of the mass culture. The light is unable to penetrate efficiently into the mass culture resulting in an unequal and sub-optimal distribution of photon absorption (Zhang et al., 2002). It has also been noted that if culture is maintained in culture bottles, due to excessive chlorophyll content, light cannot pass efficiently through two or three layer of algal culture. Thus, inner layers of cells masked away from light due to cells at the exposed surface of culture bottle.</td>
</tr>
<tr>
<td>pH and temperature Optimum pH and temperature for enhanced hydrogen production</td>
<td>• One of the strategies to overcome the low solar conversion energy of green algae is by truncating the light harvesting chlorophyll antenna size of Photosystem II and Photosystem I (Polle et al., 2002; Srirangan et al., 2011). • A lot of experiments have been done to show that a smaller chlorophyll antenna size improves the solar conversion efficiency in green algae (C. reinhardtiitii) (Beckmann et al., 2009; Polle et al., 2002, 2000, 2003; Mussunguz et al., 2007). A smaller chlorophyll antenna will avoid over absorption and wasteful dissipation of excitation energy, as well as diminish photo-inhibition of photosynthesis on the surface of mass culture (Melis, 2002). Hence truncated chlorophyll antenna antenna size will result in greater photosynthetic productivity and photo-biological hydrogen production as well as improved solar utilization efficiency in mass culture (Melis, 2002; Eregulu and Melis, 2011). • Wahal and Viamajala (2010) reported that the minimum amount of chlorophyll molecules required for Photosystem II is 37 and for Photosystem I is 95. It is also believed that a smaller chlorophyll antenna size of the photosystems (PS II and PS I) could solve the problem of fully pigmented chlorophyll antenna (Melis, 2002). • The pH is also one of the factors that influence hydrogen production as it may affect the metabolism pathway thus affecting the hydrogen production rate (Manish and Banerjee, 2006). • Hydrogen production rate is dependent on the internal pH of the cells as the pH determines the concentration of protons (Kothari, 2013). Moreover the hydrogenase enzyme which is responsible for hydrogen production is inhibited by pH shift, either to the acidic or alkaline side as pH has a direct effect on the catalytic function of the hydrogenase enzyme (Kosourova et al., 2003). • Different types of microalgae strains have different optimal pH activity for hydrogen production; most microalgae species favors neutral pH. The optimum pH for increased hydrogen yield depends largely on the type of microorganism and substrates used (Nath et al., 2006). • Jeberlin Prabina and Kumar (2010) demonstrated that Anabaena-TE 1, Fischerella-TE 1 and Nostoc-TE 1 showed higher hydrogen evolution at pH 7.5, a lower pH of 5.5, 6.5 and higher pH of 9.5 has reduced hydrogen evolution. Moreover they also showed that hydrogen production decreases more at acidic than at alkaline pH as low pH results in lower level of ATP in the cell (Ferchichi et al., 2005). Kosourova et al. (2003), on the other hand showed that C. reinhardtiiit has a maximum hydrogen production at pH 7.7 and lower hydrogen production at pH 6.5 or pH 8.2. At the optimal pH of 7.7 the rate of hydrogen evolution increased and decline slowly compared to all the other pHs. Guan et al. (2004), showed that the optimal pH for maximum hydrogen production by P. subcordiformitis at pH 8 and the lowest is at pH 5 and pH 11. Low pH results in decrease in hydrogen production due to the increase in the formation of acidic metabolites which in turn destroys the cell’s ability to maintain internal pH, resulting in lower intracellular level of ATP (Nath and Das, 2011). • Temperature regulates the cellular, morphological and physiological responses of microalgae where at higher temperatures the metabolic rates of microalgae increases (Kumar et al., 2010). Maximum growth rate of microorganism and substrate utilization during hydrogen production are also affected by temperature (Nath et al., 2006). Higher temperatures beyond the optimal temperature leads to thermal deactivation resulting to inactivation of enzymes responsible for controlling metabolic pathways in the hydrogen production process (Nath and Das, 2011).</td>
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The optimum temperature for higher hydrogen production varies considerably with different microalgae species. Studies by Jeberlin Prabina and Kumar (2010) showed that the optimum temperature for higher hydrogen production in *Anabaena*-TE 1, *Fischerella*-TE 1 and *Nostoc*-TE 1 (cyanobacteria isolates) is at 27°C.

