SUPERVISOR'S DECLARATION

"I hereby declare that I have read this thesis and in my opinion this thesis has fulfilled the qualities and requirements for the award of Degree of Bachelor of Chemical Engineering (Biotechnology)"

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FEASIBILITY STUDY ON LOCALLY ISOLATED STRAIN FOR BUTANOL PRODUCTION BY USING OIL PALM TRUNK SAP

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ABSTRACT

As the rising price of crude oil become unstoppable crisis and the shortage of resources start to fear people, the demanding of non-renewable resources gradually incline each days. The production of biobutanol which having numbers of advantages and have potential to replace the uses of petrochemical played a very important role. In biobutanol production, not only the biomass for the carbon sources in fermentation process but the microbes used also must be very precise. In order to improve the current production, the study on locally isolated strains to produce biobutanol by using local plant is proposed. The strain is isolated from soil sample taken at oil palm plantation before proceed with the fermentation process to produce biobutanol. The isolation process is done under strict anaerobic condition since the potential butanol producer strain generally is an anaerobic bacteria. For the fermentation process oil palm trunk sap is used as the carbon sources to produce the biobutanol at 37^{o}C for 5 days. From this research, there are three isolated bacteria which are; isolate 1a, isolate 2b and isolate 3b, which had been identified, can produce biobutanol and other biosolvents. Among the three of the isolated bacteria, isolate 2b was identified to be the best isolated strain in production of butanol and other biosolvents with 5.41 g/L of butanol, 15.04 g/L of acetone and 5.90 g/L of ethanol.

ABSTRAK

Sebagaimana kenaikan harga minyak mentah menjadi krisis berpanjangan dan kekurangan sumber minyak mentah kian menghantui masyarakat, namun permintaan terhadap sumber tidak boleh diperbaharui semakin bertambah setiap hari. Penghasilan biobutanol yang mempunyai banyak kelebihan dan potensi untuk menggantikan penggunaan petrokimia menjadi satu peranan yang sangat penting. Penghasilan biobutanol, bukan hanya biomas sebagai sumber karbon dalam proses fermentasi dipertimbangkan, malah microb yang digunakan juga harus diperincikan. Jadi, untuk meningkatkan penghasilan, kajian terhadap pemencilan bakteria tempatan untuk emnghasilkan butanol dengan menggunakan sumber tempatan dijalankan. Bakteria dipencilkan dari sampel tanah diambil dari tanah penanaman kelapa sawit. Pemencilan proses dijalankan dalam keadaan anaerobic sementelah bakteria yang berpotensi menghasilkan butanol umumnya adalah bakteria anaerobik. Proses fermentasi menggunakan jus batang kelapa sawit sebagi sumber karbon untuk menghasilkan butanol selama 5 hari pada 37^{o}C. Kajian ini menghasilkan tiga bakteria terpencil; terpencil 1a, terpencil 2b dan terpencil 3b, yang boleh menghasilkan butanol, aseton dan juga etanol. Dikalangan tiga bakteria terpencil ini, terpencil 2b merupakan bakteria terpencil terbaik dalam penghasilan butanol, aseton dan juga etanol dengan 5.41 g/L butanol, 15.04 g/L asetone dan 5.90 g/L etanol.

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LIST OF SYMBOL / ABBREVIATIONS

^{o}C	Degree Celsius
mg/L	Milligram per Litre
g/L	Gram per Litre
mL	Millilitre
µL	Microlitre
ABE	Acetone-Butanol-Ethanol
BTBE	Butyl-t-butylether
CO	Carbon dioxide
C4	Carbon 4 chain
HC-	Hydrogen carbon chain
OPTS	Oil Palm Trunk Sap
N_2	Nitrogen
RCM	Reinforced Clostridia Media
RCA	Reinforced Clostridia Agar

CHAPTER 1

INTRODUCTION

1.1 Background of Proposed Study

The uses of biomass fuels had already been introduced ages ago around the time of World War I and II. From the very beginning, the conversion of biomass to alcoholic biofuel such as butanol and ethanol is via acetone-butanol-ethanol (ABE) fermentation (Ranjan and Moholkar, 2011). By the 1960s the industrial ABE fermentation process starts to decline due to economical production of butanol using petrochemical route. At that time, competitive demand of substrates used for fermentation and food supply played another major role of the declination thus increase the biofuels research (Qureshi, Saha, Dien, Hector and Cotta, 2009).

