Journal of Environment & Biotechnology Research
VINANIE PUBLISHERS
www.vinanie.com/jebr

Biowaste recycling by microbes for hydrogen production – An alternative strategy for greener fuel

Shanmugam Sabarathinam,[1] Savitha Sivasubramanian,[2] Krishnasami Swaminathan,[1] Sadhasivam Subramaniam[1,*]

[1] Bioprocess and Biomaterials Laboratory, Department of Microbial Biotechnology, Bharathiar University, Coimbatore – 641046, India
[2] Department of Biotechnology, Sree Sastha Institute of Engineering and Technology, Chennai, India

REVIEW ARTICLE

ABSTRACT

The need for cleaner and greener fuel is highly desirable in near future, since fossil fuels are the known and leading cause for the environmental and health hazards like global warming and airborne toxicants. Thus far, biological processes have been engaged to produce hydrogen from biomass to replace fossil fuel economy. Development of such renewable energy technologies are considered as viable alternates to produce hydrogen using the organic substrates. However, the high cost of raw materials, problems associated with the bioconversion of lignocellulosic waste and lower hydrogen yield are several constraints behind the biohydrogen production. This review is focused on the different bioprocess methods involved in the hydrogen production, solid waste recycling with a special attention towards anaerobic bacteria mediated dark fermentation. Finally, the potential application of biohydrogen and challenges associated with its production are discussed.

KEYWORDS

anaerobes; biohydrogen; feed stock; fuel cell; renewable energy

1. INTRODUCTION

Biohydrogen production from fermentative processes is one among the most research-intensive topics worldwide as both developed and developing nations are profoundly searching for ways to avert the looming energy crisis caused by the hyper-exploitation and subsequent depletion of oil and natural gas reserves. In comparison with other thermo-chemical processes like steam reforming and pyrolysis, dark fermentation by anaerobic bacteria is not only a less energy intensive process, but also offers additional benefits of coupling waste treatment and microbe-mediated bioconversion of carbohydrate-rich substrates to significantly high yields of hydrogen gas. Since dark fermentation does not depend on solar radiation, the process is not interrupted at night, offering greater production rates compared to other biological processes (Nath et al., 2005; Hsu and Lin, 2016; Lin et al., 2016). This process is particularly suitable for agro-industrial economies like India where carbohydrate rich waste materials are generated in plenty year after year, with no uniform strategy for their disposal. However, as with all the promising novel technologies for energy storage, the commercial success of biohydrogen production largely depends on the operational parameters such as microbial inoculants, substrate suitability and pretreatment, reaction conditions, product recovery and the by-product spectrum. This review, in addition to evaluating each parameter of biohydrogen production, presents an unbiased view of the opportunities and challenges being offered in this process.

Corresponding authors: **Sadhasivam Subramaniam**
Tel: +91-7358595171
Fax: +91-422-2422387
E. mail: sadhaon@gmail.com; rahulsbio@yahoo.co.in

Recieved: 11-03-2016
Revised: 18-03-2016
Accepted: 21-03-2016
Available online: 01-04-2016

2. BIOPROCESS FOR HYDROGEN PRODUCTION

2.1. Biophotolysis

It is a process in which the photoautotrophic organisms generate the hydrogen by utilizing light and carbon dioxide as light and energy source. The production process can be distinguished into direct and indirect photolysis. In direct photolysis, water is converted into chemical energy with the aid of solar energy and photosynthetic system of algae. The light absorbed by the photosynthetic system I (PSI) and photosynthetic system II (PSII) used in the process to transport electrons to ferredoxin and hydrogen production. The light adsorbed by the PSII is used to split up the water molecules into its corresponding electrons, protons and oxygen molecules, these electrons in turn are utilized by the PSII system via electron transport chain to hydrogenase enzyme without CO_2 formation. With the aid of hydrogenase enzyme, the hydrogen gas is produced via the recombination of electrons and protons. However, in indirect photolysis, the photosynthetic system of macro algae or cyanobacteria converts the solar energy into hydrogen by a two step process. The first step involves the formation of biomass through the photosynthetic system followed by the utilization of biomass for hydrogen production (Azwar et al., 2014). The major advantage of biophotolysis is the cost effectiveness where, pure hydrogen is generated from the water, sunlight and carbon dioxide and it does not require any other additional nutrient supplements. However, the lower light energy conversion of algae or cyanobacteria makes it unsuitable for the large scale production of hydrogen.

