OPTIMISATION OF BIOHYDROGEN PRODUCTION BY LOCALLY ISOLATED *Klebsiella* sp. STRAIN PR2

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OPTIMISATION OF BIOHYDROGEN PRODUCTION BY LOCALLY ISOLATED *Klebsiella* sp. STRAIN PR2

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Alhamdulillah, this thesis dedicated to UTM, my family and my dearest friends.

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ABSTRACT

Nowadays, 80% of the world energy requirement depends on fossil fuels. This ultimately will lead to a reduction in global fossil fuel resources. Hydrogen is a sustainable, clean source of energy and can be produced through biological process by microorganisms. The biohydrogen production is important due to both environmental and economic factors, since waste substrates can be used to generate biogas rich in biohydrogen. The aim of this study was to isolate biohydrogen producing bacteria from agricultural wastewater and to optimise the biohydrogen production using enrichment medium. Out of 25 bacteria obtained, only 13 isolates had the ability to produce biohydrogen. One isolate that produced the highest biohydrogen was selected for 16S rDNA sequence identification. The bacterium was identified as *Klebsiella* sp. strain PR2. For the optimisation of biohydrogen production, fermentation was carried out in batch culture, where parameters such as carbon and nitrogen source concentration, initial pH of the medium, inoculum size, as well as incubation temperature were investigated. D-mannitol was found to be the best carbon source while peptone was the best nitrogen source for biohydrogen production with concentration of 0.2% (w/v) and 0.5% (w/v), respectively. The optimum condition for biohydrogen production was achieved at the initial pH 8 of the medium, incubation temperature of 30 °C and 10% (v/v) of inoculum. After 24 hours of incubation, the highest yield obtained ($Y_{P/X}$) was 8547.42 g/g while the highest biohydrogen productivity was 177.24 mol/L/h. Biohydrogen production using palm oil mill effluent (POME) as substrate was also conducted. Fermentation was carried out in sterile raw and final discharge POME by inoculating with 10% (v/v) bacteria inoculum and incubated at 30 ^{o}C for 5 days. The maximum biohydrogen productivity from raw and final discharge POME were 1.44 mol/L/h and 1.81 mol/L/h, respectively. The highest colour removal were 38% (initial: 18167 ADMI) for raw discharge POME and 31% (initial: 1873 ADMI) for final discharge POME were obtained after 5 days of treatment. Meanwhile, the highest COD removal were 34.8% (initial: 7320 mg/L) and 50.8% (initial: 1410 mg/L) for the raw and final discharge POME, respectively. From this study, it could be concluded that *Klebsiella* sp. strain PR2 has potential applications for biohydrogen production utilising POME as substrate besides treating the POME through biodegradation process.

ABSTRAK

Pada masa kini, 80% daripada keperluan tenaga dunia bergantung kepada bahan api fosil. Ini akhirnya akan membawa kepada pengurangan sumber bahan api fosil global. Hidrogen adalah sumber tenaga yang lestari, bersih dan boleh dihasilkan melalui proses biologi oleh mikroorganisma. Penghasilan biohidrogen adalah penting disebabkan oleh faktor alam sekitar dan ekonomi, memandangkan substrat sisa boleh digunakan untuk menghasilkan biogas yang kaya dengan biohidrogen. Tujuan kajian ini adalah untuk memencilkan bakteria yang boleh menghasilkan biohidrogen daripada air sisa pertanian dan mengoptimumkan pengeluaran biohidrogen menggunakan media yang diperkaya. Daripada 25 bakteria yang diperoleh, hanya 13 bakteria yang mempunyai keupayaan menghasilkan biohidrogen. Satu bakteria yang menghasilkan biohidrogen tertinggi dipilih untuk dikenalpasti melalui jujukan 16S rDNA. Bakteria tersebut dikenali sebagai *Klebsiella* sp. strain PR2. Bagi pengoptimuman penghasilan biohidrogen, fermentasi dijalankan dalam kultur kelompok, di mana parameter seperti kepekatan sumber karbon dan nitrogen, pH awal medium, saiz inokulum, serta suhu inkubasi telah dikaji. D-manitol dikenalpasti sebagai sumber karbon terbaik manakala pepton adalah sumber nitrogen yang terbaik untuk penghasilan biohidrogen dengan kepekatan 0.2% (b/i) dan 0.5% (b/i), masing- masing. Keadaan optimum untuk penghasilan biohidrogen dicapai pada pH awal medium 8, suhu inkubasi 30 °C dan 10% (i/i) inokulum. Selepas 24 jam inkubasi, hasil biohidrogen tertinggi diperolehi ($Y_{P/X}$) adalah 8547.42 g/g manakala produktiviti tertinggi adalah 177.24 mol/L/h. Penghasilan biohidrogen menggunakan sisa efluen kelapa sawit (POME) sebagai substrat juga dijalankan. Fermentasi telah dilakukan dalam POME mentah dan POME pelepasan akhir yang steril dengan menambah 10% (i/i) inokulum bakteria dan diinkubasi pada 30 ^{o}C selama 5 hari. Produktiviti biohidrogen maksimum dari POME mentah dan POME pelepasan akhir adalah 1.44 mol/L/h dan 1.81 mol/L/h, masing-masing. Penyingkiran warna tertinggi adalah 38% (awal: 18167 ADMI) untuk POME mentah dan 31% (awal: 1873 ADMI) untuk POME pelepasan akhir diperoleh selepas 5 hari rawatan. Sementara itu, penyingkiran COD tertinggi adalah 34.8% (awal: 7320 mg/L) dan 50.8% (awal: 1410 mg/L) untuk POME mentah dan POME pelepasan akhir, masing-masing. Dari kajian ini, dapat disimpulkan bahawa *Klebsiella* sp. strain PR2 berpotensi untuk menghasilkan biohidrogen menggunakan POME sebagai substrat di samping merawat POME melalui proses biodegradasi.

