ESTEEM Academic Journal
Vol.13, Special Issue, August 2017, 24-34

CHARACTERISTICS OF BIOFILM FORMATION FROM MIXED MICROFLORA AT MESOPHILIC AND THERMOPHILIC FERMENTATIVE HYDROGEN PRODUCTION

Lam Wai Fung[1], Nabilah Aminah Lutpi[1], Wong Yee Shian[1] and Tengku Nuraiti Tengku Izhar[1]
[1]School of Environmental Engineering, Universiti Malaysia Perlis, 02600 Arau, Perlis, Malaysia
**corresponding author: nabilah@unimap.edu.my*

ARTICLE HISTORY

Received
22 May 2017

Received in revised form
5 June 2017

Accepted
19 June 2017

ABSTRACT

Biofilm-based systems have been extensively used as immobilized cell systems as they enhance the reaction rates and population dynamics. Therefore, this study was done to characterize the biofilm formation from anaerobic sludge for fermentative hydrogen (H_2) production at mesophilic and thermophilic conditions. This study has been focusing on the development of mixed microflora biofilms whereby the physical and chemical characteristics of the biofilm will be studied. Several tests were done to characterize the biofilm formation, which included the Fourier transform infrared spectroscopy (FTIR) to test the functional group present in the immobilized cell, analysis on the gas produced by the immobilised cells using a gas analyser, and lastly analysis on the chemical compositions presence in the extracellular polymeric substances (EPS) of biofilm. This study had identified that the highest amount of gas collected which representing hydrogen is 470 ppm with the condition of 60 °C with GAC. From the FTIR analysis, the functional group exist in two peak 3308.3cm^{-1} and 1637.12cm^{-1} are the hydroxyl (O-H) group and the combination of C–N + N–H groups in proteins respectively. The transmission of each peak from the FTIR result, and the concentration of the carbohydrate and protein in the extracted EPS had increased with the increasing of the fermentation day, indicates that the physical and chemical properties of the EPS may change more than expected during biofilm growth and alteration.

Keywords: *Biofilm; hydrogen fermentation; mixed microflora*

1. INTRODUCTION

Nowadays, hydrogen (H_2) had been chosen as an alternatives for fossil fuels due to it non-polluting and environmentally friendly characteristic. According to Midilli et al. (2005), H_2 also has the highest energy content per unit weight among any known fuel which includes methane, natural gas, biodiesel, ethanol, etc (Midilli et al., 2005) H_2 can be produced by several ways, however, biological treatments are the most popular and widely used. Among those biological treatments, the most promising one is dark fermentation, which involve process of bioconversion of carbohydrate-based feedstocks by diverse group of bacteria to produce H_2 and volatile fatty acid in a dark, anaerobic condition. This method is environmentally friendly due to its capability to make use of the organic waste to reduce

p-ISSN 1675-7939; e-ISSN 2289-4934

pollutions (Das, 2008). Other than that, dark fermentation also has various advantages, such as high rate of bacterial growth, low input energy, and low costs.

The aim of this study was to characterize the biofilm formation from the anaerobic sludge for fermentative biohydrogen production at mesophilic and thermophilic conditions. The activity of attached growth system in H_2 production could be controlled by understanding the characteristics of biofilm. Biofilm has the capability to stabilize the hydrogen-producing bacteria for a good H_2 performance as they enhance the reaction rates and population dynamics during the aggregation of microorganisms. The formation of biofilm depends on several factors, which are the physical surface and chemical composition of the support carrier, the surrounding environment which include the nutrient availability, pH value, temperature, and lastly the composition of the microbial consortia (Pulcini, 2001). This study has been limited to the characterisation of the mixed microflora biofilms during the development of the immobilised cells.

2. MATERIALS AND METHODS

2.1 Mixed microflora, carrier support, and substrate

The seed sludge used in this study was palm oil mill effluent (POME) sludge that was collected from United Oil Palm Industries Sdn Bhd in Nibong Tebal, Penang. The POME sludge was preserved at 4 ^{o}C to prevent self-biodegradation and acidification (Lutpi et al., 2016). Prior to use, the seed sludge underwent tenderization by heating at 80 °C for one hr to eliminate any H_2 consuming bacteria (Lutpi et al., 2016).

The carrier material is used as support matrix for microorganism and functions as host and attached media that offer a surface area for cell growth. In this study, commercial granular activated carbon (GAC) from Carbochem Inc., USA with size in the range of 2-4 mm was chosen as the carrier material due to its physical and chemical inert characteristic (Lutpi et al., 2016).