2.2. Photo-fermentation

In photo-fermentation, hydrogen production is achieved by photosynthetic bacteria with the aid of photo-system II via photosynthesis. The process requires light as energy source and organic acids are utilized as electron donor for the powerful generation of hydrogen. The advantage of photofermentation by purple photosynthetic bacteria is the ability to produce 100% of electrons from the organic substrate to generate hydrogen and carbon dioxide with no oxygen evolution. There is no inhibition activity of nitrogenase enzyme as they are insensitive to oxygen (Wall et al., 2008).

2.3. Dark fermentation

The production of hydrogen at 30 ˚C to 80 ˚C through the fermentation by anaerobic or facultative anaerobic bacteria under the dark conditions using carbohydrate rich organic wastes is known as dark fermentation (Figure 1). Depending on the reaction process and substrates, the products of dark fermentation mainly comprises of H_2 and CO_2 and also combines with the other gases such as CH_4 and H_2S. When hydrogen concentration increases, the metabolic pathways shift to produce more reduced substrates, such as lactate, ethanol, acetone, butanol or alanine, which in turn decrease the hydrogen production (Van Niel et al., 2003). With glucose as the model substrate, maximum 4 mol H_2 is produced per mole of glucose when the end product is acetic acid. However, in practice, the 4 mol H_2 production/mol glucose cannot be achieved because the end products normally contain both acetate and butyrate (Hawkes et al., 2002). Among the above methods dark hydrogen production holds considerable promise.

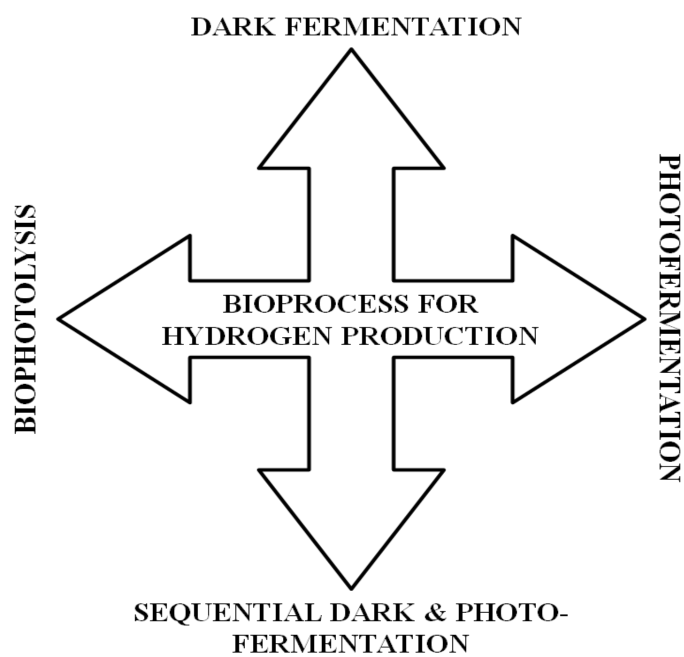

Figure 1. Bioprocess for hydrogen production.

2.4. Sequential dark and photo-fermentation

The new approach in the hydrogen production is the sequential dark and photofermentation, which offers several advantages over the single step dark or photofermentation. In this process, the effluent from dark fermentation in hydrogen production provides required amount of organic acids for the photo fermentation process. Hence, the limitation in availability of organic acids for the photofermentation is eliminated. There are several reports that state that the production yields can be substantially improved by the combination of two systems (Cheng et al., 2015). The effluent quality could be improved by the photo-fermentative bacteria through the utilization of effluent containing organic acids from dark fermentation. Ammonia concentration in the effluent from dark fermentation may inhibit the activity of photosynthetic bacteria, hence the dilution and neutralization of organic acids from dark fermentation effluents and adjusting the pH to neutral could maximize the hydrogen production (Zagrodnik and Laniecki, 2015).

