School of Industrial Technology

# Synergising Environmental Science Research & Technology for a Sustainable Tomorrow


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## PREFACE

Some of the greatest moments in scientific history were documented not in the most popular of textbooks or the most renowned of journals, but within the conscience of explorers who dedicated their lives unravelling the mysteries of the universe. As the flame of curiosity descended through apprentices and students, their burning desire for knowledge soon gave rise to a culture of exploration that transcended generations.

With that, the road towards research and development on science and sustainability was mapped; it is now up to us to nurture this flame by taking steps to explore the horizon for the benefit of mankind. And it was with this spirit that the International Conference on Research and Technology or ICERT was founded, with the aim of bringing together a diverse set of minds that would contribute to the frontiers of research in sustainability and environmental technology. Naturally, this tradition evolved to encompass researchers from across the spectrum, coming under one roof to deliberate on these frontiers.

We are proud to host participants who profess a multitude of disciplines from both the arts and sciences. Our approach towards research at the division of Environmental Technology, School of Industrial Technology, Universiti Sains Malaysia, places emphasis on multi-disciplinary endeavours, with many of our researchers actively involved in fundamental, exploratory and community development programs with an emphasis on sustainable research development.

The following pages contain the compiled papers presented and discussed during the 5th International Conference on Environmental Research and Technology (ICERT) 2017 held from 23rd to 25th August 2017 at Penang, Malaysia. With the guiding principle being sustainability in research and development, it seems apt for this year's ICERT 2017 to be themed 'Synergizing Environmental Science Research & Technology for A Sustainable Tomorrow '. We would like to highlight that all of the papers published in this proceeding were peer-reviewed.

With that, it gives us great pleasure to unveil the proceedings of the 5th ICERT 2017, and to extend our earnest appreciation for your collective effort in making this proceeding a successful one. We would like to thank all the authors for contributing their work to this proceeding and reviewers for providing constructive feedback to the authors.

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# ISOLATION AND SCREENING OF POTENTIAL BACTERIA WITH BIOFILM FORMATION ABILITY AND THEIR HYDROLYTIC ENZYMES FOR ENHANCED BIOGAS PRODUCTION

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## ABSTRACT

*Biofilm mediated anaerobic digestion of POME are to be studied to produce biogas. Out of 120 strains isolated from palm oil mill effluent (POME), palm kernel cake (PKC), and food waste compost, only 13 strains showed biofilm producing ability with significant amount at $OD_{595nm}$ (>0.01). In hydrolytic enzyme assay test, which are amylolytic enzyme assay, cellulolytic enzyme assay, lipolytic enzyme assay, and proteolytic enzyme assay all strains does not secrete protease enzyme, and strain number 1C and 23 does not secrete any hydrolytic enzymes. Strain number 23C and 30C shows positive result for cellulase, amylase and lipase enzymes to be tested as single strain bacteria for prospect research on producing biogas while other strains will be treated as mixed culture in future research.*

**Keywords:** Biofilm, hydrolytic enzyme, isolation, palm oil mill effluent.

## INTRODUCTION

Malaysia is one of the biggest palm oil producer in the world. Parallel to palm oil production, high organic waste, palm oil mill effluent (POME) is also produced. Due to the high organic content, POME is to be treated prior to discharge to any water body. On the other hand, due to its high organic content, treatment of POME can be used to produce biogas, a type of renewable energy [1]. This opens the opportunity of turning waste to wealth.

Biogas production using POME as a feedstock has been studied by many researchers. Biogas production can be increased with better pre-treatment (digestion) [2 - 4]. Pre-treatment of feedstock includes mechanical, thermal, chemical and biological method. Commonly biological pre-treatment is much cheaper than others due to its energy saving (can operate at lower temperature, no aeration, etc.) but consumes much longer time. Biological pre-treatments comes in 3 mode; aerobic digestion, anaerobic digestion and enzyme addition. It is well known that enzyme is very effective but also is very costly. Aerobic digestion could lead to uncontrollable greenhouse gases emission [5] thus anaerobic digestion system was studied in order to efficiently collect biogas. Anaerobic granular sludge and biofilm system can overcome the problem of slow growth and process instability that occurs during anaerobic digestion [6].

Biofilm based system may be able to solve the problem of biomass washout because of the continuous mixing of effluent during anaerobic treatment of POME [7]. However, biofilm mediated anaerobic digestion of POME using single or multiple identified colonies of locally isolated bacteria has yet to be explored deeply. A study done by previous researcher have resulted in 28% increase in biogas production using biofilm mediated digestion from mixed culture of anaerobic sludge digester with liquid manure as the feedstock [8]. This study aims to isolate biofilm producing bacteria from POME and palm kernel cake (PKC) as the source of bacteria that is most related to the nature of POME and food waste compost as additional source of bacteria. The isolated bacteria then were studied for their ability to produce biofilm and hydrolytic enzyme secretion in order to understand their role in pre-treating POME for future research.