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LIST OF ABBREVIATIONS

pH	-	Hydrogen ion concentration
H_2	-	Hydrogen gas
H^+	-	Hydrogen ion
dw	-	Dry weight
ADMI	-	American Dye Manufacturing Institute
C	-	Carbon
N	-	Nitrogen
$C_6H_{12}O_6$	-	Glucose
H_2O	-	Water
CH_3COOH	-	Acetic acid
CO_2	-	Carbon dioxide
CO	-	Carbon monoxide
NH_4Cl	-	Ammonium chloride
KH_2PO_4	-	Potassium dihydrogen phosphate
$MgCl_2$	-	Magnesium chloride
$CaCl_2$	-	Calcium chloride
NaCl	-	Sodium chloride
K_2HPO_4	-	Potassium hydrogen phosphate
$MgSO_4$	-	Magnesium sulphate
$MnSO_4$	-	Manganese(II) sulfate

$FeSO_4$	-	Iron(II) sulfate
HCl	-	Hydrochloric acid
MoO_3	-	Molybdenum trioxide
$ZnSO_4$	-	Zinc sulfate
$CuSO_4$	-	Copper(II) sulfate
H_3BO_3	-	Boric acid
CoCl	-	Cobalt(II) chloride
NaOH	-	Sodium hydroxide
ppm	-	Parts per million
PEG	-	Polyethylene glycol
H_2SO_4	-	Sulphuric acid
$AgSO_4$	-	Silver(II) sulphate
K_2Cr_2O	-	Potassium dichromate
DNA	-	Deoxyribonucleic acid
PCR	-	Polymerase chain reaction
EDTA	-	Ethylenediaminetetraacetic acid

LIST OF SYMBOLS

°C	-	Degree celcius
h	-	Hour
mL	-	Milliliter
L	-	Liter
%	-	Percent
v/v	-	Volume over volume
w/v	-	Weight over volume
µM	-	Micromolar
rpm	-	Rotation per minute
µL	-	Microliter

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Hydrogen gas is an ideal, clean, sustainable energy resource that is harmless to the environment (Suzuki, 1982). Hydrogen can be produced through electrochemical and thermochemical processes. However, it is more environmentally clean when produced through biological process (Nowotny *et al.*, 2016; Das, 2001). Hydrogen gas is commercially used in industries for chemicals and electronic devices production, steel processing, also hydrogenation of oils and fats in food industries (Kapdan and Kargi, 2006). Furthermore, the use of hydrogen as an energy resource is more profitable if it can be produced naturally or at a low cost. The use of wastewater such as palm oil mill effluent (POME) containing microorganisms is one of the approaches for biohydrogen production.

Biological processes of hydrogen production are normally assisted by microorganisms in water at ambient temperature and pressure. The biohydrogen generation system are usually carried out by different type of microorganisms such as photosynthetic bacteria, fermentative bacteria, green algae and cyanobacteria (Das, 2001). Microorganisms have their own unique mechanisms of biohydrogen production depending on the raw materials used. In addition, the environment condition are also important and should be well suited for microorganisms to catalyse the substrate (Das *et al.*, 2008).