3. MICROBIOLOGY OF HYDROGEN PRODUCTION

A wide variety of microorganisms that exists in nature are capable of producing hydrogen (Figure 2). Algae are capable of producing hydrogen via photosynthesis in which the water molecules can split into hydrogen and oxygen ions. The hydrogenase enzyme produced by the algae plays a key role in the conversion of hydrogen ions into hydrogen gas. Algal hydrogen production is considered to be a green approach in which the water and CO_2 are utilized as renewable resources that minimize the green house gas emission. *Chlamydomonas reinhardtii* (Hong et al., 2016), *Scenedesmus obliquus* (Florin et al., 2001), *Chlorococcum humicola* (Thomas and Jayachithra, 2015), *Playtmonas subcordiformis* (Guan et al., 2004) and *Chlorella fusca* (Winkler et al., 2002) are well-known producers of hydrogen. However, the major disadvantages associated with the algal hydrogen production are the inhibition of hydrogenase by the generated molecular oxygen and low production potential. Hence, dark fermentation by anaerobic organisms is considered to be more advantageous owing to the simultaneous waste treatment and hydrogen gas production.

The microorganisms involved in dark fermentation may be classified based on the oxygen requirement, temperature and substrate specificity. Based on the oxygen demand, the microbes can be differentiated into obligate anaerobes which strictly require anaerobic conditions and facultative anaerobes, which sustain in both aerobic and anaerobic environments. On the basis of temperature, the organisms may be distinguished into mesophiles, which requires ambient temperature for growth and thermophiles those accustomed to higher temperature. Further, based on the substrate used for the dark fermentation, these microbes may be further differentiated into pure culture, which requires single carbon source and mixed culture that can be adapted to utilize complex biowaste materials as their source of fermentation.

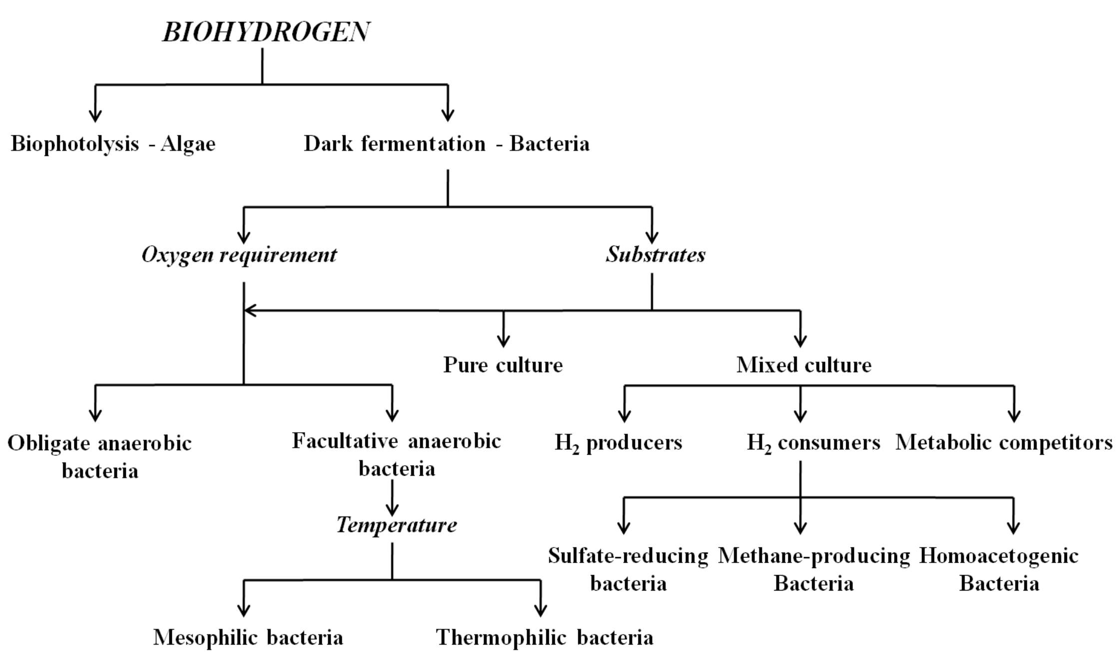

Figure 2. Microbiology of hydrogen production.

