

## Bacterial Isolation from Palm Oil Plantation Soil for Biodiesel Production: Isolation and Molecular Identification as Inferred by 16s RNA

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### Abstract

Biodiesel (methyl esters) is a clean alternative fuel which can be produced from many renewable resources. Palm oil, like other vegetable oils, can be used as feedstock for biodiesel production through transesterification to produce palm oil methyl ester. Various microorganisms like bacteria and fungi have a diversity application which could be used as catalysts in a series of degradation reactions, such as transesterification. Malaysia is rich in palm oil and therefore, lots of bacteria surviving by consuming palm oil residue resource in palm oil plantation. In this study, eighteen (18) bacterial strains were successfully isolated from local soil samples and some of their characteristics determined. The optimum temperatures of all strains were in the range of 30 to 37°C, and the optimum batch culture times of all strains were in the range of 24 to 48 hours. All strains were submitted for Gram-staining. Three (3) strains denominated as A, B and C that was involved in the most significant transesterification reaction was selected for identification by submitting them to biochemical tests using the commercial API kit. The same three (3) isolates were submitted to identification by molecular technique. Two bacteria were identified to be *Pseudomonas geniculata* (A) and *Stenotrophomonas maltophilia* (C), while the second bacteria (B) identified to be *Bacillus pseudomycoloides* B-60.

**Keywords:** Biodiesel; Transesterification; Identification; PCR

### Introduction

Microorganisms had existed on earth for billions of years before plants and animals appeared. Thus their evolutionary diversity has far outpaced that of higher organisms. This huge diversity accounts for some of the spectacular properties of microorganisms [1].

The release of microorganisms from soil samples is routine in the microbiological genetic diversity of soil bacteria is high and that soil contains many bacterial species of lineages for which no known cultivated isolate are available laboratory. The literature is extensive [2]. It has been established that the many soil bacteria are referred to as uncultured or even non-culturable [3].

The diversity of the bacterial kingdom is reflected by the diverse applications of bacteria as a cheap labour force. Normally all microorganisms produce enzymes e.g. lipase to be catalysts in each microorganisms own metabolism, so a lot of applications were based on this fact [4]. To produce biodiesel biologically is an emerging technique, with the assumption that a certain environment could be the ideal condition for some microorganism, where they are able to grow under that condition and consume the limited nutrition to produce biodiesel [5].

As a result, a lot of bacteria had been used to convert vegetable oil into biodiesel in order to find an ideal enzyme to replace the traditional chemical methods. Moreover, it is friendly to the environment and above all it is easy to separate after the reaction, as identified in previously completed research [6].

In Malaysia, the land area is covered by massive oil palm plantations, and the country is well-known for its palm oil products. Malaysian oil palm plantation soil is an ideal place for thousands of microorganisms. In addition, countless oil palm fruits were left on the surface of the earth or buried, having penetrated into soil. As a result, most probably there are a lot of microorganisms which would be able to live by consuming palm oil directly or indirectly [7].

*Pseudomonas aeruginosa*, an obligate aerobe, is found in many natural and man-made environments; it has been isolated from plants and soils [8-9]. Identification of *P. aeruginosa* in the laboratory is generally performed by growing the bacteria on either cetrimide agar or nalidixic acid-cetrimide agar. This species can grow in low-nutrient water [10] and survive for long starvation periods [11] which are often related to biofilm formation [12]. In addition to being a primary infectious agent, *P. aeruginosa* is an indicator of other opportunistic pathogens and also even promotes these microorganisms [13]. Polymerase chain reaction (PCR) methods, which allow for more rapid identification of *P. aeruginosa* by DNA amplification, have been reported [14]. *Pseudomonas luteola*, also as known as *Chryseomonas luteola*, is a Gram-negative rod [15]. *P. luteola* is aerobic, non-spore-forming, motile, oxidase-negative and catalase-positive [16]. Recent analysis of the 16S rRNA sequences of this organism suggests that *Chryseomonas* and *Pseudomonas* are related and that *Chryseomonas* is a junior subjective synonym of *Pseudomonas* [15]. A number of enzyme application have been found from *Pseudomonas* such as direct esterification, acrylate synthesis, as well as the *Ps. luteola* lipase illustrated a role of lid in interfacial activation and the conformation of the enzyme in organic media [17].