The substrate used for biohydrogen production contained 5 g/l sucrose as the sole carbon and energy source and supplements as follows: NH_4Cl, (1 g/l); NaCl, (2 g/l); $MgCl_2$.6H2O, (0.5 g/l); $CaCl_2.2H_2O$, (0.05 g/l); $K_2HPO_4.3H_2O$, (1.5 g/l); KH_2PO_4, (0.75 g/l); $NaHCO_3$, (2.6 g/l); cysteine hydrochloride, (0.5 g/l); yeast extract, (2 g/l); resazurin, (0.5 mg); and trace elements, (1 ml) (Lutpi et al., 2016) .

2.2 Methodology

Figure 1 shows the setting up of the apparatus. Duran bottle with volume of 500 ml capacity was used in this study with working volume consist of 50 % from the total volume. The seed sludge was a mixture of GAC with seed sludge at ratio of 1: 1. The mixture was used as seed in this study and the volume is 10 % of the working volume. Balance of 40 % of working volume was filled with substrate. After the mixture is ready, the pH was adjusted to 5.5 using 1 M NaOH or 1 M HCl. Next, the mixture is purge with nitrogen for five minutes to ensure

p-ISSN 1675-7939; e-ISSN 2289-4934

anaerobic condition is obtained for dark fermentation process. Once the purging is done, the tubing is inserted into the measuring cylinder to collect biogas and the duran bottle is placed inside the water bath to maintain the temperature. The temperature setting for thermophilic condition is set at 60 ^{o}C while for mesophilic condition is set at 50 ^{o}C (Karadag et al., 2009). The fermentation is done by employing repeated batch technique for a maximum of five successive batches to ensure the biofilm formed on carrier material as immobilised cells are stable enough in terms of H_2 production (Lutpi et al., 2016). The sampling was done every day after fermentation which involved the samples of biogas, liquid samples of influent, effluent and also the sample of GAC. The biogas collection from duran bottle was using water displacement method. Water displacement method is an inverted measuring cylinder filled with acidic water (≤ pH 3) that were used to trap the gas leaving from the gas outlet of duran bottle (Mamimin et al., 2012). The acidic water was used to prevent any dissolution of biogas components into the water (Mamimin et al., 2012). The gas obtained was being analyzed by using a Gas Analyzer (Geotech Model GA5000). The formation of biofilm on the GAC was analyzed to determine the functional group presence by using FTIR spectrometry (Model Nicolet 6700, Thermo Scientific, USA). Lastly, the extracted EPS was analyzed for total carbohydrate (TC) by using phenol-sulphuric acid method (Dubois et al., 1956) and protein concentration according to Bradford method (Bradford, 1976).

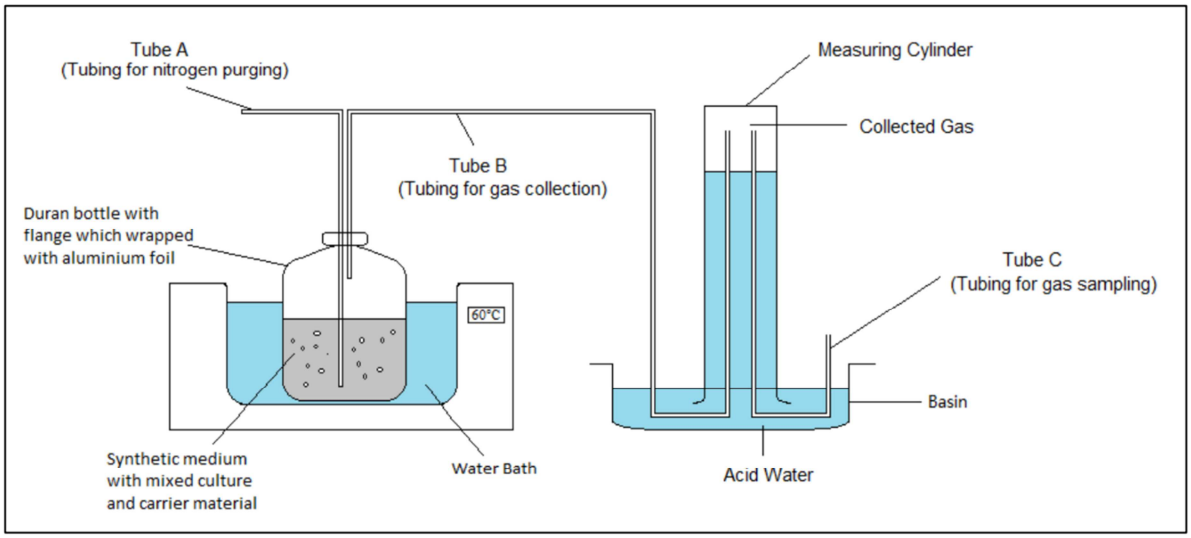

Figure 1: Setting up of the apparatus

p-ISSN 1675-7939; e-ISSN 2289-4934

The experiment is repeated with the following conditions:

Table 1 : Experiment conditions

Condition	Temperature (°C)	Support Carrier (25g)	Substrate Medium (ml)	Seed Sludge (ml)
Control A	60	Nil	225	25
A	60	GAC	225	25
Control B	50	Nil	225	25
B	50	GAC	225	25

3. RESULTS AND DISCUSSIONS

3.1 Analyses on Biogas Production

Figure 2 compares the experimental data on the volume of biogas collected based on different experimental conditions. From the graph, we can see that the highest volume of gas collected is from the fermentation at 60 °C with granular activated carbon (GAC), followed by the 60 °C without GAC, 50 °C with GAC and lastly results at 50 °C without GAC (Karadag et al., 2009). The trend of the volume of biogas collected seems quite similar at all conditions. At first it increased from day 1 to day 3 and started to get stable after the day 4 except for day 3 of 60 °C with GAC and day 2 of 50 °C without GAC, this two days shows a drop in the volume of biogas collected due to leakage was detected at the tubing connected to the duran bottle. This resulting to operation breakdown since anaerobic condition was not fully employed during fermentation, thus encourage the present of oxygen from the environment outside of the duran bottle (Lutpi et al., 2016). Thus, the volume of biogas released during the fermentation were decreased.

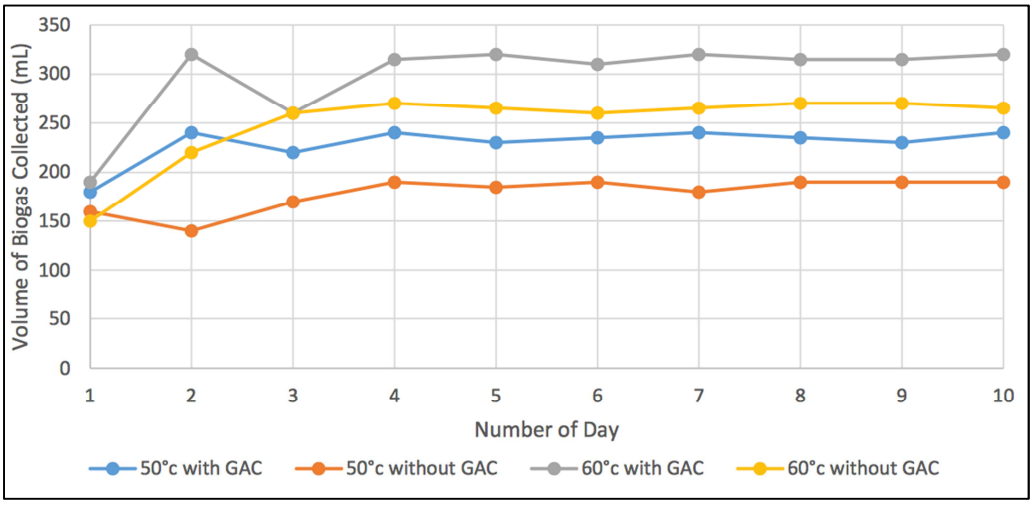

Figure 2 : Graph of volume of gas collected against number of day

p-ISSN 1675-7939; e-ISSN 2289-4934

From the results obtained, we can see that the biogas production at 60 °C which represent the thermophilic condition (60 °C-120 °C) is higher than the fermentation at 50 °C which represent the mesophilic condition (20 °C-50 °C). These results are in agreement with the finding of Kargi F. et al. (2012), who suggested that the yield and production in thermophilic fermentative H_2 production is higher than in mesophilic H_2 fermentation (Kargi et al., 2012). In addition, the presence of the GAC aid in enhancing the biogas production. These results are supported by Keskin et al. (2011) that had claimed that attached growth system could enhance H_2 production better than using suspended growth system (Keskin et al., 2011).