Malaysia is one of the world's largest exporter and producer of palm oil and its products, due to rapid development over the centuries in oil palm plantation. Nearly four million hectares or more than one-third of the land area is used for oil palm plantation in 2003 (Yusoff and Hansen, 2007). Based on the statistics reported by the Malaysian Palm Oil Board (MPOB) recently, the land use for oil palm plantation has increased to 4.49 million hectares and Sabah contributes the largest land area for oil palm cultivation. Rapid development on palm oil plantation has given impact on economic growth in palm oil mill industries but indirectly this industry is also generating a lot of wastes such as POME. Therefore, it is an advantage to use the available resources for biohydrogen production in this study. Recently, biohydrogen production using POME was conducted. Based on the research reported, the main factor that influenced the biohydrogen production was inoculum size and the optimum inoculum size was 40% of POME sludge. The highest yield of biohydrogen achieved was 28.47 ml H_2 / g COD removed (Zainal *et al.*, 2018).

1.2 Problem Statement

Nowadays, 80% of the world energy requirement depends on fossil fuels. This ultimately will lead to a reduction in global fossil fuel resources. Besides that, the combustion of fossil fuels has affects the global environment mainly due to the discharge of pollutants such as carbon dioxide, sulfur dioxide, ash, soot and other compounds that can cause greenhouse effect. With the intention to recover the limitation of fossil fuels, also to produce cleaner and environment friendly combustion effect, hydrogen has been recommended as an alternative energy resource (Das, 2001).

Energy value per unit of weight carried out by hydrogen is higher than other resources of energies such as gasoline, methane, natural gas, diesel, ethanol, methanol, coal (anthracite) and wood (Basak and Das, 2007; Lam and Lee, 2011; Azwar *et al*., 2014). Therefore, more energy can be supplied by hydrogen for combustion per unit quantity. These properties make hydrogen an attractive alternative source for the energy system (Lam and Lee, 2011). Hydrogen is a clean energy that produces water as the only by-product (Johnston *et al*., 2005). Wastewater treatment plant is one of the resources for biogas production. The hydrogen gas will be used as fuel and fed into fuel cells to generate electricity for the plant (Lam and Lee, 2011). The biohydrogen production is important due to both environmental, since the process can improve the characteristics of agroindustrial wastewater, as well as the economics, since waste substrates can be used to generate biogas rich in hydrogen, a clean energy sources.

1.3 Objectives of Research

The aim of this research were to produce clean energy as well as to reduce wastes in the environment. The objectives of this research were:

i. To isolate and screen for biohydrogen producing bacteria from agroindustrial wastewater
ii. To identify selected biohydrogen producing bacteria and optimise the hydrogen production
iii. To determine the biohydrogen production from POME using selected bacteria

1.4 Scope of Research

This research was initiated to isolate bacteria from several types of wastewater including POME, palm oil refinery effluent, tapioca mill effluent and pineapple mill effluent prior to screening of biohydrogen producing microbes. The effects of carbon and nitrogen source concentrations towards biohydrogen production on selected bacteria were investigated. The optimum condition for biohydrogen production such as pH, temperature and inoculum size were determined. Once the optimised condition were achieved, this study was further continued on determining the potential of biohydrogen production from POME using selected bacteria.

1.5 Research Significance

Hydrogen gas is a source of renewable energy, which will not cause the greenhouse effect. So, it is safe and environment friendly to use hydrogen gas as a resource to generate electricity or energy. In this study, optimum condition for hydrogen production from bacteria will be determined. The results can be used as an input to improve wastewater treatment especially for POME in term of renewable energy generation in commercial scale. Integration of waste treatment and biogas facilities has a great prospective for industries (Lam and Lee, 2011).

The production of these clean bioenergy is in compliance with the national and international policy as well as the worldwide effort to develop alternative renewable and sustainable resource of energy. Thus, creating green and sustainable environment could be achieved and at the same time, the utilisation of waste could be the key to developing a new energy carrier for the future energy resources.

REFERENCES

Abdurahman, N.H., Rosli, Y.M. and Azhari, N.H. (2013). The performance evaluation of anaerobic methods for palm oil mill effluent (POME) treatment: a review. In *International Perspectives on Water Quality Management and Pollutant Control*. InTech, pp.87–106.

Ahmad, A., Ghufran, R. and Wahid, Z.A. (2011). Bioenergy from anaerobic degradation of lipids in palm oil mill effluent. *Reviews in Environmental Science and Biotechnology*. 10(4), 353–376.

Ahmad, A.L., Chong, M.F., Bhatia, S. and Ismail, S. (2006). Drinking water reclamation from palm oil mill effluent (POME) using membrane technology. *Desalination*. 191(1–3), 35–44.