During these past four decades, the research in production of alcohols (ABE) through fermentation has been increase by using agriculturally based substrates including corn, whey permeate, molasses, and cellulosic biomass. Until recently, the use of anaerobic bacteria such as solventagonic clostridia is very popular due to its capability to convert a wide range of carbon sources (Ezeji, Qureshi and Blaschek, 2007). However, the development of potential alcohol producer strains and fermentation technologies are simultaneously being studied to improve the production of biobutanol (Qureshi *et al.*,2009).

As the rising price for the crude oils and increasing political instability in oil producing countries, the use of bio-based alcohols is being considered by people. The once prosperous ABE fermentation is again garnered interest as the increase demand of non-renewable energy resources (Nielsen, 2008). Thus, the production of alcohols from waste of locally grown plant material is crucial to agriculture country because it will support political independence through diversification and dependence on petrochemicals product can be decreased (Antoni et al., 2007).

1.2 Problem Statement

As the pollutions happened at the surrounding become more hectic each day, the responsibility of reducing the pollution in every way was vested to all the society. With the rising price of crude oils and decreasing political stability in oil producing countries, biobutanol demand had emerged its highest climate. Furthermore, the fermentation process always faced butanol toxicity, thus, in order to improve the biobutanol production, new potential alcohols-producer strains must be studied.

1.3 Research Objective

i) To study locally isolated strain for butanol production by using Oil Palm Trunk Sap.

1.4 Scope of Proposed Study

i) To determine locally isolated strain for biobutanol production and other alcoholic biosolvents.

ii) To study production of biobutanol from OPTS by using locally isolated strain.

1.5 Significant of Proposed Study

The significant of proposed study from environment aspect is biobutanol production will replace the need of butanol production from crude oil which is the non-renewable sources that will be finished later. While from the economy aspect, the cost for biobutanol production is lower because it uses the locally isolated strain that can produce same product as the commercial strain in alcohol production. The uses of palm oil trunk sap which is abundantly at Malaysia will surely lower the cost of production.

CHAPTER 2

LITERATURE REVIEW

2.1 Characteristic of Biobutanol.

Butanol (1-butanol, n-butanol) is a clear, low-viscosity, neutral C4 primary alcohol. Butanol is miscible with many commonly used organic solvents. However, the solubility in water is only around 70 g/l. (Ni and Sun, 2009). Due to its low vapour pressure, biobutanol can be blended with gasoline or gasoline containing ethanol and has a potential to be incorporated into the existing fuel supply infrastructure. Gradually, butanol has a potential to replace gasoline diesel or kerosene due to its high energy content, octane improving power and other beneficial to combustion engines (Schwarz and Gapes, 2006).

Biobutanol is an organic compound used widely as an industrial solvent. Since it is produce by using biological methods and biomass, thus to be called bio-butanol. The difference between both biobutanol and butanol produce from crude oil almost none. Nowadays, the biobutanol become more valuable and demanded compare to from the back then. In addition, biobutanol provides greater options for sustainable renewable transportation fuels as the fear of shortage fossil fuels start to develop. As the dependence on the petrochemical industry can be reduced, thus, the

greenhouse gas emission can be lowered. It can be said that biobutanol is one of the environmentally production (Aripin, 2009).

2.2 Application of Biobutanol.

Butanol is widely used as a solvent for acid-curable lacquers and baking finishes. Other important applications of butanol and its derivatives include paint thinners, solvent for dyes, brake fluids, and as an extractant in the production of drugs and natural substances such as antibiotics, hormones, vitamins, etc. Like ethanol and bio-diesel, butanol played an important application as a renewable energy carrier as well since it can be used directly as a liquid fuel (Ni and Sun, 2009). Biobutanol has a better energy density and performance compare to ethanol and can be produced from more sustainable feedstocks than biodiesel (Green, 2011). Compared with ethanol, butanol has numbers of chemical and physical properties that are particular attractive for application as a biofuel. Other than that, biobutanol also can contribute to the partially biological production of butyl-t-butylether, BTBE, as does bioethanol today with ETBE (Stoeberl, Werkmeister, Faulstich and Russ, 2011).