Figure 3 shows the amount of H_2 sulfide, H_2S detected by the gas analyzer based on the optimal biogas volume collected on each experimental condition. From the figure, we can see that the highest amount of H_2S collected is 470 ppm with the condition of 60 °C with GAC, followed by 60 °C without GAC (368 ppm), 50 °C with GAC (318 ppm) and 50 °C without GAC (245 ppm). Due to the limitation of the gas analyzer, we could not measure the amount of H_2 directly, so we can only analyzed the amount of H_2 produced based on the amount of H_2S collected. So, in order to confirm there is H_2 gas exist in the gas collected, a lighted wooden splinter is used (https://chemstuff.co.uk/analytical-chemistry/tests-for-gases/ [access on 20 April 2016]). When the lighted wooden splinter was contacted with the gas collected, a pop sound was heard. This proved that the existence of the H_2 gas in the collected gas. This results also shows that the H_2 production rate is higher at the thermophilic condition than the mesophilic condition.

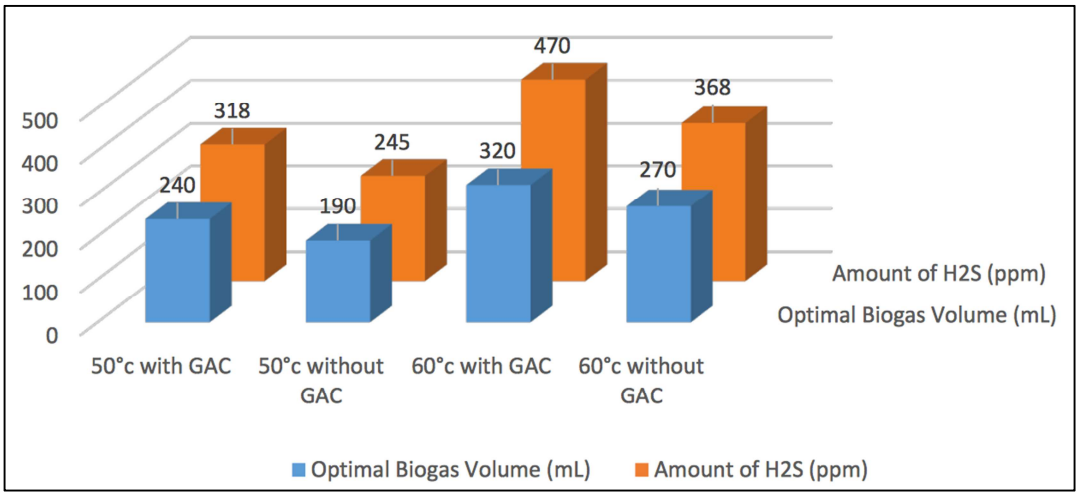

Figure 3 : Amount of H_2S collected based on the optimal biogas collected for each condition

3.2 Fourier Transform Infrared Spectroscopy (FTIR)

The procedure to collect the sample for FTIR analysis including the sample preparation technique were in accordance to Liu and Fang (Liu & Fang, 2002). Figure 4 shows the FTIR spectrum of the Extracellular Polymeric Substances (EPS) formed in the substrate medium after day 5 fermentation at 60 °C with GAC. This particular result at 60 ^{o}C is selected since

p-ISSN 1675-7939; e-ISSN 2289-4934

the condition depicted the best performance compared to mesophilic condition at 50 ^{o}C. From the figure, there are two main peak which are located at 3308.3cm^{-1} and 1637.12cm^{-1}.

