IDENTIFICATION AND PARTIAL CHARACTERISATION OF LOCALLY ISOLATED LIPOLYTIC BACTERIA

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MASTER OF SCIENCE（BIOTECHNOLOGY）

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IDENTIFICATION AND PARTIAL CHARACTERISATION OF LOCALLY ISOLATED LIPOLYTIC BACTERIA

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SUPERVISORS' DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Master of Science in Biotechnology.

Signature

Name of Supervisor:

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Position:

Date:

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for the quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for the award of any other degree.

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DEDICATION

Dedicated to my parents, my brothers, my sister and friends, who gave me everlasting inspiration, never- ending encouragements and priceless support towards the success of this study.

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ABSTRACT

Biodiesel (methyl esters) is a clean alternative fuel which can be produced from many renewable resources. It is regarded as the fuel of the future and it has the advantages of being biodegradable and non-toxic. Palm oil, like other vegetable oils, can be used as feedstock for biodiesel production. It is processed through transesterification to produce palm oil methyl ester. Enzymatic reactions catalyzed by lipases are potentially excellent processes to produce biodiesel through the transesterification reaction. Enzymes have several advantages over chemical catalysts such as mild reaction conditions, high specificity, and renewability. The production of biodiesel using a biocatalyst eliminates the disadvantages of the alkali process by producing a product of very high purity. Various microorganisms like bacteria and fungi produce different kinds of enzymes which could be used as catalysts in a series of degradation reactions, such as transesterification. 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^{o}C, and the optimum batch culture times of all strains be 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 were involved in the most significant transesterification reaction were 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 maltoplilia(C)*, while the second bacteria (B) failed to be identified. Enzymatic transesterification of crude palm oil with methanol was studied. The enzymes from the three bacterial strains with the most significant transesterification reactions were tested for yield of biodiesel by changing the molar ratio of alcohol to crude palm oil and by changing reaction temperature. The molar ratio of methanol to crude palm oil was varied in the range from 3:1 to 4:1. The reaction temperature was varied from 35^{o}C to 60^{o}C. It was found that the optimum ratio of methanol to crude palm oil is 3:1 and the optimum reaction temperature is 40^{o}C.

ABSTRAK

Biodisel (metil ester) merupakan satu bahanapi bersih alternatif yang boleh dihasilkan daripada banyak sumber yang boleh diperbaharui. Ia dianggap sebagai bahanapi masa depan dan mempunyai kelebihan biobolehurai dan tidak toksik. Minyak sawit, seperti kebanyakan minyak sayuran lain, boleh digunakan sebagai bahan suapan untuk proses penghasilan biodiesel. Ia diproses melalui transesterifikasi untuk menghasilkan metil ester minyak sawit. Tindakbalas berenzim yang dimangkinkan oleh lipase merupakan proses yang berpotensi bagi penghasilan biodisel melalui tindakbalas transesterifikasi. Enzim mempunyai beberapa kelebihan berbanding dengan mangkin kimia seperti ia tidak memerlukan sekitaran yang melampau bagi tindakbalas, ia mempunyai kememilihan yang tinggi, dan ia boleh diperbaharui. Penghasilan biodisel menggunakan biomangkin melenyapkan keburukan-keburukan proses alkali dengan menghasilkan hasil yang berketulinan tinggi. Berbagai mikroorganisma, seperti bakteria dan fungi, menghasilkan enzim-enzim yang berbeza yang boleh digunakan sebagai mangkin dalam satu siri tindakbalas penguraian, seperti transesterifikasi. Dalam kajian ini, lapan belas (18) baka bakteria telah berjaya dipencilkan daripada sampel tanah tempatan dan beberapa cirinya telah dapat ditentukan. Suhu optimum untuk semua baka adalah antara 30^{o} hingga 37^{o}C, dan jangka masa optimum untuk suatu kultur sesekumpul bakteria adalah antara 24 hingga 48 jam. Semua baka bakteria ini telah diuji menggunakan teknik pewarnaan-gram. Tiga baka yang terlibat dalam tindak-balas transesterifikasi yang paling signifikan telah dipilih untuk dikenal pasti dengan diuji secara biokimia menggunakan kit API komersial. Tiga baka yang sama telah juga diuji menggunakan teknik molekul. Dua daripada tiga bakteria tersebut telah dikenal pasti sebagai *Pseudomonas geniculata(A)* dan *Stenotrophomonas maltophilia(C)*, manakala bakteria ketiga gagal untul dikenal pasti. Transesterifikasi berenzim minyak sawit mentah dengan methanol telah dikaji. Enzim-enzim yang didapati daripada tiga (3) baka bakteria yang terlibat dalam tindakbalas transesterifikasi yang paling signifikan telah diuji untuk tahap penghasilan biodisel dengan mengubah nisbah mol alkohol kepada minyak sawit mentah dan dengan mengubah suhu tindakbalas. Nisbah mol metanol kepada minyak sawit mentah telah diubah dalam julat dari 3:1 hingga 4:1. Suhu tindakbalas telah diubah dalam julat dari 35^{o}C hingga 60^{o}C. Didapati bahawa nisbah optimum metanol kepada minyak sawit mentah ialah 3:1 dan suhu tindakbalas optimum ialah 40^{o}C.

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LIST OF ABBREVIATIONS

CPO	Crude Palm Oil
PBR	Packed Bed Reactor
NA	Nutrient agar
FAME	Fatty Acid Methyl Ester
TGs	Triglycerides
h	Hour
min	Minutes
L	Litre
M	Mole or Molar
℃	Celsius Degree
pH	Hydrogen Ion Concentration
µl	Microlitre
%	Percentage
OD	Optical Density
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
rpm	Revolutions per minute
PCR	Polymerase Chain Reaction
BLAST	Basic Local Alignment Search Tool

FAEE	Fatty Acid Ethyl Esters
GC	Gas Chromatography
HPLC	High-performance Liquid Chromatography
POB	Palm Oil Biodiesel
TAG	Triacylglycerol
MAG	Monoacylglycerides
DAG	Diacylglycerides

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

Alternative fuels research has made a lot of attention recently because a high oil price and environmental concerns (Istadi *et al*., 2010). In the among of different possible resources, diesel fuel derived from vegetable oils and animal fats triglycerides showed potential as a petroleum diesel fuel substitute. However, the direct use of vegetable oils in a diesel engine can cause a number of problems such as poor fuel atomisation, poor cold engine start-up, oil ring sticking, and the formation of gum and other deposits. Therefore, considerable efforts have been made to develop alternative which have the same properties and performance as the petroleum-based fuels, with the transesterification of triglycerides to fatty acid alkyl esters showing the most current promise (Jaturong *et al.*, 2006).

Biodiesel is commonly defined as methyl and ethyl ester of fatty acids (FAMEs). Usually the fatty acids of vegetables oils, animal fats, and even waste oils as well was mixed with alcohol (methanol and ethanol respectively) to produce biodiesel by enzymatic or chemical transesterification (Hossain and Boyce, 2009). The enzymatic transesterification method may offer many advantages and have therefore received much attention recently (Dennis *et al*., 2010).

Enzymatic transesterification method can be divided into whole cell and lipase-mediated based on the type of biocatalysts used. In the process of the traditional chemical method, it has some drawbacks compared with the method of using enzyme for the transesterification process (Yi *et al*., 2010). The moderate reaction and lower alcohol to oil ratio were the biggest advantages when enzymatic production of biodiesel was carried out (Du *et al*., 2008). Furthermore, the whole cell biocatalysts for example filamentous fungi, yeast and bacteria seem to be promising alternatives to enhance the cost effectiveness of biotechnological transesterification processes.

A number of parameters may influence the effectiveness of the transesterification reaction. Besides the chemical and physical properties of the type of lipases and the type of oil substrate used as well as the best temperature of the transesterification process, other relevant parameters are the choice of acyl-acceptors, alcohol-oil ratio, the purity of substrates, water content of the reaction mixture, solvents and enzymes (Uthoff *et al*., 2009).

It is not ecologically friendly for producing FAME in the term of classical processes. Lipases were an alternative which have been adopted as biocatalysts by converting natural oil to biodiesel for the synthesis. In nature, lipases are used in the recycling of insoluble organic materials. The advantage in the application of the alternative enzyme is the production of medium or high-value products over the traditional processes. The technique is called “clean technology” simply due to its recycling property, in which materials are systematically used and re-used to cause the drastic increase in resource productivity needed to make human activity sustainable.

However, reports have indicated that among other problems, alcohols like methanol or isopropanol can inactivate the enzymes (Moncia and Albuquerque, 2009). Furthermore, the price of commercial enzymes is quite expensive. The advantages of lipase-catalyst hydrolysis over the conventional high-temperature are their

high-pressure-steam split-quality and high-energy consumption is low. The main disadvantage of lipase hydrolysis processes is the high cost of the enzyme.

1.2 PROBLEM STATEMENT

In the biodiesel industry, the main biodiesel production utilizes the chemical method, whereby, plant and animal oils and fats with methanol or ethanol were subjected to heat (230 - 250^{o}C), in the presence of an acid or alkali catalyst (Dizge *et al.,* 2009). However, this method leads to complex mixtures, high energy use and alcohol consumption. The present proposal suggests a much superior alternative for the production of FAME.

Due to the target product is difficult to purify and the catalyst could not be recycled in the chemical transesterification; in addition, since enzymatic catalysts are reusable, energy consumption is low and overall purification of desired product is easily accomplished, the study of enzymatic transesterification presents a new dimension in catalytic study of transesterification and hold promise for creation and filing of intellectual property (Demirbas, 2008).

In sum, in the present research, an indigenous enzyme will be sought and its performance will be screened.in the whole research, the most critical step is how to get bacteria which could produce reusable and cheap price enzyme to catalyze transesterification of palm oil to produce high mass biodiesel (FAME). Commercial enzyme which is readily available tends to be the focus in this research. However a critical problem to be overcome is the successful transfer of the developed process to an upscale level as the cost of commercial enzymes is the greatest limiting factor on scaling. So far, palm oil is the most abundant natural resource around the whole world. As a result, there is tremendous potential for solving the energy crises. However, if the cost of enzymes was not solved validly, it is futile to conduct these and related studies.

1.3 RESEARCH OBJECTIVES

This research has three (3) main objectives:

1) To isolate and identify potential bacteria which are capable of producing enzymes which catalysed transesterification of palm oil.
2) To characterize potential bacteria that are capable in producing enzymes which catalysed transesterification of palm oil.
3) To screen and test the isolated characterised bacteria for potential activities of transesterification of palm oil.

1.4 SCOPE OF STUDY

In order to achieve the stated objectives, the following scope of work has been identified:

1) To gather microorganism samples from oil palm plantations, isolating the microorganisms in appropriate medium, screen the bacteria for lipase activity before identification and characterisation of bacteria.
2) To study the influence of temperature, biocatalyst concentration and agitation time of treatment process during transesterification.
3) To choose the best crude bacteria enzyme system and establish its optimum operating conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 BIODIESEL

Biodiesel is a mixture of fatty acid methyl esters, which has been developed as one of the most promising alternative fuel to fossil fuels in view of the limited resources of fossil fuels and the environment concerns (Aksoy and Becerik, 1990).

Theoretically, any form of oils and fats coming from the animals, plants or even microorganisms can be used as feedstock for biodiesel production. Currently the main biodiesel feedstocks are classified into three categories: (i) plant oil such as soybean oil (Silva *et al*., 2007), jatropha oil (Shah *et al*., 2004), palm oil (Halim *et al.*, 2009), cotton seed oil, sunflower oil, etcetera (Wu *et al.*, 2007). (ii) animal fat such as tallow (Da Cunha *et al*., 2009), lard and grease and (iii) waste cooking oil and industrial waste oil (Lara *et al*., 2003). Fatty acid distributions in some common oil sources are shown in Table 2.1.

Table 2.1: Types of Fatty Acids and Their Distributions in Raw Materials of Biodiesel (%)

Fatty Acid	Soybean	Cottonseed	Palm	Lard	Tallow	Coconut
Lauric	12:0	0.1	0.1	0.1	0.1	46.5
Myristic	14:0	0.7	0.1	1.4	2.8	19.2
Palmitic	16:0	20.1	42.8	23.6	23.3	9.8
Stearic	18:0	2.6	4.5	14.2	19.4	3.0
Oleic	18:1	19.2	40.5	44.2	42.4	6.9
Linoleic	18:2	55.2	10.1	10.7	2.9	2.2
Linolenic	18:3	0.6	0.2	0.4	0.9	0

Source: Ma and Hanna (1999)

The main raw materials used to produce biodiesel are the vegetable oils extracted from oleaginous plants. The cost of these materials currently represent about 70% of the total production costs; this means that the most suitable vegetable oils are those from crops with the highest productivity per hectare (Table 2.2) or low cost oils such as waste oils (Fairless, 2007).

Table 2.2: Oil Yield (litres/ha) from Oleaginous Species

Vegetable	Oil Yield(L/ha)
Palm	2400
Jatropha	1300
Rapeseed	1100
Sunflower	690
Soybean	400

Source: Fairless (2007)

Based on the above information, palm oil is the most potential vegetable oil which can be used as raw material to manufacture biodiesel. Oil palm is grown in abundance in Malaysia as it is a commercial crop experiencing local and international

demand as feedstock for both food and fuel industries (Jibrail *et al*., 2009). With dwindling fossil fuel resources, governments are advocating research and development of renewable resources, such as palm oil and soybean oil, as replacements for energy feedstock (Samios *et al.*, 2009). The use of biodiesel is favorable as it is not expected to create a need for drastic engine modifications. Biodiesel use is also favorable because it does not generate toxic engine emissions that will contribute to greenhouse gases. In Malaysia, the government mandated the use of 5% biodiesel (Gregore *et al*., 2008) and this is expected to increase as biodiesel becomes readily available in this country.

In Southeast Asia, palm oil is the most suitable for production of biodiesel in large scale due to its availability. Purified palm oil however is too expensive (0.74 USD per liter) to be economically feasible. Palm fatty acid (0.37 USD per liter), a by-product of palm oil refinery, on the other hand, is one of the most attractive raw materials due to its low cost. Furthermore, it is generally obtained in a purified form during the refining process of crude oil. Therefore palm fatty acid is a potential raw material for the production of biodiesel (Akaraphol *et al*., 2008).

In 2005, Malaysia and Indonesia produced nearly 80% of the 35 million tonnes total world production of palm oil. Likewise, in Southeast Asia, Malaysia has a rich oil palm industry that generates excess crude palm oil (CPO) in vast quantities for consumption. CPO is one of the four leading vegetable oils traded on the world market. It is also cheaper than canola, rapeseed or soybean oil and would reduce the overhead cost of biodiesel production and generate a ready supply of diesel fuel substitute or blend. The palm fruit as shown in Figure 2.1 had attracted attention towards to the specific Palm Oil Biodiesel (POB), POB is the centre of biofuel research not only in Malaysia which has already begun preparations to change from diesel to bio-fuels (at least 5% component of the diesel sold must be biodiesel) but also in many other countries (Ishizaki *et al*., 1999).

Figure 2.1: Palm Oil Fruit.

Demand for more POB is evolving and even though the current production of biodiesel from palm oil is insignificant (1% total production) compared to the majority of biodiesel produced worldwide from rapeseed oil (84% total production), POB remains the most attractive candidate due to the high yield of the oil palm tree and other advantages in economy and energy generation (Beckman, 2007).

The direct use of vegetable oils as biodiesel is possible only by blending them with conventional diesel fuel in a suitable ratio, but the direct usage of vegetable oils in diesel engines is not technically possible because of their: (i) high viscosity; (ii) low stability against oxidation (and the subsequent reactions of polymerization), and (iii) low volatility, which influences on the formation of a relatively high amount of ashes due to incomplete combustion (Serchelia and Vargas, 1998). Therefore, vegetable oils must be processed so as to acquire the properties necessary to be directly used in current diesel engines. The possible processes are pyrolysis (or cracking), microemulsion and transesterification (Ma and Hanna, 1999).

As the first two methods are cost intensive processes, yielding a low quality biodiesel, the most common method to transform palm oil into methyl ester is transesterificaton. This consists of the reaction between triacylglycerols (contained in the oils) and an acyl-acceptor. The acyl group acceptors may be carboxylic acids (acidolysis), alcohols (alcoholysis) or another ester (interesterification). Only alcoholysis and interesterification are of interest to produce biodiesel. The starting esters in both are triacylglycerols (oils), and if the transformation is quantitative they yield a mixture of monoalkyl esters (biodiesel) and glycerol (alcoholysis) or another triacylglycerol (interesterification) as shown in Figure.2.2.