*Stenotrophomonas maltophilia*, previously named *Pseudomonas*, and then *Xanthomonas maltophilia*, has been recently classified as the

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single species of the new genus *Stenotrophomonas*. *S. maltophilia* is a non-fermentative Gram-negative bacillus which grows readily on most bacteriological media [18-19]. Several factors confer *S. maltophilia* plays a role in the promising pathogen, especially capability to elaborate a broad span of additional cellular enzymes, for example lipase, fibrolysin, and proteases, potentially involved in the colonization process [20]. Molecular typing systems have contributed to knowledge of the epidemiology of *S. maltophilia* infections. Typing methods such as ribotyping [21], multilocus enzyme electrophoresis [22-23], random amplification of polymorphic DNA [24], enterobacterial repetitive intergenic consensus-PCR [25] and DNA macro restriction analysis [26] have been employed.

It was reported that the transesterification of several vegetable oils by bacterial lipases, including *Pseudomonas* lipase, showed a stronger activity, compared to fungal lipases such as Lipozyme TL IM [27].

The present study focuses on the isolation and identification of some unknown microorganisms from a local oil palm plantation soil. The investigation will conclude the morphology and growth characteristics of these microorganisms. In this study, three strains were investigated by API20-E kit and PCR amplification, so that the identification of three strains was carried out by sequence analysis.

## Materials and Methods

### Chemicals

Microbiological agar, liquid broth media and Gram-staining reagents were supplied by Merck, Malaysia Division. API-20 E Kit was purchased from Fisher Scientific (M) Sdn. Bhd. G-spin Genomic DNA extraction kit (for bacteria) and MEGAquick-spin PCR & Agarose Gel DNA Extraction System were purchased from Intron Biotechnology, Inc. Agarose power was purchased from Biosyntech Sdn. Bhd. SYBR safe DNA gel stain and 1 Kb plus DNA ladder were purchased from Invitrogen, USA. TAE-buffer was purchased from DKSH Technology Sdn. Bhd. Taq DNA polymerase, MgCl<sub>2</sub> and 10 X Buffer were purchased from Invitrogen, USA.

### Isolation, purification and screening of lipase bacterial

Soil samples were collected from the oil palm plantation of LKPP (Lembaga Kemajuan Perusahaan Pertanian) Lepar Hilir, Jalan Muadzam-Kuantan, Pahang, Malaysia. Two types of sampling sites were selected namely common agriculture sand and agriculture soil which has been covered with fertilizer for a very long time, as palm fruits were usually fell down from trees on the soils.

Soil samples were taken to the laboratory and stored overnight at 4°C. An amount of 10 g of each soil sample was weighed, sieved and added to 90ml prepared sterile distilled water. Each sample was stirred and diluted from 10<sup>-1</sup> to 10<sup>-8</sup> dilution factor. Then, an amount of 100 µl from each dilution was inoculated into prepared petri dishes containing sterile nutrient agar. The plates were inverted and placed in the incubator at 30°C for three (3) days.

Lipase bacterial screening was performed to find the lipase with the best catalytic activity in the transesterification of palm oil [28]. All bacteria were mixed with palm oil and methanol in a certain ratio. These initial crude activity tests were used to screen the bacterial isolates with significant activity, and to select them to carry out further experiments on. The screening experiments were intended for evaluation of the activity of the lipases from different sources. In a typical reaction, 10% V/V of crude enzymes were added to the mixture of 6 ml of palm oil, 2.4 ml methanol (3 M ratio of methanol to palm oil), with 150 rpm

constant stirring at 40°C for 5 hours. The analysis of palm oil (Fatty Acid Methyl Ester) content in the samples was carried out using Gas Chromatography (GC) by means of Inert DP WAX capillary column (30 mx0.25 mm, I.D. 0.25 µm). Helium was used as the carrier gas. Oven temperature program was as follows: 155°C for 1 min and programmed from 155 to 180°C at a rate of 2°C/min, kept for 2 min, and finally raised to 220°C at 4°C/min and maintained for 6 min. The injector was set up for 250°C and the FID detector at 260°C.