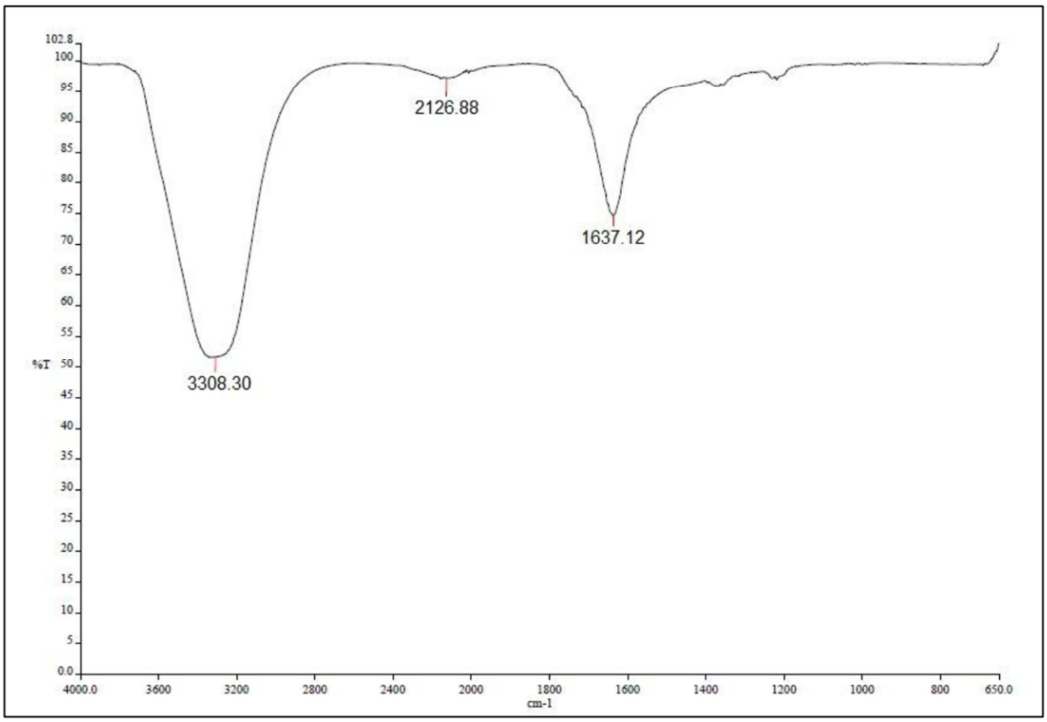

Figure 4: The FTIR spectrum of the substrate medium after the day 5 fermentation at 60°C with GAC

The functional group exist in the two peak 3308.3cm^{-1} and 1637.12cm^{-1} are the hydroxyl (O-H) group and the combination of C–N + N–H groups in proteins respectively (Sheng et al., 2010). For the hydroxyl group, the type of bond is carboxylic acids. The carboxyl present in the carboxylic acid can be charged and ionizes to release H_2 ions, H^+. When the H^+ meets another H^+, the two H^+ will eventually attract to each other and will be covalently bonded together as H-H, the H_2 gas. This will increase the amount of H_2 produced.

The hydrophilic characteristic of the hydroxyl groups existed in the EPS caused the biofilm formed to become more attractive to the bacteria living in substrate medium due to the surface of the bacteria is also hydrophilic . This enable the bacteria to make more frequent contact with the hydrophilic surface compared to a hydrophobic surface and this increases the chances of adhesion and subsequent biofilm growth. Thus this will enhance the biofilm formation. Besides that, the C-N + N-H groups in proteins also play an important role in the biofilm formation (Sheng et al., 2010).

Figure 5 shows the FTIR spectrum of the GAC after fermentation at day 1, 3 and 5 at 60 °C. From the figure, we can see that the shape of spectrum for day 1 is quite different while the shape of spectrum for day 3 and day 5 is almost similar.

p-ISSN 1675-7939; e-ISSN 2289-4934

This figure shows that, the day 1 represent the starting formation of the biofilm while day 3 and day 5 represent the complete formation of the biofilm. Besides that, we can also see that the transmission of each peak had increased from day 1 to day 5. Since these regions are mainly correlated with the formation of EPS, this indicates that the physical and chemical properties of the EPS may change more than expected during biofilm growth and alteration (Christensen & Characklis, 1990; Flemming & Wingender, 2001) . For the initial formation of the biofilm, proteins are functions as an adhesion of biofilm to the GAC surface. It allows the starting of the colonization of the bacteria to the surface and also the long-term attachment of the biofilms to the surface. Other than that, proteins also function as intermediate for aggregation of bacterial cells. It enables the bridging between cells and also the short-term immobilization of the bacterial population.