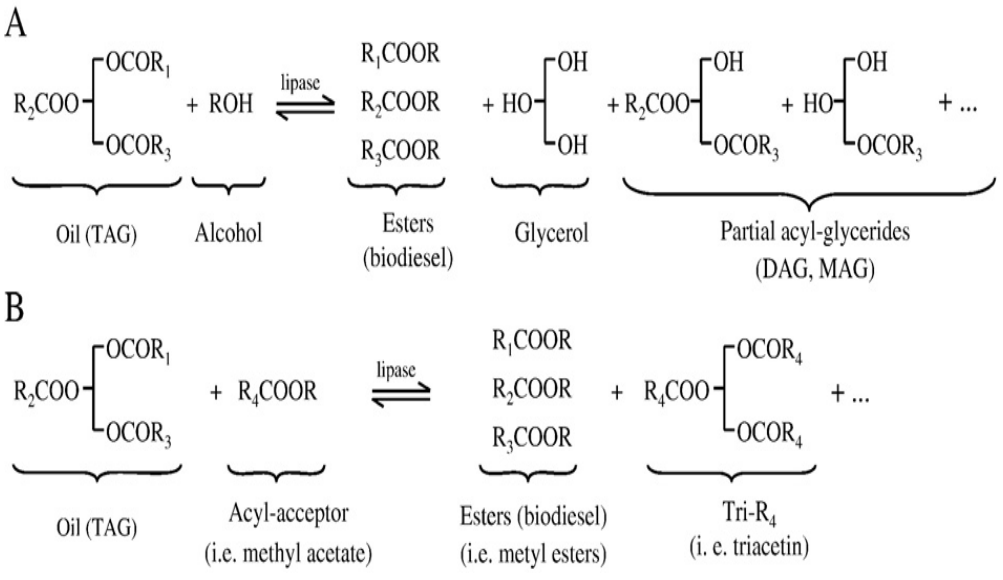

Fig.2.2: (A) Alcoholysis (B) Transesterification

Source: Hideki *et al*., 2001

The reaction occurs stepwise if the reaction equilibrium is displaced toward product formation: triacylglycerides are first converted to diacylglycerides, then to monoacylglycerides and finally to glycerol. R, R1, R2, R3, R4 are acyl groups.

Alcohols are the most frequently used acyl-acceptors, particularly methanol and,

to a lesser extent, ethanol. Other alcohols can be used, e.g. propanol, methanol, butanol, isopropanol, tert-butanol, branched alcohols and octanol but the cost is much higher. Regarding to the choice between methanol and ethanol, the former is cheaper, more reactive and the fatty acid methyl esters (FAME) are more volatile than those of the fatty acid ethyl esters (FAEE) (Kumari *et al.*, 2004).

The quality control of biodiesel is greatly important to this fuel and its blends (Knothe, 2006). On this point, it is worth to note that the type (chain length, degree of unsaturation and presence of other chemical functions) and concentration of fatty esters as well as the structure of the ester derived from the alcohol have an outstanding effect on biodiesel properties, which will also influence its storage and oxidation (Pinto *et al*., 2005). Besides, biodiesel composition is completely dependent on the source used to produce it (Stauffer and Byron, 2007). Further, since some contaminants come up from the transesterification reaction, it is important to monitor the status of biodiesel production to recognize and correct problems at an early stage. Fernando *et al.* (2007) described the complete reaction to mono-alkyl esters, the removal of free glycerine, catalysts, alcohol, and free fatty acids in biodiesel as critical issues of the quality control. Chromatography and spectroscopy are the most used analytical methods on biodiesel analyses (Knothe, 2006).

2.2 METHODS OF PRODUCTION OF BIODIESEL

2.2.1 Catalytic Conversion Processes

The conversion of catalytic transesterification can be carried out by different catalytic processes (Marchetti *et al*., 2007). The catalyst used may be classified as: (i) alkaline-catalyst such as sodium hydroxide, NaOH, Potassium hydroxide, KOH, sodium metoxide, NaOMe; (ii) acid-catalyst such as sulphuric acid, phosphoric acid, hydrochloric acid, sulfonic acid and (iii) enzymatic-catalyst such as lipase.

The alkaline-catalyst is the best known and most widely used in the process of producing biodiesel (Demirbas, 2008). Acid catalysts should be the method when using oils extracted from micro-algal biomass (Nagle and Lemke, 1990). Enzymatic-catalyst using lipases for biodiesel production are mainly to overcome the issue involving recovery and treatment of the by-product that requires complex processing equipment (Ha *et al*., 2007).

2.2.2 Alkaline Catalysis and Transesterification

Alkaline metal alkoxides are the most active catalysts in the transesterification process, since they give very high yields in short reaction times (30 min) even if they are applied at low molar concentrations (0.5mol%) (Schwab *et al.*, 1987). Next come hydroxides (Stavarache *et al*., 2005), as well as sodium or potassium carbonates (Varghaa and Truterb, 2005).

The alkaline catalysts normally show high performance when high quality vegetable oil is used. However, when the oils contain significant amounts of free fatty acids, they cannot be converted into biodiesels but to a lot of soap (Furuta *et al.*, 2004). These free fatty acids react with the alkaline catalyst to produce soaps that inhibit the separation of biodiesel, glycerin and wash water (Canakci and Gerpen, 2003).

Triglycerides are readily transesterified batch wise in the presence of alkaline catalyst at atmospheric pressure and at a temperature of approximately 60–70 ℃ with an excess of methanol (Srivastava and Prasad, 2000). It often takes at least several hours to ensure the alkali (NaOH or KOH) catalytic transesterification reaction is complete. Removal of these catalysts is technically difficult and it brings extra cost to the final product (Demirbas, 2003).

During transesterification, the glycerin that is formed needs to be removed so that it is not converted into formaldehyde or acetaldehyde when burned because both would pose a health hazard (Freier, 2005).

Alkaline-catalyzed transesterification is much faster than acid-catalyzed transesterification and is the most commonly used method commercially (Ma *et al.*, 1998). Putting that together with the fact that the alkaline catalysts are less corrosive than acidic compounds, industrial processes usually favor base catalysts such as alkaline metal alkoxides and hydroxides as well as sodium or potassium carbonates (Soumanou, 2003).

2.2.3 Acid Catalysis and Transesterification

One advantage of acid catalysts over base catalysts is their low susceptibility to the presence of free fatty acid in the starting feedstock. However, acid-catalyzed transesterification is especially sensitive to water concentration. It was demonstrated, previously, that as little as 0.1% water in the reaction mixture was able to affect ester yields in transesterification of vegetable oil with methanol, with the reaction almost completely inhibited at 5% water concentration (Canakci and Gerpen, 1999).

Palm oil with high free fatty acid content can be converted to biodiesel using acid catalysis, which is the second approach for handling high free fatty acid content feedstock. This technique uses a strong acid. Soap formation is not a problem because there are no alkali metals in the reaction medium.

Acid catalysts can be used for transesterification of the triglycerides, but the reaction might take several days to complete. This is too slow for industrial processing.

Acid catalysis requires a high alcohol to free fatty acid ratio (20:1 or 40:1 mole

ratio) and a large amount of catalyst (5-25 %). Sulphuric acid and phosphoric acid are the most common acid catalysts. The feedstock is sometimes dried to 0.4 % water and filtered before the reaction. Then, an acid and methanol mixture are added to the feedstock. Once the conversion of the fatty acids to methyl esters has reached equilibrium, the methanol, water and acid mixture is removed by settling or centrifugation. Fresh methanol and base catalyst are added into the remaining oil for transesterification. The rest of the process is the same as base catalysis. Reaction times of 10 minutes to 2 hours have been reported (Kusdiana and Saka, 2004).

2.2.4 Enzymatic Transesterification

There are two major categories of enzymatic biocatalysts: (i) extracellular lipase. The major producer microorganisms are *Pseudomonas cepacia, Rhizopus oryzae, Candida antarctica and Mucor miehei;* (ii) intracellular lipase, which still remains either inside the cell, or in the cell walls.

The pros and cons of using lipases as biocatalysts as opposed to conventional alkaline catalysts for biodiesel production are summarized in Table 2.3.

Table2.3: Comparison between Enzymatic and Conventional Alkaline Technology

Key Issue	Enzymatic Process	Alkaline Process
Presence of free fatty acid in the starting oil.	Free fatty acids are transformed to biodiesel.	Free fatty acids are transformed to soaps.
Water content of starting oil.	It is not deleterious for lipase.	Impact on catalyst by forming soaps. May hydrolyze the oil and more soaps are formed.
Biodiesel yield.	High, usually around 90%.	High, usually > 96%.
Glycerol recovery.	Easy, high grade glycerol.	Complex, low grade glycerol.
Catalyst recovery and re-usage.	Easy or not necessary when operating in Packed-Bed Reactors (PBR).	Difficult or not profitable, usually neutralized by adding an acid after transesterification.
Energy costs.	Low. Temperature range 20-50^{o}C.	Medium. Temperature range 60-80^{o}C.
Catalyst cost.	High.	Low.
Environmental impact.	Low, waste water treatment not needed.	Medium. Alkaline and saline effluents are generated.
Process productivity.	Low	High.

Source: Robles and Gonzalez (2009)

The enzymes can be used in solution or immobilized onto a support material, which allows for the use of fixed-bed reactors. The reaction can be performed at 35 to 45^{0} C. However, the reaction is very slow, requiring from 4 to 40 hours. Because of the high cost of the enzymes, this process is not economically feasible for biodiesel production at this time (Vasudevan and Briggs, 2008).

To date, practically the only microorganism employed in this type of biocatalyst is the previously mentioned *R. oryzae*, a filamentous fungus that produces a specific lipase. Culture conditions of this fungus are well established in the literature;

100 mL of basal medium with about 10^6 spores were cultivated for 40 h at 35 ℃ in an orbital shaker at 130 rpm. Kumar *et al*. (2005) cultivated the fungus in a similar way, but once sufficient fungus biomass had been obtained in the shaking flask, they transferred the culture into a draught tube airlift bioreactor where the fungus continued to grow.

Recently, enzymatic conversion technology seems to be starting its application in industrial scale. It has been claimed that this technology has been applied at industrial scale with a capacity of 20,000 tonnes / year in China, and this is the first industrial scale with lipase as the catalyst in the whole world to date (Du *et al.*, 2008).

2.3 SOURCES OF ENZYMES FOR ENZYMATIC CONVERSION

Microorganisms produce a large variety of enzymes, most of which are made in only small amounts and are involved in cellular processes. However, certain enzymes are produced in much larger amounts by some organisms, and instead of being held within the cell, they are excreted into the medium (Kirk *et al.*, 2002).

Extracllular enzymes (exoenzymes) are capable of digesting insoluble polymers such as cellulose, protein, and starch. The products of digestion are then transported into the cell where they are used as nutrients for growth. Some of these exoenzymes are used in food, dairy, pharmaceutical, and textile industries and are produced in large amounts by microbial synthesis (Bataillon *et al.*, 2000).

Enzymes are especially useful biocatalysts because they often act on single chemical functional groups, as they can easily distinguish between similar functional groups on a single molecule, and in many cases, they catalyze reactions in a stereospecific manner producing only one of two possible enantiomers.

The majority of the enzymes studied for transesterification reactions are lipases. Lipases play an important role in the metabolism of all living organisms. They can be divided into intracellular and extracellular lipases and are easily obtained biotechnologically in high yield by fermentation and purification. Lipases enzymes are widely employed to catalyze hydrolysis, alcoholysis, esterification and transesterification of carboxylic esters. Lipases have excellent catalytic activity and stability in non-aqueous media, which facilitate the esterification and transesterification process during biodiesel production (Villeneuve *et al*., 2000).

Many microorganisms such as bacteria, yeast, and fungi are known to secrete lipase during growth on hydrophobic substrates, which renders the lipid substrates available to the cells (Lee, 2006).

2.4 BACTERIA AS A SOURCE OF LYPOLYTIC ENZYME

Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer. Only about 2% of the world's microorganisms have been tested as enzyme sources. Bacterial strains are generally more used as they offer higher activities compared to yeasts and tend to have neutral or alkaline pH optima and are often thermostable (Wiseman, A. 1995).

Genetic and environmental manipulation to increase the yield of cells, to increase the enzyme activity of the cells by making the enzyme of interest constitutive, or by inducing it, or to produce altered enzymes, may be employed easily using

microbial cells because of their short generation times, their relatively simple nutritional requirements, and since screening procedures for the desired characteristic are easier (Fariha *et al*., 2006).

Lipolytic enzymes are currently attracting an enormous attention because of their biotechnological potential (Benjamin and Pandey, 1998). They constitute the most important group of biocatalysts for biotechnological applications. The high-level production of microbial lipases requires not only the efficient over expression of the corresponding genes but also a detailed understanding of the molecular mechanisms governing their folding and secretion. Lipases from a large number of bacterial, fungal, plant and animal sources have been purified to homogeneity (Saxena *et al*., 2003).

Lipases isolated from different sources have a wide range of properties depending on their sources with respect to positional specificity, fatty acid specificity, thermo-stability, pH optimum, etc. (Huang, 1984). One could probably find a lipase from nature that would be suitable for desired application. Most of the lipases in use are from fungi and other eukaryotic microorganisms. Only a few have been purified from bacteria such as *Burkolderia cepacia, Chromobacterium viscosum, Enterobacter aerogenes, Pseudomonas cepacia* and *Pseudomonas fluorescens* [Orcaire *et al*., (2006), Shah *et al*., (2004), Kumari *et al*., (2009), Zhao *et al*., (2007)]

2.5 METHODS OF ISOLATION OF BACTERIA

Microorganisms outnumber all other organisms in both biomass and diversity. They are a major part of global ecosystem services and natural capital in the world. However, they are the least known species, and we know only 10% of their names, habits, structures, functions, and forms. They are the first occurring living organisms on earth. However, our knowledge of them remains poor largely because approximately 99% of them are not readily culturable (Zhou *et al*., 2009).

In order to obtain a pure culture of any microorganism, any method to be used must ensure the introduction of a single cell into a sterile growth medium in a suitable culture vessel. The isolates will be firstly collected Accurately weighed samples consisting of about 10 g freshly sieved soil were added to 90-ml prepared sterile distilled water in 150-ml conical flasks and dispersed by stirring with Teflon-coated magnetic bars (6 mm in diameter, 30 mm long) for 20 min at approximately 200 rpm, and then diluted the mixed solution according to the following concentration from 10-1until10-8 . From each dilution tube (but not the 10-2) place 0.1 ml of dilution fluid into each of two sterile Petri plates. All Petri dish were placed in the incubator or at 30℃ for at least 3 days. Find several different surface colonies that are well isolated from other colonies, then the independent colonies will be streaked one generation by one generation, finally kept well in the refrigerator at 4 ℃ for the further research.

2.6 METHODS OF IDENTIFICATION OF BACTERIA

Identification of unknown microorganisms at species level with conventional biochemical tests, such as sodium chloride tolerance, citrate utilization, and tobramycin susceptibility, takes days to weeks and can be laborious (Yakrus *et al.*, 2001). For species-level identification of isolates, nucleic acid amplification tests such as polymerase chain reaction (PCR) restriction analysis or DNA sequencing are used widely nowadays (Heller *et al*., 2008).

2.6.1 Conventional Method

The traditional method to identify unknown bacteria is the biochemical test. In the course of this method, a single colony is used to streak a nutrient agar plate to determine if their unknown bacteria are Gram positive or Gram negative. The Gram-staining dye will enter the Gram positive bacteria and inhibit growth, but Gram negative bacteria are protected by their enhanced cell wall.

For the physiological characteristics, two aspects will be evaluated. Firstly, the oxidation tests will be conducted to determine if a bacterium produces certain cytochrome c oxidases. It uses disks impregnated with a reagent such as *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) or *N,N*-Dimethyl-*p*-phenylenediamine (DMPD), which is also a redox indicator. The reagent is a dark blue to maroon colour when oxidized, and colorless when reduced. Strains may either be oxidase positive (OX+) or negative (OX-). OX+ normally means that the bacterium contains cytochrome c oxidase and can therefore utilize oxygen for energy production with an electron transfer chain. Typically the *Pseudomonadaceae* are OX+. OX- normally means that the bacterium does not contain cytochrome c oxidase and therefore cannot utilize oxygen for energy production with an electron transfer chain.

Secondly, bacteria are unable to carry out phagocytosis for acquiring food materials and therefore they excrete exoenzymes that split larger molecules into smaller units. A variety of proteases degrade protein molecules, such as casein and gelatin, into amino acids, and fats or triglycerides are split into fatty acids and glycerol by various lipases. Bacteria also hydrolyze small molecules, producing signature compounds that can be used in identifying them. Tryptophan is split biochemical test specific for the indole ring. Likewise, some bacteria degrade urea producing carbon dioxide and ammonia, which causes a colour change in pH indicators owing to the alkaline conditions produced by ammonia.

Apart from the above biochemical tests, there are several additional physiological tests used in unknown bacterial identification. They include tests for hydrogen sulfide production, citrate utilization, phenylalanine deaminization, and litmus milk reactions (Alfred, 2005).

2.6.2 Biochemical Test by Kit

The commercial kit used in this project is profile index kit which consists of 20 tubes. These tubes were developed for clinical use to identify bacteria. Each tube with a different type of media will be tested for the presence of a different enzyme or set of enzymes in the unknown bacteria. The compartments are inoculated with the unknown bacteria and the tubes are placed in the 37 ℃ incubator. After overnight incubation, each compartment is examined to determine the colour of the media. The colour of the compartments is compared with a reference guide from the suppliers to determine if the colour indicates a positive or negative result for the presence of that particular enzyme(s). Each positive result is used in generating a three-digit number. This three-digit number, or "biocode," can then be looked-up in tube code book, as appropriate; the number will correspond to a species of bacteria that produces that particular combination of enzymes. Usually the unknown bacteria are identified at the completion of this test (Jaak *et al*., 1999).

