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BIOHYDROGEN PRODUCTION USING DISTILLERY SPENT WASH IN A CSTR UNDER MESOPHILIC AND THERMOPHILIC CONDITIONS

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ABSTRACT

To identify the viability and performance, distillery effluent having very high organic content was studied on continuous stirred tank reactor (CSTR). Under mesophilic and thermophilic temperatures, different organic loading rates (OLR), optimum conditions for highest chemical oxygen demand (COD) removal and biogas generation was found at OLR of 3.72 to 42.16 kg COD/(m^3·d) and 4.21 to 43.17 kg COD/(m^3·d). Highest COD exertion efficiency was found to be around 70% for OLR of 25.82 kg COD/(m^3·d), when hydraulic retention time (HRT) reduced from 24 to 12 h at mesophilic condition. The steady state of hydrogen production was 1360 ml/l·d obtained at the OLR of 25.82 kg COD/(m^3·d) with Volatile fatty acids to alkalinity ratio of 2.2 to 0.5 and the hydrogen production of 1130 ml/l.d obtained at the OLR of 28.52 kg COD/(m^3·d) with a conversion coefficient of 1.8 to 0.5 Volatile fatty acids to alkalinity ratio were recorded in these stages. The maximum of 70% of the Distillery Spent Wash was converted to hydrogen at the OLR of 25.82 kg COD/(m^3·d) at mesophlic conditions with HRT of 12 h and hence the mesophilic condition favoured the hydrogen production from Distillery Spent Wash.

KEY WORDS : CSTR, Distillery Spent Wash, Anaerobic Mixed Sludge, COD removal, H_2 production, SEM analysis.

INTRODUCTION

Hydrogen (H_2) is considered as a promising uncontaminated renewable energy resource due to its high energy yield (122 kJ g^{-1}) (Nandi R and sengupta 1998; Mizuno *et al.*, 2000; Tuna E *et al.*, 2009). The contamination of environment as well as the increase of fossil fuels cost is the driving forces for explore of new, environmental friendly and cheap sources of energy. For this kind of energy transformation, hydrogen is considered to be a promising vector, as it is a clean fuel which releases no carbon dioxide during combustion and it can be used in fuel cells for generating electricity. In addition, in comparison with hydrocarbon fuels, hydrogen has the highest energy value (122 kJ g^{-1}). However, this fuel is created at high temperatures during energy intensive processes such as non-catalytic fossil fuels partly oxidizing methane and hydrocarbon renovation (Kapdan and Kargi 2006). Thus, in recent years, significant attention has been paid to biological hydrogen production, photo and dark fermentation, direct and indirect Photolysis. Bio-H_2 development through Dark Fermentation has become a promising technology. In this regard, the use of feed stocks made higher rates of hydrogen production (100-400 ml-$H_2L^{-1}h^{-1}$) than other biological technologies and also the simplicity of the reactor technology is comparatively similar to mature anaerobic digestion technology (Lin *et al.*, 2018). Chemical waste has a high potential for improving the production of biohydrogen by the dark fermentation process. One of the key reasons for effective biohydrogen process is the good use of raw material, cheaper and widely available (Arimi *et al.*, 2015). Sugarcane molasses is a halfway of ethanol production and is produced in high amounts, about 40-60 kg/ton of sugarcane processed. It has relatively high organic matter content with approximately 60% of dissolved solids combined by sucrose, glucose and fructose (Otero rambla *et al.*, 2009) plus nutrient minerals. Because of this high organic content and nutrient minerals, sugarcane molasses can be converted by dark fermentation processes into hydrogen, ethanol and volatile fatty acids (VFAs). Nevertheless, there are few reports on the utilization of molasses as a sole carbon source for hydrogen production (Ren *et al.*, 2006; Li J *et al.*, 2007; Wang *et al.*, 2013). According to Urbaniec and Bakker (2015), at this stage of the advancement of hydrogen fermentation technologies, the discovery of substrates for potential economic applications in industrial scale had become a priority mission. Mixed- culture systems are an enticing alternative to pure/co-cultural systems for food waste and hydrogen production (Kleerebezem and Van loosdrecht, 2007, and Laxman pachapur *et al.*, 2016). In order to inoculate acidogenic reactors for hydrogen processing instead of pure cultures, anaerobic sludge (mixed cultures) was commonly used in order to suit more for environmental stresses including restriction of Nutrients, pH and temperature increases. The microflora in anaerobic sludge consists typically of both H_2-consuming and H_2-producing bacteria (Shizas and Bagley, 2005). Pre-treatment is widely used to enrich the sludge with bacteria that produce hydrogen and to kill micro-organisms that consume hydrogen. The heat pretreatment of sludge is the most commonly used method (Temudo *et al.*, 2008; Shizas and Bagley 2005). The pH of the next stage of batch reactor for base treatment was approximately 5.5, which reduced methanogens activity and was optimally suitable for the production of hydrogen (Zhu and Beland, 2006). 5–6 acidic pH is optimal for production of hydrogen and the optimum pH is reduced and increased, resulting in a metabolic change with volatiles fatty acids (VFA) (Laxman pachapur *et al.*, 2015 and Laxman Pachapur *et al.*, 2015).

