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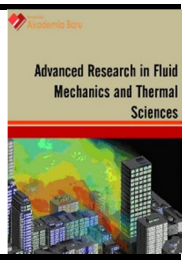

Development of Indigenous biofilm for enhanced biogas production from palm oil mill effluent

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ABSTRACT

Palm oil mill effluent (POME), high content of organic substances can be a great source for production of renewable energy i.e. biogas through microbial biodegradation. The production of biogas has widely been discovered, however, it shows a problem with the low yield of biogas produced. Thus, in order to overcome this problem, a process was used by using indigenous biofilm as a simultaneous pretreatment (hydrolysis) and biogas production. The studies on isolation, purification and screening of biofilm producing bacteria were conducted to evaluate the simultaneous pretreatment and biogas production. The results showed that the developed biofilm enhanced the biogas production with the operating pH, total suspended solid (TSS) and biofilm which were found to be 4-8, 1.5- 4.5 % and 2-4 g respectively. Based on the experiment conducted, the optimum conditions for the biogas production were at pH 8, TSS of 3.0% and biofilm used of 3 g and the highest biogas yield was 382 ml within 5 days of anaerobic digestion. Thus, anaerobic digestion of POME using indigenous biofilm to produce biogas at high yield could be an alternative and effective solution to reduce the source of pollution to the environment.

1. Introduction

Recently, palm oil industry has grown very vast around the Asian country, especially Malaysia and Indonesia due to the massive consumption of palm oil around the world. The production of palm oil has been increased rapidly in the last 10 years and by 2020, this industry can continue to rise up to 78 million tons to meet the demand from people all around the world for fats and oils [1]. During palm oil processing and production, one ton of fresh fruit brunch produces 0.5 to 0.7 tonnes of palm oil mill effluent (POME) [1]. The generation of such by-product as POME along with empty fruit bunch (EFB), palm kernel shell (PKS) and mesocarp fibre had been a serious issue to handle [2]. These wastes need proper treatment to be handled as without proper management it can create significant impact towards the environment. However, high management cost for the treatment of

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POME as high strength wastewater finds an alternative process of recovering of value added products especially renewable energy (biogas) that suitable for societies and industries.

Nowadays biogas production from palm oil mill effluent (POME) has gained its popularity in the industries due to the generation of renewable energy. It cannot denies the fact that they are facing problem with the low production yield of biogas due to the rate limiting steps of hydrolysis that is not properly implemented. There are various ways that could overcome the problem for example by using biofilm, changing type of reactor, etc. There are lacks of existing researches on the development of biofilm as a pretreatment (hydrolysis) for POME. From the previous study, many of the researches focused on the anaerobic system which is widely used due to its low cost and easily treated or ponding system [2]. However, Ahmed *et al*. [3] emphasized that ponding system was not encouraged to use as it has long retention time in term of degradation and poor operational control.

In anaerobic digestion, microorganism like bacteria or archaea attached to biofilm carriers and formed the biofilm. The biofilm formed were composed of microorganism that already attached to a surface in an extracellular polymer structure that also known as EPS and act as corporative consortium. The processes involve three stages that were biofilm maturation, complex microcolonies and the leaving of highly motile planktonic cells [4].

Despite the issues arise, this study focuses on the formation of indigenous biofilm that uses different groups of microbes based on the enzymatic systems (i.e. hydrolytic enzymes such as cellulolytic, proteolytic, lipolytic, amylolytic, etc) could increase the hydrolysis process of POME which is considered as a polymer based complex substance and enhanced the biogas production in single step in order to overcome the problem. First, research works focus on developing the biofilm that is ready to act as a pretreatment for POME. Then, it moves into the aerobic and anaerobic processes to enhance the biogas production while controlling the parameters like pH, oxygen level, temperature and the substrate, POME. The processes are optimized to evaluate the biogas production in biofilm system.

2. Materials and Methods

2.1 Sample Collection

POME sample collected from Sime Darby, Carey Island, Selangor, Malaysia and placed inside a sterile container in the ice box to ensure that the temperature stays around 4^{o}C. Then, the sample was tested for its characterization like chemical oxygen demand (COD), total suspended solids (TSS), pH and temperature [5].

2.2 Isolation and Purification of Bacteria

The diluted sample (10^{-2}-10^{-3}) was distributed onto the agar plate and incubated for 48 hours at 37^{o}C. After 48 hours, the bacterial growth was observed based on the shape of the cells and colony morphology on the solid nutrient agar. The microbes were isolated according to their single cell into new agar plate to make it as pure culture.