### Biochemical characterization of bacterial strains and Genomic PCR amplification

The biochemical characterization of potential bacterial isolates was performed as per standard protocol for biochemical tests [29]. After the successful isolation from soil, a number of bacteria were sub-cultured and their morphology was observed. Eighteen bacterial isolates were subjected to Gram-staining procedure and morphological characteristics. The API-20E test kits were performed following the manufacturer's instructions. The results were interpreted with the APILAB PLUS software (version 3.3.3).

Modified 16S r-RNA sequence [30] primers for PCR based on the API -20E results was used, these sequences were filed in the international gene banks. Two oligos universal primers were designed for use in PCR. The first forward primer (F: 5' AGA GTT TGA TCC TGG CTC AG3') and one reverse primer (R: TAC GGY TAC CTT GTT ACG ACT T3') for use in initial PCR amplification. The concentration of samples DNA was estimated by measurement of absorbency at 260 nm on a spectrophotometer. Amplification conditions were run for 30 cycles. A typical reaction mixture in each PCR tube for 20 µl total volume, containing 1 µl of the appropriate dilutions of DNA, 2 µl of 10X PCR reaction buffer, 0.5 µl of DNA polymerase, 0.5 µl of d-NTPs, 1 µl of MgCl<sub>2</sub>, 1 µl of each primer. PCR involved initial denaturation at 94°C for 1 min, five cycles with a low annealing temperature of 50°C for 5 s, extension at 72°C for 30 s and heated to 94°C for 5 s, and additional 25 cycles of denaturation at 92°C for 2 s, annealing temperature at 55°C for 2 s, extension at 72°C for 30 s and final extension at 72°C for 2 min. The PCR products were then separated on a 1% agarose gel containing 1 mM ethidium bromide for visualization on a Bio-imaging machine. Interesting DNA fragment was cut out with a sharp scalpel after PCR product electrophoresis and was taken carefully as much as agarose gel as possible. The mixture was shaking and incubate at 55°C for 10 minutes or until the gel is completely dissolved. The dissolved gel mixture was transferred to the kit column assembly and centrifuged for 1 minute; the flow-through was discarded after centrifuge. 700 µl of washing buffer was added to column and centrifuged at 13,000 rpm for 1 minute and discarded the flow-through. The column was placed to a clean 1.5 ml micro-centrifuge tube after centrifuge for 1 min at 13,000 rpm to dry the spin membrane. 60 µl of the elution buffer was directly applied to the centre of the column without touching the membrane with the pipette tip. The tube was incubated at room temperature for 1 min and centrifuged for 1 min at 13,000 rpm. Lastly tubes were stored at -20°C.

### Sequence Analysis and Blast Analysis

The PCR products were sent to expert company (1st BASE, USA) to analyse the sequence, and then Blast the sequence with Gene bank.

### Results and Discussion

All the samples were collected from 2 sampling sites. The two groups of samples have a distinctive colour difference. Group 1 is from agriculture sand soil and is shown in Figure 1, the group 2,



Figure 1: Group 1 of Soil Samples.



Figure 2: Group 2 of Samples.

| Isolates Code | Colour       | Isolate Colony | Form            | Gram-stain | Cell Shape       | Sample Group |
|---------------|--------------|----------------|-----------------|------------|------------------|--------------|
|               |              | Size (µm)      |                 |            |                  |              |
| A             | White        | 10             | Floral          | Positive   | Bacilli          | 1            |
| B             | Pale yellow  | 5              | Cream/Irregular | Negative   | Strepto, bacilli | 2            |
| C             | Yellow       | 10             | Oval            | Negative   | Bacilli          | 1            |
| D             | White        | 10             | Irregular       | Negative   | Bacilli          | 1            |
| E             | Light Yellow | 10             | Oval            | Negative   | Worm Shape       | 2            |
| F             | Brown        | 5              | Irregular       | Negative   | Cocci            | 1            |
| G             | White        | 5              | Irregular       | Positive   | Bacilli          | 1            |
| H             | White        | 10             | Flat            | Positive   | Bacilli          | 1            |
| I             | Yellow       | 5              | Oval            | Positive   | Bacilli          | 2            |
| J             | Pale yellow  | 10             | Irregular       | Negative   | Cocci            | 1            |
| K             | White        | 5              | Cream/Irregular | Negative   | Bacilli          | 1            |

Table 1: Morphological Characteristics of 11 Bacterial Isolates.

form fertilized agriculture soil is shown in Figure 2. It was observed that samples from group 2 have a darker colour than group 1 which indicates a rich in nutrient from palm oil waste fertilizer.

