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VARIATION OF CARBON SOURCES IN PRODUCTING RHAMNOLIPID BY *Pseudomonas aeruginosa* FOR MICROBIAL ENHANCED OIL RECOVER'S APPLICATION

VARIASI SUMBER KARBON PADA PRODUKSI RHAMNOLIPID OLEH *Pseudomonas aeruginosa* DALAM APLIKASI *MICROBIAL ENHANCED OIL RECOVERY* (MEOR)

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ABSTRAK

Menurunnya produksi minyak bumi disebabkan karena sumur produksi yang sudah tua. Teknologi enhanced oil recovery (EOR) terbukti mampu meningkatkan cadangan dan produksi lapangan minyak mature. Salah satu teknologi EOR yang dikenal efisien dalam meningkatkan perolehan minyak adalah microbial enhanced oil recovery menggunakan biosurfaktan. Biosurfaktan yang paling efektif adalah rhamnolipid yang dihasilkan oleh bakteri Pseudomonas aeruginosa yang dapat menurunkan tegangan antarmuka antara minyak bumi dengan air. Dalam produksi biosurfaktan oleh bakteri ini, diperlukan substrat sebagai sumber karbon dalam proses fermentasi. Sumber karbon yang digunakan pada penelitian ini adalah glukosa, gliserol, molase, kulit pisang, dan minyak jelantah. Penelitian ini bertujuan untuk mengetahui sumber karbon yang paling optimum dalam menghasilkan biosurfaktan dari Pseudomonas aeruginosa dengan menggunakan busnell hass medium sebagai media cair pertumbuhan bakteri. Produksi biosurfaktan yang dihasilkan adalah 74mg/L dari glukosa; 63mg/L dari kulit pisang; 66mg/L dari gliserol; 85mg/L dari minyak jelantah; dan 64mg/L dari molase dengan penurunan tegangan permukaan berturut-turut: 33,55 mN/m dari glukosa; 32,51 mN/m dari kulit pisang; 27,55 mN/m dari gliserol; 22,46 mN/m dari minyak jelantah; dan 31,49 mN/m serta memiliki penurunan tegangan antarmuka dari glukosa; kulit pisang; glisero; minyak jelantah; dan molase berturut-turut adalah 15,2 mN/m; 13,78 mN/m; 8,15 mN/m; 0,14 mN/m; dan 11,2 mN/m.

Kata kunci: *Biosurfaktan, pseudomonas aeruginosa; rhamnolipid; tegangan permukaan; tegangan antarmuka*

ABSTRACT

The decrease in oil production is caused by the ageing of oil production wells. The enhanced oil recovery (EOR) technology is proven to increase oil reserves and production in mature oil fields. One EOR technology that has proven to be efficient in increasing oil production is microbial EOR by using biosurfactant. The most effective *biosurfactant* is rhamnolipid produced by *Pseudomonas aeruginosa,* the bacteria of which can lower the interfacial tension between the petroleum and water. In *biosurfactant*'s production thanks to these bacteria, the substrate as the source of carbon in the fermentation process is needed. The sources of carbon used in this study are glucose, glycerol, molasses, banana peels, and waste

cooking oil. This research aims to determine the most optimum carbon sources to produce biosurfactant from *Pseudomonas aeruginosa* by using Busnell Hass medium as a liquid medium of bacterial growth. Biosurfactant's production results are; 74mg/L from glucose; 63mg/L from banana peels; 66mg / L from glycerol; 85mg/L from waste cooking oil; and 64mg/L of molasses with the following decreasing surface tension: 33.55 mN/m from glucose; 32.51 mN/m from banana peels; 27.55 mN/m from glycerol; 22.46 mN/m from waste cooking oil; and 31.49 mN/m from molasses. In addition, the decrease of interface tension of glucose; banana peels; glycerol; waste cooking oil; and molasses are as follows : 15.2 mN/m; 13.78 mN/m; 8:15 mN/m; 0.14 mN/m; and 11.2 mN/m respectively.

Keywords: Biosurfactant; *pseudomonas aeruginosa;* rhamnolipid; surface tension; interface tension.

I. INTRODUCTION

Currently the oil reserves in many countries continues to decline due to the age of the fields. To overcome this, there is a technology that is used in an effort to increase oil recovery, or as it is often known Enhanced Oil Recovery (EOR). EOR technology is a popular method used in the oil industry today. This technology involves injecting a material into the reservoir. EOR could increase oil recovery by up to 40-45% (Sen 2008). Nowadays, the development of biotechnology EOR leads to more environmentally friendly result at a lower cost. This technology is microbial enhanced oil recovery (MEOR) (Bordoloi & Konwar, 2008). In term of the MEOR technology, bioproduct injection is the most effective technique. One example is the injection of bioproduct biosurfactant. The interfacial tension of a biosurfactant work to lower the voltage between water and oil is release the oil which was adhesing to the rocks. (Sen 2008).