The main purpose of the present study was to assess the influence of mesophilic and thermophilic temperature with different organic loading rates on the treatment efficiency of a continuous stirred tank reactor (CSTR) relating the process parameters, COD, and the hydrogen production. The experiment was conducted at various concentrations, HRTs of 24, 16, 12 and 8 h, mesophilic (35 °C) and thermophilic (55°C) temperature and pH 5 to 6. The results of this study may be useful in investigating the performance and overall feasibility of CSTR for the treatment of spent wash. The various factors affect the performance of the digestion process. The activity of microorganisms, substrate utilization and biogas formation are the key factors in anaerobic digestion. The important process parameters required and to be considered which includes Alkalinity, VFA and COD exertion (Bhavik K *et al.*, 2008).

MATERIALS AND METHODS

Experimental setup

The schematic representation and experimental setup with overview of the Continuous Stirred Tank Reactor have been shown in the figure 1 and 2. The system includes four automated units, feeding tank, main body of the reactor, gas measuring sensor unit and automated temperature control system. The temperature was automatically maintained at 35°C and 55°C with agitation speed of 120 rpm. The influent flow rate was controlled by a feed pump to regulate the HRT and Organic Loading Rate (OLR) in the reactor. The CSTR was constructed from stainless steel which consists of feed tank and has the feed supply volume of 10 L and the total volume

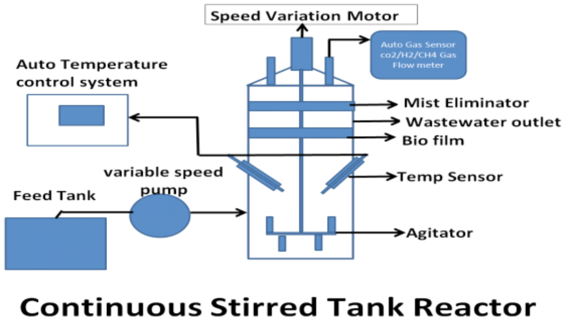


Fig. 1. Schematic diagram of Continuous Flow Stirred Tank Reactor

Fig. 2. Experimental set up of Continuous Stirred Tank Reactor

of the reactor was 21.78 L. Out of which 5 L volume meant for gas collection chamber located at the top of the reactor and 16.34 L as working volume to perform the bioconversion. The bioreactor dimensions were measured as diameter of 0.215 m and height of 0.6 m. The entire bioconversion mechanism took place in 4 different segments of the bioreactor namely, seed sludge introduction area at the bottom, substrate configuration part at the middle, bio-film placed in between the substrate and the gas collection chamber at the top of the reactor.

Substrate and inoculums

The Distillery Spent Wash (molasses) was collected from the industry at Cuddalore, Tamil Nadu, India and it was diluted with tap water. The COD value found to be varying between 4000 mg/l to 100000 mg/l for raw molasses. The Anaerobic sludge used for biological hydrogen production was collected from anaerobic digester unit treating Distillery Wastewater (sugar industry) at Cuddalore. The sludge was pre-treated by boiling at 95 °C for 15 minute (Sreethawong *et al.*, 2010) to suppress the activities of hydrogen-consuming bacteria. The reactors were inoculated with 4L of anaerobic sludge with Volatile Suspended Solids (VSS) of 4.5 g/l and the working volume was completed with diluted Distillery spent wash.