2.6.3 Identification by Molecular Biology Method

In the past decade or so, molecular techniques have proven beneficial in overcoming some limitations of traditional phenotypic procedures for the detection and characterization of bacterial phenotypes. Several non-culture based methods have emerged in the past 15 years. PCR and microarrays are currently the most commonly employed molecular techniques. PCR is highly sensitive and allows identification of bacteria at species level. Microarray based bacterial identification relies on the hybridization of pre-amplified bacterial DNA sequences to arrayed species-specific oligonucleotides. Each probe is tagged with a different coloured dye which fluoresces upon hybridization.

Primers targeting the internal transcribed spacer of the unknown bacteria

including a unique reverse primer forward primer, DNA extraction and multiplex, PCR with SYBR green detection dye on PCR instrument are performed as described by Kevin and Xuan (2001). Amplified products in the melting curve plot are analyzed for their intensity and specificity. Peaks with intensities of greater than 30 U are considered positive.

In the final exercise, the electrophoresis is viewed from their sequencing reaction, and the sequence is then used in a BLAST search limited to a bacterial data base. The unknown bacteria are identified by examining the top-scoring sequences from the BLAST search results.

2.7 PARAMETERS INFLUENCING THE BIOCONVERSION OF PALM OIL TO METHYL ESTERS BY LIPOLYTIC ENZYME FROM BACTERIA

The process of transesterification is affected by various factors depending upon the reaction conditions used. The effects of these factors are described below.

2.7.1 Enzyme Concentration

In the case of base catalysed transesterification conversions, catalyst concentration can affect the yield of the biodiesel product. As mentioned before, the most commonly used catalyst for the reaction is sodium hydroxide. However, Freedman *et al*. (1984) found that sodium methoxide was more effective than sodium hydroxide because upon mixing sodium hydroxide with methanol a small amount of water will be produced, which will affect the product yield because of the hydrolysis reaction (Guo, 2005). This is the reason why the catalyst should be added into the methanol first and then mixed with the oil. As the catalyst concentration increases the conversion of triglyceride, the yield of biodiesel increases. This is because an insufficient amount of catalysts result in an incomplete conversion of the triglycerides

into the fatty acid esters (Leung and Guo, 2006).

Usually, the yield reaches an optimal value when the catalyst concentration reaches 1.5% and then decreases a little with a further increase in catalyst concentration. The reduction of the biodiesel yield is due to the addition of excessive alkali catalyst causing more triglycerides to react with the alkali catalyst and form more soap (Eevera *et al*., 2009).

How enzyme concentration affect the yield of enzyme-catalysed transesterification conversions will be studied in this research.

2.7.2 Influence of Reaction Time and Temperature

The enzyme conversion yield increases with reaction time. Freedman *et al*. (1984) investigated transesterification by peanut, cotton-seed, sunflower and soybean oils under the conditions of methanol–oil molar ratio of 6:1, 5% lipase catalyst and temperature of 60.8^{o}C. An approximate yield of 80% was observed after 1 min for soybean and palm oils. Similar final results were obtained for all four oils (soybean oil, palm oil, sunflower oil, peanut oil: 93–98%). Ma *et al*. (1998) studied the effect of reaction time on enzymatic transesterification of beef tallow with methanol. The reaction was very slow during the first minute due to mixing and dispersion of methanol into beef tallow. From 1 to 5 min, the reaction proceeded very fast. The production of beef tallow methyl esters reached the maximum value at about 15 min.

Transesterification can occur at different temperatures, depending on the oil used. For the transesterification of refined oil with methanol (6:1) and 1% lipase, the reaction was studied at three different temperatures (Samios *et al.*, 2009). After 1 h, ester formation was identical for 60^{o}C and 45.8^{o}C runs and only slightly lower for the 32. 8^{o}C run. Ma and Hanna (1999) asserted that temperature clearly influenced the

reaction rate and yield of esters, different catalyst reaction has its own optimum temperature, at this temperature, the production of biodiesel will be maximum achieved, in addition, it could be able to save materials and energy and keep the enzyme at a high level activity.

2.7.3 Mixing Intensity

Mixing is very important in the transesterification reaction, as oils or fats are immiscible with catalyst–methanol solution. Once the two phases are mixed and the reaction is started, stirring is no longer needed. Initially the effect of mixing on transesterification of beef tallow was studied by Ma *et al.* (1998). No reaction was observed without mixing and when catalyst was added to the melted beef tallow in the reactor while stirring, the effect of stirring speed was insignificant. Reaction time was the controlling factor in determining the yield of methyl esters. This suggested that the stirring speeds investigated exceeded the threshold requirement of mixing.

2.7.4 Molar Ratio of Alcohol to Oil and Type of Alcohol

One of the most important variables affecting the yield of ester is the molar ratio of alcohol to triglyceride. The stoichiometric ratio for transesterification requires 3 moles of alcohol and 1 mole of triglyceride to yield 3 moles of fatty acid alkyl esters and 1 mole of glycerol.

However, transesterification is an equilibrium reaction in which a large excess of alcohol is required to drive the reaction to the right. For maximum conversion to the ester, a molar ratio of 6:1 should be used. The molar ratio has no effect on acid, peroxide, saponification and iodine value of methyl esters. However, the high molar ratio of alcohol to vegetable oil interferes with the separation of glycerin because there is an increase in solubility. When glycerin remains in solution, it helps to drive the

equilibrium back to the left, lowering the yield of esters.

The transesterification of palm oil with ethanol was studied at molar ratios between 3:1 and 15:1. The ester yield increased as the molar ratio increased up to a value of 12:1. The best results were for molar ratios between 3:1 and 6:1. For molar ratios less than 6:1, the reaction was incomplete.

For a molar ratio of 3:1 the separation of glycerine is difficult and the apparent yield of esters decreased because a part of the glycerol remains in the biodiesel phase. Therefore, molar ratio 4:1 seems to be the most appropriate (Farooq, 2010).

The base catalyzed formation of ethyl ester is difficult compared to the formation of methyl esters. Specifically the formation of stable emulsion during methanolysis is a problem. The alcohol materials that can be used in the transesterification process include methanol, ethanol, propanol, butanol, and amyl alcohol. Among these alcohols, methanol and ethanol are used most frequently. Methanol is especially used because of its lower cost and its physical and chemical advantages. Ma and Hanna (1999) reported that methanol can react with triglycerides quickly and the alkali catalyst is easily dissolved in it. However, due to its low boiling point, there is a large explosion risk associated with methanol vapors which are colorless and odorless. Both methanol and methoxide are extremely hazardous materials that should be handled carefully. It should be ensured that one is not exposed to these chemicals during biodiesel production.

2.8 METHOD OF DETERMINATION OF METHYL ESTER

Both GC and HPLC analyses and combinations thereof have been reported for biodiesel production. Generally, GC has been the most widely used method for the analysis of biodiesel owing to its generally higher accuracy in quantifying minor

components. However, accuracy of GC analyses can be influenced by factors such as baseline drift, overlapping signals, and aging of standards and samples (Knothe, 2006).

To date, most chromatographic analyses have been applied to methyl esters and not to higher esters such as ethyl and isopropyl. Most methods would likely have to be modified to analyze the higher esters properly. For example, when using GC, temperature program changes or other alterations may be necessary. The original work (Freedman *et al.,* 1986) on GC analysis reported the investigation of methyl and butyl esters of soybean oil. Not all individual components were separated there in the analysis of butyl soyate, but classes of compounds were analyzed. HPLC analysis was applied to some ethyl, isopropyl, 2-butyl, and isobutyl esters of soybean oil and tallow (Foglia and Jones, 1997).

CHAPTER 3

ISOLATION, IDENTIFICATION AND OPTIMIZATION OF PERFORMANCE OF BACTERIA ISOLATES AND ITS ACTIVITY IN CONVERSION OF PALM OIL TO METHYL ESTERS

3.1 INTRODUCTON

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 (Staley *et al.*, 1997).

The release of microorganisms from soil samples is routine in the microbiological laboratory. The literature is extensive (Riis *et al.,* 1997). It has been established that the genetic diversity of soil bacteria is high and that soil contains many bacterial species of lineages for which no known cultivated isolate are available. Many soil bacteria are referred to as uncultured or even non-culturable. A range of methods have been developed to study these organisms directly in their habitats and are extremely useful for studying their characteristics (Perer *et al.,* 2002).

To understand the differences between the different types of organisms, it is necessary to isolate them into individual colonies. In order to obtain pure colonies, it is necessary firstly to remove all living material from the growth media. The streak plate

technique can be used to obtain pure cultures of bacteria from a sample of mixed organisms. Inoculation consists of removing the bacteria from a starter plate and transferring it to a sterile, nutrient agar plate. This spreading of bacteria with a loop is called streaking and results in single colonies of bacteria appearing which can then be identified by different methods.

Once the bacterial cultures have grown, a small sample can be taken for Gram-staining. The Gram-stain is the most important and universally used staining technique in the bacteriology laboratory. It is used to distinguish between Gram-positive and Gram-negative bacteria, which have a distinct and consistent difference in their cell walls (Bergey *et al.,* 1994). Stain or dye reacts chemically with the bacterial cell but not with the background, allowing the bacteria to be distinguished. Shapes of the cell become very obvious, consisting of cocci (spherical), bacillus (rod-like), or spiral (curved), with some indeterminate types.

Likewise, all microorganisms have a unique growth condition, which consist of optimum temperature, pH, water activity, osmotic pressure and oxygen as well. These parameters could reflect and help in research on bacteria.

Bacteria have many properties that have useful applications. 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 (Bull, 2001).

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 (Helwani and Othman, 2009).

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 (Dizge *et al*., 2009). Chapter 4 will give further evidence on enzymatic catalysis in biodiesel production.

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 (Kansedo *et al*., 2009).

The identification of bacteria is a careful and systematic process that uses many different techniques to narrow down the types of bacteria that are present in an unknown bacterial culture.

Traditional methods of bacterial identification rely on phenotypic identification of the causative organism using Gram staining, culture and biochemical methods. The techniques used at the earliest stages are relatively simple. Biochemical tests identify the main biologically important chemical compounds. However, these methods of bacterial identification suffer from two major drawbacks. First, they can be used only for organisms that can be cultivated in *vitro*. Second, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic of any known genus and species.

In the recent 25 years, molecular biology has developed rapidly and it is now possible to sequence the proteins from different bacterial species, make large database of the sequences, and use them as very powerful identification tools. Similar database

have been developed for bacterial DNA and bacterial RNA, particularly the RNA that forms the structural components of bacterial ribosomes. Agarose gel electrophoresis is a method used in molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments by charge (Kryndushkin, 2003).

The polymerase chain reaction (PCR) represents a major advance in terms of the speed, sensitivity and specificity of diagnostic methods, and has been increasingly used to identify several bacterial species from food and clinical samples. Another advantage is that PCR is not dependent on utilization of a substrate or the expression of antigens, thereby circumventing the phenotypic variations in biochemical patterns and lack of detectable antigens (Hoorfar *et al*., 2003).

Pseudomonas aeruginosa, an obligate aerobe, is found in many natural and man-made environments; it has been isolated from plants and soils (Walker *et al.*, 2004). Identification of *P. aeruginosa* in the laboratory is generally performed by growing the bacteria on either cetrimide agar or nalidixic acid-cetrimide agar. Although this method is reliable, the time required for performing it is up to 48 h. Polymerase chain reaction (PCR) methods, which allow for more rapid identification of *P. aeruginosa* by DNA amplification, have been reported (Jaffe *et al.*, 2001).

Pseudomonas luteola, also as known as *Chryseomon luteola,* is a Gram-negative rod (Anzai *et al.*, 1997). *P. luteola* is aerobic, non-spore-forming, motile, oxidase-negative and catalase-positive (Kiska and Gilligan, 1999). The organism was originally described as *P. luteola.* Based on low levels of DNA-DNA hybridization, these organisms were subsequently referred to as *C. luteola* (Holmes *et al.*, 1987). 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* (Anzai *et al*., 1997).

Aeromonas hydrophila is the most well known of the six species belonging to the genus *Aeromonas*. It is rod- shaped, non- spore forming, oxidase- positive, glucose-fermenting, facultative anaerobic, Gram- negative bacterium, *Aeromonas hydrophila* is an opportunistic pathogen of a wide variety of hosts (Yin and Xu, 1995). PCR application was carried out for detection of pathogenic strains of *A. hydrophila* in human health care (Lior and Johnson, 1991).

Stenotrophomonas maltophilia, previously named *Pseudomonas*, and then *Xanthomonas maltophilia*, has been recently classified as the single species of the new genus *Stenotrophomonas.' S. maltophilia* is a non-fermentative Gram-negative bacillus which grows readily on most bacteriological media (Marty, 1997). Molecular typing systems have contributed to knowledge of the epidemiology of *S. maltophilia* infections. Typing methods such as ribotyping (Gerner *et al.*, 1995), multilocus enzyme electrophoresis (Schable *et al*., 1991), random amplification of polymorphic DNA (Yao *et al.*, 1995), enterobacterial repetitive intergenic consensus-PCR (Denton *et al.*, 1998) and DNA macrorestriction analysis (Talon *et al.*, 1994) have been employed.

The aim of the study referred in this chapter was to isolate and identify the dominant soil bacteria. 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. The activity of producing biodiesel from palm oil and the identification of the bacteria with such activity will be reported in the subsequent chapters. In this study, three strains were investigated by API20-E kit and PCR amplification, so that the identification of three strains were carried out by sequence analysis.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Microbiological agar, liquid broth media and Gram-staining reagents were supplied by Merck, Malaysia Division. API 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, $MgCl_2$ and 10 X Buffer were purchase from Invitrogen, USA.

3.2.2 Equipment and Instrumentation

The 50L autoclave was supplied by Hirama (Toyono, Japan). The microbiological incubator was supplied by Heraeus (Hanau, Germany). The laminar air flow chamber was supplied by Esco (Hatboro, USA). The invert microscope was supplied by Carl Zeiss (New York, USA). Sterile petri dish plate was supplied by Mikro Makmur Enterprise (Johor Bahru, Malaysia). The Bio-imaging system was supplied by Alpha Innotech (USA). The PCR machine was obtained from Eppendorf. The Microcentrifuge was supplied by Heraeus (Hanau, Germany). The Microwave Oven was purchased from Sharp (Tokyo, Janpan). Electrophoresis Apparatus was supplied by Major Science (Saratoga, USA). Clear tips and tubes were supplied by Science Valley Sdn. Bhd.

3.2.3 Samples Collection

Soil samples were collected from the oil palm plantation of LKPP Lepar Hilir,

Jalan Muadzam-Kuantan, Pahang, Malaysia. Two types of sampling sites (Figures 3.1 and 3.2) 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, the soils must contain some bacteria which could be able to degrade palm fruit. Both sampling sites were located under oil palm trees (Because a lot of palm fruits fell down from trees and rotted in the soils) and at a distance of 200 m from each other moving along a direction chosen randomly. The samples were collected at various depths 10cm to 50cm from the soil surface.

Aseptic techniques were used all the way during the sampling process. During sampling, ten (10) disinfected 20-cm-spatulas were used to obtain soil samples from points at varying depths of between 10 and 50 cm from the surface of each hole. An external 3 mm of each core was discarded and approximately 30 g of soil were collected in prepared sterile bottles. In total ten (10) bottles, comprising of five (5) bottles from each sampling site, were collected. All together, an amount of 300 g of soil from the oil palm plantation were collected and marked accordingly. Then, the samples were kept in a cardboard box surrounded with ice bags to keep them at 4 ℃.

Figure 3.1: The Sand Soil Hole

Figure 3.2: The Fertilizer Soil Hole

3.2.4 Media Preparation and Initial Isolation

The commercial sources of media and the composition of the culture media used in this research are given in appendix A. The preparation of media from commercial products is simple and straightforward. Normally, to prepare agar plates, 20g/L agar media powder and a specific amount of broth are weighed by using a balance, and then suspended in 1L distilled water. The suspension is mixed thoroughly in an Erlenmeyer flask. Then it is dispensed and sterilized for 20 minutes at 121^{o}C. Then the agar is poured into petri plates, each containing 15 to 16 ml of media.

To prepare a broth medium, 8g/L broth media powder is weighed by using a balance, and then suspended in 1L distilled water. The suspension is mixed thoroughly in an Erlenmeyer flask. Then it is dispensed and sterilized for 20 minutes at 121^{o}C. After the temperature has cooled down the broth medium liquid is kept in the freezer

until used.

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

Independent colonies that appear to be composed of only one cell type were chosen. An inoculating loop was flamed and cooled, and used to gently transfer the colony by touching, and re-streaking onto a new nutrient agar plate. The plates were then incubated at 30^{o}C for two (2) days.

3.2.5 Cultivation and Morphological Characteristics of Bacterial Isolates

After the successful isolation from soil, a number of bacteria were sub-cultured and their morphology were observed. Eighteen (18) bacterial isolates were subjected to the Gram-staining procedure in order to classify them. The morphology observation and gram-staining results were analyzed together.

The general characteristics of bacterial colonies were then described in terms of size, shape, margin, elevation, consistency, colour, transparency and gave an accurate description of the colonies.