Cell immobilization is defined as the physical localization of a viable microbial cell on a particular material in a way that limits the free migration of the cells while still retaining their catalytic activities for repeated and continuous use (Kilonzo and Bergougou, 2012). Immobilization involves the attachment or entrapment of cells onto a particular polymer matrix and the type of polymer matrices that can be used are polyacrylamide gel, agarose gel, alginate, chitosan, porous glass, polyurethane and so on.

Immobilization of cells on solid matrices for greater hydrogen production has been reported to be more advantageous than free floating cell suspension as immobilized cells occupy less space, requires small volume of growth medium, easier to handle and can be used repeatedly for product generation (Eroglu and Melis, 2011). Moreover bound cells can shift more readily between the oxygenic photosynthesis (growth phase) and hydrogen production phase, which are controlled by shifting the cells between sulfur containing and sulfur free culture media (Eroglu and Melis, 2011; Hahn et al., 2007). Cell immobilization also provides robustness against cell washout under hydraulic shock loadings (Keskin et al., 2006).

Laurinavichene et al. (2006) used immobilized *C.reinhardtii* on Al-borosilicate porous glass sheets and saw an increased on the hydrogen production rate from 2.5 up to 4.3 mL/L/h. Kosourov and Seibert (2009) used alginate beads to immobilize *C. reinhardtii* and revealed higher cell densities and hydrogen production rates (12.51 mol/mg/Chl/h). They also reported that the alginate polymer helped to boost the hypoxic environment within the cells promoting hydrogen production conditions.

Hence, the rate of hydrogen production can be greatly enhanced by using immobilized algae cells compared to free floating algae cells.

Light intensity is also an important factor for bio-hydrogen production especially for photosynthetic microorganisms (green algae and photosynthetic bacteria) and different strains require different light intensities for enhanced hydrogen production (Kothari, 2013).

Microalgal cultures pre-grown under low light intensities and exposed to high light intensities during sulfur deprivation produced higher hydrogen production as these cultures are able to transition more rapidly to anaerobiosis. During the pre-growth phase with low light intensities, microalgae have higher chlorophyll content, decreased hydrogen evolution and CO₂ fixation capacities per chlorophyll, compared to being grown under high light intensities. The reason for accelerated an aerobic conditions and increase in hydrogen production is because when pre-grown under low light intensities the damage to PSII D1 protein takes place and its repair rate decreases under sulphur deprivation hence they experience additional photo inhibition when placed under high light intensities and sulphur deprived condition (Tsygankov et al., 2006).

Jeberlin Prabina and Kumar (2010) reported that *Anabaena*-TE1, *Fischerella*-TE1 and *Nostoc*-TE1 (cyanobacteria) produced maximum hydrogen at 3500 lux and a higher light intensity than 3500 lux decreases the hydrogen production of all three cyanobacteria’s. For sulphur deprived *C. reinhardtii*, Kim et al. (2006) reported that the maximum production of hydrogen was produced at 200 µE m⁻² S⁻¹ of light intensity with a maximum hydrogen volume of 2.01 mL H₂ g⁻¹ cell h⁻¹. Also observed was that the initiation time for hydrogen production decreased from 62 to 22 hours with increasing light intensity.

The hydrogen production phase was accelerated by high light intensity resulting in prolonged production time as higher light intensities quickly induced sulphur deprived conditions so that the culture becomes more anaerobic resulting in hydrogenase enzyme to be activated sooner (Kim et al., 2006). With increasing light intensity the cell number and chlorophyll concentration increases, as when light is absorbed by chlorophyll antenna more electron are released which will then combine with proton to produce hydrogen (Kim et al., 2006).

However hydrogen production decreased at 300 µE m⁻² S⁻¹ because the cell number and chlorophyll concentration decreased sharply due to rapid destruction of Photosystem II at very high light intensity (Kim et al., 2006).