Biosurfactant is mostly produced by microorganisms such as bacteria. Some microbes can produce surfactant when grown on different substrates, ranging from carbohydrates, fats, to hydrocarbons (Korsaic 2001). The use of different substrates leads to change in the chemistry of the structure of the surfactant. Knowledge of the surfactant will be very useful in designing products with properties that correspond to the desired application. The biosurfactant most widely studied is a type of glycolipid biosurfactant, which rhamnolipid producesfrom bacteria *P.aeruginosa* (Moussa et al. 2014)

Although rhamnolipid biosurfactant is effective and suitable for application a wide variety of applications such as bioremediation of oil pollutants. But there are still many problems and obstacles to overcome, including the fact that biosurfactant production is still low and there also high production costs due to the use of expensive substrates such as pure glucose. However, this can be minimized by the use of waste in production (Silva, Farias, Rufino, Luna, & Sarubbo 2010). Various kinds of organic waste from processing argoindustri such as glycerol and molasses as well as household waste such as waste cooking oil and banana peels can be used as an alternative carbon source to produce biosurfactant. Waste from the biodiesel industry, namely glycerol and sugar industry waste, such as molasses is agroindustrial waste, are abundant in Indonesia. Glycerol and molasses have also been used as a carbon source for the microorganisms in the fermentation process. Cooking oil is a large part of household waste, but it has not been put to good use. Waste cooking oil can be a source of carbon needed by the bacteria (Shanda et al. 2014). In addition banana peel is waste that is rarely used by humans. Based on it physical and chemical properties, banana peel waste that has been hydrolyzed by strong acid is a potential source of carbon, because it contains a fair amount of glucose (Asteria and Franky 2013).

Factors that influence the production of biosurfactant, such as the type of substrate, bacterial source of nutrition, and environmental condition, are of concern in this research (Desai and Banat, 1997) and (Milena et al. 2012). Based on the above explanation, this research compares the effectiveness of employing several kinds of carbon source in the production of rhamnolipid produced from bacteria *P. aeruginosa,* the terms of the rate of bacterial growth of any carbon source, biosurfactant produced and biosurfactant activity. A decrease in surface tension and interfacial tension are also important in this study because both of these are a major factor in the performance of biosurfactant to mobilize the oil in the well.

II. METHODOLOGY

A. Materials and Tools

The bacteria used were *Pseudomonas aeruginosa ATCC* from Microbiology Laboratory FK UI, solid medium agar slant (agar), the liquid medium busnell hass medium consisting of 1 g KH_2PO_4; 1

g K_2HPO_4; 1g NH_4NO_3; 0.2 g $MgSO_4.7H_2O$; 0.05 g $FeCl_3$; 0.02 g $CaCl_2.2H_2O$; 0.6% (v/v) $NaNO_3$, 10 g (w/v) molasses; banana peels; glucose, waste cooking oil, glycerol, orcinol, diethyl ether, L-rhamnose monohydrate, and concentrated HCL. The equipment used in this study is a set of tools fermentation, shacker incubator, laminar airflow, vortex, spectrophotometer, analytical balance, and pH meters.

B. Rejuvenation of *Pseudomonas aeruginosa* Isolates

Refreshing of isolates was done for two medium: Luria Broth medium (LB) and also Busnell hass medium. A total of 50 mL of LB was put into a 250 mL Erlenmeyer, then 50 mL Busnell hass medium, was added, and then sterilized in the autoclave for 15 minutes and cooled. The culture was shaken in the incubator shacker for 24 hours at room temperature with a speed of 150 rpm.

C. Fermentation of *Pseudomonas aeruginosa*

Fermentation is done with a batch system in a 500 mL Erlenmeyer flask with a working volume of 300 mL. By combining medium busneel hass 300 mL, 10% (v/v) inoculum, each variation subtract 10 gr (w/v), and 0.6% (v/v) $NaNO_3$ as nitrogen nutrition. The fermentation was carried out for 7 days in the incubator shaker at 150 rpm, at room temperature. Then 40 mL culture samples were taken every 24 hours.

D. Determination of Growth Curve

Take 1 mL sample diluted fermentation culture in 9 mL of 0.85% NaCl solution and shaked. Repeat these steps to obtain the solution until you reach the desired dilution ratio. 0.1 mL dilutions into a petri dish and spread by using tryglasky. Petri dishes are then incubated at a temperature of 34-36^oC for 24-48 hours with the plated cup in the inverted position. Count the number of colonies (CFU) that exist in a petri dish using a colony counter.