3.1. Facultative anaerobes and thermophiles

Facultative anaerobic bacteria can produce ATP during the anaerobic environment which contains either [Fe-Fe] hydrogenase or formate hydrogen lyase, which is responsible for the higher yield and production rate of hydrogen (Sinha et al., 2015). *Enterobacterieaceae* are extensively studied facultative anaerobic bacteria and the representative bacteria were *Enterobacter aerogenes* and *Enterobacter cloacae* which are capable of higher yields and production rates (Zhang et al. 2011; Ramprakash and Muthukumar, 2016). In comparison, obligate anaerobes produce higher rate of hydrogen by utilizing the wide range of substrates such as solid biomass waste and different kinds of waste water. These organisms are able to switch over to solvent production during the stationary phase (Han and Shin, 2004). *Clostridia* species shows promise among spore-forming hydrogen producers under anaerobic conditions. Thermophiles are found in the geothermally heated regions of the earth atmosphere such as hot springs, deep sea volcanic and hydrothermal vents and mostly found as obligate anaerobes. Based on the source of isolation, their culture requirements differs from high concentration of sodium chloride (from deep sea volcanoes) or high concentration of sulphur (from volcanic vents) in the production media for the enhanced production of hydrogen. Typical examples of this group include the genera *Caldicellulosiuptor*, *Thermoanaerobacter* and *Thermotoga* which are able to utilize the complex carbohydrates and proteins for their growth and hydrogen production (Van Niel et al., 2002; Nguyen at al., 2008).

With regard to biohydrogen production from complex feedstock, the exploitation of mixed cultures showed promising results over the pure culture. The mixed or co culture in the presence of facultative anerobes are able to consume trace amounts of oxygen in the medium and provides the anaerobic environment to the obligate anaerobes, which prevents the use of expensive reducing agents like L-cysteine and makes the process economical. In industrial point of view, the utilization of cheap and non-sterile complex feedstock for the biohydrogen production was easily attained by this mixed microbial culture. Mixed cultures as inocula for hydrogen production can be isolated from various sources, such as anaerobic sludge from municipal wastewater plants and cow dung composts, cattle or dairy residue composts, sludge from palm oil mill effluent, soil, rice straw compost, fermented soy bean meal as well as landfill lixiviates. However, the major problem associated with the mixed cultures is the presence of hydrogen consumers (Sulfate-reducing bacteria, the Methane-producing bacteria, and the Homoacetogenic bacteria) and metabolic competitors (Lactic acid bacteria) within the community which lowers the production of hydrogen. This problem can be overcome by the pretreatment of mixed consortia which removes these microbes.

4. SOLID BIO-WASTE RECYCLING

The economics of the biological hydrogen production depends on the selection of the raw materials. The raw materials should be abundant, readily available, cheap, rich in carbohydrate and biodegradable in nature. The above criteria are satisfied by biological waste materials of organic origin and can be distinguished into: waste generated from agricultural (crop) residues, livestock waste (animal manure), food industry waste (molasses), industrial waste (glycerol and palm oil waste), and municipal waste (sewage sludge). The worldwide production of agricultural crop residues like straw, stover, peelings, cobs, stalks, bagasse and other lignocellulosic biomass has been evaluated to be approximately 200 billion tons worldwide (Ren et al., 2009). These crop residues can be easily converted biologically into biohydrogen via dark fermentation.

The variability in hydrogen production is based on the varied composition of carbohydrates viz., cellulose, hemicelluloses and lignin in the substrates (Li et al., 2008). Owing to the complex nature of biomass, biodegradability and hydrogen production are greatly affected. Hence, prior to use, these agro based wastes require some pretreatment process (heat, acid, base) for the effectual conversion of complex sugar molecules into simple sugars. Thus far, different types of pretreatment such as pH, acid or base were found to improve the biohydrogen production via dark fermentation (Song et al. 2012). It is estimated that 1500 million tons of animal manure is produced annually and these can be distinguished into slurry or liquid manure, solid manure and wastewater from farms (Burton and Turner, 2003). The production of biohydrogen from the livestock is comparatively low than that of the agricultural feedstock due to the strong inhibition by ammonium, nitrogen and sulfate concentration present in the manure (Chen et al., 2008). Interestingly, the production of hydrogen from the livestock can be substantially improved by the co-digestion of animal manure with carbohydrate rich agricultural waste (Callaghan et al., 2002).