The reason this research propose to produce biobutanol is because there are numbers of advantages of butanol as fuel or blending component over ethanol and other biofuel. One of the advantages is lower vapour pressure which can reduce the chance of vapour lock. The ability to be blended at higher concentrations without retrofitting vehicles is very beneficial to the user because any additional cost can be avoided.

Biobutanol is an oxygenated fuel that can reduce HC- and CO-emissions as the combustion efficiency is improved. Additional of butanol to ethanol-benzene-mixtures also resulted positive effect on the vapour pressure of the mixture (Stoeberl *et al.,* 2011).

2.3 Production of Biobutanol.

2.3.1 ABE Fermentation

Acetone–butanol–ethanol (ABE) fermentation was intoduced industrially throughout the United States during the first half of last century, but was discontinued in the early 1960s due to unfavourable economic conditions brought about by competition with the petrochemical industry (Ezeji *et al.,* 2004). Until now, fermentation substrate is an important factor influencing the cost of butanol production (Qureshi and Blaschek, 2000). Futhermore, ABE fermentation also has long been subjected to a high potential of end-product inhibition which usually due to butanol, which somehow has affected the economics especially in large scale production. The main problem of ABE fermentation lies on the toxic effects that butanol has on bacteria that leads to low solvent productivity. Therefore, it was important to search for high butanol-tolerant microorganism bacteria as well as adaption of in situ toxicity removal technologies in large scale production to overcome the toxicity problem, thus, increase the yield (Yen *et al.,* 2011).

2.3.2 Biomass for Butanol Production.

The utilization of potato for butanol production is reports by Gutierrez *et a*l. (1998). The ability of this substrate to produce butanol at the ratio of 3:6:1 at the total broth concentration of around 20g/L is one of the achievement that been made back then. At the early era of biobutanol production, the uses of agriculture feedstock such as sorn, sugar, wheat, sorghum and sugarcane are causing a lot of cost due to the competition of the substrate for butanol production and food supply.

Thus, in an attempt to reduce the cost in biobutanol production, the feedstock used was replaced with variety of low cost feedstock such as soy molasses and cracked corn. Agriculture residues also been used including, barley straw, wheat straw and byproducts left over from the corn milling processes. These types of materials are economically available, however must undergo pre-treatment process before proceed with the fermentation to produce butanol (Nielsen *et al.*, 2008).

Generally, biomass that had been used in biobutanol production contains a mixture of carbohydrates including starch, hemicelluloses, sucrose and other carbohydrates that can be utilized by the butanol-producer strains (Kalil *et al*., 2003). Previous study had stated that corn fiber which one of the biomass, contains about 69% fermentable sugars, of which approximately 20, 14, and 35% is in the form of starch, cellulose, and hemicellulose fractions, respectively. Typically, 2.04 kg (4.5 lb) of corn fiber is obtained from 25.40 kg (one bushel or 56 lb) of corn, which can be converted to about 1.36 kg (3.0 lb) of fermentable sugars (Ezeji, Qureshi and Blacshek, 2007).

In Table 2.1 on the next page, it shows the production of biobutanol by various type of biomass and butanol production for the fermentation process.

Table 2.1: Biobutanol Production by Various Kinds of Biomass and Strains.

Biomass	Microbes	Butanol (g/L)	References
Potato starch	*C. Acetobutylicum*	0.17	Yen et al. (2011)
Whey	*C. Acetobutylicum*	12.00	Stoeberl et al. (2011)
Barley straw	*C. Beijerinckii*	13.62	Qureshi et al. (2010)
Wastewater algae	*C. Saccharoperbutylacetonium*	5.61	Ellis et al. (2012)
Seaweed extract	*C. Acetobutylicum*	10.40	Huesemann et al. (2007)
Corn fibre	*C. Beijerinckii*	13.20	Qureshi et al. (2007)
Maple wood	*C. Acetobutylicum*	0.20	Sun and Liu (2010)
Wheat straw	*C. Beijerinckii P260*	5.50	Qureshi et al. (2007)
Palm empty fruit bunches	*C. Acetobutylicum*	0.82	Noomtin and Cheirsilp (2011)
Date palm fruits	*C. Acetobutylicum*	7.90	Abd-Alla and El-Enany (2012)

2.3.2 Microbes for Butanol Production.

During the last century, the production of biobutanol by clostridial solvent fermentation was the second largest industrial fermentation process besides yeast-based production of ethanol until its declination due to the sky rocketing of oil industry demand in the 1980s (Antoni, Zverlov and Schwarz, 2007).