Ahmad Kamal, S., Mansor, M.F., Mohd Jahim, J. and Anuar, N. (2011). Effect of pre-treatment palm oil mill effluent POME on biohydrogen production by local isolate *Clostridium butyricum*. *Advanced Materials Research*. 236–238, 2987-2992.

Allakhverdiev, S.I., Thavasi, V., Kreslavski, V.D., Zharmukhamedov, S.K., Klimov, V. V., Ramakrishna, S., Los, D.A., Mimuro, M., Nishihara, H. and Carpentier, R. (2010). Photosynthetic hydrogen production. *Journal of Photochemistry and Photobiology C: Photochemistry Reviews*. 11(2–3), 101–113.

American Public Health Association (APHA), American Water Works Association and Water Environment Federation (2005). *Standard Methods for the Examination of Water and Wastewater 21st Edition*,

Assas, N., Ayed, L., Marouani, L. and Hamdi, M. (2002). Decolorization of fresh and stored-black olive mill wastewaters by *Geotrichum candidum*. *Process Biochemistry*. 38(3), 361–365.

Atif, A., Fakhrulrazi, A., Ngan, M., Morimoto, M., Iyuke, S. and Veziroglu, N. (2005). Fed batch production of hydrogen from palm oil mill effluent using anaerobic microflora. *International Journal of Hydrogen Energy*. 30(13–14), 1393–1397.

Azman, N.F., Abdeshahian, P., Al-Shorgani, N.K.N., Hamid, A.A. and Kalil, M.S. (2016). Production of hydrogen energy from dilute acid-hydrolyzed palm oil mill effluent in dark fermentation using an empirical model. *International Journal of Hydrogen Energy*. 41(37), 16373–16384.

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.

Badiei, M., Jahim, J.M., Anuar, N. and Sheikh Abdullah, S.R. (2011). Effect of hydraulic retention time on biohydrogen production from palm oil mill effluent in anaerobic sequencing batch reactor. *International Journal of Hydrogen Energy*. 36(10), 5912–5919.

Bae, S., Kwak, K., Kim, S., Chung, S. and Igarashi, Y. (2001). Isolation and characterization of CO_2-fixing hydrogen-oxidizing marine bacteria. *Journal of Bioscience and Bioengineering*. 91(5), 442–448.

Barbosa, M.J., Rocha, J.M.S., Tramper, J. and Wijffels, R.H. (2001). Acetate as a carbon source for hydrogen production by photosynthetic bacteria. *Journal of Biotechnology*. 85(1), 25–33.

Basak, N. and Das, D. (2007). The prospect of purple non-sulfur (PNS) photosynthetic bacteria for hydrogen production: the present state of the art. *World Journal of Microbiology and Biotechnology*. 23(1), 31–42.

Berman, H.M., Jeffrey, G.A., Rosenstein, R.D. and IUCr (1968). The crystal structures of the α’ and β forms of D-mannitol. *Acta Crystallographica Section B Structural Crystallography and Crystal Chemistry*. 24(3), 442–449.

Boodhun, B.S.F., Mudhoo, A., Kumar, G. and Kim, S.-H. (2017). Research perspectives on constraints, prospects and opportunities in biohydrogen production. *International Journal of Hydrogen Energy*. 42(45), 27471–27481.

Borja, R. and Banks, C.J. (1994). Treatment of palm oil mill effluent by upflow anaerobic filtration. *Journal of Chemical Technology and Biotechnology*. 61(2), 103–109.

Cai, J. and Wang, G. (2012). Hydrogen production by a marine photosynthetic bacterium, *Rhodovulum sulfidophilum* P5, isolated from a shrimp pond. *International Journal of Hydrogen Energy*. 37(20), 15070–15080.

Chong, M., Rahim, R., Shirai, Y. and Hassan, M. (2009). Biohydrogen production by *Clostridium butyricum* EB6 from palm oil mill effluent. *International Journal of Hydrogen Energy*. 34(2), 764–771.

Chookaew, T., Thong, O. S. and Prasertsan, P. (2014). Biohydrogen production from crude glycerol by immobilized *Klebsiella* TR17 in a UASB reactor and bacterial quantification under non-sterile conditions. *International Journal of Hydrogen Energy*. 39(18), 9580–9587.

Chookaew, T., Thong, O. S. and Prasertsan, P. (2012). Fermentative production of hydrogen and soluble metabolites from crude glycerol of biodiesel plant by the newly isolated thermotolerant *Klebsiella pneumoniae* TR17. *International Journal of Hydrogen Energy*. 37(18), 13314–13322.