Analytical methods

Chemical analysis was performed for both influent as well as effluent. The parameters like Total Solids (TS), Volatile Solids (VS), Suspended Solids (TSS), COD and BOD_5 were determined based on the standard methods (APHA, 2005). The total volume of biogas produced was measured by using Gas Measuring Sensor Unit which comprises of three Sensors (H_2, CO_2, and CH_4). It helps to sense and measures the amount of biogas generated from the reactor and the reading will be shown in the LED display in ppm. After the biogas passed through the sensor unit, it reaches the water displacement unit. For confirmation of the biogas, it was injected into the GC using syringe and biogas composition was analyzed by GAS Chromatography (GC 7410) equipped with a Thermal Conductivity Detector (TCD) and Stainless steel column packed with nitrogen gas was used as a carrier gas for biogas analysis. The temperature of injector and column was maintained at 80 ^{o}C.

RESULTS AND DISCUSSION

Bio H_2 production at mesophilic and thermophilic condition

Initial usage of OLR 3.54 and 4.21 kg COD / m^3 d, bacteria should use the carbon source primarily for the growth of biomass and not for the production of hydrogen, and thus it produces minimum hydrogen (Zhang *et al.*, 2007a). The steady state production rates of hydrogen were 1253, 1266, 1293, 1305, 1354 and 1360 ml/d on 38, 39, 40, 41, 42 and 43rd day at 35^{o}C, 1097 ml/d on 38th day, 1090 ml/d on 39th day, 1101 ml/d on 40th day, 1110 ml/d on 41st, 1113 ml/d on 42nd day and 1130 ml/d on 43rd day obtained at 55^{o}C respectively as shown in the figure.3. Hydrogen was derived from anaerobic degradation of organic matter, so the production rate of hydrogen was in tandem with OLR. Therefore, the increase in OLR from 3.54 and 4.21 Kg COD/m^{3}d to 42.16 and 43.17 Kg COD / m^{3}d also increased the production rate of hydrogen. The maximum hydrogen production at 35^{o}C was 1360 ml/d; it is comparatively higher than 1130 ml/d obtained at 55^{o}C. The variation in the hydrogen production rate can be attributed to variation in the microbial population and OLR (Hussy *et al.*, 2005). During dark fermentation, the medium endure pH changes that can significantly affect bio-H_2 production. Thus, in continuous process with pH control, a distinction between initial and operational pH must be done. The final pH reached during this study was 5 to 6 for both temperature regimes. This value was in agreement with the optimal ones reported for other industrial wastewaters such as rice winery wastewater (Yu *et al.*, 2002), food industry (Chu *et al.*, 2013), and dairy wastewater (Gadhe *et al.*, 2013). (Azbar *et al.*, 2009) During the bio-H_2 production from cheese whey wastewater at 36 ^{o}C and 55 ^{o}C. They observed a higher bio-H_2 yield at 36 ^{o}C (206 ml-H_2 gCOD^{-1}) than 55 ^{o}C (178 mL-H_2 gCOD^{-1}). They (Lee *et al.*, 2008) (Borges T *et al.*, 2018) also reported that the temperature at 37 ^{o}C is preferably compared

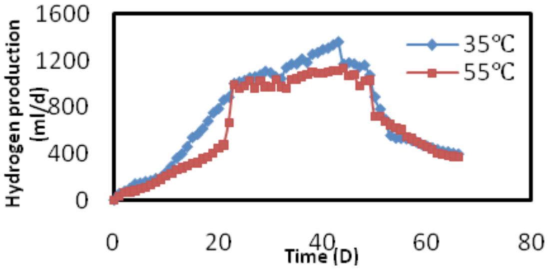


Fig. 3. Biological hydrogen production at 35^{o}C and 55^{o}C

to 55°C for the bio-H_2 production from cassava starch wastewater. (Han *et al.*, 2012) reported that the OLR between 24-32 kg COD/m^3 d enhanced the HPR of 326 mL/L.h at 35°C in a continuous stirred tank reactor (CSTR). Finally, it was reported that the mesophilic (35 °C) was more efficient than thermophilic (55 °C) for H_2 production from Distillery spent wash in anaerobic Continuous Stirred Tank Reactor.