The fermentation process usually known as ABE fermentation is namely after its main components of acetone, butanol and ethanol. Four distinct species of clostridia were identified among the industrial production strains which are among the most reliable butanol-producer strains: *Clostridium acetobutylicum*, *Clostridium beijerinckii, Clostridium saccharoperbutylacetonicum* and *Clostridium saccharobutylicum beijerinckii* (Shaheen and Jones, 2011). Based on Table 2.1, bacteria used for the production of biobutanol also depend on the type of the biomass in order to produce higher yield of biobutanol.

2.3.3. Isolation of Potential Butanol Producer Microbes.

The characteristic of the microbes must be identified before handling with it. Biobutanol is produced by anaerobes microbe that converted biomass to alcohol in anaerobic condition. From the previous study, it is said that the genes Clostridium is a heterogeneous collection of Gram-positive, obligate anaerobic, non-sulfide reducing, spore forming and rod-shaped bacteria. However, due to the spore forming characteristics, the toxicity of butanol is always encountered (Dubourguier *et al*., 1986).

Clostridia contain large number of saccharolytic and easily isolated from soil samples at various place. Isolation of solvent producer at slightly higher acidic pH of soil offers a better potential compare to normal soil (Aripin, 2009). For this research the uses of soil strains as the locally isolated strains for the potential butanol-producer bacteria is chosen due to this reason.

The method that been used for the isolation is Hungate method. The soil sample from various agriculture places is taken and dried. Reinforced Clostridia Medium (RCM) is used to grow the strains. By using Gram Staining method, the characteristics of the strain is identified under microscope.

2.3 Biobutanol from Oil Palm Trunk Sap.

Malaysia is one of the biggest palm oil producers in the world. However, compare to the other type of agriculture biomass, palm oil industry leaved behind large numbers of biomass residue from its plantation and milling process. Oil palm trunk is used to make a lumber unfortunately only the outer part of the trunk can be used due to its harder properties. Thus the inner part which is the soft and weak part is not suitable for the plywood manufacturing (Dalibard, 1999). Oil palm trunk sap (OPTS) is one of leaved behind biomass squeezed from the palm oil trunk that is useful in biobutanol production. It is because it contents high amount of carbon sources in the form of cellulose and hemicelluloses.