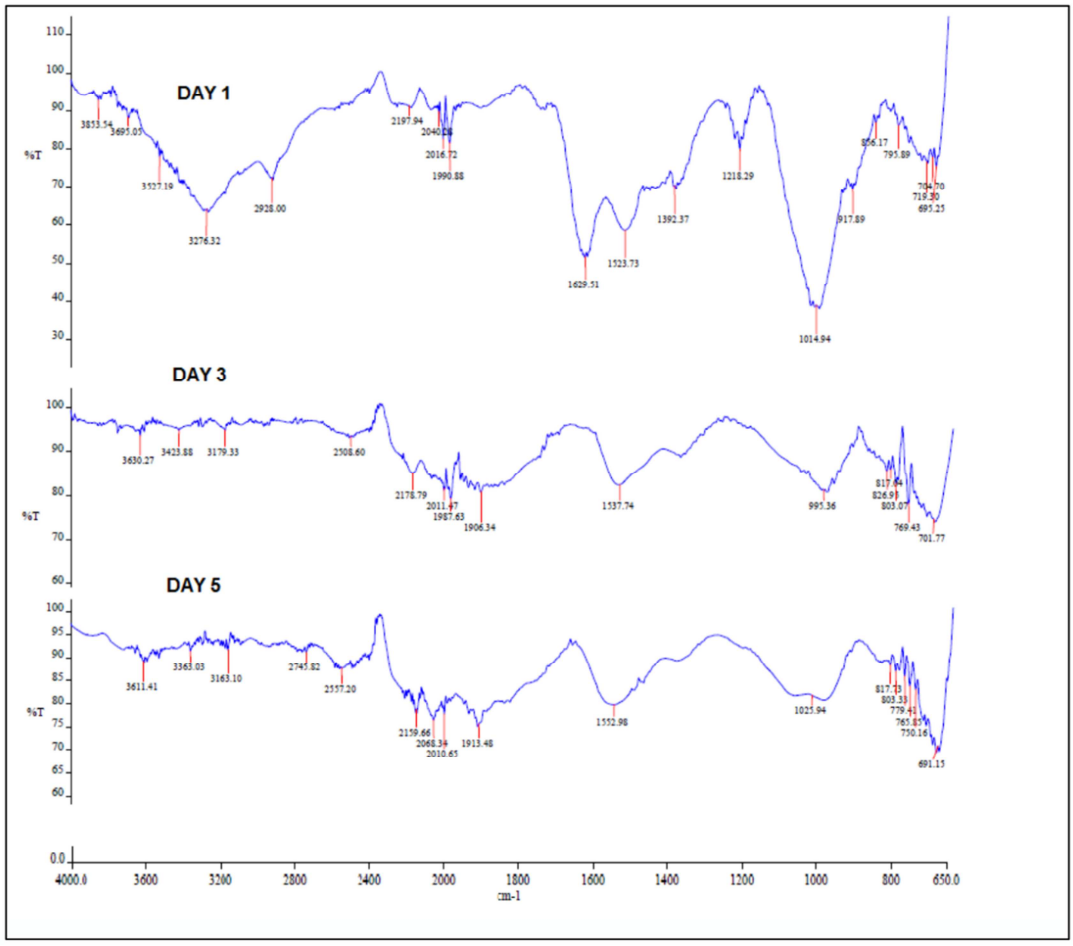

Figure 5 : The FTIR spectrum of the GAC after fermentation at day 1, 3 and 5 at 60°C

3.3 Extraction and chemical composition of EPS

Extracellular polymeric substances (EPS) are components of the aggregation of H_2 producing bacteria (HPB) which accumulated in the biofilm located on the support carrier which is the granular activated carbon (GAC) in this study. EPS that were produced on the GAC surface

p-ISSN 1675-7939; e-ISSN 2289-4934

helps to enhance the microbial adhesion by changing the physicochemical characteristics of the GAC surface. They created scaffolds with suitable physical characteristics and an interconnected GAC pore structure which promote cell attachment (Sheng et al., 2010).

In this study, the procedure to collect the sample for EPS analysis including the sample preparation technique were in accordance to Liu and Fang (Liu & Fang, 2002).

The total carbohydrate (TC) and also the total protein concentration in the extracted EPS had been determined by summing up the TC and protein concentration of the soluble EPS, loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS).

For the TC of the extracted EPS, the phenol sulphuric acid method was used to determine the glucose concentration while Bradford method was employed to determine the protein concentration. Figure 6 and Figure 7 show the total carbohydrate (TC) and the protein concentration in extracted EPS at both 50 °C and 60 °C from day 1 to day 5 respectively.