## METHODS

### Sample collection

Palm oil mill effluent (POME) and palm kernel cake (PKC) sample were collected at Sime Darby Sdn. Bhd. Banting, Selangor. Food waste compost was collected from composter in International Islamic University Malaysia (IIUM). Then the collected samples were taken for bacterial isolation.

### Bacteria Isolation

One ml of POME sample was cultivated overnight in 10 ml of Luria Bertani (LB) broth at 37°C, and 150 rpm agitation. PKC and food compost waste were cultivated overnight by adding 0.1 gram of the sample into 100 ml LB broth and incubated at 37°C with 150 rpm agitation. The culture was diluted in LB broth up to $10^{-6}$ dilution factor and plated on LB agar ($10^{-3}$ to $10^{-6}$) and then incubated at 37°C for 24 to 48 hour. Any visible colony was transferred to the new fresh plate. Isolates from POME were labelled with numbers, isolates from PKC were labelled with numbers and letter P and isolates from food waste compost were labelled with numbers and letter C.

### Biofilm Producer Screening

The isolated bacteria undergoes biofilm assay screening. Method of biofilm assay test was adapted from Djordjevic (2002) [9]. 5 ml of overnight cultures were prepared for each isolates and incubated at 37°C with 150 rpm shaking. The overnight cultures were diluted by adding 0.1 ml of culture into 10 ml LB broth and then mixed. The diluted cultures then were liquated 100 µl into each wells of sterile 96 wells microtiter plate, triplicated for each culture. After 24 hour and 48 hour of incubation, the growth of bacteria were taken at optical density (OD) 595 nm with 96 wells microtiter plate reader.

The plate then washed 5 times with sterile distilled water, and dried for 45 minutes. Each well of the plate then stained with 150 µl of 1% crystal violet solution for 45 minutes then washed again with sterile distilled water 5 times. After drying for 45 minutes, the wells of the plate were destained with 200 µl 95% ethanol solution and read $OD_{595\ nm}$. Positive biofilm producing bacteria were used for hydrolysis enzyme assay test. Four hydrolysis enzyme assay were cellulolytic enzyme assay, lipolytic enzyme assay, amylolytic enzyme assay, and proteolytic enzyme assay.

**Hydrolytic Enzyme Screening**

Carboxymethylcellulose (CMC) agar was prepared to detect cellulose enzyme. The formula of the CMC agar was adapted from Gohel et. al (2014) [10]. 1% of CMC and 2% of bacteriological agar were prepared. The isolated bacteria was streaked on CMC agar plate and incubated for 24 hour. After 24 hour, the plates were stained with 2 ml Gram's iodine solution for 5 minute, and then washed with distilled water.

Lipase enzyme was detected using phenol red agar with olive oil as the indicator (0.01% w/v phenol red, 0.1% v/v olive oil, 0.1% w/v calcium chloride, and 2% w/v agar). The bacteria were streaked on agar plate and incubated at 37°C for 24 hour and 48 hour [11]. Colour changes were observed.

Amylase was detected using starch medium. The formulation of the agar is as follow; 2 grams soluble starch, 1 gram peptone, 1 gram yeast extract, 20 grams agar, and 1 litre distilled water [12]. After 24 hour to 48 hour incubation, the plates were stained with 2 ml Gram's iodine solution and for 5 minutes and then washed with distilled water.

The method of testing the presence of protease enzyme was adopted from Vijayaraghavan et. al (2013) [13]. For 1 litre of distilled water, 5 grams of peptic digest of animal tissue, 1.5 grams of beef extract, 1.5 grams of yeast extract, 5 grams of calcium chloride, 15 grams of agar and 0.0015% w/v of bromocresol green (BCG) reagent were added. BCG dye was prepared adding 0.56% w/v succinic acid, 0.1% w/v sodium hydroxide, 0.028% w/v BCG dye and 1% Brij-35. The pH then adjusted to pH 4.15 and the solution must be stored at 2-8°C. Protease producing bacteria will be screened on by streaking on the prepared plate and incubated at 37°C for 48 hour. Any colour changes will be recorded.