Food waste consists of 85-95% of volatile solids and 75-85% moisture which favors the microbial development and thus it acts as a potential source for the biohydrogen production (Li et al., 2008). Among the food waste, molasses, rice slurry from sugar and starch based industries serves as a better source since, they contains good amount of sucrose and it can be degraded anaerobically (Chong et al., 2009). Food waste is usually disposed as landfills which pose serious threats on environment like fouling and leachates polluting the ground water. In this view, the exploitation of food waste for the biohydrogen production through dark fermentation appears to be highly promising and economically viable alternative in the waste management (Cantrell et al., 2008). The utilization of industrial waste for the hydrogen production shows promising results which also simultaneously minimizes the problem of environmental pollution. Glycerol, the major by-product obtained from the biodiesel industry poses disposal threat. Hydrogen production from the substrate glycerol in an immobilized bioreactor at thermophillic and mesophilic temperatures showed promising production of hydrogen and 86.7% conversion of the organic carbon from the glycerol (Yang et al., 2008). Palm oil effluent obtained from the crude palm oil extraction process are significantly rich in carbohydrates which act as a substrate for the hydrogen production with thermophilic microorganism produces up to 6.5 liter of H_2 per liter. (Sompong et al., 2007).

Increasing global population has resulted in an alarming rise in sewage sludge generation which acts as a major source of solid waste that needs an effective waste management system. Sewage sludge is rich in several macro (carbohydrate, proteins, lipids) and micronutrients (vitamins and minerals). Hence, it may be used as a suitable candidate for the biohydrogen production. In spite of all these advantages, sewage sludge receives much lower attention for use as a seed or substrate for hydrogen production due to the presence of hydrogen consuming microbes. These microbes lower the hydrogen production and make the process highly unprofitable. This problem can be overcome by a simple pretreatment process before dark fermentation for the enhanced production of hydrogen by using hydrogen producing anaerobic/facultative anaerobic bacteria (Lay et al., 1999).