2.3 Biofilm Formation

The isolated colonies were tested for the formation of biofilm using biofilm assay where each colony from positive and negative control placed in microtiter plate wells and observed the formation of the ring around the wells as well as the optical density. After that, each of the wells

was washed with distilled water and dried at 37°C. Methanol was added to the wells to fix the biofilm that might be developed in the wells and left for few minutes. After methanol was removed with distilled water and dried, crystal violet solution was added to the wells and remain at room temperature for 30 minutes for staining purpose. After taking out the crystal violet, the wells were washed again with distilled water. Lastly, a mixture solution of ethanol and acetone (4:1) was added into the wells to dissolve the pellet. Then, the OD was taken and recorded. Method was adopted by the Djordjevic *et al.* [6].

2.4 Immobilization Process

The chosen potential microbes that produced biofilm were immobilized with Polyethersulfone (PES) beads inside shake flask for 72 hours before anaerobic digestion for the collection of biogas. Then, the immobilized biofilm was tested with starch and phenol red agar to find whether the microorganisms secrete starch and lipase enzymes or not. Method was modified based on Lowry [7].

2.5 Single Stage Anaerobic Digestion for Biogas Production using Developed Biofilm

The biogas produced from the anaerobic digestion was collected using water displacement method and measured daily. The biogas produced as a result from the digestion process in the scotch bottle was transferred into the cylinder through the vinyl tubing and displace the water in the cylinder. The amount of water in the cylinder was decreased as it was displaced by the biogas and can be measured by the level changes as shown in Figure 1. Then, the process was optimized with different parameters such as pH, total suspended solids (TSS) of POME and amount of biofilm by face centered central composite design (FCCCD) under response surface methodology (RSM). The analyses were conducted by using ANOVA, p-test and t-test, regression coefficient, R^2, 2D and 3D surface plots. Optimum parameter was determined by using a numerical solution in design expert software.

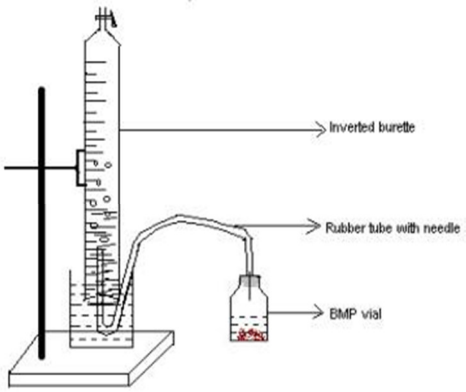

Fig. 1. Set up for water displacement method for biogas production

3. Results and Discussion

3.1 Characterization

Raw POME collected was viscous, dark brown in color and had a strong odor. It had pH of 4.34, TSS content of 57000 mg/L and high COD content of 103000 mg/L as shown in Table 1. The readings

found was in the range stated in the general characteristic of POME by Ahmed et al. (2015) where pH in the range of 4-5, TSS and COD had the concentration of 5000 – 54,000 mg/L and 15,000–100,000 mg/L respectively.

Table 1
Characterization of Palm oil mill effluent

Characteristics	Unit
pH	4.34
Total Suspended Solids (TSS)	57000 mg/L
Chemical Oxygen Demand (COD)	103000 mg/L

3.2 Isolation, Purification, Biofilm Formation and Secrete Extracellular Enzymes

A total of 32 colonies was isolated and sub-cultured into new plates based on the incubated the sample. Biofilm assay is tested to the isolated strains in order to find the possible strong biofilm producer. Based on the biofilm assay test, the strong biofilm producer formed a clear blue ring and the optical density obtained from the microtiter plate reader was higher than other strains as shown in Figure 2 (a-b). The first biofilm assay test showed that there were 9 possible biofilm producers. The possible biofilm producers are shown in the figure (shaded) where the color difference can be seen clearly and selected for the second testing as only strong biofilm producer is selected. Thus, from the second test, it is found that there were only 3 strong biofilm producers with the strain number of 14, 15 and 16 where these strains formed a clear blue ring at the wells and the optical density obtained are high with the range value of 1.5 to 3.04. The highest optical density obtained is 3.04 from strain 14 as shown in Fig. 2 (b)

Apart from biofilm formation, these strains were also tested using starch and phenol red agar. Starch agar provides the test to find the ability of an organism that has the ability to secrete extracellular enzymes (exoenzymes) which are α-amylase and oligo-1,6-glucosidase and diffuse into the starch agar. The glycosidic linkages between glucose subunits are broken by the enzymes hydrolyze starch and create passage for the starch hydrolysis product to enter the cell [8]. Figure 2 shows that there is clear zone surrounding the bacterial growth indicates positive starch hydrolysis due to the secretion of exoenzymes as it formed yellow zone after addition of iodine.