Gram-positive organisms retained the initial violet stain, while Gram-negative organisms were decolorized by the organic solvent and hence showed the pink counter-stain. The difference between Gram-positive and Gram-negative bacteria lies in the ability of the cell wall of the organism to retain the crystal violet.

In the Gram-stain, the cells were first heat-fixed and then stained with a basic dye, crystal violet, which was taken up in similar amounts by all bacteria. The slides were then treated with an I_2-KI mixture (mordant) to fix the stain, washed briefly with 95% ethanol (destained), and finally counter-stained with a paler dye of different colour (safranin).The smears on glass slides were covered with a few drops of mixture of methyl violet and crystal violet. The primary stain rendered all the bacteria uniformly violet. After a minute of exposure to the staining solution, the slide was washed in water. The smear was treated with a few drops of Gram's Iodine and allowed to act for a minute. The slides were again washed in water and then decolorized in absolute ethyl alcohol or acetone. A mixture of acetone-ethyl alcohol (1:1) would also be used for decolorization. A mixture of ethanol and acetone acts more slowly than pure acetone.

After the smears have been decolorized, they were washed in water without any delay. The smears were finally treated with few drops of the counter-stain safranin. The slides were then washed in water; excess water was removed using a blotting paper, and the slides were dried in air and heat-fixed before observing under a microscope. Those bacteria that held on to primary dye iodine complex and remained violet are called Gram-positive and those which were decolorized and subsequently took up the counter-stain (pink/red) are called Gram-negative.

3.2.6 Growth Experiments on Agar Medium

The lowest temperature at which a particular species will grow is the minimum growth temperature, while the maximum growth temperature is the highest temperature at which they will grow. The temperature at which their growth is optimal is called the optimum growth temperature. Most bacteria grow at temperatures around 30-40 ℃ and incubation period of 24-48 h.

In this study, all bacteria isolates were cultured on nutrient agar medium to look for the optimum growth temperature in the range of 30^{o}C to 40 ℃. A triplicates of agar

plates were prepared for each samples. Observations were made for 24 h to 48h one plate was divided into four quarters, symbol + was used to indicate the concentration of colonies, one + stands for 25% plate was covered by colony, so under different conditions, it is clearly to observe the status of cultivation.

3.2.7 Screening of Lipases Activity

All bacteria were mixed with palm oil and methanol in a certain ratio, the specific method of which would be described in the section 4.2.4, 4.2.5. 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.

Three distinctively positive reactions occurred under the prevailing conditions. Each individual bacteria, namely A, B and C respectively have shown better activities in contrast to the rest of the bacteria. The research performed after the results of these initial crude activity tests were obtained would focus on these three bacteria.

3.2.8 API -20E Kit Test

The API-20E test kit for the identification of enteric bacteria provides an easy way for the identification of bacteria. A plastic strip holding twenty mini-test tubes is inoculated with a saline suspension of a pure culture (as per manufacturer's directions). This process also rehydrates the dessicated medium in each tube. A few tubes were completely filled (CIT, VP and GEL)(Appendix C), and some tubes were overlaid with mineral oil such that anaerobic reactions can be carried out (ADH, LDC, ODC, H_2S, URE) (Appendix C). After incubation in a humidity chamber for 18-24 hours at 37℃, the colour reactions were read according to reading table（Appendix C）, and the reactions were converted to a seven-digit code. The code can be fed into the manufacturer's database via email, and the manufacturer emailed back the identification,

usually either at the genus or species level. An on-line database can also be accessed for the identification.

3.2.9 Genomic DNA Extraction

The DNA of three bacteria were extracted by using a commercial kit (Appendix G), the detail steps as following: Harvest 1.5ml of cells ($OD_{600:}$ 0.8-1.0) by centrifuging at 13,000rpm for 1 min. Supernatant was removed and completely res-suspend by vortex or tapping after centrifugation. If the bacterial was Gram-positive, add 50µl or pre-buffer and 3µl of lysozyme solution and mix well. Incubate at 37℃ for at least 15min, and then continue the procedure as Gram-negative bacteria. Add 300µl of G-buffer solution, and invert-mix well. Incubate at 65℃ in a constant temperature water bath for 15min. Then add 250µl of binding buffer, and completely mix well by pipetting (at least 10 times) or gently vortexing. Then the mixture is transferred to a column containing silica-membrane with a polypropylene tube for 2 ml and centrifuged at 13,000rpm for 1min. 500µl of washing buffer A was added to column and centrifuge for 1min at 13,000rpm. The washing buffer solution was removed and then tube was centrifuged for 1min at 13,000rpm. The column was placed in a clean 1.5ml microcentrifuge tube, and 50µl of elution buffer was directly added onto the membrane. The tube was incubate at room temperature for 1min, and then was centrifuged for 1min at 13,000rpm. Lastly sample tubes were kept sample at - 20℃ freezer.

3.2.10 PCR Amplification with Universal Primers and PCR Product Purification

DNA concentration was determined by using UV-VIS spectrophotometer under 260nm wavelength on samples diluted 50 times by mixing 1µl samples with 49µl distilled water, and then DNA concentration was calculated as follows:

DNA concentration = OD_{260} X 50ng/µl X 50 (Dilution Factor)

Published 16S rRNA sequence was available on three *Pseudomonas* for the use in design of primers for PCR, 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 20µl total volume, containing 1µl of the appropriate dilutions of DNA, 2µl of 10 X PCR reaction buffer, 0.5µl of DNA polymerase, 0.5µl of dNTPs, 1µl of $MgCl_2$, 1µl of each primer. The mixture was brought to 20µl with sterile deionised distilled water. All PCR reactions were run with a negative control (no DNA). PCR involved initial denaturation at 94℃ for 1 min, five cycles with a low annealing temperature of 50℃ for 5 s, extension at 72℃ for 30 s and heated to 94℃ for 5 s, and additional 25 cycles of denaturation at 92℃ for 2 s, annealing temperature at 55℃ for 2 s, extension at 72℃ for 30s and final extension at 72℃ 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 gel slice was weighed in a 1.5 ml tube and then 3 volumes of BNL buffer was added to 1 volume of gel (300µl per 100mg of agarose gel). The mixture was shaking and incubate at 55℃ for 10 minutes or until the gel is completely dissolved. The dissolved gel mixture was transferred to the kit column assembly and centrifuged for 1minute; the flow-through was discarded after centrifuge. 700µl 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 1.5ml

micro-centrifuge tube after centrifuge for 1 min at 13,000 rpm to dry the spin membrane. 60µl of the elution buffer was directly applied to the centre of the column without touching the membrane with the pipette tip. The tube was incubate at room temperature for 1 min and centrifuged for 1 min at 13,000rpm. Lastly tubes was stored the at minus 20 ℃.

3.2.11 Visualisation of Extracted DNA and PCR Products

Agarose gel electrophoresis is the easiest and commonest way of separating and analyzing DNA. The purpose of the gel might be to look at the DNA, to quantify it or to isolate a particular band. The DNA is visualized in the gel by addition of ethidium bromide. This binds strongly to DNA by intercalating between the bases and is fluorescent, meaning that it absorbs invisible UV light and transmits the energy as visible orange light. So the detail steps as follows: weigh 0.3g agarose power, suspended in 30ml 1X TAE buffer, and thoroughly dissolve in a microwave oven for 2 min. Add 3µl ethidium bromide before pouring the gel when the agarose has cooled to about 55 °C. The proper comb was inserted for the particular gel rig. The gel should be allowed to cool until it has set. The comb was carefully removed and placed the gel in the gel rig with the wells closest to the cathode (black) end. The gel was covered with 1X TAE running buffer. 3µl of glycerol loading dye was placed onto the waxy side of parafilm for each sample. Keeping samples on ice, 1µl of a sample was added to one of the drops of loading dye. Mix the sample and loading dye by filling and emptying the pipette a few times then load the mixture into a well. The rest of the samples were loaded one by one as same as the first one sample. 4µl of 1 Kb ladder was loaded at left end of the series of samples. The cover was placed on the gel rig and samples were run towards the anode (red) end at 90V for 40min. The power pack was turn off and the gel was removed out. Visualization was carried out with U.V. light and photograph was taken with a polaroid Photo documentation camera. Lastly the waste gel was disposed properly.

3.2.12 Sequence Analysis and Blast Analysis

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

3.3 RESULTS AND DISCUSSION

3.3.1 Soil Samples and Sampling Sites

The locations of the sampling sites were situated under oil palm trees where the temperature is 30^{o}C. 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 Fig 3.3 and the group 2, form fertilized agriculture soil is shown in Fig 3.4. 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.

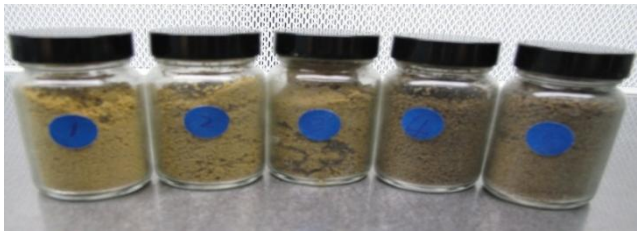

Figure 3.3: Group 1 of Soil Samples from Common Agriculture Sand Soil.

Figure 3.4: Group 2 of Samples from Fertilized Agriculture Soil.

After the gross samples have been collected, much care was taken to avoid contamination and to prevent the occurrence of further chemical and biochemical reactions (as much as sterile technique possible). The samples were preserved as much as possible in its original condition, as they were necessary to maintain the properties and the identity of the samples at all stages of sample preparation.

3.3.2 Morphological Characteristics and Gram-staining

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. The cultures generated from the highest dilution series consisted mostly of one single cell type, whereas those from the lower dilution series were generally mixed cultures with large cells.

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 3.1.

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.

Table 3.1: Morphological Characteristics of 11 Bacterial Isolates

Isolates	**Isolate Colony**			**Gram-stain**	**Cell**	**Sample**
Code	Colour	Size(μm)	Form	**Reaction**	**Shape**	**Group**
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

The morphology and Gram-staining results of bacteria A, B and C were shown in sequence in Figures 3.5, 3.6 and 3.7, as the three isolates have positive activity in the screening of lipase from bacteria. Namely the importance of bacteria A, B and C over the rest is covered in the section 4.3.1 towards the end of this chapter.

Bacterial isolate A gave a flowery-like shape on nutrient agar plate whereas in the Gram-staining procedure appeared Gram-positive rod shaped (Figure 3.5). For bacterial isolate B, the shape was round in nutrient agar plate whereas in Gram staining it was in Gram-negative rod shape (Figure 3.6). 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 3.7). In terms of colour they were white, violet and light brown respectively.

In Gram reaction, isolate A gave a purple colour which indicated that it was a Gram-positive bacteria whereas isolates B and C gave pink colour which suggested they were Gram-negative bacteria.

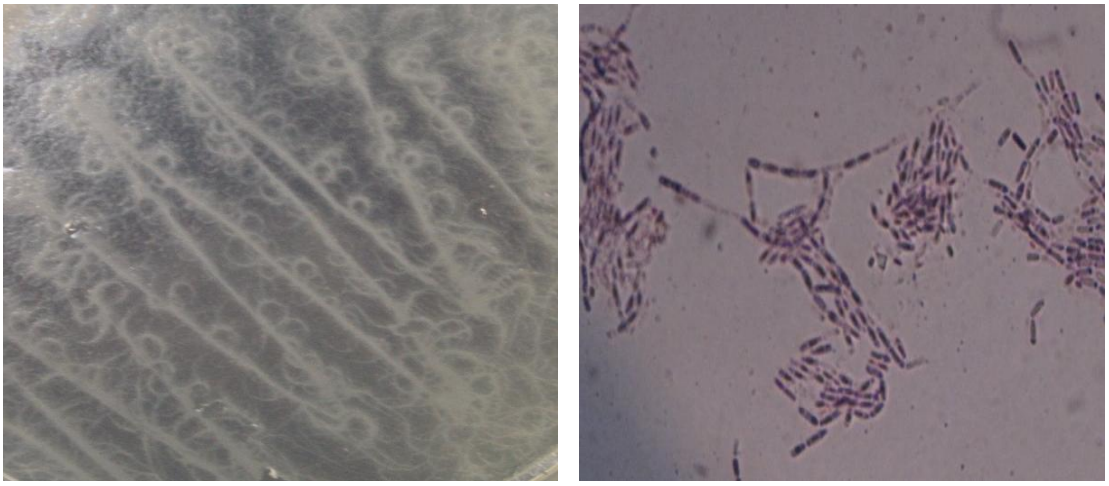

Figure 3.5: Isolate A on Nutrient Agar (left) and Gram-reaction (right) is Positive

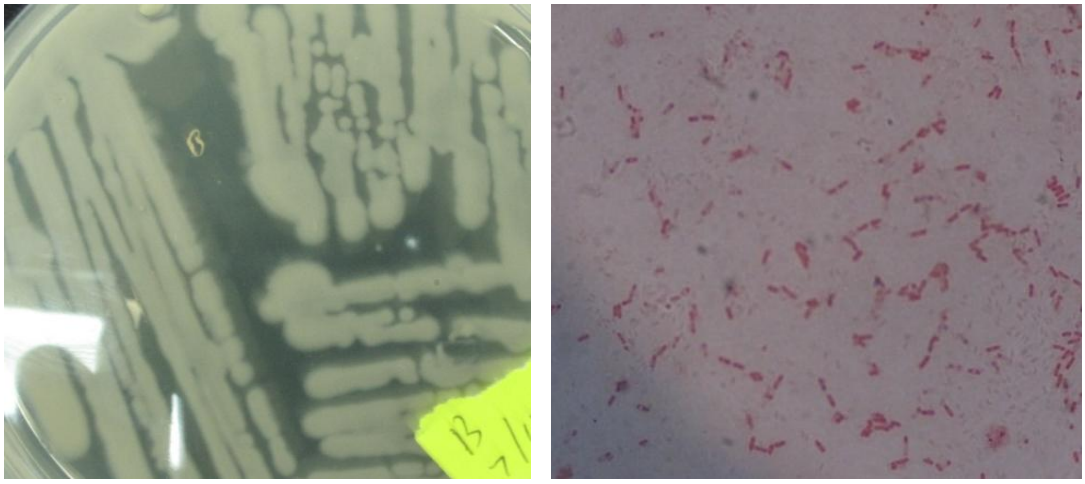

Figure 3.6: Isolate B on Nutrient Agar (left) and Gram-reaction (right) is Negative

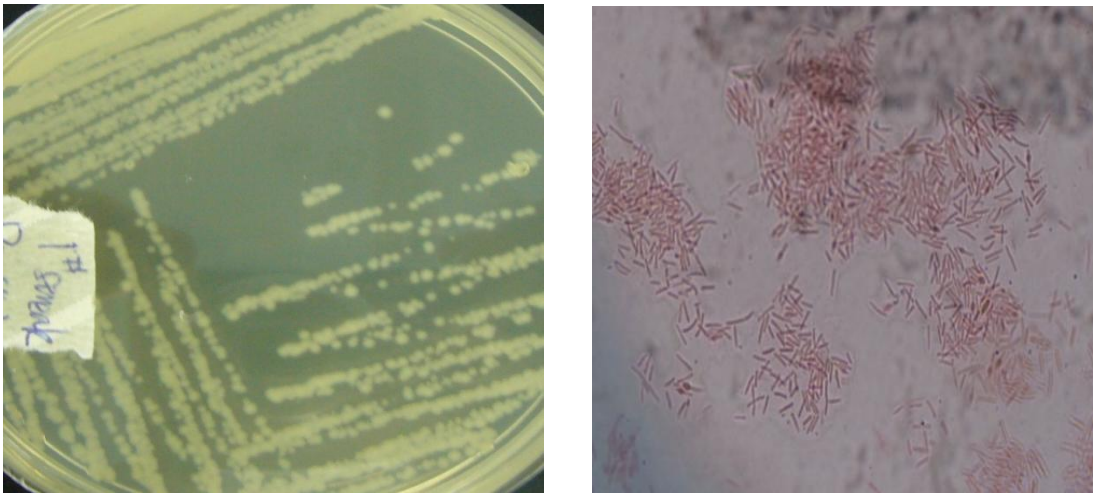

Figure 3.7: Isolate C on Nutrient Agar (left) and Gram-reaction (right) is Negative

3.3.3 The Growth Experiments on Agar Medium

The growth conditions of eleven (11) bacterial isolate are summarized in Table 3.2 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^{o}C .The result was in line with the concept, the incubation temperature of bacterial isolate in this research was either 30^{o}C or 37^{o}C. For the incubation period, the longest was no more than 48h. Inversely, the lowest incubation period was at least 24h, with most bacteria can survive from 24- 48h on the agar plates.

Table 3.2: Number of Colony Produced at Different Conditions

Isolate Code	Number of colony Temperature (℃) on Agar Medium		Incubation Time (h)					
			Agar Medium			Broth Medium (OD)		
	30	37	24	30	48	24	30	48
A	++++	+++	+++	++++	++++	155	140.9	120
B	++++	++	+++	++++	++++	125.7	146	136.7
C	+++	++++	++	+++	++++	121	152.5	148
D	++++	+++	++	+++	++++	123.5	138	110.1
E	+++	++	++	+++	++++	117.6	128.4	120
F	++	++++	++	+++	++++	112.2	140.4	138.4
G	++++	+++	++	+++	++++	115.6	132.8	129.8
H	+++	++	++	+++	++++	124.7	134.9	130.1
I	+++	++++	++	+++	++++	118	125.5	111.4
J	++	++++	+++	+++	++++	120.2	129.6	118.8
K	++++	++	++	+++	++++	117.1	130.5	120.4

++: Weak Growth; **+++:** Moderate Growth; **++++:** Heavy Growth.