The use of serial dilution to obtain bacterial isolates which are significant in or at least typical of a habitat has been employed to isolate micro-bacteria. Several dominant cell types were observed in the different plates in which growth occurred. In various series dilution, mixture of cell types was present. Besides very small and quite large rod-shaped organisms and cocci shape could also be recognized under microscope. As in the previous description of the methods, a lot of colonies were cultured in the first three days. From these, independent and single colonies were chosen for sub-culture; the sub-culturing process proceeded along with the same methods and using the same

medium. As many as eighteen (18) bacterial isolates were successfully isolated from soil samples and labelled from A to R. On the basis of the taxonomical characteristics of all 18 strains, some of them are not only identical in morphology, but also similar in Gram-staining. As a result, eleven (11) isolates were selected as typical species and their morphological characteristics are shown in Table 1. Of all the isolated isolates, as many as 13 of them were from group 2 soil, namely the fertilized agriculture soil, with the rest was from the group 1.

The growth conditions of eleven (11) bacterial isolate are summarized in Table 2 below. The first step was monitoring the parameters of culturing on the nutrient agar; from these results it was found that they all could easily survive on the nutrient agar; and their growth were fast. Most bacteria have growth temperature of 30-37°C. The result was in line with the concept; the incubation temperature of bacterial isolate in this research was either 30°C or 37°C. For the incubation period, the longest was no more than 48h. Inversely, the lowest incubation period was at least 24 hours, with most bacteria can survive from 24- 48 hours on the agar plate.

In this study, all eleven (11) bacteria were tested for the conversion reaction under the same conditions. After determination by Gas Chromatography, only three bacteria gave positive result, namely bacterial A, B and C. Further all these lipases were screened for biodiesel synthesis from crude palm oil and methanol. The catalytic activities of three lipases on transesterification were compared in Figure 3; all three

| Isolate Code | Bact. growth Temperature (°C) on Agar Medium |     | Incubation Time (h) |     |                   |       |
|--------------|--|-----|---------------------|-----|-------------------|-------|
|              | 30   | 37  | Agar Medium         |     | Broth Medium (OD) |       |
|              |  |     | 24                  | 30  | 48                | 24    |
| A            | +++  | ++  | ++                  | +++ | +++               | 155   |
| B            | +++  | +   | ++                  | +++ | +++               | 125.7 |
| C            | ++   | +++ | +                   | ++  | +++               | 121   |
| D            | +++  | ++  | +                   | ++  | +++               | 123.5 |
| E            | ++   | +   | +                   | ++  | +++               | 117.6 |
| F            | +  | +++ | +                   | ++  | +++               | 112.2 |
| G            | +++  | ++  | +                   | ++  | +++               | 115.6 |
| H            | ++   | +   | +                   | ++  | +++               | 124.7 |
| I            | ++   | +++ | +                   | ++  | +++               | 118   |
| J            | +  | +++ | ++                  | ++  | +++               | 120.2 |
| K            | +++  | +   | +                   | ++  | +++               | 117.1 |

Table 2: Determination of bacterial growth at Different Conditions.

+: Weak Growth; ++: Moderate Growth; +++: Heavy Growth

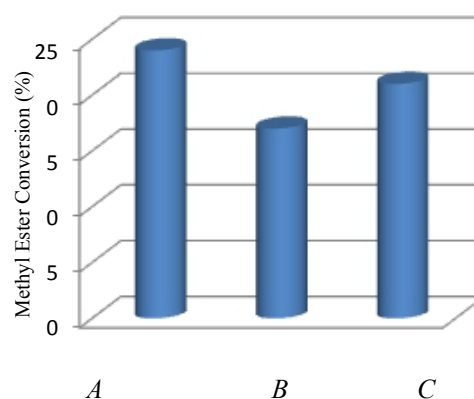


Figure 3: Methyl Ester Conversion Catalysed by enzyme from Bacterial A, B and C.

lipases show conversion activity during the transesterification from palm oil to methyl esters. The methyl ester conversion of oil by using lipase from *bacterial A* is around 24% and methyl ester conversion of lipase from *bacterial B* showed comparable higher catalytic activity around 21% as compared to lipase from *bacterial C* around 17%.