Chung, S.Y.Y., Maeda, M., Song, E., Horikoshij, K. and Kudo, T. (1994). A gram-positive polychlorinated biphenyl-degrading bacterium, *Rhodococcus erythropolis* strain ta421, isolated from a termite ecosystem. *Bioscience, Biotechnology and Biochemistry*. 58(11), 2111–2113.

Correia, R.T.P., McCue, P., Magalhães, M.M.A., Macêdo, G.R. and Shetty, K. (2004). Production of phenolic antioxidants by the solid-state bioconversion of pineapple waste mixed with soy flour using *Rhizopus oligosporus*. *Process Biochemistry*. 39(12), 2167–2172.

Das, D. (2001). Hydrogen production by biological processes: a survey of literature. *International Journal of Hydrogen Energy*. 26(1), 13–28.

Das, D., Khanna, N. and Veziroğlu, N. (2008). Recent developments in biological hydrogen production processes. *Chemical Industry and Chemical Engineering Quarterly*. 14(2), 57–67.

Devereux, R. and Wilkinson, S.S. (2004). Amplification of ribosomal RNA sequences. *Molecular Microbial Ecology Manual*. 01, 509–522.

Drancourt, M., Bollet, C., Carta, A. and Rousselier, P. (2001). Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with description of *Raoultella ornithinolytica* comb. nov., *Raoultella terrigena* comb.

nov. and *Raoultella planticola* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*. 51(3), 925–932.

Estevam, *et. al*., (2018). Production of biohydrogen from brewery wastewater using *Klebsiella pneumoniae* isolated from the environment. *International Journal of Hydrogen Energy*. 43(9), 4276–4283.

Fang, H.H.P., Zhang, T. and Liu, H. (2002). Microbial diversity of a mesophilic hydrogen-producing sludge. *Applied Microbiology and Biotechnology*. 58(1), 112–118.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*. 39(4), 783–791.

Forsberg, C.W. (2007). Future hydrogen markets for large-scale hydrogen production systems. *International Journal of Hydrogen Energy*. 32(4), 431–439.

Fouts, D.E., *et. al*., (2008). Complete genome sequence of the N_2-fixing broad host range endophyte *Klebsiella pneumoniae* 342 and virulence predictions verified in mice D. S. Guttman, ed. *PLoS Genetics*. 4(7), e1000141.

Gerardi, M.H. (2010). *Troubleshooting the Sequencing Batch Reactor*, Hoboken, NJ, USA: John Wiley & Sons, Inc.

Grady, C.P.L., Grady, C.P.L. and Grady, C.P.L. (2011). *Biological Wastewater Treatment*, CRC Press.

Hach, C.C., Robert L. Klein, J. and Gibbs, C.R. (1997). *Introduction to Biochemical Oxygen Demand*, U.S.A: Hach Company.

Hallenbeck, P.C. (2009). Fermentative hydrogen production: Principles, progress, and prognosis. *International Journal of Hydrogen Energy*. 34(17), 7379–7389.

Hallenbeck, P.C., Hashesh, M. A. and Ghosh, D. (2012). Strategies for improving biological hydrogen production. *Bioresource Technology*. 110, 1–9.

Hanson, R., Phillips, J. and Gherhardt, P. (1981). Manual of methods for general bacteriology. *American Society for Microbiology*.

Hillmer, P. and Gest, H. (1977). H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas* capsulata: H_2 production by growing cultures. *Journal of Bacteriology*. 129(2), 724–31.

Holladay, J.D., Hu, J., King, D.L. and Wang, Y. (2009). An overview of hydrogen production technologies. *Catalysis Today*. 139(4), 244–260.

Igwe, J.C. and Onyegbado, C.C. (2007). A review of palm oil mill effluent (POME) water treatment. *Global Journal of Environmental Research*. 1(2), 54–62.

Ismail, I., Hassan, M.A., Abdul Rahman, N.A. and Soon, C.S. (2010). Thermophilic biohydrogen production from palm oil mill effluent (POME) using suspended mixed culture. *Biomass and Bioenergy*. 34(1), 42–47.

Jamal, P., Alam, M.Z. and Mohamad, A.B. (2007). Microbial bioconversion of palm oil mill effluent to citric acid with optimum process conditions. In *3rd Kuala Lumpur International Conference on Biomedical Engineering 2006*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp.483–487.

Jang, S., *et. al.*, (2015). Hydrogen fermentation of food waste by alkali-shock pretreatment: Microbial community analysis and limitation of continuous operation. *Bioresource Technology*. 186, 215–222.