The bacterial isolate were then sub-cultured on agar to keep their viability for the next step of the research. Considering that typical isolate A, B and C, respectively have a series of optimum parameters, presumably these bacteria have a similar culture conditions. On the contrary, the isolate C took quite long time no matter what type medium, and required quite a high temperature to grow.

With respect to culture in the broth medium, all bacteria have an ability to survive. There was a tendency for the broth to be free of turbidity and form a firm pellicle at the surface or of cottony growth in the depths of the flask. Growth in shaken broth cultures seemed to be more variable. Strains differed in growth habit, varying from spiny, compact spherules resembling those of fungi, to loose flocs ranging in size down to a few cells.

Shaken cultures were more apt to produce lipase material than static cultures (Peter *et al*., 1997), although this characteristic differed according to the strain. In the

progress of broth culture, bacteria A gave a unique shape, like snow flowers, the supernatant is clear. In contrast, bacteria B and C gave a common suspended fluid, cloudy and not clean. By carefully observing them, some very tiny and light brown or white colour particles were found.

3.3.4 Screening of Lipase from Bacteria

Three distinctively positive reactions occurred under the prevailing conditions. Each bacterial isolate A, B and C have shown better activities in contrast to the rest of all isolates. The research performed after the results of these initial crude activity tests were obtained would focus on these three bacteria. The details of result will be described in the section 4.3.1.

3.3.5 Bio-chemical Test by API Kit

The API KIT results for biochemical test were given in Table 3.3, 3.4 and 3.5 below. The API kit identify bacteria A either as potential *Pseudomonas aeruginosa* or *Pseudomonas fluorescens* with strongly 97% test against oxidase test. For bacteria B, the API kit identified it as potential *Aeromonas hydrophila/caviae* at strongly 98 % test against ONPG and 100% against oxidase. However, for bacteria C, the API kit give fairly percentage at CIT test at 94% and GLU at 84%.Bacteria C was fairly identified as either *Pseudomonas luteola* or *Stenotropomonas maltophilia*.

Table 3.3: The API Result of Bacterial Isolate A

As in Appendix C

Significant Taxa	% ID	T	Test Against
Pseudomonas aeruginosa	77.5	0.77	OX 97%
Pseudomonas fluorescens/putida	15.6	0.65	OX 99%

Table 3.4: The API Result of Bacterial Isolate B

As in Appendix C

Significant Taxa	Test Against			
Aeromonas hydrophila/caviae/sobria 1	ONPG98% OX 100%	LDC25%	CIT 25%	IND85%

Table 3.5: The API Results of Bacterial Isolate C

As in Appendix C

Significant Taxa	% ID	T	Tests Against			
Pseudomonas luteola	61.7%	0.44	CIT 94%	GEL 13%	GLU 84%	ARA 85%
Stenotrophomonas maltophilia	25.7	0.34	CIT 75%	LDC 75%	ADH 0%	

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 20NE 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.

3.3.6 DNA Extraction of Bacteria

The OD values of the three bacteria were tested before DNA extraction, to quantify the bacteria, and the results are listed in Table 3.6. In 1.5ml samples, under the 260nm of UV-VIS detection, the average of two readings for bacteria A has the lowest value of OD at 0.86, the value for bacteria B is 1.89, meanwhile the bacteria C gives the highest value of 2.01.

Table 3.6: The OD Value of Bacteria

Bacteria	**OD Value (600nm)(24h)**		**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

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 24h. 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 3.8. In Figure 3.8, M is 1kb maker ladder, its range can be referred in the appendix D; the rest are the three bacteria, namely A, B and C. A has a brilliant band of 5,000bp and a fade band of 12,000bp; the bacteria B has a faded band; by contrast, the bacteria C has two brilliant bands, both at 12,000bp and 5,000bp.

Gel electrophoresis is a procedure that separates molecules on the basis of their rate of movement through a gel under the influence of an electric field. 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 (Sambrook and Russel, 2001).

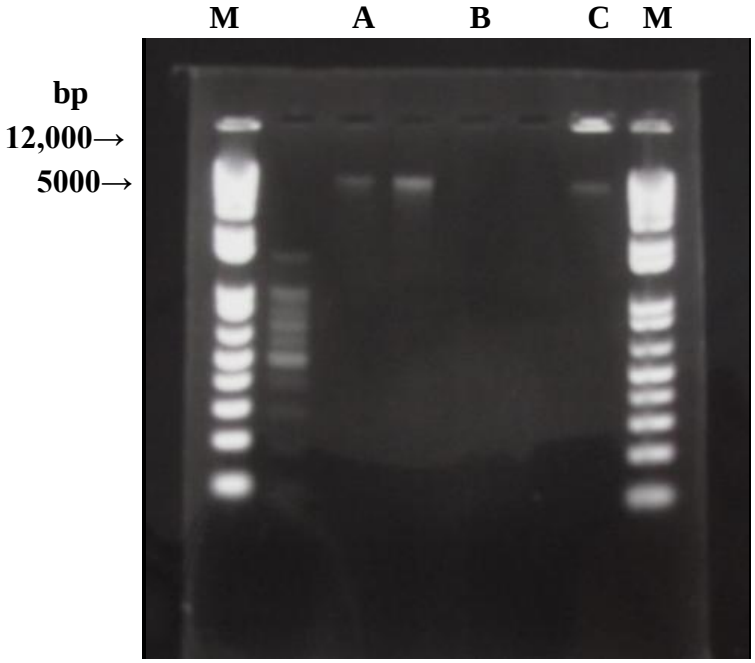

Figure 3.8: Aga-rose Gel Electrophoresis of Extracted DNA

3.3.7 PCR Amplification of Bacteria and DNA Purification

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 3.7. In the 50 times diluted sample of bacteria, under the 260nm of UV-VIS detection, the calculation showed that bacteria A has the lowest value of around 121.7ng/µl, the bacteria C is around 147.5ng/µl, meanwhile the bacteria B gives the highest concentration of around 166.7ng/µl.

Table 3.7: The Concentration of Bacterial DNA

Bacteria	**OD Value (260nm)**		**Average Value**	**DNA Concentration**
	Reading 1	**Reading 2**	**±（SD）**	**(ng/µl)**
Control (Distilled Water)	0.000	0.000	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	0.060	0.058	0.059±0.0001	147.5

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 3.9. The 16S rRNA size of three bacteria were all between 1650bp to 2000bp, is mostly around 1650bp.

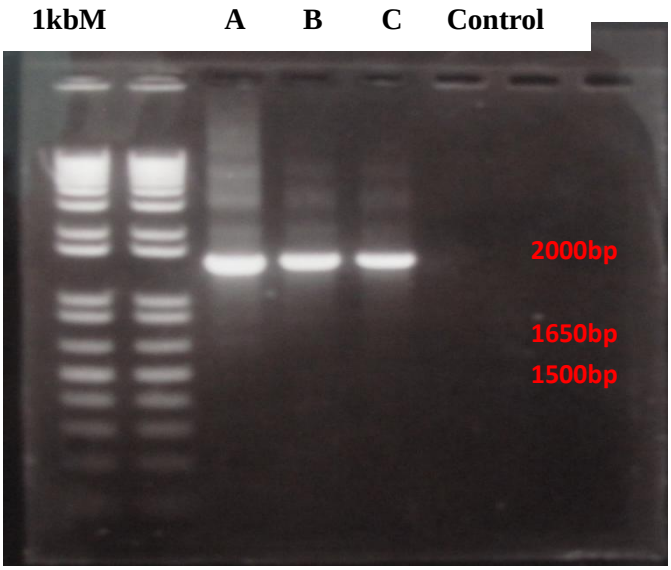

Figure 3.9: Aga-rose Gel Electrophoresis of PCR Amplification

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 3.8. The concentrations of 16S rRNA were all very much lower than the concentration of bacterial DNA in Table 3.7. The isolate A has a concentration of 78.8ng/µl; B has the highest concentration of 93.8ng/µl; and C has the lowest concentration of 65.0 ng/µl.

Table 3.8: The Concentration of Purified PCR Product

Bacteria	**OD Value (260nm)**		**Average**	**16S rRNA**
	Reading 1	**Reading 2**	**Value±(SD)**	**Concentration (ng/µl)**
Control (Distilled Water)	0.000	0.000	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	65.0

The electrophoresis of PCR product after purification were carried out, the results are as shown in the Figure 3.10; 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.

In Figure 3.10, 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.

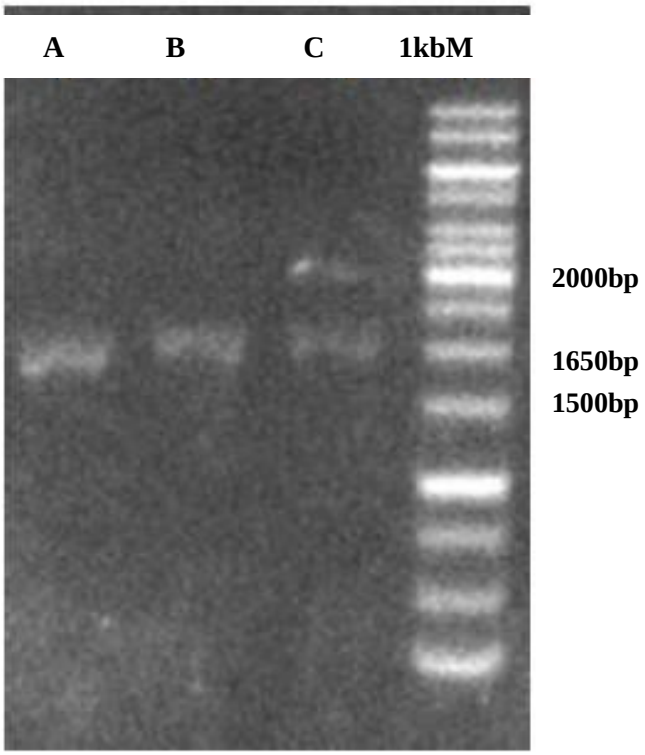

Figure 3.10: Purified PCR Product of Unknown Bacteria

3.3.8 DNA Sequencing and Blast

After DNA purification, DNA cycle sequencings were performed for the three unknown bacterial isolate in both forward and reverse directions. Each forward and reserve primer will generate a different electropherogram. As a result, there were six ABI Electropherograms generated from DNA cycle sequencing and each was shown in Appendix F (1-6).

The overall performance of the sequence data was considered good. All peaks were not cut from the first nucleotide until the final nucleotide because the peaks of

two ends were not only flat but also overlapping seriously. This might have been caused by the lower concentration of samples or primer is not matched with them.

Among all Appendix F (1-6)3.14, the bacteria A and C gave evenly distributed peaks without much overlapping. In the comparison, the bacteria B did not show good peaks, most peaks were lower and the gap between two nucleotides was not even. On the base of all figures, the middle parts possessed an even and identical height of peaks; it had been noticed that there was quite big gap at the last part of all figures. The causes of this phenomenon include low quality, low concentration, or degradation of the primer or template.

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 blast result could be seen in the Appendix E. Actually, 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 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 pseudomycoides 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 *Stenotrophononas maltophilia AQN2,* It has a best relationship with biochemical test by API kit 20E, so probably this primer is suitable with this bacteria.

CHAPTER 4

CONVERSION OF PALM OIL TO METHYL AND ETHYL ESTER USING ISOLATE A, B & C

4.1 INTRODUCTION

In recent years, demand for fatty acid methyl esters (biodiesel) being used as fuel in diesel engines and heating systems increased due to rises in petroleum prices, increase in earth population and their energy needs, and development of government measures such as The EU Directive 2003/30/EC and The US Energy Policy Act (Vicente *et al*., 2007).

Transesterification of triglycerides with alcohol or other acyl acceptors in the presence of a chemical catalyst or biocatalyst leads to the formation of alkyl ester commercially known as biodiesel. Biodiesel can be produced from vegetable oils like soybean oil, jatropha oil, rapeseed oil, palm oil, sunflower oil, corn oil, peanut oil, canola oil and cottonseed oil (Sharma and Singh, 2008). Other oils used for biodiesel production include algal oil, waste cooking oil and animal fats. Among the vegetable oils with a potential to obtain biodiesel, palm oil stands out for being the second most abundant vegetable oil in the world next to soybean oil. Further, palm oil is characterized as having superior productivity among all the crops (Kalam and Masjuki, 2002).

Transesterification reaction can be catalyzed by both homogeneous (basic or acidic) and heterogeneous (basic, acidic or enzymatic) catalysts (Mittelbach, 1990). Recent studies showed that biodiesel can be produced enzymatically by lipase-catalyzed

transesterification which has become more attractive in biodiesel production since the glycerol can be recovered easily and the purification process for biodiesel is simple (Vicente *et al*., 1998). In addition, the use of lipase in biodiesel production tolerates the water content of oil and increases biodiesel yield by avoiding the soap formation.

Microbial lipases are diversified in their properties and substrate specificities, which improve their biotechnological importance and justify the search for novel lipases possessing entirely new properties and specific substrate specificities depending on their applications. Extracellular lipases have been proven to be efficient and selective biocatalysts in many industrial applications such as biosensors, pharmaceuticals, foods, cosmetics, detergents (Pandey *et al*., 1999).

Most of the lipases are produced commercially from fungi (Gao *et al.*, 2000), yeast (Dalmou *et al.*, 2000) and bacteria by using a variety of methods involving ammonium sulphate precipitation and ion exchange chromatography followed by gel filtration (Ferreira *et al.*, 1999). Isolation of lipases from new sources is being carried out for a variety of applications because of their ability to withstand wide pH range and their high thermal stability. In this sense, bacterial lipases have gained importance as they are found to be more stable at higher temperatures and tolerant to a wide range of pH, compared to the lipases extracted from other microorganisms. Hence, proper screening of organisms, isolation and characterization become important for the production of a highly active and stable lipase. Although a number of lipase producing bacterial sources are available, only a few are commercially exploited. Of these, the important genera are *Achromobacter, Alcaligenes, Arthrobacter, Aeromonas Hydrophila, Bacillus, Burkholderia, Chromobacterium* and *Pseudomonas* (Palekar *et al.*, 2000).

The *Pseudomonas* enzymes have found application in a number of processes, namely the production of detergents, glycerolysis of fats and oils, direct

transesterification, chiral resolution and acrylate synthesis (Martin *et al.*, 1992). A number of bacterial lipases have been purified and characterized. The information with respect to their three dimensional structure is however limited. To date the three dimensional structures of the lipase of only three species of bacteria are known, namely *Pseudomonas cepacia (Burkholderia cepacia), Chromobacterium viscosum* and *Pseudomonas glumae* (Noble *et al.*, 1993).

In this chapter, an evaluation of the suitability of palm oil as a feedstock in transesterificaion is reported. Three crude lipases extracted from selected microbial isolate were used as catalysts; the effects of different levels of some parameters were investigated.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

The analytical grade methanol and ethanol were purchased from Fisher Scientific. Hexane (GC grade) was obtained from Fisher Scientific.

4.2.2 Equipment and Instrumentation

The shaking incubator (2435) was supplied by Ecotron (Switzerland). The gas chromatography (4720) was supplied by Agilent tech (Creek Blvd Santa Clara CA, USA). Flasks were used from Schott (Germany).

4.2.3 Palm Oil Pre-treatment

The crude palm oil (1L) was collected from LKKP Corporation Sdn. Bhd, Malaysia. The crude palm oil was filtered at reduced pressure with filter paper to eliminate the impurities, and then the palm oil was stored under room temperature for

further research.

4.2.4 Microorganism Cultivation

Three bacterial isolates were finally confirmed as having a certain activity of degrading palm oil. These three microorganisms were *Pseudomonas aeruginosa, Aeromonas hydrophila and Pseudomonas luteola.* They were cultured in broth medium for 24-48 hours at 150 rpm in shaking incubator at 37℃. They were harvested by centrifugation at 4℃ with 12,000 rpm in a high speed centrifuge. The supernatant contained the extracellular enzyme lipases were used in the experiment.

4.2.5 Screening of Lipases

Lipase screening was performed to find the lipase with the best catalytic activity in the transesterification of palm oil. 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. The experiments were conducted under a preliminary set of reaction conditions, which may not have been the optimum set for all the lipases. 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℃for 5h (Noureddini *et al.*, 2005).

4.2.6 Reaction Conditions and Optimization

The mixture of enzyme in water and the defined molar ratio of methanol to palm oil were investigated for biodiesel synthesis. The enzymatic transesterification reactions were carried out in a 50 ml conical flask, and optimized with respect to temperature and

the molar ration of methanol to palm oil in an orbital shaker.