As the three isolates have positive activity in the screening of lipase from bacteria. Namely the importance of bacteria A, B and C is over the rest. So the following identifications also focus on these three screens

Bacterial isolate A gave a flowery-like shape on nutrient agar plate whereas in the Gram-staining procedure appeared Gram-positive rod shaped (Figure 4). For bacterial isolate B, the shape was round in nutrient agar plate whereas in Gram staining it was in Gram-negative rod shape (Figure 5). While bacterial isolate C the shape was also round in nutrient agar plate and bigger than isolate B but rod and Gram-negative reaction (Figure 6). In terms of colour they were white, violet and light brown respectively.

The API 20-E KIT results for biochemical test were given in Table 3. The identify of bacterial A is either as potential *Pseudomonas aeruginosa* or *Pseudomonas fluorescens* with strongly 97% test against oxidase test. For bacteria B, it is as potential *Bacillus pseudomycooides* at strongly 98% test against ONPG and 100% against oxidase. However,

| Bacterial No. | Significant Taxa                      | % ID | T    | Test Against                     |
|---------------|---------------------------------------|------|------|----------------------------------|
| A             | <i>Pseudomonas aeruginosa</i>         | 77.5 | 0.77 | OX 97%                           |
|               | <i>Pseudomonas fluorescens/putida</i> | 15.6 | 0.65 | OX 99%                           |
| B             | <i>Pseudomonas luteola</i>            | 70.8 | 0.71 | ONPG 98% LDC 25% CIT 25% IND 85% |
|               | <i>Stenotrophomonas maltophilia</i>   | 21.5 | 0.25 | OX 100%                          |
| C             | <i>Pseudomonas luteola</i>            | 61.7 | 0.44 | CIT 94% GEL 13% GLU 84%ARA 85%   |
|               | <i>Stenotrophomonas maltophilia</i>   | 25.7 | 0.34 | CIT 75% LDC 75% ADH 0%           |

Table 3: The API Results of Bacterial A, B and C.

| Bacterial isolate         | OD Value (600 nm) (24 hours) |           | Mean ± (SD) |
|---------------------------|------------------------------|-----------|-------------|
|                           | Reading 1                    | Reading 2 |             |
| Control (Distilled water) | 0.002                        | 0.001     | 0.01 ± 0.01 |
| A                         | 0.869                        | 0.846     | 0.86 ± 0.01 |
| B                         | 1.896                        | 1.879     | 1.89 ± 0.01 |
| C                         | 2.013                        | 2.014     | 2.01 ± 0.01 |

Table 4: The OD Value of Bacteria.

isolate C gave fairly percentage at CIT test at 94% and GLU at 84%, so fairly identified as either *Pseudomonas luteola* or *Stenotrophomonas maltophilia*.

Concerning the additional assays tested, some biochemical tests showed false negatives and false positives. Consequently, this procedure should be discarded as a confirmative test. In contrast, the API 20-E system proved a powerful tool as it correctly identified all the confirmed isolates. This commercial system could be used by qualified laboratories when dealing with isolates of dubious identification [31].

The OD values of the three bacteria were tested before DNA extraction, to quantify the bacteria, and the results are listed in Table 4. In 1.5 ml samples, under the 260 nm of UV-VIS detection, the average of two readings for isolate A has the lowest value of  $0.86 \pm 0.01$ , the value for isolate B is  $1.89 \pm 0.01$ , meanwhile the isolate C gives the highest value of  $2.01 \pm 0.01$ .

These values were in line with the lowest value by the DNA extraction protocol from the manufacture of the commercial kit. Hence it proved that all three bacteria were at suitable optical densities after incubation period for 24 h. They were all suitable to be used for the DNA extraction, since the high cells density gave an assurance of the extraction.

The DNA extraction was carried out by agarose gel electrophoresis and the results are shown in the Figure 7 M is 1 kb maker ladder; the rest are the three bacteria, namely A, B and C. A has a brilliant band of 5,000 bp and a fade band of 12,000 bp; the bacteria B has a faded band; by contrast, the bacteria C has two brilliant bands, both at 12,000 bp and 5,000 bp. From the bands results, bacteria B has a faded band, the reason was probably due to DNA molecular mass being too big to move through the gel. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving [32].