Substrate degradation at mesophilic and thermophilic bio H_2 production

The steady state COD removal efficiencies obtained at 35 °C were 56% on 24th to 28th day, 68% on 38th to 40th day and 72% obtained on 46th to 58th day respectively. Whereas, the maximum COD removal of 55% obtained on 57th to 73rd day for 55 °C were presented in figures 4 and 5. The lowest efficiency in COD removal in both mesophilic and thermophilic temperature studies may be attributed to the minimal acclimatization period available (Mullai *et al.*, 2013a). As the biomass concentration was increased by the use of the substratum with the maximum COD removal of 72% achieved on 46th day at 35 °C. The COD removal efficiency was found to be higher at 35 °C, as compared to 55 °C. Gradual growth in the OLR created a new environment for micro-organism adaptation, resulting in a fluctuation before achieving stable efficiency in COD removal (Chang and Lin, 2014).

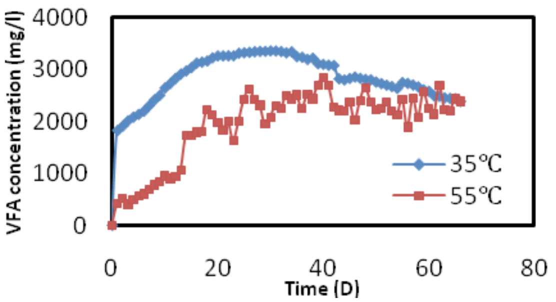


Fig. 4. COD removal percentage in a CSTR runs at 35°C and 55°C

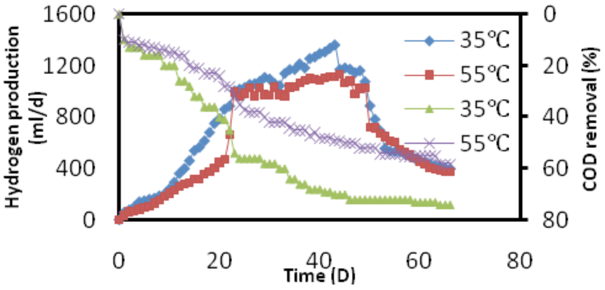


Fig. 5. Hydrogen production vs COD removal in a CSTR runs at 35°C and 55°C

Metabolic pathways involved in bio H_2 production

The VFA concentrations of acidogenic reactor were presented in the figure 6, acetic and propionic acids make up the bulk of volatile acids accumulated inside the reactor (Show *et al.*, 2004). The maximum VFA concentration was found at HRT of 16 hours with the hydrogen production of 1094 ml/d and minimum VFA concentration was found at HRT of 24 hours with hydrogen production of 56 ml/d at 35 °C. However, the VFA production of 426 to 2654 mg/l with hydrogen production of 1130 ml/d occurs at VFA concentrations of 2197 mg/l at 55 °C. VFA concentration defines the hydrogen production rate with respect to the reactor pH as illustrated in the Figure 7. VFA plays a major role in the production of hydrogen and also to maintain the reactor in the acidic condition. Hydrogen production by acidogenic microorganisms is always accompanied by the production of VFA. Therefore, changes in the VFA production reflect changes in the metabolism of the microorganisms. Moreover, pH has been confirmed as an important factor influencing the activities of hydrogen producing bacteria because it may affect the hydrogenase activity as well as metabolic pathways (Wang and Wan, 2009). The physiochemical stability of the reactor was continuously monitored by measuring alkalinity in the reactor. Figure.6 shows the alkalinity of acidogenic reactor; it was clear that the alkalinity in the acidogenic reactor was between 826 to 5827 mg/l at 35 °C and 242 to 4977 mg/l at 55 °C was attained. The maximum hydrogen production of 1360 ml/d gained at alkalinity of 4940 mg/l at 35 °C with CSTR. The Fig. 7 shows the VFA/Alkalinity ratio for acidogenic reactors run at 35 °C and 55 °C; it ranges between 2.2-0.5 and 1.8-0.5. Hence, the effluent VFA/Alkalinity ratio of the acidogenic reactor was more or less equal to 2, which demonstrate the proper functioning of the anaerobic