A standard set of conditions were used as the baseline in the optimization studies. The initial conditions set were 6 ml palm oil, 2.4-3.0 ml methanol (methanol to oil molar ratio of 3 or 4), 10% volume crude lipases, 40℃, 150 rpm and 5 h reaction time. During optimization studies, each parameter was varied one at a time. For example, when the effect of methanol to palm oil ratio was investigated, the remaining reaction conditions were unchanged at 6 ml palm oil, 10% V/V crude lipases, 40℃, 150 rpm and 5h reaction time (Noureddini *et al.*, 2005). In order to compare which is better acyl-acceptors between methanol and ethanol, ethanol was also run same reaction under same conditions with methanol, ethanol will replace methanol in reaction.

4.2.7Analytical Method

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 (30m x 0.25 mm, I.D. 0.25 um). Helium was used as the carrier gas. Oven temperature program was as follows: 155 ℃ for 1 min and programmed from 155 to 180 ℃ at a rate of 2 ℃/min, kept for 2 min, and finally raised to 220 ℃ at 4 ℃/min and maintained for 6 min. The injector was set up for 250 ℃ and the FID detector at 260 ℃.

4.3 RESULT AND DISCUSSION

4.3.1 Screening of Lipase from Bacteria

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 the non-specific lipases from *Pseudomonas*

qeniculata (pseudomonas aeruginosa), Aeromonas hydrophila (*Bacillus pseudomycoides* strains or *Bacillus sp*) and *Stenotorophomonas maltophilia* (*Pseudomonas luteola).* 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 4.1, all three lipases show conversion activity during the transesterification from palm oil to methyl esters. The methyl ester conversion of oil by using lipase from *Pseudomonas qeniculata (pseudomonas aeruginosa)* is around 24% and methyl ester conversion of lipase from *Stenotorophomonas maltophilia* (*Pseudomonas luteola)* showed comparable higher catalytic activity around 21% as compared to lipase from *Aeromonas hydrophila* (*Bacillus pseudomycoides* strains or *Bacillus sp*) around 17%.

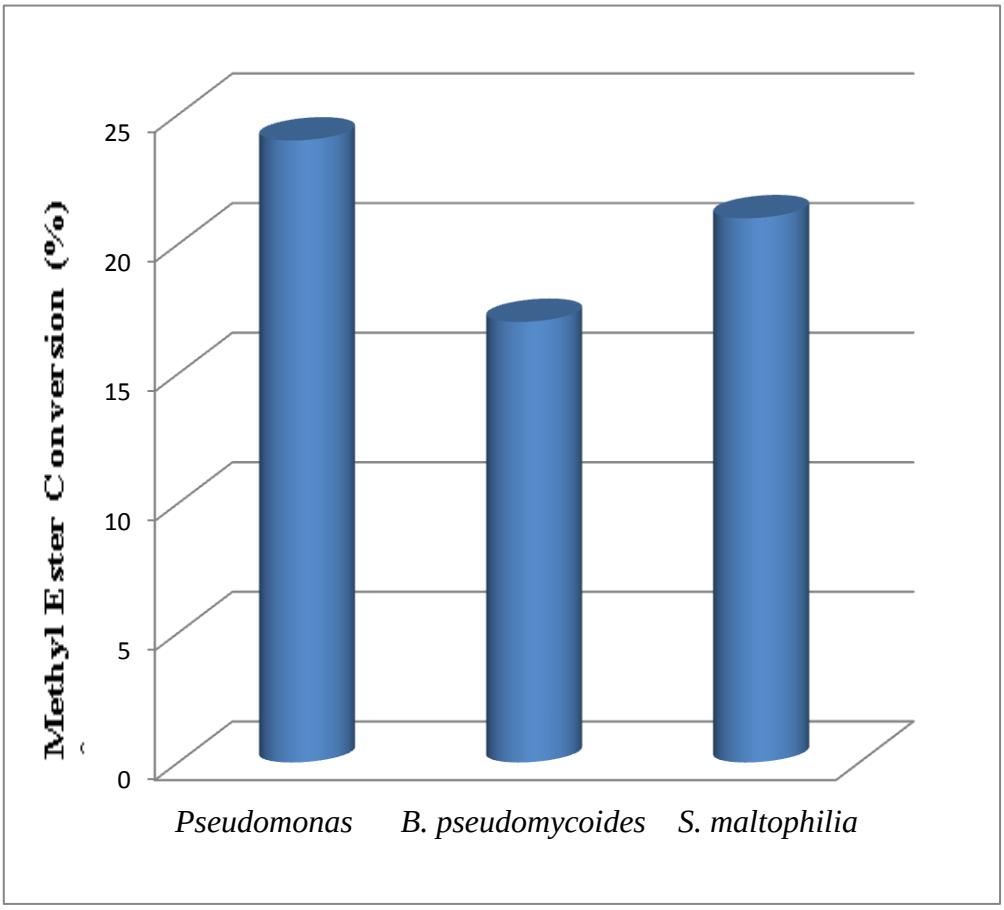

Figure 4.1: Methyl Ester Conversion Catalysed by Enzyme from *Pseudomonas, Bacillus (Pseudomycoides) and Stenotorophomonas maltophilia*

It was reported that the transesterificatin of several vegetable oils by bacterial lipases, including *Pseudomonas* lipase, showed a stronger activity, compared to fungal lipases such as Lipozyme TL IM (Ketsara and Benjiamas, 2010). In this study, since the final results of methyl ester conversation rate by lipase from *Pseudomonas qeniculata (pseudomonas aeruginosa)* (24%) and lipase from *Stenotorophomonas maltophilia (Pseudomonas luteola)* (21%) were higher than that by lipase from *Aeromonas hydrophila (Bacillus pseudomycoides)* (17%); therefore, lipase from *Pseudomonas aeruginosa* and lipase from *Stenotorophomonas maltophilia (Pseudomonas luteola)* were considered as the most suitable lipase for transesterification reaction of crude palm oil and methanol to methyl ester.

4.3.2 Effect of Temperature on the Transesterification Reaction

Enzyme catalytic reaction temperature is an important parameter, and the selection of the appropriate reaction temperature not only enhances the reaction rate, but also helps extend the life of the enzyme. At certain temperature range, temperature, reactant collision frequency increases, causing the enzyme reaction to increase in speed; but when the temperature exceeds a certain range, the enzyme protein denatures, enzyme activity to decrease, which cause the enzyme reaction rate to decrease.

Figure 4.2 shows that, in the studied temperature range (20-65℃), the enzymatic reaction speed increases with increasing temperature, up to the maximum at the temperatures of 50 ~ 60℃, where the product of the methyl ester at this degree was the highest. At lower temperatures, given a longer reaction time, the methyl ester content (%) produced could reach the same level as same as the optimum degree.

This finding is correspond with the work of Wu and Zong (2004) who concluded that after taking into account the speed, the reaction temperature in the range of 35 ~ 40℃ was more appropriate.

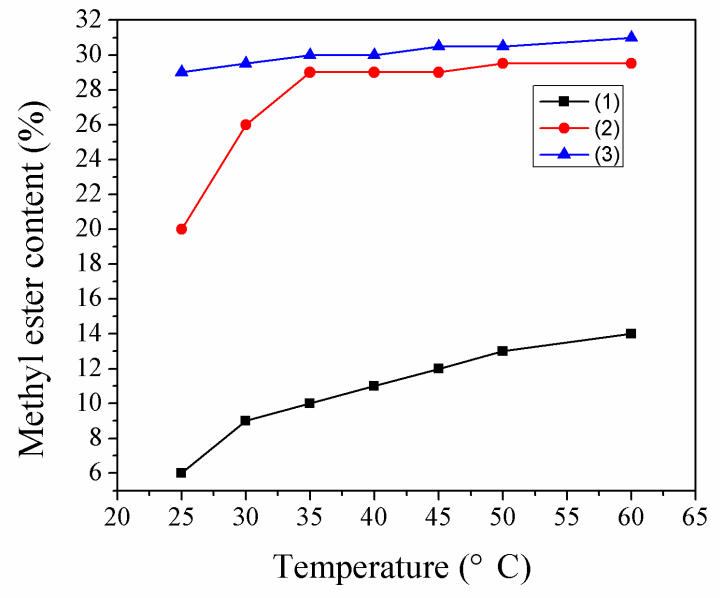

Figure 4.2: (1) *Bacillus pseudomycoides*, (2) *Stenotorophomonas maltophilia*, (3) *Pseudomonas qeniculata.* Effect of Different Temperature Values on the Enzymatic Transesterification of Crude Oil

4.3.3 Effect of Methanol to Oil Molar Ratio

The methanol to oil molar ratio is one of the most important parameters in methyl ester production. Experiments were performed to optimize the synthesis of biodiesel by varying the molar ratio of methanol to palm oil. Optimum methanol requirements were determined as shown in Figure 4.3. The amount of methanol added was varied from 3 to 4 molar ratios to crude palm oil.

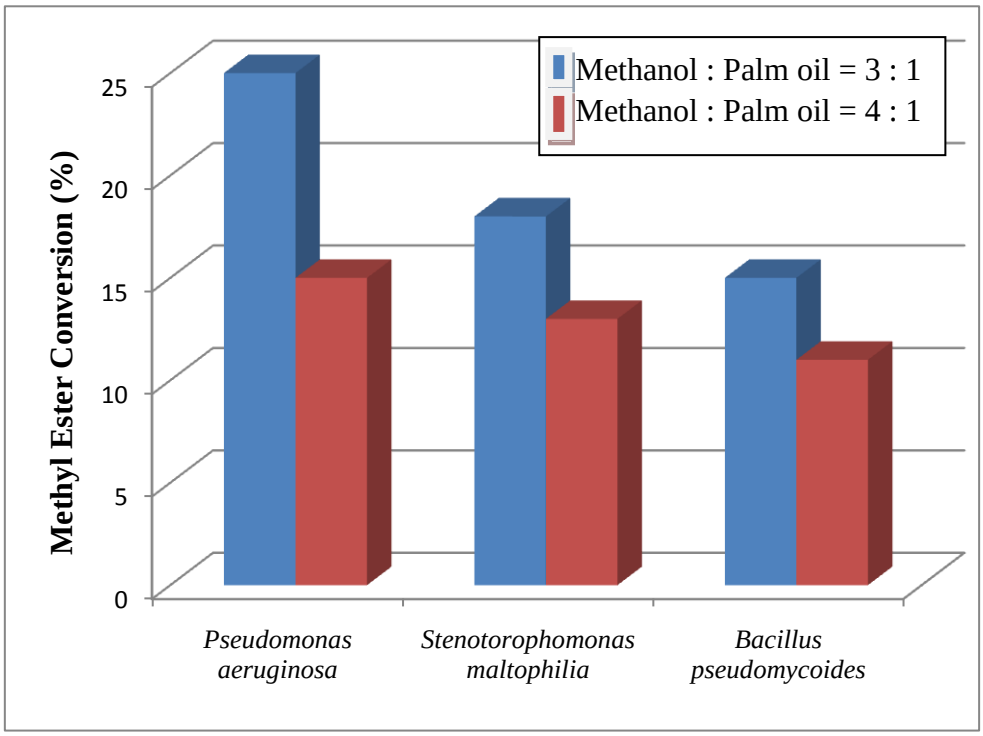

Figure 4.3: Effect of Molar Ratio of Methanol to Oil on Biodiesel Synthesis：3:1 and 4:1

The three bacterial strains *Pseudomonas qeniculata (pseudomonas aeruginosa)*, *Stenotorophomonas maltophilia(Pseudomonas luteola)* and *Bacillus pseudomycoides (Aeromonas hydrophila)*, yield of biodiese at 4 molar methanol / palm oil ratio was lower than that at 3 molar ratio. This could be due to the inhibitory effect of high amount of methanol on the activity of the enzyme. Fatty alcohols, in which the carbon lengths are more than 3, completely dissolve in the oil in a stoichiometric amount, but the solubility of methanol was only 1/2 of the stoichiometric amount (Shimada *et al*., 2002). Therefore, lipases could be deactivated by insoluble methanol, which exists as drops in the oil. Soumanou and Borscheuer (2003) reported that some *Pseudomonas* strains developed substantial methanol resistance. They may also be tolerant to methanol.

Although, the mixed lipases in this study gave especially high conversion with

three molar equivalents of methanol, one of the problems faced in the enzyme-based route for methyl ester preparation has been the inactivation of the lipase by exposure to methanol; hence the stepwise addition of methanol was also attempted. It was observed that it made no difference to methyl esters whether the whole of the methanol was added in a single step. In this respect, the single step is a good choice since stepwise addition of the substrate does complicate the process design.

4.3.4 Comparison between Methanol and Ethanol as Acyl-acceptors

Alcohols are the most frequently used acyl-acceptors, particularly methanol and, to a lesser extend, ethanol. Other alcohols can be used, such as butanol, isopropanol, and octanol but the cost is much higher. Although the use of different alcohols presents some differences with regard to the reaction kinetics, the final yield of esters remains more or less inalterable. Therefore, selection of the alcohol is based on cost and performance consideration (Encinar *et al*., 2007). Regarding the choice between methanol and ethanol, Methanol was dominating in most of the literature reviewed (Yuan *et al*., 2008), Methyl, rather than ethyl, ester production was modeled because methyl esters are the predominant commercial products, methanol is considerably cheaper and more available than ethanol (Pinto *et al*., 2005) and the downstream recovery of unreacted alcohol is much easier (Zhou and Boocock, 2006).

However, ethanol is less toxic and it can be considered more renewable because it can be easily produced from renewable sources by fermentation. In addition to the entirely agricultural nature of the ethanol, the extra carbon atom brought by the ethanol molecule slightly increases the heat content and the cetane number. Also, ethanol, as extraction solvent, is preferable to methanol because of its much higher dissolving power for oils. Finally, another important advantage in the use of ethanol is that the ethyl esters have cloud and pour points that are lower than the methyl esters (Encinar *et al*., 2007).

However, the utilization of ethanol also presents inconveniences. Also, the formation of stable emulsion during ethanolysis is a problem. During the reaction, emulsions are usually formed. In the case of methanolysis, these emulsions break down quickly and easily to form a lower glycerol rich layer and upper methyl ester rich layer. In ethanolysis, these emulsions are more stable and severely complicate the separation and purification of esters (Zhou and Boocock, 2006). In contrast, methanol is mainly produced from non- renewable fossil sources, such as natural gas (Robles and Gonzalez, 2009).

The base-catalyzed formation of ethyl ester is difficult compared to the formation of methyl esters; reaction rate with ethanol is slightly slower than with methanol, what is attributed to the more difficult formation of the ethoxide anion (Omtapanes *et al.*, 2008).

In this study, experiments on transesterification reactions were carried out by using palm oil and ethanol under the same conditions as has been done with methanol. The yield showed no significant gap between these two alcohols, as shown in the Table 4.1. In the process, the both of two alcohols could mix well with oil and crude lipase. The conversion yields of using two alcohols were very close. So both of the two alcohols could be used as acyl-acceptors, and will give a positive result

Table 4.1: Comparison of the Conversion Rate with Ethanol and Methanol

Lipases Resource	**Conversion with Ethanol (%)**	**Conversion with Methanol (%)**
Control without Lipase	0	0
Pseudomonas qeniculata (pseudomonas aeruginosa)	24	22
Bacillus pseudomycoides (Aeromonas hydrophila)	17	16
Stenotorophomonas Maltophilia (Pseudomonas luteola)	21	20

CHAPTER 5

FINAL CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

The experiments have produced the outcomes that can be concluded as listed below. The conclusions were based on the experimental data and analysis, and discussion made in Chapter 3 and 4.

1) From the subsequent 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.

2) In this research all isolated strains have different growth parameters, and it is useful to identify each strain according to this prelimanary information.

3) Only three strains exhibited the ability to produce extracellular lipases, and these lipases could catalyze the transesterification of raw palm oil to methyl esters.

4) The API-20E test was chosen as the biochemical test for initial identification, as it is a simple and faster way to identify bacteria. The results indicated a possibility that the strains were *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida*,

Aeromonas hydrophila, and *Pseudomonas luteola* or *Stenotropomonas maltophila*. However, biochemical tests are unable to differentiate these species, in the present study, merely allow us to identify species in the biotype level, so it is not exactly compared with the molecular tests, the molecular test could identify at genus level, from the results of these three bacteria were conducted for the identification at this level. It might be found that some difference between biochemical (API KIT 20E) and molecular test, the probably reasons were caused as follows: (i): API 20 E kit is not suitable the target species; (ii) 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, the unknown bacteria were identified as molecular test results mentioned above, meanwhile, as a result, the consequent of this research should be continued in this aspect.

5) The final results of methyl ester produced by lipase from *Pseudomonas qeniculata (Pseudomonas aeruginosa)* and se*torophomonas maltophilia* strains or *Pseudomonas sp. cb-1* strains were higher than that from *Bacillus pseudomycoides* strains or *Bacillus sp*, the former bacteria has a conversion of approximately 24%, the second bacteria has an approximately conversion rate of 21%, for lipase from bacteria *Bacillus pseudomycoides* strains or *Bacillus sp* which has a lower conversion rate, around 17%; therefore the lipases from *Pseudomonas qeniculata (Pseudomonas aeruginosa)* and from *Stenotorophomonas maltophilia* or *Pseudomonas sp. cb-1*were considered as the most suitable catalyst in the conversion reaction. Compare with the commercial lipase the chemical method which used alkaline or acid lipase, all three bacteria

were lower activity, because the conversion rate of commercial and chemical lipases could even achieved 90% or 100% according to the previously published research papers and same research using chemical methods.