Jin, H., Xu, Y., Lin, R. and Han, W. (2008). A proposal for a novel multi-functional energy system for the production of hydrogen and power. *International Journal of Hydrogen Energy*. 33(1), 9–19.

Johnston, B., Mayo, M.C. and Khare, A. (2005). Hydrogen: The energy source for the 21st century. *Technovation*. 25(6), 569–585.

Jung, G.Y., Jung, H.O., Kim, J.R., Ahn, Y. and Park, S. (1999). Isolation and characterization of *Rhodopseudomonas palustris* P4 which utilizes CO with the production of H_2. *Biotechnology Letters*. 21(6), 525–529.

Kapdan, I.K. and Kargi, F. (2006). Bio-hydrogen production from waste materials. *Enzyme and Microbial Technology*. 38(5), 569–582.

Kapdan, I.K., Kargi, F., Oztekin, R. and Argun, H. (2009). Bio-hydrogen production from acid hydrolyzed wheat starch by photo-fermentation using different *Rhodobacter International Journal of Hydrogen Energy*. 34(5), 2201–2207.

Kim, D.H. and Kim, M.S. (2011). Hydrogenases for biological hydrogen production. *Bioresource Technology*. 102(18), 8423–8431.

Kim, J.S., Iti, K. and Takahashi, H. (1980). The relationship between nitrogenase activity and hydrogen evolution in *Rhodopseudomonas palustris*. *Agricultural and Biological Chemistry*. 44(4), 827–833.

Kohlmiller, E.F. and Gest, H. (1951). A comparative study of the light and dark fermentations of organic acids by *Rhodo-spirillum rubrum*. *Journal of*

Bacteriology. 61(3), 269–82.

Koku, H., Eroğlu, I., Gündüz, U., Yücel, M. and Türker, L. (2003). Kinetics of biological hydrogen production by the photosynthetic bacterium *Rhodobacter sphaeroides* O.U. 001. *International Journal of Hydrogen Energy*. 28(4), 381–388.

Krishnan, S., *et. al.*, (2016). Process enhancement of hydrogen and methane production from palm oil mill effluent using two-stage thermophilic and mesophilic fermentation. *International Journal of Hydrogen Energy*. 41(30), 12888–12898.

Kumar, S., Stecher, G. and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular biology and evolution*. 33(7), 1870–1874.

Lam, M.K. and Lee, K.T. (2011). Renewable and sustainable bioenergies production from palm oil mill effluent (POME): Win-win strategies toward better environmental protection. *Biotechnology Advances*. 29(1), 124–141.

Lane, D.J. (1991). *Nucleic acid techniques in bacterial systematics*, Univ New Brunswick.

Lazaro, C.Z., Varesche, M.B.A. and Silva, E.L. (2015). Effect of inoculum concentration, pH, light intensity and lighting regime on hydrogen production by phototrophic microbial consortium. *Renewable Energy*. 75, 1–7.

Lee, C. (2002). Photohydrogen production using purple nonsulfur bacteria with hydrogen fermentation reactor effluent. *International Journal of Hydrogen Energy*. 27(11–12), 1309–1313.

Li, L., Li, A. and Ma, F. (2011). Isolation, Identification and Optimization of Culture Conditions of Photosynthetic Bacteria. In *2011 5th International Conference on Bioinformatics and Biomedical Engineering*. Wuhan,Hubei,China: IEEE, pp.1–4.

Liu, F. and Fang, B. (2007). Optimization of bio-hydrogen production from biodiesel wastes by *Klebsiella pneumoniae*. *Biotechnology Journal*. 2(3), 374–380.

Mahmod, S.S., Jahim, J.M. and Abdul, P.M. (2017). Pretreatment conditions of palm oil mill effluent (POME) for thermophilic biohydrogen production by mixed culture. *International Journal of Hydrogen Energy*. 42(45).

Martínez, J., Martínez, L., Rosenblueth, M., Silva, J. and Martínez-Romero, E. (2004). How are gene sequence analyses modifying bacterial taxonomy? The case of *Klebsiella*. *International Microbiology*. 7(4), 261–268.

Meher Kotay, S. and Das, D. (2008). Biohydrogen as a renewable energy resource-Prospects and potentials. *International Journal of Hydrogen Energy*. 33(1), 258–263.

Minnan, L. *et. al.*, (2005). Isolation and characterization of a high H_2-producing strain *Klebsiella oxytoca* HP1 from a hot spring. *Research in Microbiology*. 156(1), 76–81.