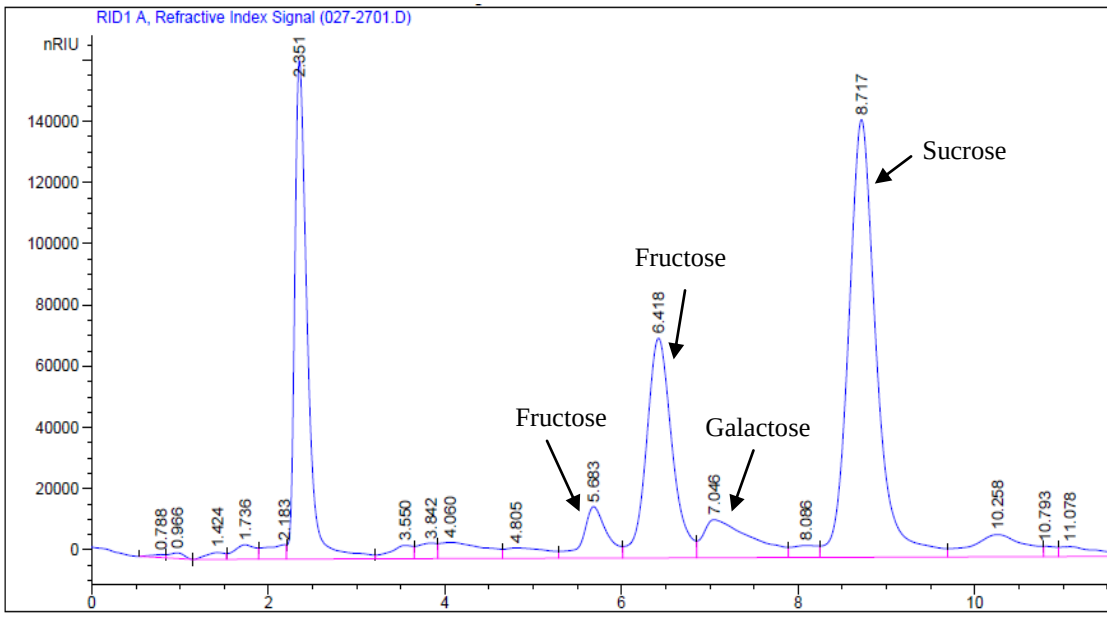

Figure 2.1: HPLC Profile on Sugar Analysis on OPTS.

Table 2.2: Type of Sugar in OPTS

Type of Sugar	Concentration (g/L)	%
Sucrose	47.77	67.17
Glucose	15.47	21.75
Galactose	3.37	4.73
Fructose	4.51	6.34
Total	71.12	100.00

From the Figure 2.1 and Table 2.2, they show the glucose concentration in the OPTS is the highest compare to the other sugar in the sap. This is shows that OPTS is suitable for production of biobutanol due to high glucose concentration.

Table 2.3: Production of OPT and OPTS

Biomass	Production	Journal
Oil Palm Trunk	Hydrogen rich gas	Mohammed et al. (2011)
	Bioethanol	Jung et al. (2011)
	Bioplastic	
	Lactic acid	Mori (2007)
	Glucose	Chin et al. (2011)
Oil Palm Trunk Sap	Biohydrogen	Noparat et al. (2012)
	Bioethanol	Kosuki et al. (2010)
	Lactic Acid	

Based on Table 2.3, it shows several type of product from oil palm trunk. From previous study based on the table, there is a possibility of biobutanol can be produced from OPTS because it is stated that OPTS capable in producing glucose, ethanol and others.

In this study OPTS is used as the biomass to produce the locally isolated strains. By using this abundantly available source that are most economically, the potential of locally isolated butanol-producer strains can be studied.