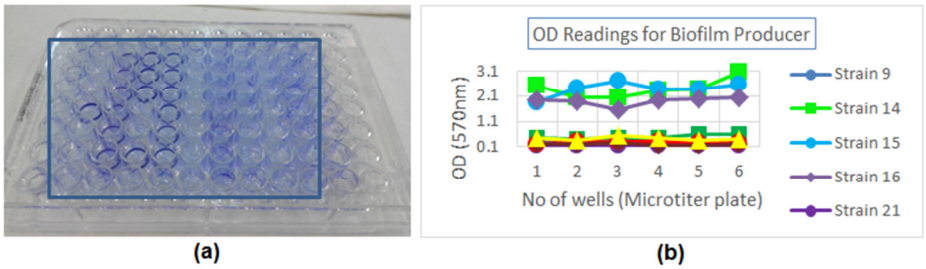

Fig. 2. (a) Biofilm assay test; (b) OD Readings for Biofilm Producer [blue colors present biofilm producers]

Meanwhile, phenol red agar test shown in the Figure 3 is usually uses for simple staining protocol for rapid detection of lipases where it uses the concept of the detection of pH drop due to fatty acid release following lipolysis. The test was done to check whether these microorganisms have the ability to secrete lipolytic enzymes. The initial pH of the chromogenic substrate was set neat the end point of the dye. As a result, a slight change of immediately changed the color [9]. For

this case, the red color in the plate surrounding the colonies was changed to yellow indicated that fatty acid is released by the microorganism.

After the biofilm formation the strain 14 and 16 were immobilized with Polyethersulfone (PES) beads under the optimum conditions. The results showed that the maximum biomass was produced by the strain 14 with 40-50 mg/g of beads within 72-96 hours of biofilm formation. According to Geng *et al*. [10], PES beads have an average size of 3 mm and a surface dense layer of 20 microns and it has numerous holes about 1.5 micrometers in diameter to help adhesion and proliferation of bacterial cells.

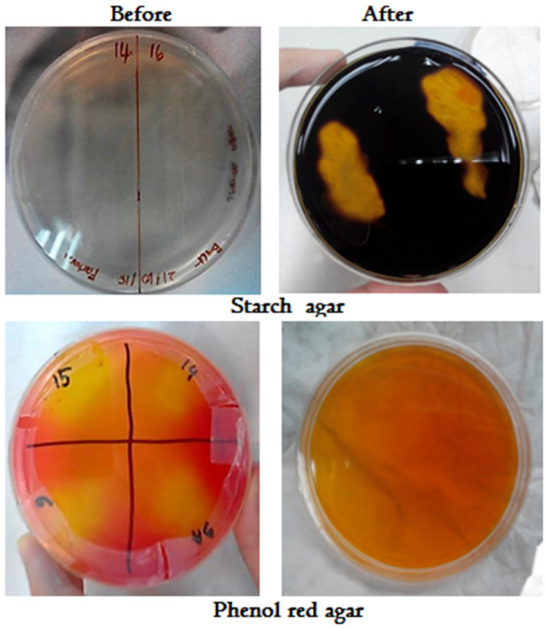

Fig. 3. Test using starch agar and phenol red agar

3.3 Biogas Production using Developed Biofilm Formation

The biogas production using biofilm formation in a water displacement method with different process conditions designed by statistical approach is shown in the Table 2. The anaerobic digester operated for 5 days when the biogas production was highest (382 ml). The optimization designs were based on three variables such as pH, TSS and biofilm. Based on the optimization results, it was found that the optimum condition for biogas production was at pH 8, TSS of 3% and biofilm of 2 g (including beads). The optimization of biogas production with three factors was predicted by following the polynomial equation based on the experimental data:

$$F(\text{biogas}) = 13.41 + 5.12A + 0.48B - 0.26C - 1.53A2 - 5.22B2 + 0.72C2 + 1.73AB - 0.38AC + 0.29BC \quad (1)$$

where F is biogas in mL and A, B and C are the pH, TSS and biofilm respectively.

Analysis of variance (ANOVA) is based on the results obtained and a summary is included. The quadratic model F-value of 5.69 implies the model is significant. There is only a 0.60% chance that a "Model F-Value" could happen due to noise. Values of "Prob >F" less than 0.0500 indicate the model terms are significant. In this case A, B2 are significant model terms where A is pH, B2 is square of total suspended solids. The R2 value predicted was 0.84 shows an acceptable level of regression in the model.