Mohammadi, P., Ibrahim, S., Mohamad, M.S.A and Law, S. (2011). Effects of different pretreatment methods on anaerobic mixed microflora for hydrogen production and COD reduction from palm oil mill effluent. *Journal of Cleaner Production*. 19(14), 1654–1658.

Najafpour, G.D., Hashemiyeh, B.A., Asadi, M. and Ghasemi, M.B. (2008). Biological treatment of dairy wastewater in an upflow anaerobic sludge-fixed film bioreactor. *American-Eurasian J. Agric. and Environ. Sci.* 4(2), 251–257.

Neoh, C.H., Yahya, A., Adnan, R., Abdul, Z. M. and Ibrahim, Z. (2013). Optimization of decolorization of palm oil mill effluent (POME) by growing cultures of *Aspergillus fumigatus* using response surface methodology. *Environmental Science and Pollution Research*. 20(5), 2912–2923.

Niu, K., Zhang, X., Tan, W.S. and Zhu, M.L. (2010). Characteristics of fermentative hydrogen production with *Klebsiella pneumoniae* ECU-15 isolated from anaerobic sewage sludge. *International Journal of Hydrogen Energy*. 35(1), 71–80.

Norfadilah, N., Raheem, A., Harun, R. and Ahmadun, F.F. (2016). Bio-hydrogen production from palm oil mill effluent (POME): A preliminary study. *International Journal of Hydrogen Energy*. 41(28), 11960–11964.

Nowotny, J., *et. al.*, (2016). Towards sustainable energy. Generation of hydrogen fuel using nuclear energy. *International Journal of Hydrogen Energy*. 41(30), 12812–12825.

Prasertsan, P., Choorit, W. and Suwanno, S. (1993). Isolation, identification and growth conditions of photosynthetic bacteria found in seafood processing wastewater. *World Journal of Microbiology and Biotechnology*. 9(5), 590–592.

Pugazhendhi, A. and Thamaraiselvi, K. (2017). Optimization of fermentative hydrogen production by *Klebsiella pneumoniae* KTSMBNL 11 isolated from municipal sewage sludge. *Environmental Science and Engineering (Subseries: Environmental Science)*. (9783319484389), 267–278.

Rasdi, Z., *et. al.*, (2009). Statistical optimization of biohydrogen production from palm oil mill effluent by natural microflora. *The Open Biotechnology Journal*. 3(1), 79–86.

Rasdi, Z., Mumtaz, T., Abdul Rahman, N. and Hassan, M.A. (2012). Kinetic analysis of biohydrogen production from anaerobically treated POME in bioreactor under optimized condition. *International Journal of Hydrogen Energy*. 37(23), 17724–17730.

Reungsang, A. and Sreela-or, C. (2013). Bio-hydrogen production from pineapple waste extract by anaerobic mixed cultures. *Energies*. 6(4), 2175–2190.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*. 4(4), 406–425.

Sasaki, K. (1998). Hydrogen and 5-aminolevulinic acid production by photosynthetic bacteria. In *BioHydrogen*. Boston, MA: Springer US, pp.133–142.

Seengenyoung, J., Prasertsan, P. and Sompong, O.T. (2013). Biohydrogen production from palm oil mill effluent pretreated by chemical methods using thermoanaerobacterium-rich sludge. *Iranica Journal of Energy & Environment IJEE an Official Peer Reviewed Journal*. 4(4), 312–319.

Seghezzo, L., Zeeman, G., Van, J.B.L., Hamelers, H.V.M. and Lettinga, G. (1998). A review: The anaerobic treatment of sewage in UASB and EGSB reactors. *Bioresource Technology*. 65(3), 175–190.

Singh, L., *et. al.*, (2013). Application of immobilized upflow anaerobic sludge blanket reactor using *Clostridium* LS2 for enhanced biohydrogen production and treatment efficiency of palm oil mill effluent. *International Journal of Hydrogen Energy*. 38(5), 2221–2229.

Singh, P. and Thakur, I. (2004). Removal of colour and detoxification of pulp and paper mill effluent by microorganisms in two step bioreactor. *Journal of Scientific* *and Industrial Research*. 63(11), 944–948.

Sinha, P. and Pandey, A. (2011). An evaluative report and challenges for fermentative biohydrogen production. *International Journal of Hydrogen Energy*. 36(13), 7460–7478.

Sivashanmugam, K. and Jayaraman, G. (2013). Production and partial purification of extracellular tannase by *Klebsiella pneumoniae* MTCC 7162 isolated from tannery effluent. *African Journal of Biotechnology*. 10(8), 1364–1374.