The DNA extracted of the three bacterial isolates were quantified by measuring the OD at 50 times dilution before PCR amplification, and the results are listed in the Table 5. In the 50 times diluted sample

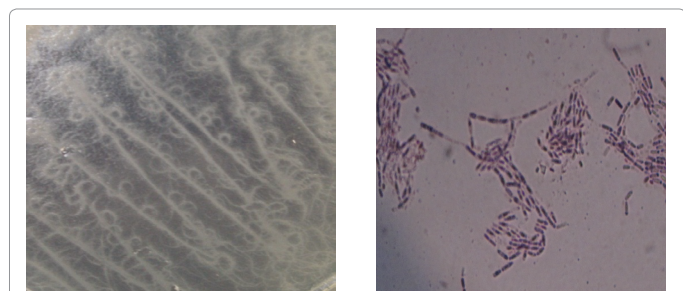


Figure 4: Isolate A on Nutrient Agar (left) and Gram-reaction (right) is Positive.

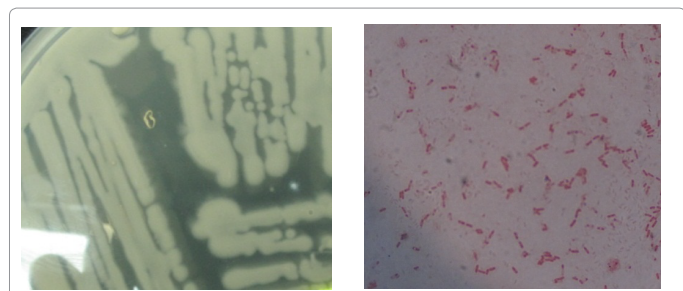


Figure 5: Isolate B on Nutrient Agar (left) and Gram-reaction (right) is Negative.

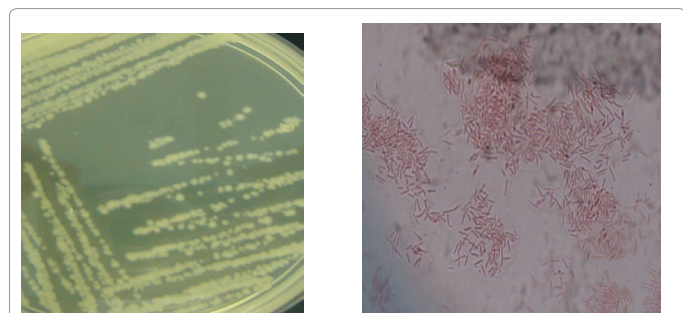


Figure 6: Isolate C on Nutrient Agar (left) and Gram-reaction (right) is Negative.

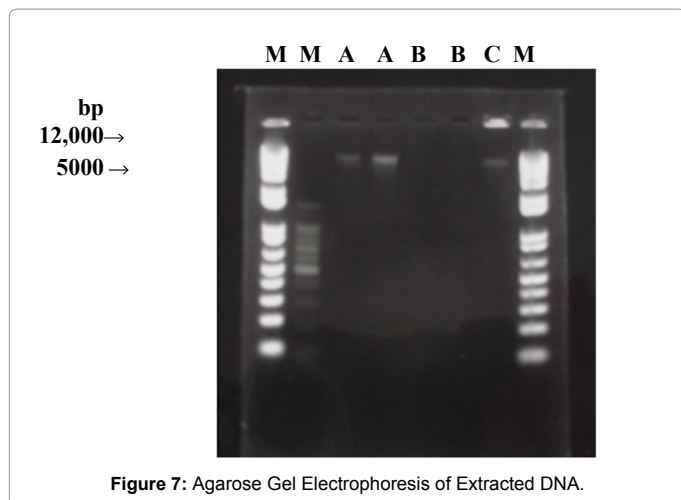


Figure 7: Agarose Gel Electrophoresis of Extracted DNA.

| Bacteria isolate          | OD Value (260 nm) |           | Average Value ± (SD) | DNA Concentration (ng/μl) |
|---------------------------|-------------------|-----------|----------------------|---------------------------|
|                           | Reading 1         | Reading 2 |                      |                           |
| Control (Distilled Water) | 0                 | 0         | 0                    | 0                         |
| A                         | 0.048             | 0.051     | 0.0495 ± 0.0001      | 121.7                     |
| B                         | 0.066             | 0.069     | 0.0675 ± 0.0001      | 166.7                     |
| C                         | 0.06              | 0.058     | 0.059 ± 0.0001       | 147.5                     |

Table 5: The Concentration of Bacterial DNA.