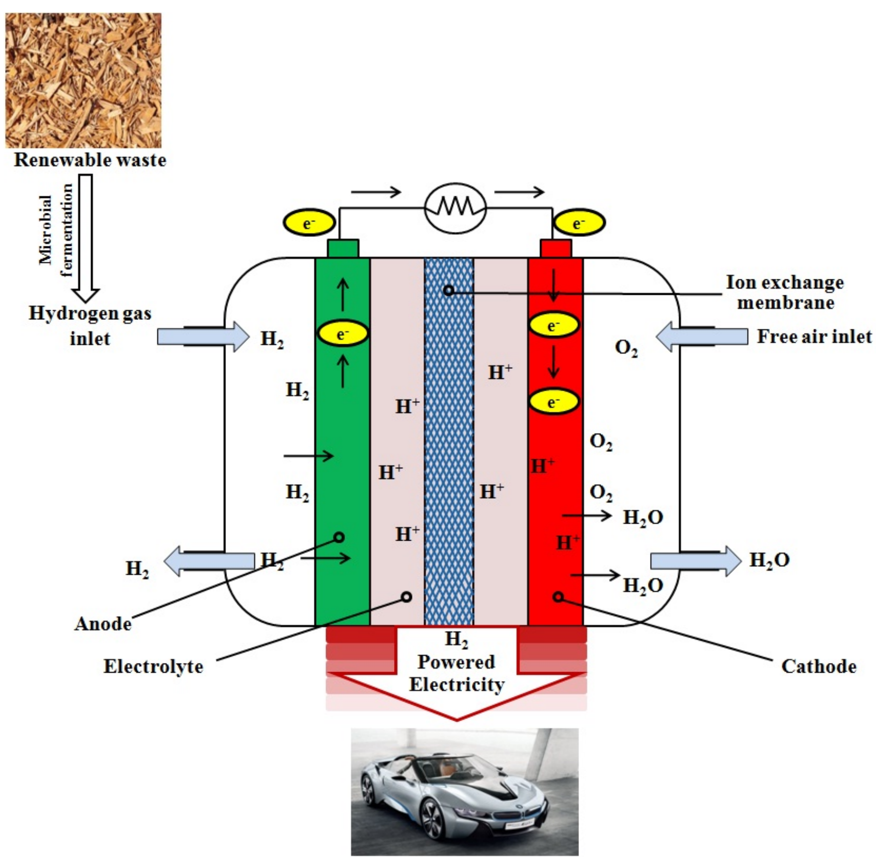

Figure 3. Biohydrogen fuel cell.

5. POSSIBLE APPLICATIONS OR USES OF H_2

Hydrogen as alternative fuel is recently gaining importance since it is regarded as near-zero net emission fuel (Kathe et al. 2016). During the hydrogen combustion, a significant amount of energy is released in the form of heat and electricity. Fuel cells are much more efficient than combustion in terms of energy capture and also free of air pollution. Moreover, fuel cell is an electrochemical energy conversion device that generates electricity from the fuel and oxygen with heat and water as a by-product. Based on the applications the development of the fuel cell differentiate into polymer electrolyte membrane fuel cell (PEMFC), alkaline fuel cell (AFC), phosphoric acid fuel cell (PAFC), molten carbonate fuel cell (MCFC), solid oxide fuel cells (SOFC) and direct methanol fuel cell (DMFC). Biohydrogen from *Rhodobacter sphaeroides* ZX-5, *Rhodopseudomonas palustris* CQK 01 and *Rhodopseudomonas faecalis* RLD-53 is sufficient to provide enough hydrogen power up the 5W to 100W of PEMFC for different application on continuous basis. Thus the integrated photo fermentation system may be applicable in generating hydrogen of portable PEMFC power generator with power output less than 100W (Orozco et al., 2010). An alternative to fossil fuel driven vehicles, prototype hydrogen powered vehicles have been developed (Figure 3). Recently, the feasibility of using hydrogen with gasoline in combustion engine was demonstrated to run BMW hydrogen cars, however the large-scale production is not currently in pipeline. The core limitation of producing hydrogen from the biomass is affected by the lack of efficient utilization of electrons and energy in biomass to H_2. Hence, increasing the yield of the biomass is crucial for expanding its application.

6. CHALLENGES TO BE ADDRESSED

For the past several decades, various efforts have been made to make H_2 fermentation technology more feasible. In spite of the several research works, the main problems associated with the H_2 production remain unaddressed. At present, the low substrate conversion efficiency and low yield are considered to be the major hurdles for the industrial-scale production. Due to the varied H_2 yields reported in the literature it is unfeasible to identify the suitable feedstock for hydrogen production. On the other hand, the pretreatment methods and microorganism known to affect the degradation of the feedstock plays a vital role in hydrogen production. The bioconversion of agroindustrial feedstock into biohydrogen is primarily affected by the pretreatment and fermentation method in which the composition of the feedstock is known to affect the activity of the microbial metabolic pathways and subsequently the yield of H_2 production (Monlau et al., 2013). Hence, it is necessary to develop a cost-effective conversion methodology which includes various chemical, thermal, enzymatic microbial, fungal and multi stage combined treatment for the effective conversion of raw material into a suitable substrate that aids the successful exploitation by the microbes, which facilitates the H_2 production. Once these approaches has been finalized, the other process parameters such as pH, hydraulic retention time, temperature and reactor design on which the hydrogen production was highly reliant on production have to be optimized. In the future, the integration of biohydrogen production process to the green waste may have the simultaneous beneficial advantages such as waste water treatment and generation of clean energy. With technology advancement, biohydrogen production may offer a sustainable alternative energy resource in the near future.

ACKNOWLEDGEMENTS

The author Dr. Sadhasivam Subramaniam acknowledges DBT, India for the financial support provided by the Ramalingaswami Re-entry fellowship (Order No. BT/RLF/Re-entry/55/2013).