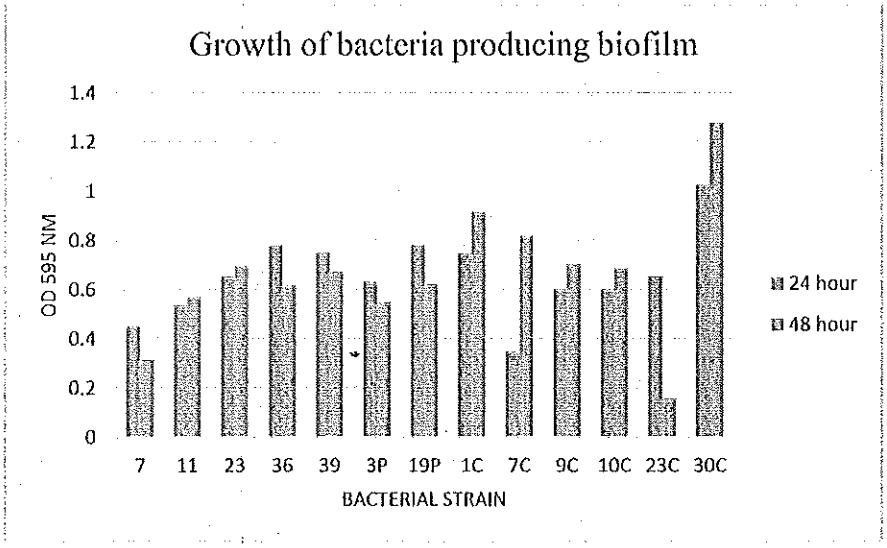
## RESULTS

**Isolation and screening for biofilm formation**

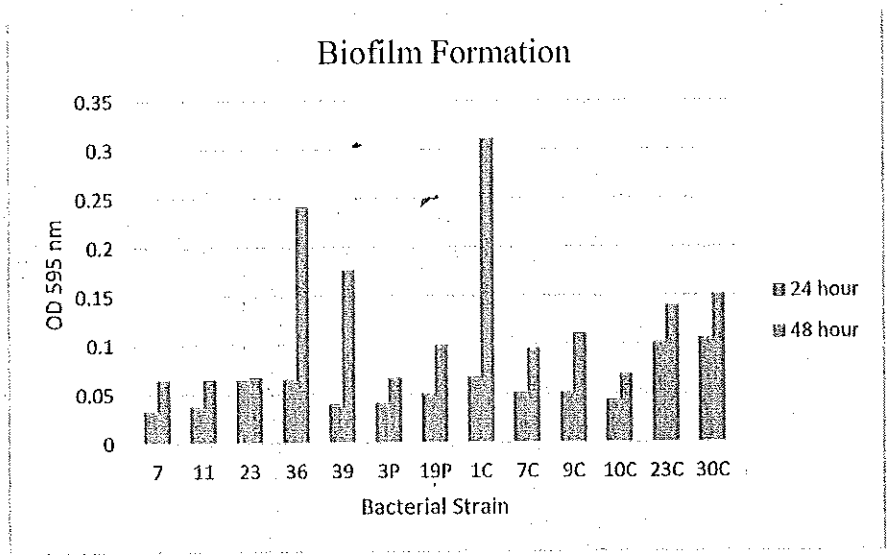
120 isolates were acquired from POME, PKC and food waste compost. Out of all isolates obtained, only 13 strains were chosen to undergo hydrolysis enzyme screening. Table 1 shows the isolates and the source of bacteria. The strains chosen have significant amount of biofilm spectrophotometry reading at optical density (OD) 595 nm (<0.01). Figure 1 (a) and (b) shows the results of bacterial growth and biofilm amount at 24 hour and 48 hour.

**Table 1.** Isolates and its sources.

| Source | Isolates |
|---|---|
| POME | 7, 11, 23, 36, 39 |
| PKC | 3P, 19P |
| Food waste compost | 1C, 7C, 9C, 10C, 23C, 30C |




(a)




(b)

**Figure 1.** (a) Growth of bacteria producing biofilm (b) Biofilm growth from isolated bacteria

**Hydrolysis enzyme screening**

Table 2 summarizes the result of hydrolysis enzyme assay for all 13 chosen isolated strains. For cellulase and amylase assay test, after the plate was stained with Gram's iodine, bacteria with positive enzyme assay will have clear hue around the colony on the plate. The result can be seen as shown in Figure 2. In case of lipase enzyme assay test, the reddish – orange colour of the agar will change to yellow in the presence of lipase enzyme as shown in Figure 3. Positive proteolytic enzyme assay test will also show clear hue around the colony on the agar when compared to the milky colour of the agar without the need to stain the agar due to the presence of BCG dye in the agar.