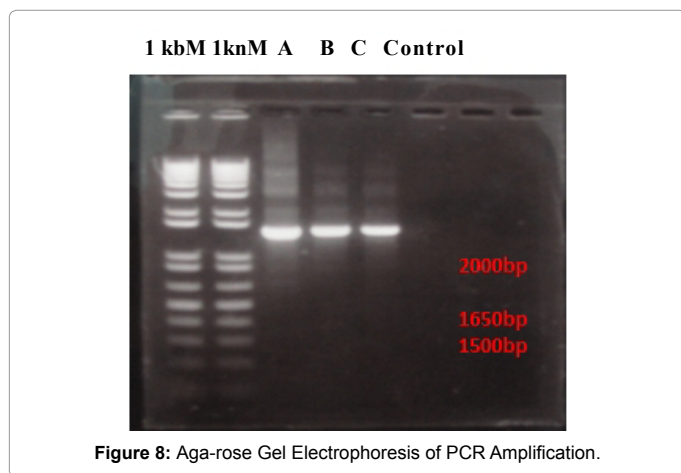


Figure 8: Agarose Gel Electrophoresis of PCR Amplification.

of bacteria, under the 260 nm of UV-VIS detection, the calculation showed that bacteria A has the concentration at 121.7 ng/μl, the bacteria C was 147.5 ng/μl, meanwhile the bacteria B gives the highest concentration up to 166.7 ng/μl.

In fact, the UV-VIS result is not only able to test the DNA concentration, but also test the purity of DNA, the purpose is to detect the DNA samples which were suitable to ensure the possibility of success of or the good results the PCR reaction. To evaluate the sensitivity of the species specific primers, extracted chromosomal DNA were amplified by PCR. The results of PCR amplification was shown in Figure 8. The 16S rRNA sizes of three bacteria were all between 1650 bp to 2000 bp, is mostly around 1650 bp.

The bands were very bright, which are indicates that the PCR amplification was successful and 16S rRNA was gotten after the

running of PCR. The purification of PCR product was carried as well. The quantification indicator is the test for the concentration of PCR products, their results were shown in Table 6. The concentrations of 16S rRNA were all very much lower than the concentration of bacterial DNA in Table 6. The isolate A has a concentration of 78.8 ng/μl; B has the highest concentration of 93.8 ng/μl; and C has the lowest concentration of 65.0 ng/μl.

The electrophoresis of PCR product after purification were carried out, the results are as shown in the Figure 9; in the comparison with the standard, bacteria A has band of around 1700bp, the band of bacteria B seems identical with the band of bacteria C is around 1710bp. The bands that appeared confirmed that the desired gene was contained in each PCR product which has been purified. All bands were very faint compared with the standard; this was due to the fact that the amount of DNA was reduced after purification. For this reason, the unknown bacteria gene was more difficult to be amplified. In addition, the following DNA purification procedure further reduced the amount of the unknown bacterial gene. Therefore, it was normal that the gene bands became faint.

The most important result, the identification by molecular test, is the BLAST on the gene bank website. BLAST for all bacteria was carried out to prove what kind of bacteria they are. The sequences producing significant alignments, concluded a lot of species genus. For bacteria A, it has a description of *Pseudomonas geniculata* strain in the Max identification, though it is not exactly in line with the API-20E kit result. It nearly approached the *Pseudomonas aeruginosa*, normally the molecular test has an exact identification, so the unknown bacteria A is presently named as *Pseudomonas geniculata* NBG2 strain for

| Bacteria isolate          | OD Value (260 nm) |           | Average Value ± (SD) | 16S rRNA Concentration (ng/μl) |
|---------------------------|-------------------|-----------|----------------------|--------------------------------|
|                           | Reading 1         | Reading 2 |                      |                                |
| Control (Distilled Water) | 0                 | 0         | 0                    | 0                              |
| A                         | 0.032             | 0.031     | 0.0315 ± 0.01        | 78.8                           |
| B                         | 0.038             | 0.037     | 0.0375 ± 0.01        | 93.8                           |
| C                         | 0.025             | 0.027     | 0.026 ± 0.01         | 65                             |

Table 6: The Concentration of Purified PCR Product.