Microalgae and cyanobacteria contain pigments (chlorophylls, carotenoids and phycobilins) which each has different absorption ranges (Gutierrez-Wing et al., 2014). Chlorophylls, the most plentiful found pigments in microalgae has two major absorption ranges which are blue light (450-475 nm) and red light (630-675 nm) (Gutierrez-Wing et al., 2014).

Carotenoids have an absorption range of 400 to 500 nm and phycobilins have an absorption range of 500 to 650 nm (Gutierrez-Wing et al., 2014).

Although the primary photosynthetic pigment for all microalgae and cyanobacteria is chlorophyll “a”, different species responds to different distinct wavelength (Gutierrez-Wing et al., 2014).

Experiments conducted have found that microalgal growth and bio-products are obtained more efficiently using red light. Uyar et al. (2007), reported that using *Rodobacter sphaeroides* highest yield of hydrogen was produced using red and infrared light (Uyar et al., 2007). The red light spectrum improves the bio-hydrogen production as the photons in that spectrum provides the energy that matches the energy needed by the chlorophyll to reach its first excited stage hence greater photosynthetic activity will be obtained (Carvalho et al., 2006).

The light and dark photo-periods are two phases of photosynthesis. The light phase is used as the storage phase and the dark phase is used as the catabolism phase, depleting oxygen leading to an increase in the hydrogenase activity to produce hydrogen in the dark. Studies have shown that the light and dark cycles help to increase the hydrogen production yield. According to Jeberlin Prabina and Kumar (2010), 16 hours of darkness followed by 8 hours of light gave the best hydrogen production yield by *Fischerella*-TE1, while 24 hours of light without a dark period gave minimal hydrogen yield. Moreover those two photoperiods had no oxygen co-production.Hence the best photoperiod would be 16 hours dark: 8 hours light as *Fischerella*-TE1 will not be able to survive with only 24 hours of darkness and no exposure to light (Jeberlin Prabina and Kumar, 2010).

Koku et al. (2003), also reported that even when little or no hydrogen was produced during the dark period, the total amount of hydrogen gas produced in the cycle (14 h light:10 h dark) yielded more hydrogen than a continuously illuminated reactor. The reason why the overall amount of hydrogen produced in the cycle culture is significantly higher could be due to the high cell densities on the cycle cultures or due to the beneficial effect of illumination cycles on nitrogenase (Lazar et al., 2013).
A contradicting report on the other hand by Oncel and Vardar Sukan (2011) stated that when *C. reinhardtii* were used, the hydrogen production declined in the light/dark cycles that when compared to continuously illuminated cultures.

Microalgae require carbon dioxide for photosynthesis. The level of hydrogen production by microalgae using carbon dioxide as the sole carbon source is low (Rashid et al., 2009). The microalgae are also able to store carbon in the form of starch during photosynthesis and can use it during anaerobic condition, however, the amount of starch that they can accumulate are low hence only low level of hydrogen is produced (Rashid et al., 2013).

Exogenous carbon sources can be added in order to significantly increase the hydrogen production (Rashid et al., 2011). Exogenous carbon sources either organic or inorganic carbon can be used and the selection of type of carbon sources is important as the hydrogen yields varies with the type of carbon sources and algal strain (Rashid et al., 2013).

Burrows et al. (2008) used concentration of bicarbonate as a carbon source and saw an increase (2 fold) in the hydrogen produced by *Synechocystis* sp. Jeberlin Prabina and Kumar (2010) reported that *Anabaena* TE1, *Fischerella* TE1 and *Nostoc* TE1 recorded maximum amount of hydrogen produced when 0.3 % carbon dioxide in gas phase with 50% argon was used, however above 0.3 % the hydrogen production starts to decline which may be due to inhabitation of nitrogenase.

Glucose can also be used as an exogenous carbon source and Rashid et al. (2009) reported that when 30 mM of glucose were used the hydrogen production increased till all the glucose had been consumed.