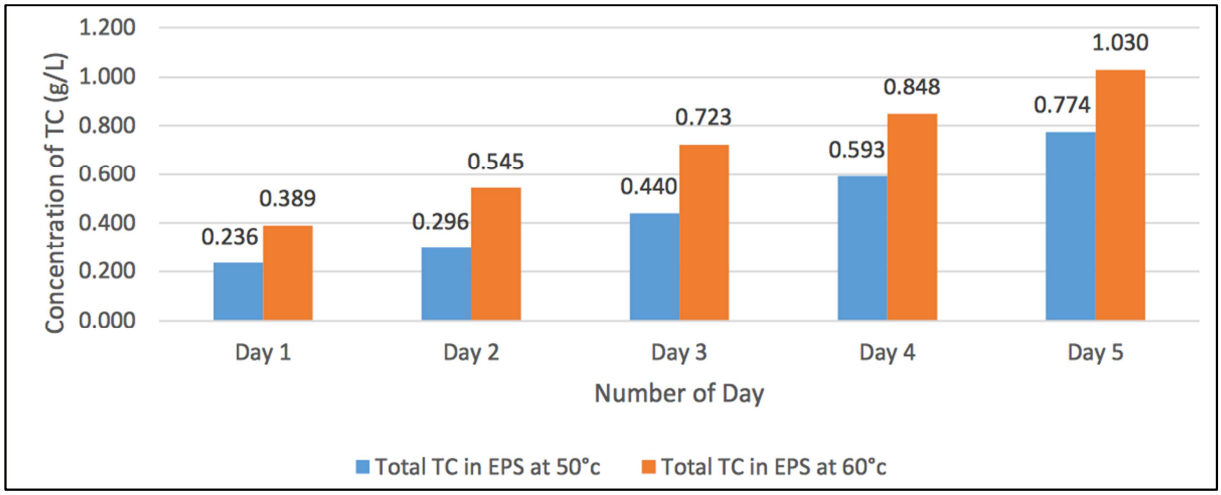

Figure 6 : Graph of total carbohydrate (TC) in extracted EPS against number of day

p-ISSN 1675-7939; e-ISSN 2289-4934

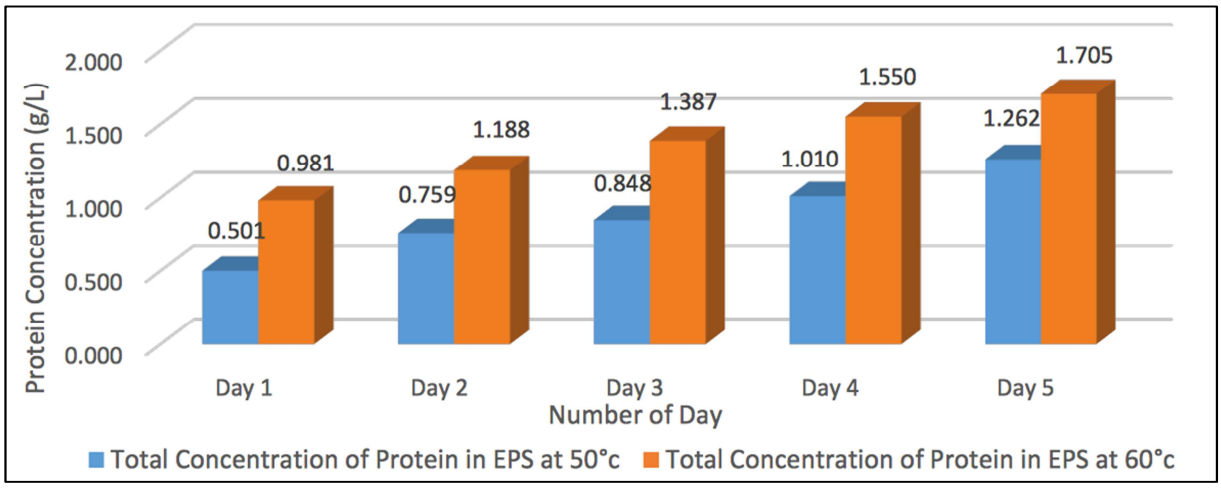

Figure 7 : Graph of total protein concentration in the extracted EPS against number of day

Based on the data in Figure 6 and Figure 7, we can see that the concentration of the carbohydrate and protein in the extracted EPS had increased with increasing fermentation day. These results may be explained by the fact that the enhanced adhesion between the bacteria and the support carrier GAC during the biofilm formation (Lutpi et al., 2016).

Overall, if ratio of protein over carbohydrate (P/C) is determined, the ratios were in the range from 1.6 to 2.6. These results are in agreement with Ras et al. (2011) findings which showed the protein/polysaccharide ratio of biofilm EPS are between 1.8 and 5.4 (Ras et al., 2011).

The EPS secreted by mixed microflora can be subdivided into two main types which are the bound EPS and the soluble EPS. The bound EPS includes the loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) which represents the attached organic materials. While according to Infantes et al. (2011) findings, the soluble EPS do reflects to the hydrogenase metabolic pathway (Infantes et al., 2011). A hydrogenase is an enzyme that catalyzes the reversible oxidation of molecular H_2 which contribute a lot to the H_2 production performance. Thus, the concentration of the EPS directly affects the functional and structural integrity of microbial biofilms and had a direct relationship with H_2 production (Branda et al., 2005). However, the types of soluble EPS and the degree of microbial adhesion on the support carrier will be different and mainly depends on the microbial species and operational conditions, such as pH value and temperature.