CHAPTER 3

Material and Methodology

3.1 Flowchart of Research Methodology

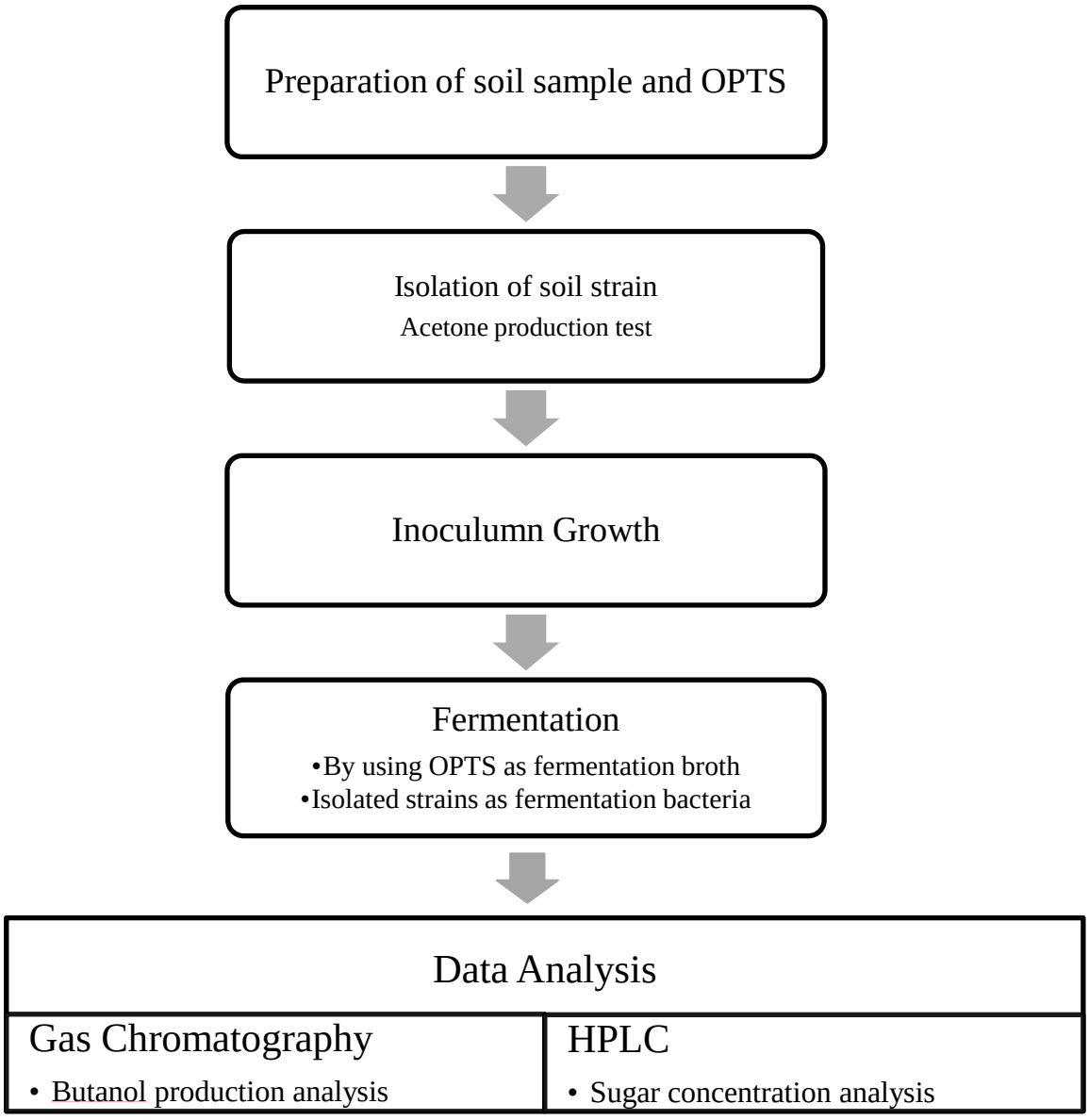

Figure 3.1: Flowchart of Research Methodology

3.2 Palm Oil Trunk Sap, Strain and Media Growth.

Palm Oil Trunk Sap (OPTS) was obtained from Palm Oil Plantation, Jengka 21, Temerloh. The OPTS juice was extracted by pressing the frond using a conventional sugarcane press machine. As for the strain, *Clostridium acetobutylicum* and *Clostridium beijerinckii* strains were purchased and used as reference. The bacteria were growth in anaerobic condition in Reinforced Clostridial Medium (RCM) for 48 h at 37^{o}C (Kalil et al, 2003).

3.3 Source of Soil Strains.

Soil sample were collected from oil palm plantation site. The sample was let to air dry for 1 week and sieve. 10 g of sample was dried at 80°C until the constant weight to determine the humidity. While the pH was determined by using pH meter after re-suspending and homogenizing 1 g dry sample for 20 min in 10 ml of distilled water (Montoya *et al*., 2000).

Figure 3.2: Soil sample was collected and sieved.

3.4 Isolation of solvent-producing bacterial isolates.

The counterselection against the non-sporeformers was done by suspending 1 g soil in 10 ml anaerobic RCM . The tube was intubated in a water bath at 70°C for 10 min to inactivate vegetative cells. The RCM medium was prepared under anerobic condition by using by purging the media with N_2 to remove oxygen from medium and headspace of the containers (Aripin, 2009). The tube was then incubated for five days at 37°C and daily checked for growth and gas production.

Figure 3.3: Soil Sample Culture in RCM media.

100 µL of growing cultures were spread plated on RCA agar to grow soil mixture bacteria. The plates were incubated inside the anaerobic jar at 37°C for five days. Morphology of the colonies grew inside the plates were observed recorded. Visible colonies from the plates were streak onto the fresh RCA agar plate. This method was done for several times to ensure that the cultured bacteria were purified. (Montoya *et al*., 2000).