Nitrogen is also an important element for microalgae as it is directly associated with the primary metabolism of microalgae (Kumar et al., 2010). Nitrogen is essential for long term hydrogen production as they are important for nitrogen fixation and cell metabolism, however nitrogen inhibits some nitrogenase mediated hydrogen production in some cyanobacteria (Jeberlin Prabina and Kumar, 2010). Limitations of nitrogen supply to microalgae are known to alter its photosynthetic metabolism and direct it more towards the release of excess energy and reducing power in the form of hydrogen (Koku et al., 2003).

The source of nitrogen is also an important consideration. When nitrate it used as nitrogen source in cyanobacteria, it requires reducing equivalents to be reduce to ammonia, hence elimination of nitrate from the growth media increases the reductant flow to hydrogen production for hydrogen production (Guthmann et al., 2007). Toshina et al. (2002) reported that when non-nitrogen fixing cyanobacterium *G. alpica* was grown in limited nitrate, they observed an increase in the rate of hydrogen production and specific hydrogenase activity.

Addition of ammonium instead of nitrate results in an increase in hydrogen production as the electrons are not directed towards the reduction of nitrate to ammonium hence all electrons are directed towards hydrogenase for hydrogen production (Burrows et al., 2008).

A mix culture of green algae and photosynthetic bacteria is able to enhance the overall hydrogen production rates, as photosynthetic bacteria are able to evolve hydrogen in the dark and light by utilizing the fermentation products of green algae. Integration of photosynthetic hydrogen production by microalgae that uses visible region of light spectrum (400-700nm) with hydrogen producing photosynthetic bacteria using near infrared region (700-950 nm) are able to improve the solar energy utilization and widening the range of solar spectrum to include the wavelengths from 400 to 950 nm (Eroglu and Melis, 2011). Moreover co-culturing holds a promise of metabolic integration where microalgae generate organic carbon from CO₂ and H₂O while photosynthetichbacteria generate organic nitrogen via nitrogenase, when both are producing hydrogen (Eroglu and Melis, 2011).

In the mixed culture, the fermentation products of the *C. reinhardtii* accumulated during the first 6 hours of dark, hereafter the formate concentration starts to decrease as the *R. rubrum* evolves hydrogen by using the formate formed by *C. reinhardtii* hence the hydrogen evolution by mixed culture increased four times compared to *C. reinhardtii* alone (Eroglu and Melis, 2011).

Wu et al. (2012) co-cultured *B. japonicum* a nitrogen-fixing bacterium species with *C. reinhardtii* strain-849, which is a cell wall deficient mutant algal strain and another *C. reinhardtii* transgenic algal strain (transgenic 1ba strain). Their findings showed that the hydrogen production in the co-culture increase 14.2 times higher for transgenic 1ba strain and 5.5 times higher for strain-849 compared to the hydrogen production level of the green algae alone. It was noticed that the oxygen content of both the co-cultures decreased more quickly than the single algae culture (Wu et al., 2012).

The rapid decrease of oxygen content is due to the rapid respiration rate, promoting anaerobic conditions in the co-cultured which might lead to lower consumptions of aerobic respiration metabolism, higher Fe-hydrogenase activity leading to higher hydrogen production (Wu et al., 2012). Hence co-culturing of photosynthetic bacteria with green algae is able to promote the enhancement of the green algae hydrogen production.

Microwaves (MW) are non-ionizing radiation and part of the electromagnetic spectrum with frequencies ranging from 300 MHz to 300 GHz corresponding to wavelength range of 1 mm to 1 m (Mishra et al., 2013).

Microwave irradiation produces thermal and also non-thermal effects. When microwave irradiation is subjected to microalgae and bacteria, the thermal effect among others can cause the whole organism or major portions of them participate in heat transfer process (Mishra et al., 2013). The effect is generated from vibrational energy due to penetration of electromagnetic waves into biological materials heating up intra- and extra- cellular fluids by transfer of vibrational energy (Mishra et al., 2013).