Table 2
Experimental result of biogas optimization

Run	Input Factor			Response: Total biogas produced, mL	
	pH	Total suspended solids (TSS), %	Biofilm, g	Actual	Predicted
1	4	1.5	2	10	13.6
2	6	3.0	3	180	179.8
3	6	3.0	3	194	179.8
4	4	3.0	3	10	45.7
5	6	3.0	3	180	179.8
6	8	4.5	4	230	206.5
7	8	3.0	3	382	289.0
8	6	1.5	3	148	59.4
9	6	3.0	3	194	179.83
10	4	4.5	2	10	0.40
11	6	4.5	3	10	75.2
12	8	1.5	2	95	126.0
13	6	3.0	2	180	207.1
14	8	4.5	2	240	226.8
15	6	3.0	4	190	192.1
16	6	3.0	3	194	179.8
17	4	4.5	4	10	2.02
18	4	1.5	4	10	11.1
19	8	1.5	4	50	87.6
20	6	3.0	3	194	179.8

The graphical representation of the parameters related to the optimization of biogas production is illustrated in the contour and 3-Dimensional response surface plots (pH vs TSS) as shown in the Figure 4. The contour and 3-Dimensional plots are based on the function of two parameters with the third parameter is at its optimum level. The interaction between the corresponding variables is significant as it can be indicated by an elliptical or saddle nature of the contour plots [11]. pH has significant effect on the production of biogas. Total suspended solid (TSS) increased with high pH to its optimum value for any value of biofilm used. Highest biogas is produced at pH 8 where the pH values increase gradually from pH 4 to 8. The interations of pH vs

biofilm and TSS vs biofilm are evaluated (data not shown) and found that biofilm has not much significant within the range studied and TSS shows high significant with biofilm treatment.

The primary factors that affected biogas production are temperature, pH, retention time, organic loading and viable bacteria population [12]. The authors also suggested that the optimal pH for biogas production under anaerobic condition betwwen pH 6.8 to 7.3. The results (data not shown) without biofilm in the POME treatment showed the amount of biogas at 100—150 ml after 2 weeks of treatment.

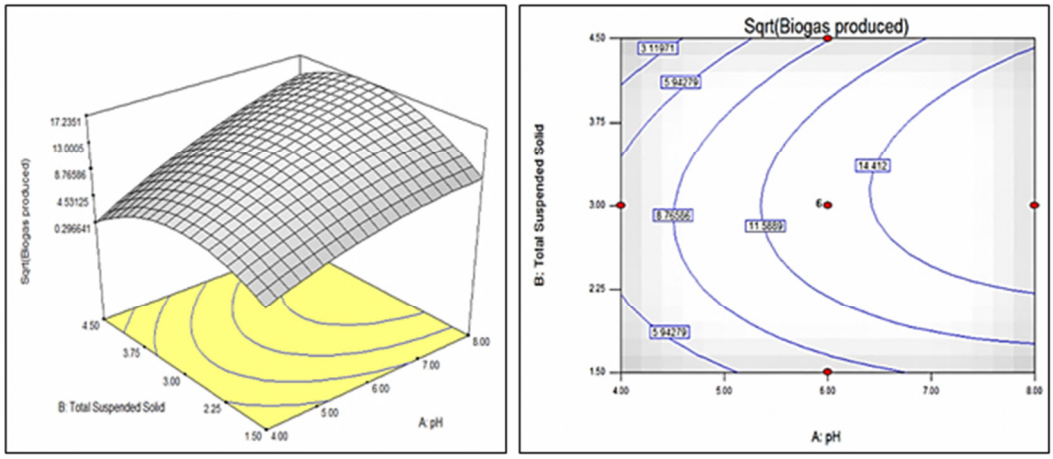

Fig. 4. Optimization of biogas with different variables: pH and TSS

Sadaka and Engler [13] found that higher solid content in feed will lessen cumulative biogas production without depend on the type of feed used. As for the biofilm, there are not much usage biofilm for biogas production compared to biofilm-based reactors thus the main reason behind it cannot yet be found. However, Langer *et al*. [14] and Fernández *et al.* [15] stated that biofilm of high cell densities had higher chances to enhance organic waste digestion and the effects on biogas production. It also stated that biofilm favored high organic loading where the thickness of biofilm layers are higher compared tolow organic loading and biofilms were completely embedded within seven to extracellular polymer substance (EPS).

Validation experiments are conducted to verify the obtained models' predictability. There are three validation experiments that designed and needed to be tested (data not shown). The validation experiment is done by following the conditions of pH 8, total suspended solid of 3.2% and biofilm of 2 g. Based on the validation result, the biogas produced within five days of experiment was 242 ml which is lower than the predicted value that shows error of 10-15%.

4. Conclusion

The research work has demonstrated that biogas production by single-stage anaerobic digestion using indigenous biofilm as a pretreatment by using the substrate of POME is viable and making possible to solve pollution problems and lead to implement a source of renewable energy. However, extensive study needs to be done on the biofilm as there is not much application of biofilm for biogas production as compared to biofilm-based wastewater treatment processes.

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