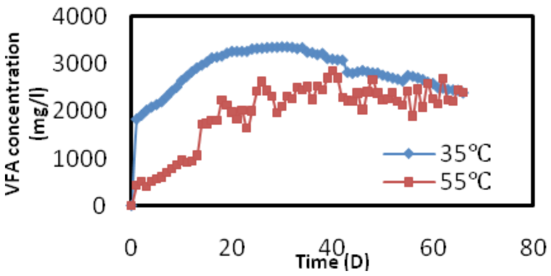


Fig. 6. VFA concentration in a CSTR runs at 35°C and 55°C

process. (Simpson, 1960) reported that the Volatile fatty Acid/Alkalinity ratio must be very low in range for stable anaerobic digester. It reveals that throughout the experimental period, volatile fatty acid accumulation was under the control and the reactor was in a stable condition.

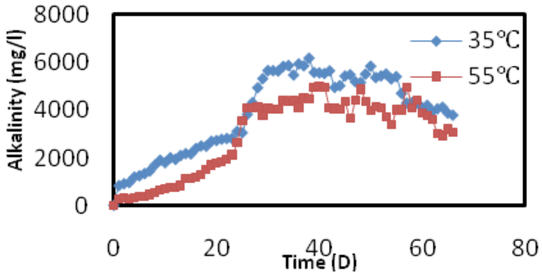


Fig. 7. Alkalinity concentration in a CSTR runs at 35°C and 55°C

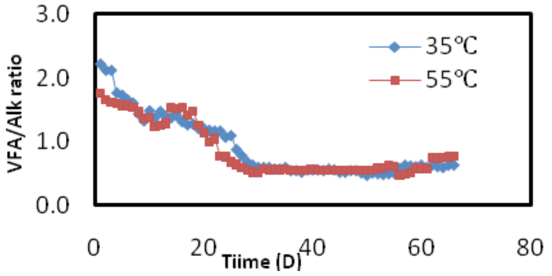


Fig. 8. VFA/Alkalinity ratio in a CSTR runs at 35°C and 55°C

In a stable reactor, Volatile Fatty Acid content will be low in proportion to the available alkalinity.

Biomass concentration and microbial identification

The biomass at different substrate concentrations after the cessation of hydrogen production is presented in the figure.9. It was apparent from the figure that the final biomass level concentration increased with an increasing COD concentration. The initial VSS concentration of 4.5 g/l was continuously increasing and decreasing in the biomass concentration and finally reduced in the VSS concentration. The maximum hydrogen production occurs at the biomass concentration of 5.5 g/l at 35 °C and 4.7 g/l at 55 °C with constant pH maintained between 5 and 6. However, a lower final pH was observed at a higher COD concentration. It reflects that the removed mixed wastewater was used by hydrogen-producing bacteria for their growth and organic acid production. This finding was in close agreement with that of (Heyndrickx *et al.*, 1987).

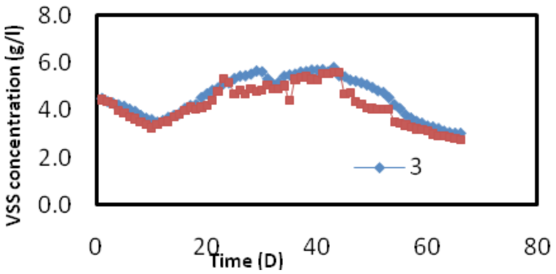
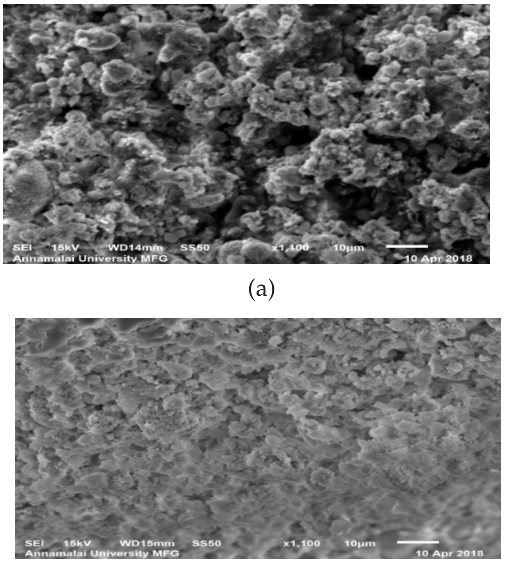