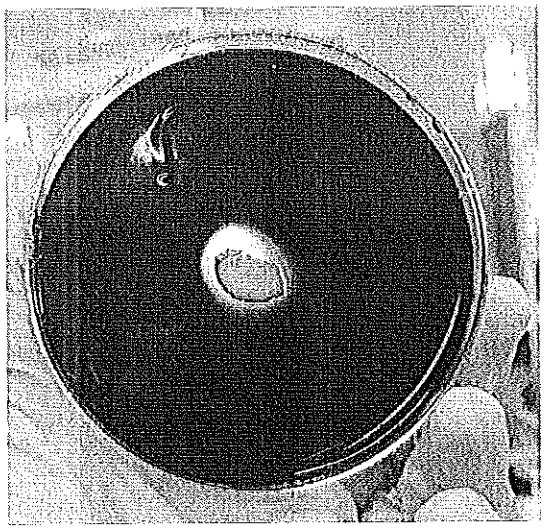


**Figure 2.** Example for cellulase/amylase enzyme test result after staining

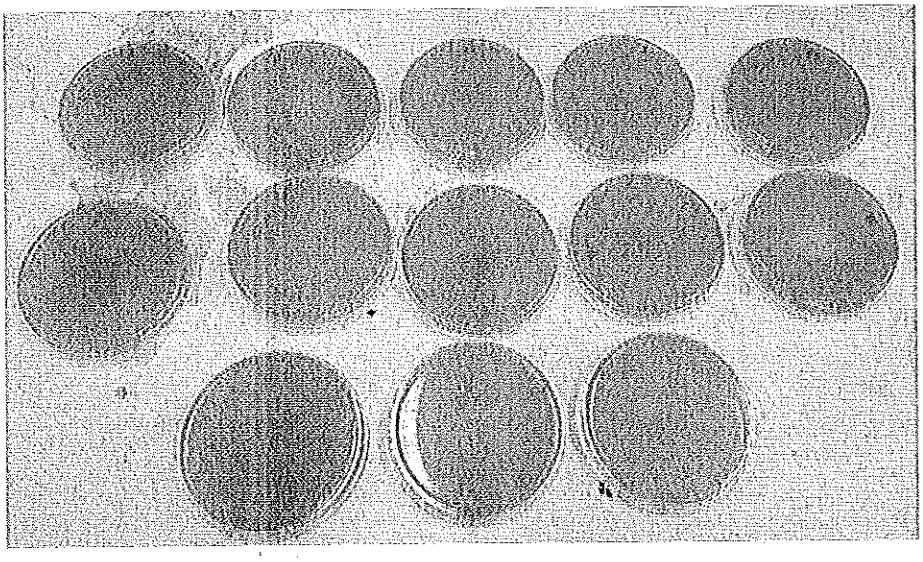


**Figure 3.** Results of lipase enzyme assay test

**Table 2.** Summary for the result of hydrolytic enzyme assay test.

| Strain | Cellulase assay | Amylase assay | Protease assay | Lipase assay |
|---|---|---|---|---|
| 7 | Positive | Positive | Negative | Negative |
| 11 | Positive | Positive | Negative | Positive |
| 23 | Negative | Negative | Negative | Negative |
| 36 | Negative | Positive | Negative | Negative |
| 39 | Negative | Positive | Negative | Negative |
| 3P | Negative | Positive | Negative | Negative |
| 19P | Positive | Positive | Negative | Negative |
| 1C | Negative | Negative | Negative | Negative |
| 7C | Negative | Positive | Negative | Negative |
| 9C | Positive | Positive | Negative | Positive |
| 10C | Negative | Positive | Negative | Negative |
| 23C | Positive | Positive | Negative | Positive |
| 30C | Positive | Positive | Negative | Positive |

## DISCUSSION

All 13 isolated bacteria has been tested for its ability to produce biofilm and it hydrolysis enzyme production. From Figure 1 (a) and (b), there are no conclusive relationship between the growth of bacteria and the amount of biofilm produced by the bacteria. This result is in agreement with previous study [2] that concludes growth rate of bacteria does not correlate with biofilm formation when grown under the same environmental condition. A study done on Salmonella strains also found that different strain have different strength of producing biofilm [15]. The most attractive result from the graph is from strain 1C, which exhibit the highest amount biofilm. Strain 30C have the highest growth but not the highest biofilm producer. The amount of biofilm produced is higher at 48 hr compared to that at 24 hour which are similar to previous studies [2, 15].
All isolated bacteria are negative for protease enzyme assay. This result is good for hydrolysis process, where the absence of protease means that it cannot degrade other enzymes. In a previous research, it was stated that degradation process was slowed down due to the protease breaking down lipase enzyme [14].

Strain number 1C and 23 exhibit negative results for all assay test thus will be excluded for future research. Strain number 11, 9C, 23C, and 30C shows positive results for all cellulase, amylase, and lipase enzyme and will be used for single strain biofilm for simultaneous pre-treatment and enhanced biogas production from POME. The other strains will be mixed for future research.

## CONCLUSION

Biofilm producing bacteria are very limited compared to the total bacteria isolated. The biofilm producing bacteria also does not secrete the same enzymes. The depth study need to be done in order to understand the biofilm work for future research on pre-treatment of POME.

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