4. CONCLUSION

This study had identified that characterization of such biofilms based on the functional group present in the immobilized cell, biogas produce, and total carbohydrate and protein composition in EPS of biofilm could contribute the knowledge on the enhancement of biohydrogen production by stabilizing the hydrogen-producing bacteria on the biofilm. This study also had depicted granular activated carbon (GAC) has the capability to favour the adhesion of hydrogen-producing bacteria because of its high specific surface properties that

p-ISSN 1675-7939; e-ISSN 2289-4934

provide a great number of available binding sites for immobilizing and development of biofilm for a good H_2 performance.

5. ACKNOWLEDGEMENT

This study was supported by a Fundamental Research Grant Scheme (FRGS No: 9003-00511) from Institution of Higher Learning Malaysia (KPT).

REFERENCES

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-254.

Branda S. S., Vik A., Frieldman L. & Kolter R. (2005). Biofilms: the matrix revisited. *Trends Microbiol*., 13, 20–26.

Christensen, B. & Characklis, W.G. (1990). In: Charaklis, W.G., Marshall, K.C. (Eds.). *Physical and chemical properties of biofilms*. Biofilms, Wiley Interscience, New York, pp. 93-130.

Das, D. & Veziroglu, T. (2008). Advances in biological hydrogen production processes. *Int. J. Hydrogen Energy* 33, 6046-6057.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry* 28(3), 350-356.

Flemming HC & Wingender J. (2001). Relevance of microbial extracellular polymeric substances (EPSs) – part I: structural and ecological aspects. *Water Sci Technol* 43,1–8.

https://chemstuff.co.uk/analytical-chemistry/tests-for-gases/ [access on 20 April 2016].

Infantes D., Campo A. G., Villase˜nor J. & Fern´andez F. J. (2011). Influence of pH, temperature and volatile fatty acids on hydrogen production by acidogenic fermentation, *Int. J. Hydrogen Energy* 36, 15595–15601.

Karadag, D., Mäkinen, A.E., Efimova, E. & Puhakka, J.A. (2009). Thermophilic biohydrogen production by an anaerobic heat treated-hot spring culture, *Bioresource Technology*, 100 (23), 5790-5795.

Kargi, F., Eren, N. S., & Ozmihci, S. (2012). Bio-hydrogen production from cheese whey powder (CWP) solution: Comparison of thermophilic and mesophilic dark fermentations. *Int. J. Hydrogen Energy* 37, 8338–8342.

p-ISSN 1675-7939; e-ISSN 2289-4934

Keskin, T., Aksoyek, E. & Azbar, N. (2011). Comparative analysis of thermophilic immobilized biohydrogen production using packed materials of ceramic ring and pumice stone. *Int. J. Hydrogen Energy* 36, 15160–15167.

Liu, H. & Fang, H.H.P. (2002). Extraction of extracellular polymeric substances (EPS) of sludges. *Journal of Biotechnology* 95(3), 249–256.

Lutpi, N.A., Jahim, J.A., Mumtaz, T., Harun, S. & Abdul, P.M. (2016). Batch and continuous thermophilic hydrogen fermentation of sucrose using anaerobic sludge from palm oil mill effluent via immobilisation technique. *Process Biochemistry* 51 (2) 297-307.

Mamimin, C., Thongdumyu, P., Hniman, A., Prasertsan, P., Imai, T. & O-Thong, S. (2012). Simultaneous thermophilic hydrogen production and phenol removal from palm oil mill effluent by Thermoanaerobacterium-rich sludge. *International Journal of Hydrogen Energy* 37(20), 15598–15606.

Midilli, A., Ay, M., Dincer, I. & Rosen, M.A. (2005). On hydrogen and hydrogen energy strategies: I: Current status and needs. *Renew Sustain Energy Rev* 9, 255–71.

Pulcini, E. D. L. (2001). Bacterial biofilms: A review of current research. *Nephrologie*, 22, 439–441.

Ras M., Lefebvre D., Derlon N., Paul E. & Neuhauser E. G. (2011). Extracellular Polymeric Substances diversity of biofilms grown under contrasted environmental conditions. *Water Res*. 45, 1529–1538.

Sheng, G., Yu, H. & Li, X. (2010). Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems : A review. *Biotechnology Advances* 28(6), 882–894.

p-ISSN 1675-7939; e-ISSN 2289-4934