6) For the transesterification reactions, a number of parameters had effects on the yield of the conversion reactions. In this research, focus was given on the effect of reaction temperature, the performance of two different alcohols (methanol and ethanol) as acyl-acceptors, the effect of palm oil to methanol ratio, and the effect of different lipases concentration. Each of the four parameters had a relatively unique optimum range. The optimum temperature range is $35^{o}C$ – $40^{o}C$. The optimum palm oil to methanol ratio is 1:3. The most suitable alcohol is methanol on the basis of a series of comparisons.

5.2 CONTRIBUTION

It is expected to find a locally occurring microorganism which could produce some enzymes, specifically lipases, to fulfil the final aim of this research, which is to solve the drawbacks in the conversion of palm oil to methyl ester. On the other hand, the knowledge of local microorganisms is also a supplement for microbiology, such as, it proved that what kind of bacteria is surviving in local area as different place has unique environment, therefore, from the results, concluded the bacteria has a variety of characteristics under the environment.

Several contributions could be expected from this research as I outline here in the below paragraph. Firstly, the economic value and cost effective of using lipases from locally isolated microorganisms are very promising. It is well known that the traditional method to produce biodiesel is either using chemical method or using

commercially-available lipase. Although the former method possesses a high conversion yield, nevertheless it is seriously harmful for our environment, and this is a critical problem, which raised concerns around the globe. By comparison, the commercially lipase gave a fairly advantageous on the conversion reaction, but its shortcomings is also a problem in terms of expenditure. As a result, looking for a cheap-priced and ecologically friendly lipase is a merging research focus in recent years.

This research focus on the Malaysia palm oil plantation soil to isolate some microorganisms which is suitable to solve the above mentioned conversion problems. A lot of strains were already screened for application around the world; what is more, different application conditions were also tested to find the optimum conditions for particular strains. The results showed potential for new industrial application of the identified bacteria; which serve as a basis for further research.

Secondly, hundreds of hectares of oil palm plantations are distributed across the entire country of Malaysia, making the country abundant in crude palm oil. In general, this abundant crude palm oil is used as feedstock for processing into cooking oil; while on the other hand, the globe is facing a shortage of fossil fuel, and more and more vegetable oils are regarded as the feedstock for producing engine fuels. Palm oil is one of the promising materials for this application. In short, this research took crude palm oil as experimental material for producing biodiesel, and proved the feasibility of using palm oil as feedstock in some modified method, therefore giving a reference valued in similar studies in this field.

Thirdly, the characteristics of bacteria are always an important research scope since early history, and thousands of microorganisms were generally isolated, classified, and identified. However, a lot of work remains to be done on either side of microorganisms. In this research, some bacteria were isolated and identified according

to their basic characteristics information, and compared with the results of the previous research with the same bacteria. The bacteria isolated from local soils has few unique traits, the probable reason being that the same family of bacteria has more or less similar respective characteristics in different environments, since they have to get used to the surroundings. So, the characteristics of microorganisms and their identifications as found in this research are an excellent supplement in microbiology, which will be useful in the further reference.

In conclusion, all these contributions from this study are centred around on how to isolate a potentially lypolytic microorganisms which could catalyze crude palm oil into methyl esters, initially studied as the catalytic possibility of lipases; which gave the research a useful skills and experiences throughout this project.

5.3 RECOMMENDATIONS FOR FUTURE WORK

Though the research successfully investigated a series of experiments on transesterification, a number of recommendations are proposed to enhance the whole research as listed below:

1) The soil properties should be studied in the further research, including the count of soils, the moisture o, pH, and temperature of soils. These works could be helpful to know the reason why it could be a habitat of microorganisms.

2) For the further research, more work is needed in the isolation. For example, the use of different media should be tested, by adding or removing some compounds to verify what bacteria could be living or be dead in each case.

3) The identification should be supplemented in terms of some basic conventional biochemical test, in order to complement the result from API kit. In the same way, a thorough and complete Gram-staining and biochemical test for all the eighteen bacterial isolates should be conducted to investigate the potential lypolytic of each and every bacteria found in this research.

4) The purification of lipases should be carried out; it could improve the conversion rate and easily separate the mixture after reaction. In addition, the immobilization of lipase is worth investigating and a lot of parameters after immobilization can be investigated as well.

5) The comparison between extracellular enzyme and intracellular enzyme should be investigated in the future research, since there are in fact some enzymes from intracellular.

6) Lastly, the transesterification studies should be complemented with more work, so that the optimum enzymes conditions can be determined more accurately.

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APPENDIX A

RECIPE OF CULTURE MEDIA

RECIPE OF CULTURE MEDIA

A.1: Recipe of nutrient agar (pH 7.0) (Thermo Fisher Scientific, USA)

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g
Sodium chloride	8.0 g
Distilled water	1,000.0ml

A.2: Recipe of nutrient broth (pH 7.0) (Merck Scientific, Germany)

Peptone	5.0 g
Beef extract	3.0 g
Sodium chloride	2.5g
Distilled water	1,000.0ml

APPENDIX B

GRAM – STAINING REAGENTS

GRAM -STAINING REAGENTS

B.1: Crystal violet

Crystal violet 85% dye	2 g
95% ethyl alcohol	20 ml

Mix and dissolve.

Ammonium oxalate	0.8 g
Distilled water	80.0 ml

B.2: Gram's iodine solution

Iodine crystals	1 g
Potassium iodide	2 g
Distilled water	300 ml

Store in an amber bottle; discard when the color begins to fade.

B.3: Safranin solution

Safranin	2.5 g
95% ethyl alcohol	100.0 ml

For a working solution, dilute stock solution 1/10 (10 ml of stock safranin to 90 ml of distilled water).

APPENDIX C

API KIT READING TABLE

API KIT READING TABLE

C.1: Reading table

Test	**Active Ingeredients**	**Qty (Mg /Cup)**	**Reactions/Enzymes**	**Results**	
				Negative	Positive
ONPG	2-nitrophenyl -β D-Galactopyranoside	0.223	β-galactosidase (Ortho nitrophenyl-β D-Galactopyranisidase)	Colorless	Yellow
ADH	L-arginine	1.9	Arginine Dihydrolase	Yellow	Red /orange
LDC	L-lysine	1.9	Lysine Decarboxylase	Yellow	Red /orange
ODC	L- Ornithine	1.9	Ornithine DeCarboxylase	Yellow	Red /orange
CIT	Trisodium citrate	0.756	CITrate utilization	Pale green/yellow	Blue-green/blue
H_2S	Sodium thiosulfate	0.075	H_2S production	Colorless/grayish	Black deposit/thin line
URE	urea	0.76	UREase	Yellow	Red /orange
TDA	L-tryptophane	0.38	TRYptophane DeAminase	Yellow	Raddish brown
IND	L-tryptophane	0.19	INDole production	Colorless pale green/green	Pink
VP	Sodium pyruvate	1.9	Acetoin production (Voges Proskauer)	Colorless/pale pink	Pink/red
GEL	Gelatin (bovine orgin)	0.6	GELatinase	No diffusion	Diffusion of black pigment
GLU	D-glucose	1.9	Fermentation/ oxidation (GLU cose)	Blue/blue green	Yellow/grayish yellow

MAN	D-mannitol	1.9	Fermentation/ oxidation (MANnitol)	Blue/blue green	Yellow
INO	inositol	1.9	Fermentation/ oxidation (INOsitol)	Blue/blue green	Yellow
SOR	D-sorbitol	1.9	Fermentation/ oxidation (SORbitol)	Blue/blue green	Yellow
RHA	L-rhamnose	1.9	Fermentation/ oxidation (RHAmnose)	Blue/blue green	Yellow
SAC	D-sucrose	1.9	Fermentation/ oxidation (SACcharose)	Blue/blue green	Yellow
MEL	D-melibiose	1.9	Fermentation/ oxidation (MELibiose)	Blue/blue green	Yellow
AMY	amygdalin	0.57	Fermentation/ oxidation (AMYgdalin)	Blue/blue green	Yellow
ARA	L-arabinose	1.9	Fermentation/ oxidation (ARAbinose)	Blue/blue green	Yellow

APPENDIX D

1 Kb PLUS DNA LADDER STANDARD PICTURE

1 Kb PLUS DNA LADDER STANDARD PICTURE

D.1:

1 Kb Plus DNA Ladder
0.7 µg/lane
0.9% agarose gel
stained with ethidium bromide

Structure of Fragments in 1-Kb Increments:

5′-GATCC——— G
G ——— CCTAG-5′
(996 bp)$_n$

Notes:
During 1% agarose gel electrophoresis with Tris-acetate (pH 7.5) as the running buffer, bromophenol blue migrates together with the 500 bp band.

The 1650 bp band is generated from pUC. The bands smaller than 1000 bp are derived from lambda DNA.

APPENDIX E

PCR SEQUENCES BLAST RESULT OF BACTERIA

PCR SEQUENCES BLAST RESULT OF BACTERIA

E.1: Blast result of bacteria A

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ256559.1	Pseudomonas geniculata strain NBG2 16S ribosomal RNA gene, partia	1845	1845	100%	0.0	100%

E.2: Blast result of bacteria B

Sequences producing significant alignments:

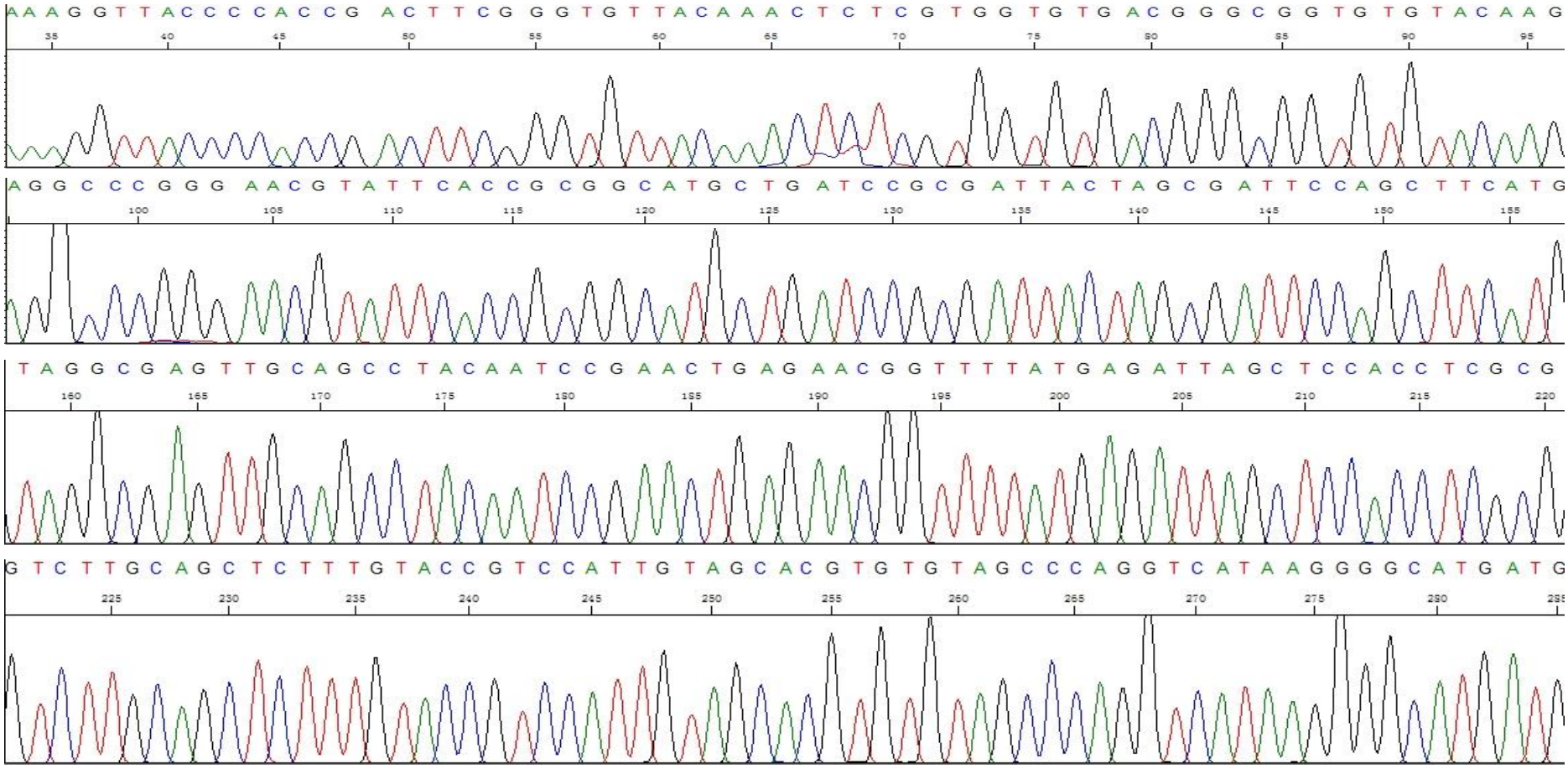
Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU593762.1	Bacillus sp. 00763 16S ribosomal RNA gene, partial sequence	1862	1862	100%	0.0	98%
GU391527.1	Bacillus pseudomycoides strain NI4 16S ribosomal RNA gene, partial s	1840	1840	100%	0.0	97%

E.3: Blast result of bacteria C

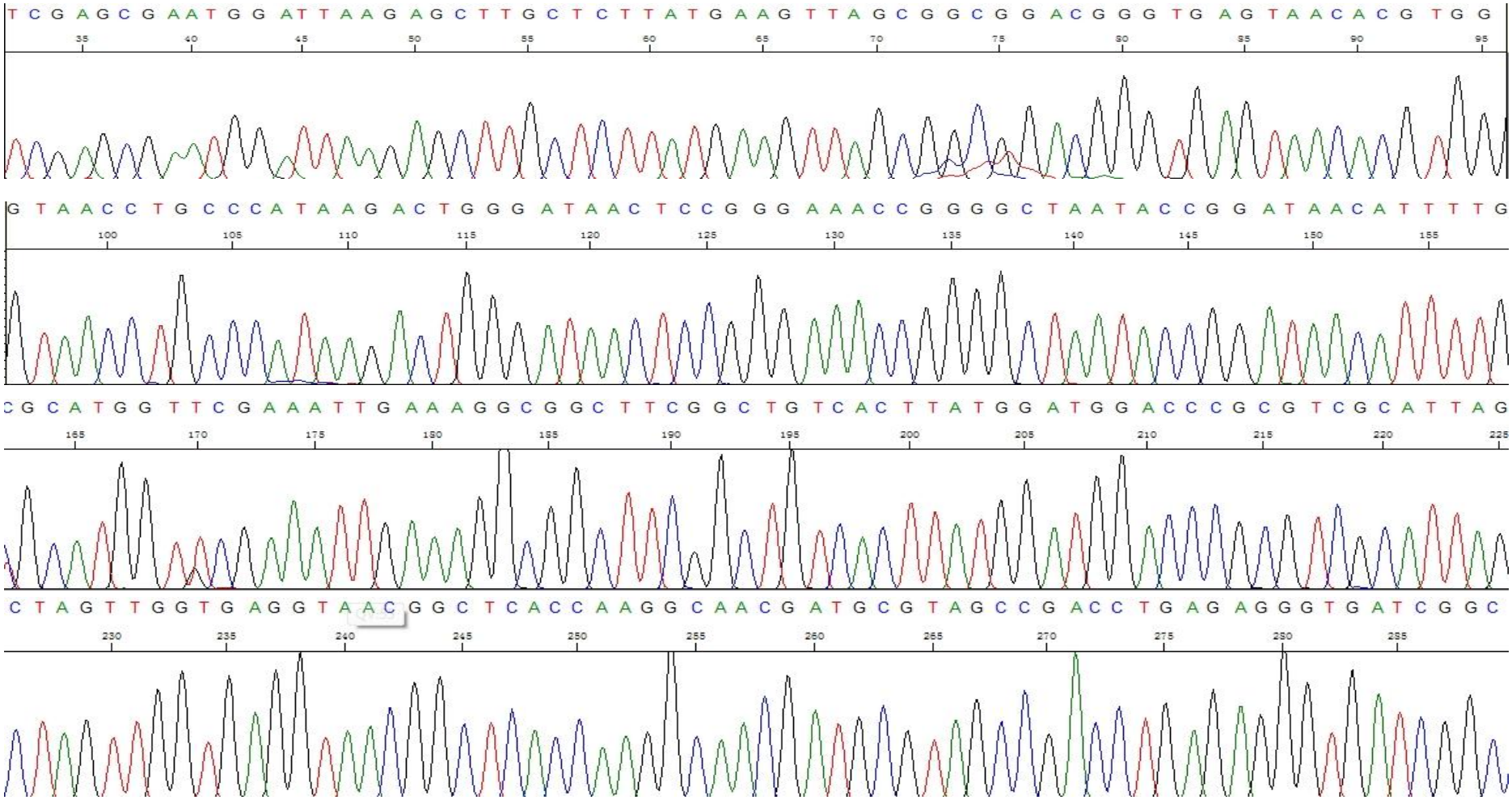
Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ457015.1	Stenotrophomonas maltophilia strain AQN2 16S ribosomal RNA gene,	1890	1890	100%	0.0	100%