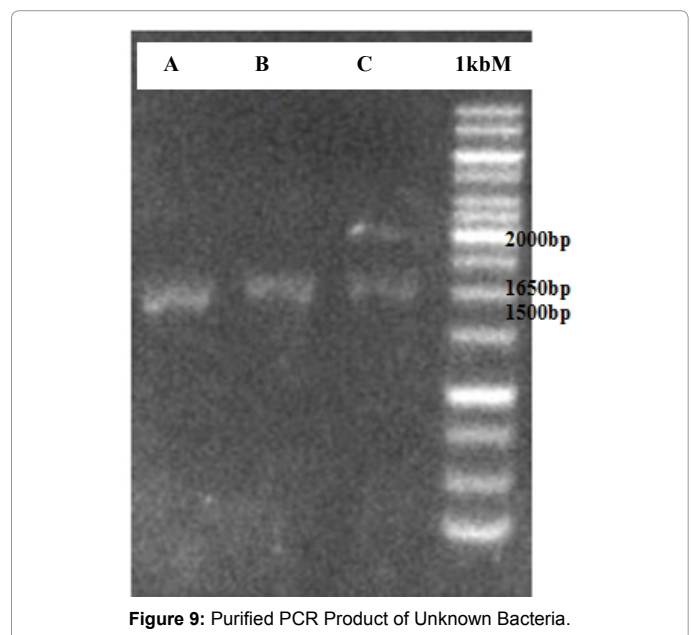


Figure 9: Purified PCR Product of Unknown Bacteria.

which the max identification is 100%. For unknown bacteria B, its identification by molecular test has no connection with biochemical test by API kit 20E. The molecular test proved that the bacterial isolate is probably *Bacillus Sp.* or *Bacillus pseudomycooides B-60*, for which the max identification is above 97%. The third unknown bacteria C, has a max identification of 100% in the description of *Stenotrophomonas maltophilia AQN2*, It has a best relationship with biochemical test by API-20E, so probably this primer is suitable with this bacteria.

To date, Laboratory accurate identification of *P. aeruginosa* has not been reported an imperative component of patient management and nosocomial infection control, there has been no single test that can reliably identify *P. aeruginosa* [30]. Previously reported the limitations of phenotypic methodologies including biochemical tests [33-34].

The identification of *S. maltophilia* by random primer is in line with random PCR which has proven effective in *S. maltophilia* typing when compared to gel electrophoresis (GE), it is a considerable pathway of choice [35], PCR is always a much powerful way to prove result reliable extent amongst two different amplification systems [36].

In conclusion, from results, most of the successfully isolated bacteria were from the fertilizer soil. The reason is probably that fertilization has a positive effect to help bacteria in degradation activities. As a result, the fertilizer soil is comparatively an excellent environment for bacteria. The universal primer designed was not efficient for the bacteria, moreover, only bacteria is absolutely not in line with the biochemical test. But by contrast, the molecular test gave an exact and persuaded finding on the identification, in the combination of the two methods for identification, It was reported that the transesterification of several vegetable oils by bacterial lipases, including *Pseudomonas* lipase, showed a stronger activity, compared to fungal lipases such as Lipozyme TL IM [27]. In this study, since the final results of methyl ester conversation rate by lipase from *Pseudomonas qeniculata (pseudomonas aeruginosa)* (24%) and lipase from *Stenotrophomonas maltophilia (Pseudomonas luteola)* (21%) were higher than that by lipase from *Aeromonas hydrophila (Bacillus pseudomycooides)* (17%); therefore, lipase from *Pseudomonas aeruginosa* and lipase from *Stenotrophomonas maltophilia (Pseudomonas luteola)* were considered as the most suitable lipase for transesterification reaction of crude palm oil and methanol to methyl ester.

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