Stuart, H. (2005). *Essential Microbiology*, The University of Glamorgan, UK: Johh Wiley & Son, Ltd.

Suzuki, Y. (1982). On hydrogen as fuel gas. *International Journal of Hydrogen Energy*. 7(3), 227–230.

Taifor, A.F., *et. al.*, (2017). Elucidating substrate utilization in biohydrogen production from palm oil mill effluent by *Escherichia coli*. *International Journal of Hydrogen Energy*. 42(9), 5812–5819.

Tamura, K., Nei, M. and Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences*. 101(30), 11030–11035.

Tao, Y., *et. al.*, (2008). Characteristics of a new photosynthetic bacterial strain for hydrogen production and its application in wastewater treatment. *International Journal of Hydrogen Energy*. 33(3), 963–973.

Torsvik, V. and Øvreås, L. (2008). Microbial diversity, life strategies, and adaptation to life in extreme soils. In *Microbiology of Extreme Soils*. Springer Berlin Heidelberg, pp.15–43.

Wang, J. and Wan, W. (2008). Effect of temperature on fermentative hydrogen production by mixed cultures. *International Journal of Hydrogen Energy*. 33(20), 5392–5397.

Wang, L., Hung, Y., Lo, H. and Yapijakis, C. (2004). *Handbook of industrial and hazardous wastes treatment*, New York: Marcel Dekker, Inc.

Wang, X., Hoefel, D., Saint, C.P., Monis, P.T. and Jin, B. (2007). The isolation and microbial community analysis of hydrogen producing bacteria from activated sludge. *Journal of Applied Microbiology*. 103(5), 1415–1423.

Wang, X., Ren, N., Shengxiang, W. and Qianguo, W. (2007). Influence of gaseous end-products inhibition and nutrient limitations on the growth and hydrogen production by hydrogen-producing fermentative bacterial B49. *International Journal of Hydrogen Energy*. 32(6), 748–754.

Wu, K.-J. and Chang, J.-S. (2007). Batch and continuous fermentative production of hydrogen with anaerobic sludge entrapped in a composite polymeric matrix. *Process Biochemistry*. 42(2), 279–284.

Wu, K.J., Lin, Y.H., Lo, Y.C., Chen, C.Y., Chen, W.M. and Chang, J.S. (2011). Converting glycerol into hydrogen, ethanol, and diols with a *Klebsiella* HE1 strain via anaerobic fermentation. *Journal of the Taiwan Institute of Chemical Engineers*. 42(1), 20–25.

Wu, T.Y., Mohammad, A.W., Jahim, J.M. and Anuar, N. (2010). Pollution control technologies for the treatment of palm oil mill effluent (POME) through end-of-pipe processes. *Journal of Environmental Management*. 91(7), 1467–1490.

Xiao, Y., Zhang, X., Zhu, M. and Tan, W. (2013). Effect of the culture media optimization, pH and temperature on the biohydrogen production and the hydrogenase activities by *Klebsiella pneumoniae* ECU-15. *Bioresource Technology*. 137, 9–17.

Xu, J.F., Ren, N.Q., Wang, A.J., Qiu, J., Zhao, Q.L., Feng, Y.J. and Liu, B.F. (2010). Cell growth and hydrogen production on the mixture of xylose and glucose using a novel strain of *Clostridium* HR-1 isolated from cow dung compost. *International Journal of Hydrogen Energy*. 35(24), 13467–13474.

Yacob, S., Ali Hassan, M., Shirai, Y., Wakisaka, M. and Subash, S. (2006). Baseline study of methane emission from anaerobic ponds of palm oil mill effluent treatment. *Science of the Total Environment*. 366(1), 187–196.

Yu, L., *et. al.*, (2017). Simultaneous decolorization and biohydrogen production from xylose by *Klebsiella oxytoca* GS-4-08 in the presence of azo dyes with sulfonate and carboxyl groups. *Applied and Environmental Microbiology*. 83(10).

Yun, Y. M., *et. al.*, (2017). Biohydrogen production from food waste: Current status, limitations, and future perspectives. *Bioresource Technology*., 79–87.

Yusoff, S. and Hansen, S.B. (2007). Feasibility study of performing an life cycle assessment on crude palm oil production in Malaysia. *International Journal LCA*. 12(1), 1–9.

Zainal, B.S., Zinatizadeh, A.A., Chyuan, O.H., Mohd, N.S. and Ibrahim, S. (2018). Effects of process, operational and environmental variables on biohydrogen production using palm oil mill effluent (POME). *International Journal of Hydrogen Energy*. 43(23), 10637–10644.