REFERENCES

Azwar, M.Y., Hussain, M.A. and Abdul-Wahab, A.K. (2014) Development of biohydrogen production by photobiological, fermentation and electrochemical processes: A review. Renewable and Sustainable Energy Reviews, 31, 158–173.

Burton, C. and Turner, C. (2003) Manure management: Treatment strategies for sustainable agriculture. Maulden, Bedford, UK: Turn-Around Typesetting Ltd.

Callaghan, F.J., Wase, D.A.J., Thayanithy, K. and Forster, C.F. (2002) Continuous co-digestion of cattle slurry with fruit and vegetable wastes and chicken manure. Biomass and Bioenergy, 22, 71-77.

Cantrell, K.B., Ducey, T., Ro, K.S. and Hunt, P.G. (2008) Livestock waste to bioenergy generation opportunities. Bioresource Technology, 99, 7941-7953.

Chen, Y., Cheng, J.J. and Creamer, K.S. (2008) Inhibition of anaerobic digestion process: a review. Bioresource Technology, 99, 4044-4064.

Cheng, J. Ding, L. Xia, A. Lin, R. Li, Y. Zhou, J. Cen, K. (2015) Hydrogen production using amino acids obtained by protein degradation in waste biomass by combined dark- and photofermentation. Bioresource Technology, 179, 13-19.

Chong, M.L., Rahim, R.A., Shirai, Y. and Hassan, M.A. (2009) Biohydrogen production by *Clostridium butyricum* EB6 from palm oil mill effluent. International Journal of Hydrogen Energy, 34, 764–771.

Florin, L., Tsokoglou, A. and Happe, T. (2001) A novel type of iron hydrogenase in the green alga *Scenedesmus obliquus* is linked to the photosynthetic electron transport chain. Journal of Biological Chemistry, 276, 6125–6132.

Guan, Y., Deng, M., Yu, X. and Zang W. (2004) Two stage photo-production of hydrogen by marine green algae *Platymonas subcordiformis*. Biochemical Engineering Journal, 19, 69–73.

Han, S.K. and Shin, H.S. (2004) Biohydrogen production by anaerobic fermentation of food waste. International Journal of Hydrogen Energy, 29, 569–577.

Hawkes, F.R., Dinsdale, R., Hawkes, D.L. and Hussy, I. (2002) Sustainable fermentative hydrogen production: challenges for process optimization. International Journal of Hydrogen Energy, 27, 1339-1347.

Hong, M.E. Shin, Y.S., Kim, B.W. and Sim, S.J. (2016) Autotrophic hydrogen photoproduction by operation of carbon-concentrating mechanism in *Chlamydomonas reinhardtii* under sulfur deprivation condition. Journal of Biotechnology, 221, 55–61.

Hsu, C.W. and Lin, C.Y. (2016) Commercialization model of hydrogen production technology in Taiwan: Dark fermentation technology applications. International Journal of Hydrogen Energy, 41, 4489–4497.

Kathe, M.V., Empfield, A., Na, J., Blair, E. and Fan, L.S. (2016) Hydrogen production from natural gas using an iron-based chemical looping technology: Thermodynamic simulations and process system analysis. Applied Energy, 165, 183–201.

Lay, J.J., Lee, Y.J. and Noike, T. (1999) Feasibility of biological hydrogen production from organic fraction of municipal solid waste. Water Research, 33, 2579–2586.

Li, M., Zhao, Y., Guo, Q., Qian, X. and Niu, D. (2008) Bio-hydrogen production from food waste and sewage sludge in the presence of aged refuse excavated from refuse landfill. Renewable Energy, 33, 2573-2579.

Lin, R., Cheng, J., Ding, L., Song, W., Liu, M., Zhou, J. and Cen, K. (2016) Enhanced dark hydrogen fermentation by addition of ferric oxide nanoparticles using *Enterobacter aerogenes*. Bioresource Technology, 207, 213-219.