For the non-thermal effect, it is postulated that there is a direct stabilizing interaction of microwave with specific (polar) molecules in reaction medium with no rise in temperature (Herrero et al., 2008). The effect of the MW on the cell are influenced by multiple factors, such as MW frequency, duration of exposure, pulsed or continuous MW treatments and the medium/matrix in which the cells are embedded during MW exposure (Banik et al., 2003; Herrero et al., 2008). Different microwave frequencies in continuous waves and modulated modes produced significantly different physiological effects (Banik et al., 2003; Herrero et al., 2008).
towards the 21st century due to increasing energy demand and increasing greenhouse gas emission makes it important to develop alternative energy carriers that are renewable, clean and environmentally friendly. Hydrogen holds an increasing role as a future fuel and renewable source of energy however the current classical methods of producing hydrogen are energy intensive, costly and are not environmentally friendly. Major technical challenge in achieving practical applications of bio-hydrogen would be lowering the cost of production, delivery, storage, conversion and practical applications. Bio-hydrogen production employing renewable biomass may be a potential answer to overcome some of the economic constraints to fulfil many of our energy needs. Other challenge of the bio-hydrogen production includes unstable hydrogen production possibly attributed to the metabolic shift of hydrogen producing organisms.

Bio-hydrogen production from biological processes using microalgae holds an alternative to hydrogen production from classical methods as it offers promising advantages such as hydrogen can be produced from renewable sources and eliminates environmental pollutants. However bio-hydrogen yield from microalgae are relatively low to compete with the classical methods of hydrogen production. Hence for it to be commercially competitive, sustained hydrogen production and improvement on the yields of hydrogen has to be achieved. In order to achieve that, further research and development on the optimization of key parameters for enhanced hydrogen production has to be done. The optimization of key experimental factors, genetic modification and metabolic engineering of microalgae are the ultimate approaches to make hydrogen production cost-effective and sustainable. Bio-hydrogen yields and production rates must at least surpass considerably the present achievements for realistic applications. Technological breakthrough must be sought after to extract most of hydrogen from various substrates. Investigation addressing this challenge should be one of focuses of future research.

Table 5: Continue

- Non-thermal physiological effects of continuous waves and modulated microwaves MW irradiation is currently a rapidly growing area of research. Research by Asadi et al. (2011) showed that when Phormidium spp. Kutzing ISC31 (a cyanobacterium) was treated with a frequency of 2450 MHz by combining five different frequencies intensities (180, 360, 540, 720 and 900 W/cm²) and three pretreatments (10, 20 and 30 s), the content of the chlorophyll a decreased with increase in intensity and exposure time.
- In MW irradiation, the treatment time rather than temperature is the key factor to obtain high hydrogen production. This is because regardless of the temperature if the microorganism is irradiated for too long the hydrogen production decreases as the cell will lyse drastically due to harsh ambient conditions causing inhibition of hydrogen production (Bakonyi et al., 2014).
- Recently, Hsia and Chou (2014) studied ultrasonic effect on bio-hydrogen production based on four changeable parameters (frequency, intensity, duration and starch concentration). They reported that the optimal hydrogen production rate occurred with ultrasonic energy 4 joules, exposure for 15 minutes followed by no exposure for 15 minutes, transduces 0.5MHz and starch concentration 30 g/L.
- The research on the effects of microwave irradiation on microalgae to enhance hydrogen production is still in the early stages. More work is required in this area to better understand the MW effects.

CONCLUSION

In conclusion, the fossil fuel reserves shortage towards the 21st century due to increasing energy demand and increasing greenhouse gas emission makes it important to develop alternative energy carriers that are renewable, clean and environmentally friendly. Hydrogen holds an increasing role as a future fuel and renewable source of energy however the current classical methods of producing hydrogen are energy intensive, costly and are not environmentally friendly. Major technical challenge in achieving practical applications of bio-hydrogen would be lowering the cost of production, delivery, storage, conversion and practical applications. Bio-hydrogen production employing renewable biomass may be a potential answer to overcome some of the economic constraints

ACKNOWLEDGMENT

The authors would like to thank the Malaysian Ministry of Education through the Fundamental Research Grant Scheme (FRGS) (20150203FRGS) for the financial grant to carry out this research. We also would like to thank University Tenaga Nasional for the research facilities.

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