Fig. 9. VSS concentration in a CSTR runs at 35°C and 55 °C

The morphology of microbes in the sludge granules collected from the reactor. The sludge samples were first fixed for 2 h by soaking in an equal volume of 6% glutaraldehyde. After fixation, it was centrifuged and washed at least thrice in a phosphate buffer and kept overnight at 4°C. The samples were sequentially dehydrated using ethanol solutions of increasing concentrations: 10%, 15%, 30%, 50%, 75%, and 90%. And thereafter exposed to 100% ethanol wash for two times. This sample was dried at about 37 °C for two days and mounted on the sample holder of the SEM and coated with gold in a sputter coating unit. The SEM images of CSTR sludge granules showed rough and uneven surface with spherical and rod shaped, which closely resembles to *Clostridium sp*. (densely packed spherical shaped) and *bacillus sp.* (rod shaped) were dominant species in the granules as shown in the Figure 10.



(a)

(b)

Fig. 10. Scanning electron microscopy of the suspended bacteria in the CSTR reactor at various temperature 35°C (a) and 55°C (b)

CONCLUSION

Hydrogen produced from Distillery spent wash using a CSTR runs at mesophilic and thermophilic temperature with HRT of 24, 16, 12 and 8 h and pH maintained in the range 5 to 6. The steady state hydrogen production of 1360 ml/L·d was obtained at the OLR of 25.82 kg COD/(m3·d) with a COD removal of 70% at 35 °C and hydrogen production of 1130 ml/L.d with COD removal of 49% was obtained at the OLR of 28.52 kg COD/(m^3·d) at 55 °C. Through the continuous hydrogen production, a maximum 70% of the Distillery spent wash was converted to hydrogen in the mesophilic temperature (35 °C). This study provides suitable operational conditions for industrial application of the continuous process to maximize energy recovery from Distillery wastewater under mesophilic condition. These records are predominantly significant, when operating the anaerobic biodigesters for treating the distillery effluent along with the production of hydrogen as an energy sources. CSTR can effectively be employed in treatment of this effluent. However, post bio-digestion effluent still contains considerable COD. To meet the pollution norms and standards, it needs to be treated further. To understand the complex biological treatment process of this effluent, further trials are required to be conducted.

REFERENCES

APHA. 2005. *Standard Methods for the Examination of Water and Wastewater*, 20th ed., Washington, DC, USA: American Public Health Association.

Arimi, M.M., Knodel, J., Kiprop, A.; Namango, S.S., Zhang, Y. and Geiben, S.U. 2015. Strategies for improvement of biohydrogen production from organic-rich wastewater: A review. *Biomass and Bioenergy, Oxford*. 75 : 101-118.

Azbar, N., Dokgeoz, F.T., Keskin, T., Eltem, R., Korkmaz, K.S. and Gezgin, Y. 2009. Comparative evaluation of bio- hydrogen production from cheese whey wastewater under thermophilic and mesophilic anaerobic conditions. *Int J Green Energy*. 6: 192-200.

Bhavik K. Acharya, Sarayu Mohana, Datta Madamwar, 2008. Anaerobic treatment of distillery spentwash - A study on upflow anaerobic fixed lm bioreactor. *Bioresource Technology*. 99 (11) : 4621-4626.

Borges, T., Catucci, G., Rodrigues, L., Kimiko, I., Lima, L., Oliveira, D. 2018. Selection of metabolic pathways for continuous hydrogen production under thermophilic and mesophilic temperature conditions in anaerobic fluidized bed reactors. *Science Direct*. vol. 3.