APPENDIX F
ABI ELECTROPHEROGRAMS

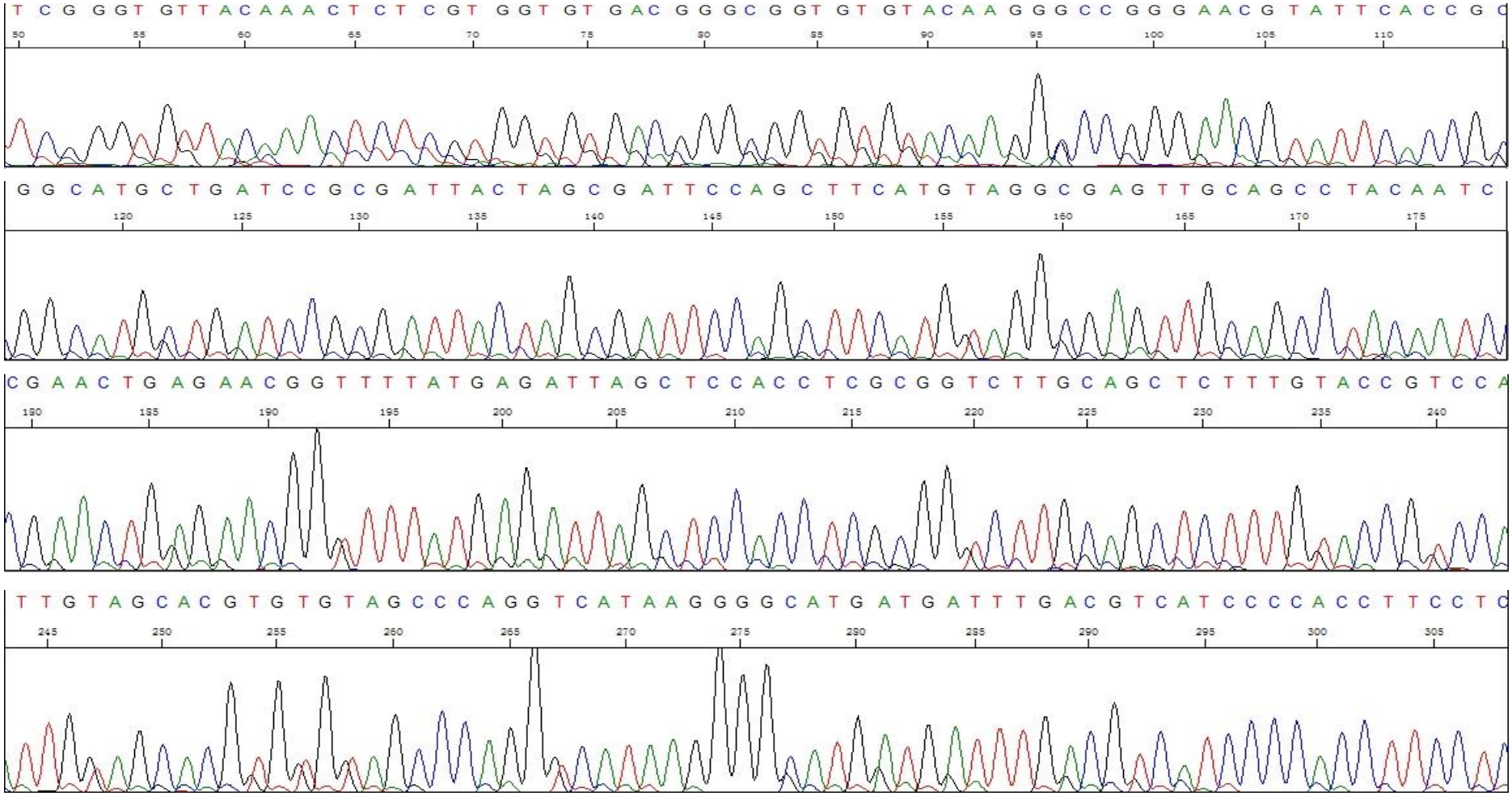
ABI ELECTROPHEROGRAMS



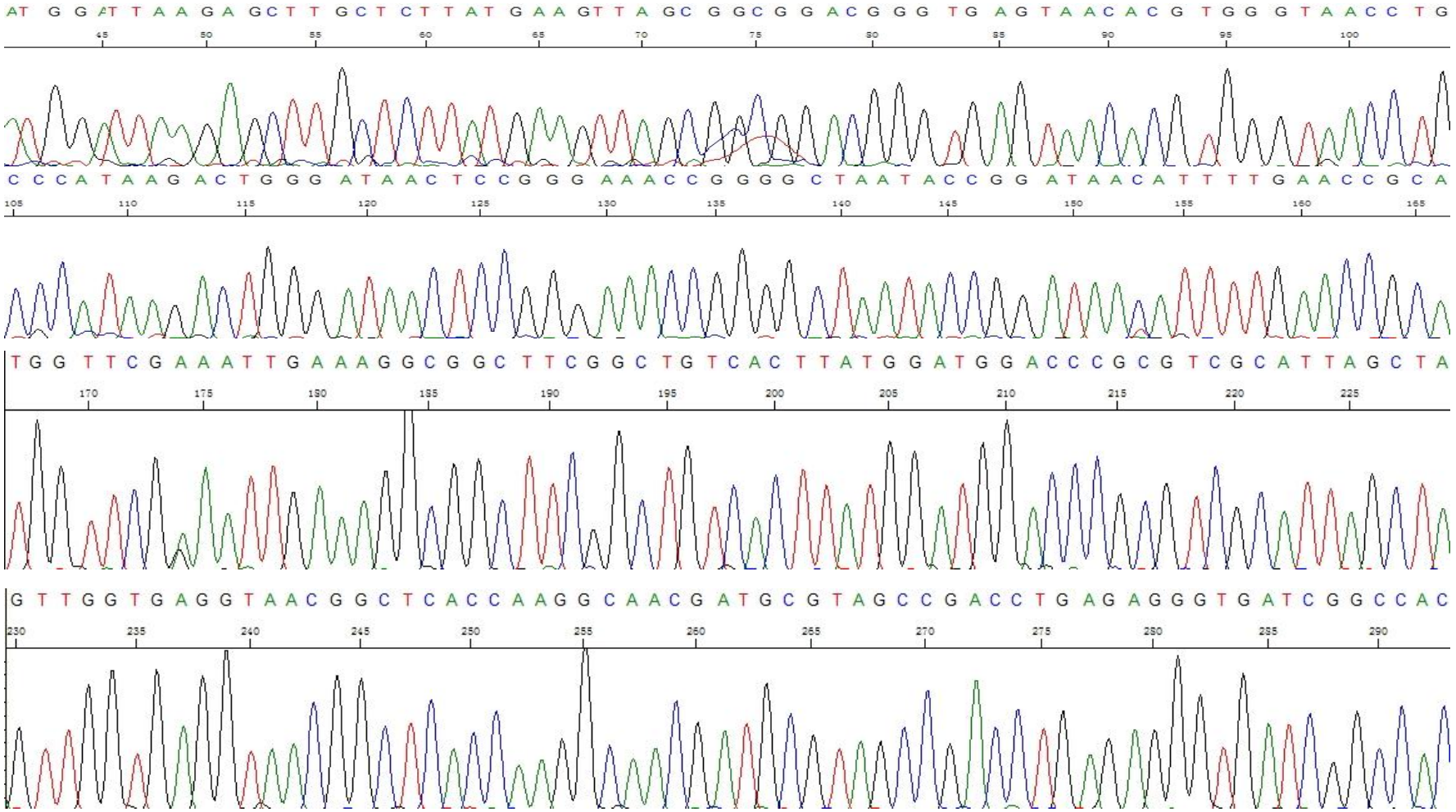
F.1: ABI electropherogram of bacteria A (Reverse)



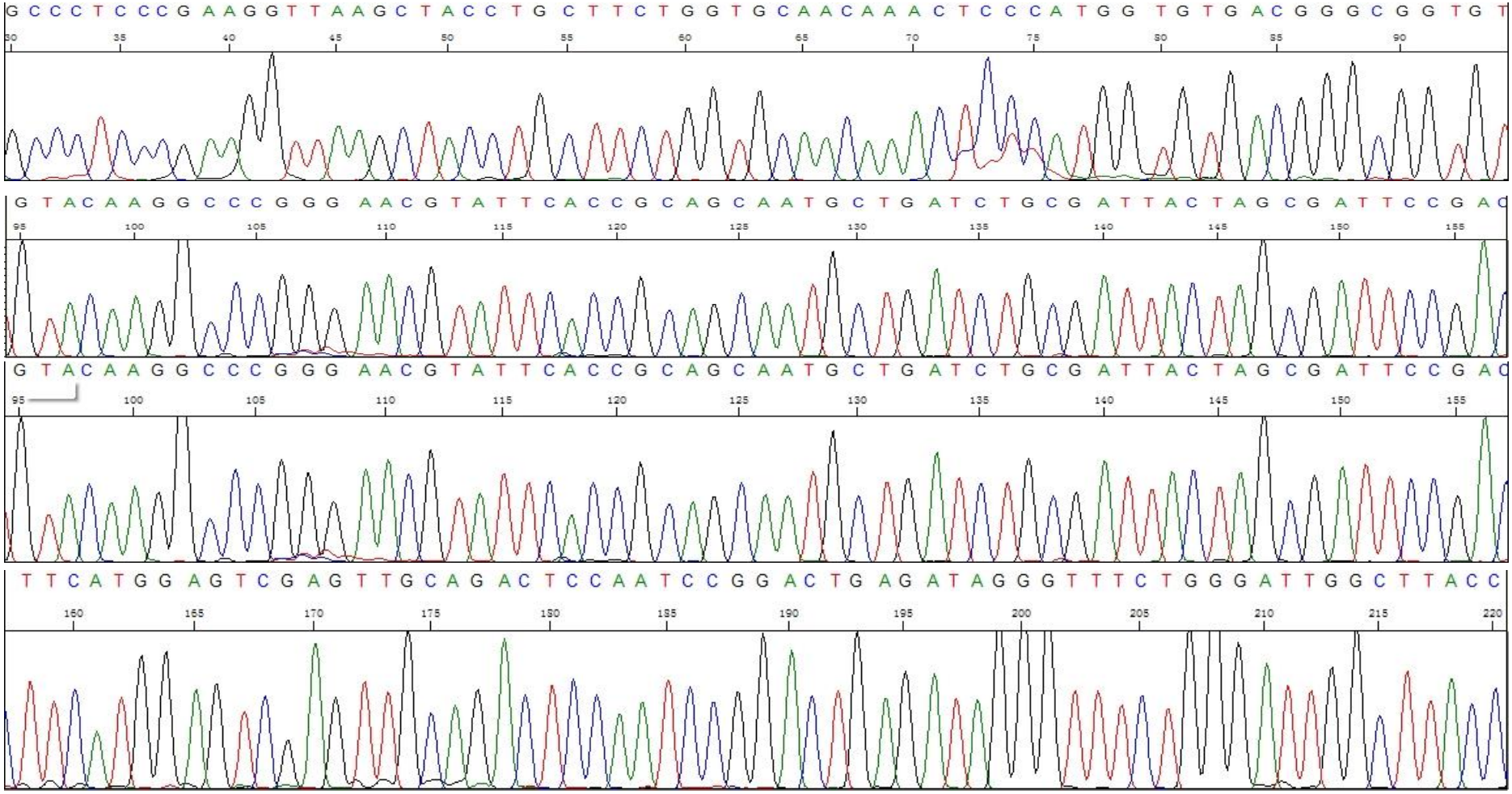
F.2: ABI electropherogram of bacteria A (Forward)



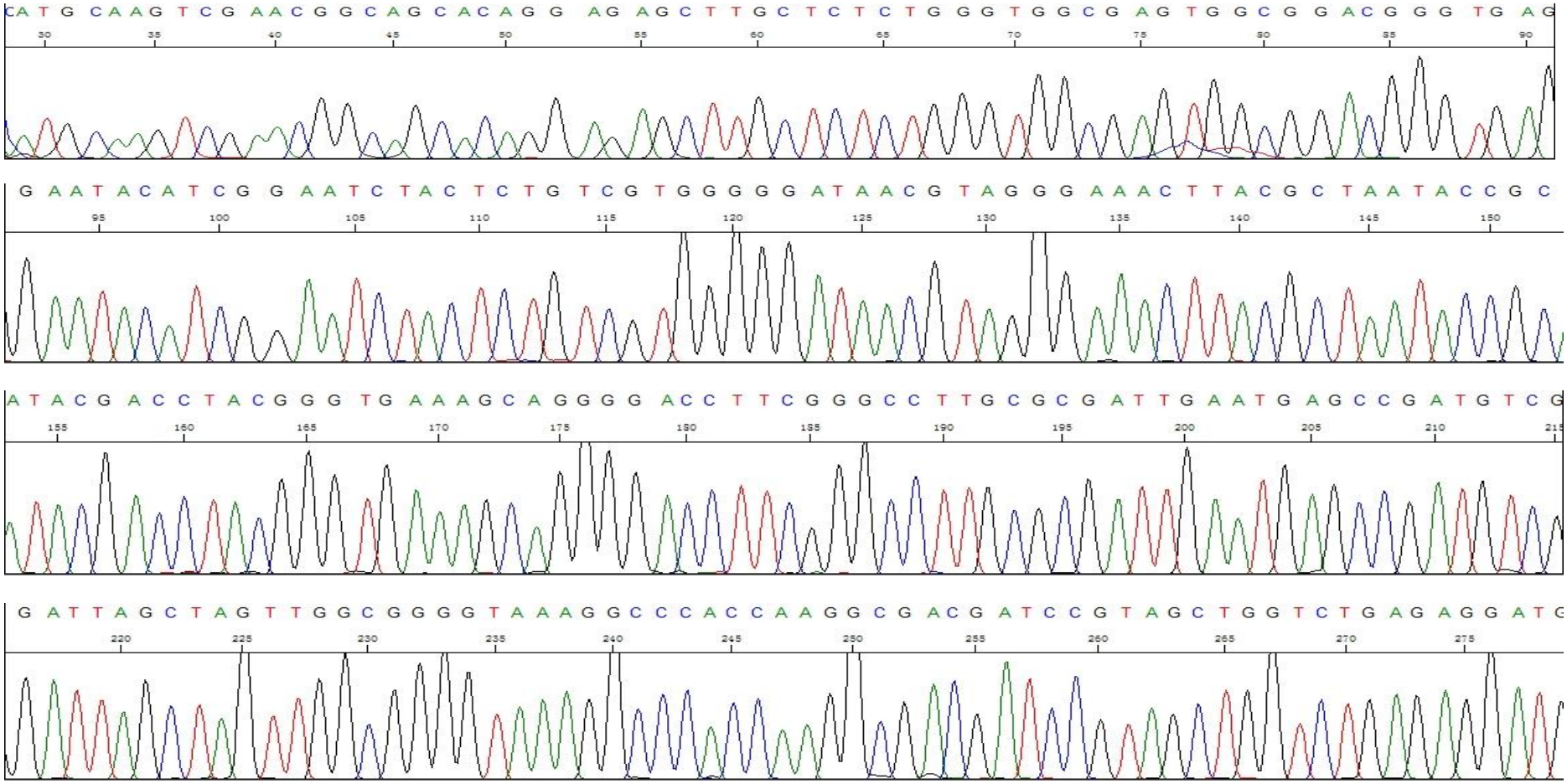
F.3: ABI electropherogram of bacteria B (Reverse)



F.4: ABI electropherogram of bacteria B (Forward)



F.5: ABI electropherogram of bacteria C (Reverse)



F.6: ABI electropherogram of bacteria C (Forward)

APPENDIX G

COMMERCIAL KITS FOR PCR

COMMERCIAL KITS FOR PCR

G.1: G-Spin Genomic DNA Extraction Kit (For Bacterial) (iNtRON Biotenology, INC, 2010)

Kit Contents: Pre-buffer, G-buffer, Binding Buffer, Washing Buffer A, Washing Buffer B, Elution Buffer, Columns Containing Silica-Membrane, Collection Tubes (Polypropylene Tube), Lysozyme Stock Solution, Protainase K Stock Solution, Protainase K Stock Solution.

G.2: MEGAquick-spin PCR & Agarose gel Extaction System (iNtRON Biotenology, INC, 2010)

Kit Contents: Agarose Gel Lysis Buffer, Washing Buffer, Elution Buffer, Nucleic Acid Binding Column, Polypropylene Tube.

APPENDIX H

LIST OF PUBLICATIONS

LIST OF PUBLICATIONS

H.1: Purification of Novel Enzymes from Newly Isolated Indigenous Soil Bacterial, Oral presentation - National Conference of Post Graduate Research (NCON-PGR), 1st of October 2009, Universiti Malaysia Pahang, organized by UMP Post Graduate Office and Jabatan Hal Ehwal Akademik dan Antarabangsa (JHEAA).

H.2: The Isolation and Identification of Locally lipolytic Bacteria from Pahang, Oral presentation – National Postgraduate Seminar (NPS2010), 30th of December 2010, Universiti Kebangsaan Malaysia, organised by Malaysian Society for Microbiology (MSM).