Monlau, F., Barakat, A., Trably, E., Dumas, C., Steyer, J.P. and Carrère, H. (2013) Lignocellulosic materials into biohydrogen and biomethane: Impact of structural features and pretreatment. Critical Reviews in Environmental Science and Technology, 43, 260-322.

Nath, K., Kumar, A. and Das, D. (2005) Hydrogen production by Rhodobacter sphaeroides strain O.U.001 using spent media of *Enterobacter cloacae* strain DM11. Applied Microbiology and Biotechnology, 4, 533–541.

Nguyen, T.A.D., Kim, J.P., Kim, M.S., Oh, Y.K. and Sim, S.J. (2008) Optimization of hydrogen production by hyperthermophilic eubacteria, *Thermotoga maritime* and *Thermotoga neapolitana* in batch fermentation. International Journal of Hydrogen Energy, 33, 1483–1488.

Orozco, R.L., Redwood, M.D., Yong, P., Caldelari, I., Sargent, F. and Macaskie, L.E. (2010) Towards an integrated system for bio-energy: hydrogen production by *Escherichia coli* and use of palladium-coated waste cells for electricity generation in a fuel cell. Biotechnology Letters, 32, 1837–1845.

Ramprakash, B. and Muthukumar, K. (2016) Biohydrogen production from rice mill wastewater using mutated *Enterobacter aerogenes*. Engineering in Agriculture, Environment and Food, 9, 109-115.

Ren, N., Wang, A., Cao, G., Xu, J. and Gao L. (2009) Bioconversion of lignocellulosic biomass to hydrogen: potential and challenges. Biotechnology Advances, 27, 1051-1060.

Sinha, P., Roy, S. and Das D. (2015). Role of formate hydrogen lyase complex in hydrogen production in facultative anaerobes. International Journal of Hydrogen Energy, 40, 8806-8815.

Sompong, O., Poonsuk, P., Nugul, I., Srisuda, D. and Nils-Kåre, B. (2007) Improvement of biohydrogen production and treatment efficiency on palm oil mill effluent with nutrient supplementation at thermophilic condition using an anerobic sequencing batch reactor. Enzyme and Microbial Technology, 41, 583–590.

Song, Z.X., Dai, Y., Fan, Q.L., Li, X.H., Fan, Y.T. and Hou, H.W. (2012) Effects of pretreatment method of natural bacteria source on microbial community and bio-hydrogen production by dark fermentation. International Journal of Hydrogen Energy, 37, 5631–5636.

Van Niel, E.W.J., Budde, M.A.W., de Haas, G.G., van der Wal, F.J., Claassen, P.A.M. and Stams, A.J.M. (2002) Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*. International Journal of Hydrogen Energy, 27, 1391–1398.

Van Niel, E.W., Claassen, P.A. and Stams, A.J. (2003) Substrate and production inhibition of hydrogen production by the extreme thermophile *Caldicellulosiruptor saccharolyticus*. Biotechnology and Bioengineering, 81, 255-262.

Thomas. J. and Jayachithra, E.V. (2015) Growth kinetics of *Chlorococcum humicola* - A potential feedstock for biomass with biofuel properties. Ecotoxicology and Environmental Safety, 121, 258-262.

Wall, J.D., Harwood, C.S. and Demain, A. (2008) Bioenergy, ASM Press, Washington.

Winkler, M., Heil, B., Heil, B. and Happe T. (2002) Isolation and molecular characterization of the [Fe]-hydrogenase from the unicellular green alga *Chlorella fusca*. Biochimica et Biophysica Acta (BBA): Gene Structure and Expression, 1576, 330–334.

Yang, Y., Tsukahara, K. and Sawayama, S. (2008) Biodegradation and methane production from glycerol-containing synthetic wastes with fixed-bed bioreactor under mesophilic and thermophilic anaerobic conditions. Process Biochemistry, 43, 362–367.

Zagrodnik, R. and Laniecki, M. (2015) The role of pH control on biohydrogen production by single stage hybrid dark- and photo-fermentation. Bioresource Technology, 194, 187-195.

Zhang, C., Lv, F.X. and Xing, X.H. (2011) Bioengineering of the *Enterobacter aerogenes* strain for biohydrogen production. Bioresource Technology, 102, 8344–8349.