Chang, S.H., Wu, C.H., Chang, D.K. and Lin, C.W. 2014. Effects of mediator producer and dissolved oxygen on electricity generation in a baffled stacking microbial fuel cell treating high strength molasses wastewater. *International Journal of Hydrogen Energy*. 39 (22) : 11722-11730.

Chu, C.Y., Tung, L. and Lin, C.Y. 2013. Effect of substrate concentration and pH on biohydrogen production kinetics from food industry wastewater by mixed culture. *Int J Hydrogen Energy*. 38 : 15849-15855

Gadhe, A., Sonawane, S.S. and Varma, M.N. 2013. Optimization of conditions for hydrogen production from complex dairy wastewater by anaerobic sludge using desirability function approach. *Int J Hydrogen Energy*. 38 : 6607-6617.

Han, W., Chen, H., Jiao, A., Wang, Z., Li, Y. and Ren, N.Q. 2012. Biological fermentative hydrogen and ethanol production using continuous stirred tank reactor. *Int J Hydrogen Energy*. 37 : 843-847.

Heyndrickx, M., De Vos, P., Hibau, B., Stevens, P. and De Ley, J. 1987. Effect of various external factors on the fermentative production of hydrogen gas from glucose by Clostridium butyricum strains in batch culture. *Systematic and Applied Microbiology*. 1; 9(1-2) : 163-168.

Hussy, I., Hawkes, F.R., Dinsdale, R. and Hawkes, D.L. 2005. Continuous fermentative hydrogen production from sucrose and sugarbeet. *International Journal of Hydrogen Energy*. 30 : 471-483.

Kapdan, I.K. and Kargi, F. 2006. Bio-hydrogen production from waste materials. *Enzym Microb Technol*. 38 : 569-582.

Kleerebezem, R. van Loosdrecht, M.C. 2007. Mixed culture biotechnology for bioenergy production. *Curr. Opin. Biotechnol.* 18 : 207-212.

Laxman Pachapur, V., Jyoti Sarma, S., Kaur Brar, S., Le Bihan, Y., Ricardo Soccol, C., Buelna, G. and Verma, M. 2015. Co-culture strategies for increased biohydrogen production. *Int. J. Energy Res.* 39: 1479-1504.

Lee, K.S., Hsu, Y.F., Lo, Y.C., Lin, P.J., Lin, C.Y. and Chang, J.S. 2008. Exploring optimal environmental factors for fermentative hydrogen production from starch using mixed anaerobic microflora. *Int J Hydrogen Energy*. 33 : 1565-1572.

Li, J., Li, B., Zhu, G., Ren, N., Bo, L. and He J. 2007. Hydrogen production from diluted molasses by anaerobic hydrogen producing bacteria in an anaerobic baffled reactor (ABR). *Int J Hydrogen Energy*. 32 : 3274-3283.

Lin, C.Y., Nguyen, T.M.L., Chu, C.Y., Leu, H.J. and Lay, C.H. 2018. Fermentative biohydrogen production and its byproducts: a mini review of current technology developments. *Renew Sustain Energy*

Rev. 82 : 4215-4220.

Mizuno, O., Dinsdale, R., Hawkes, F.R., Hawkes, D.L. and Noike, T. 2000. Enhancement of hydrogen production from glucose by nitrogen gas sparging. *Bioresour Technol*. 73 : 59-65.

Mullai, P. and Sobiya, E. 2014. Industrial Phytopesticide Wastewater Treatment using Methanogenic Consortium. *International Journal of Chem Tech Research*. 6 (12) : 4977-4983.

Nandi, R. and Sengupta, S. 1998. Microbial production of hydrogen: an overview. *Crit Rev Microbiol.* 24 : 61-84.

Otero-Rambla, M.A., Garcia, R., Perez, M.C., J.A. Martinez, Vasallo, M.C., Saura, G. and Bello, D. 2009. Producci_on de bioetanol a partir de mezclas de jugos-melazas de ca˜na de azu´ car ICIDCA. nu´m. 1, enero-abril. Sobre los Derivados de la Ca˜na de Azu´ car 2009;XLIII:17-22. Instituto Cubano de Investigaciones de los Derivados de la Ca˜na de Azu´ car. Disponible en:

Pachapur, V.L., Kutty, P., Brar, S.K. and Ramirez, A.A. 2016. Enrichment of Secondary Wastewater Sludge for Production of Hydrogen from Crude Glycerol and Comparative Evaluation of Mono-, Co- and Mixed-Culture Systems. *Int. J. Mol. Sci.* 16 (17): 92.

Pachapur, V.L., Sarma, S.J., Brar, S.K., Le Bihan, Y., Buelna, G. and Verma, M. 2015. Biological hydrogen production using co-culture versus mono-culture system. *Environ. Technol. Rev.* 4 : 55-70.

Ren, N., Li, J., Li, B., Wang, Y. and Liu, S. 2006. Biohydrogen production from molasses by anaerobic fermentation with a pilot-scale bioreactor system. *Int J. Hydrogen Energy*. 31 : 2147-21459

Shizas, I. and Bagley, D.M. 2005. Fermentative hydrogen production in a system using anaerobic digester sludge without heat treatment as a biomass source. *Water Science and Technology*. 52 (1-2) : 139-144.

Show, K.Y., Tay, J.H., Yang, L., Wang, Y. and Lua, C.H. 2004. Effects of stressed loading on startup and granulation in upflow anaerobic sludge blanket reactors. *Journal of Environmental Engineering.* 130 (7) : 743-750.

Simpson, J.R. 1960. Some aspects of bio-chemistry of anaerobic digestion in wastewater treatment, in Issac, P.C.G. (Ed.): *Waste Treatment*, Pub Pergamon Press, Oxford, pp. 31-51.

Sreethawong, T., Chatsiriwatana, S., Rangsunvigit, P. and Chavady, S. 2010. Hydrogen production from cassava wastewater using an anaerobic sequencing batch reactor: Effects of operational parameters, COD:N ratio, and organic acid composition. *International Journal of Hydrogen Energy, Oxford.* 35 : 4092-4102.

Temudo, M.F., Poldermans, R., Kleerebezem, R. and van Loosdrecht, M. 2008. Glycerol fermentation by (open) mixed cultures: A chemostat study. *Biotechnol. Bioeng.* 100 : 1088-1098.

Tuna, E., Kargi, F. and Argun, H. 2009. Hydrogen gas production by electrohydrolysis of volatile fatty acid (VFA) containing dark fermentation effluent. *Int J Hydrogen Energy*. 34 : 262-269.

Urbaniec, K. and Bakker, R.R. 2015.Biomass residues as raw material for dark hydrogen fermentation – A review. *International Journal of Hydrogen Energy, Oxford*. 40 : 3648-3658.

Wang, B., Yongfeng, L. and Nan-Qi, R. 2013. Biohydrogen from molasses with ethanol-type fermentation: effect of hydraulic retention time. *Int J Hydrogen Energy*. 38: 4361-4367.

Wang, J. and Wan, W., 2009. Factors influencing fermentative hydrogen production: a review. *International journal of Hydrogen Energy*. 34 (2) : 799-811.

Wang, J. and Wan, W. 2008. Comparison of different pretreatment methods for enriching hydrogen-producing bacteria from digested sludge. *Int. J. Hydrogen Energy*. 33 : 2934-2941.

Yu, H., Zhu, Z., Hu, W. and Zhang, H. 2002. Hydrogen production from rice winery wastewater in an upflow anaerobic reactor by using mixed anaerobic cultures*. Int J Hydrogen Energy*. 27 : 1359-1365.

Zhang, Z.P., Tay, J.H., Show, K.Y., Yan, R., Liang, D.T., Lee, D.J. and Jiang, W.J. 2007. Biohydrogen production in a granular activated carbon anaerobic fluidized bed reactor. *Int J Hydrogen Energy*. 32 : 185-191.

Zhu, H. and Beland, M. 2006. Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge. *Int. J. Hydrogen Energy*. 3 : 1980-1988.