E. Analysis of Content Rhamnonse by Orcinol Methods

The biosurfactant concentration that was formed was analyzed by a modified orsinol. First, create a standard curve rhamnosa to make rhamnosa with concentrations of 0, 10, 50, 100, and 200 ppm which were diluted in sodium bicarbonate ($NaHCO_3$) 0.05 M. Then insert 2 mL into each test tube. Then, in each solution 3.6 mL orsinol was added, heated to boiling, cooled at room temperature for 15 min and analyzed by spectrophotometer at a wavelength of 421 nm. The regression line rhamnosa standard curve is determined by the method of least squares (Diah et al. 2010).

Liquid from the incubation medium was centrifuged at 6000 rpm for 10 minutes to separate the culture medium from the bacteria and the supernatant was taken. A total of 4 mL of the supernatant was extracted with a 2 mL dietilether for 5 minutes. A layer of ether was taken with a pipette, dried and redissolved in 2 mL of a solution of sodium bicarbonate ($NaHCO_3$), 0.05 M solution of the sample was homogenized by vortex and 3.6 mL orsinol added, heated to boiling, cooled at room temperature for 15 min and the analyzed by spectrophotometer (Diah et al. 2010)

F. Analysis Surface Tension and Interface Tension

Interfacial tension measurement was performed with a processor tensiometer (Kruss) equipped with a ring plate. Testing the light phase was the first stage. A total of 40 mL of crude oil used as a put on the light phase, then the plate was cleaned by using n-hexane, acetone, distilled water and mounted in place and the tool was closed. Tools are calibrated beforehand and then the reaserchers choose the appropriate method for analyzing surface tension and interfacial tension. The plate is immersed in the oil, the relevant data is then recorded.

The second stage is to clean the plate and prepare a fermentation culture that serves as a heavy phase. Stages are firsly placing a 15 mL culture fermentation vessel into the thermostat and then cleaning the plate which is already installed on the device and closed. Vessel thermostat placed or promoted to the plate until the distance between plate and the sample surface is very near but not touching. Furthermore, the tool will work automatically to indicate that the researcher should cover the enclose plate with a light phase (crude oil). Plate covered with crude oil to close completely, with a ratio of 1: 1 between crude oil and culture fermentation, the next is to wait until stable. If it is stable, press start and the tension was antermuka value and standard deviation values will appear on the monitor screen and the paper will be printed.

A surface tension test was performed using the same tool to test the interfacial voltage. Enter 35 mL from the fermentation vessel into the thermostat. A dry clean plate is then carefully installed and the

tool is blown on, so as to cool it down. The vessel thermostat is the placed or promoted to the plate until the distance between the plate and the surface of the sample are very close together but not touching. The tool will work automatically, and of surface tension and standard deviation values will appear on the monitor screen.

III. RESULT AND DISCUSSION

A. Determination of the Growth Curve

The growth curve is useful to provide an overview of the bacterial growth phase for biosurfactant a method which is based on the theoretical relationship that the colonies are derived from a single cell bacteria and the assumption that the number of colonies on a petri dish of bacteria is closely related to the actual number (Salle 1961). This curve also serves to relate the growth of bacteria to the activity of biosurfactant. Figure 1 is an overall picture of the growth phase of *Pseudomonas aeruginosa* on various carbon sources.

Figure 1 above shows that the growth of Pseudomonas aeruginosa varies according to the type of carbon source. It can be seen that the growth curve decreased on the first day. This is due to undergoing a process of adaptation because the inoculum was transferred from one medium to Busnell Hass medium inoculum. In this process the bacteria a major change because the medium in which they grow has little nutrient compared to current inokulas process that decreases the amount of bacterial growth. However, on the second day to the seventh day the number of bacteria tended to increased. This proves that the *P. aeruginosa* bacteria can grow in the medium. In bacterial growth curves cooking oil has the greatest number of bacteria. This is became cooking oil are a long hydrocarbon chain which is a good source for the growth of bacteria. (Prastikasari, 2000). This condition is more easily degraded by bacteria. Cooking oil generally contains fatty acids which are hydrophobic so that the carbon source is favored by *Pseudomonas aeruginosa* (Fatimah, 2013). In the waste cooking oil it can be seen that the growth of bacteria increased but not significantly and decreased on day 6. This is because the bacteria had already experienced the earlier stage of pre-culture (Ciccyliona and Refdinal 2012) and also had resulted in a large number of bacteria which is not significant in term of growth the phase (log phase). Bacterial growth on carbon sources such as glucose, molasses, banana peels, and glycerol through a phase of growth (log fase) significant from day one but has a number of bacterial initiations smaller than cooking oil. This is because the carbon source sugars have adapted over a longer time in coating the sugar in the fermentation culture to make the substrate of the bacteria. However, the peak phase (the highest peak) of the carbon source is at 7 days due to the carbon source sugars having a reducing sugar content (sugar and derivatives) that are more than the waste cooking oil so the log phase condition had a longer time than the waste cooking oil. This growth curve shows the deadth phase by day 8 and 9.

B. Production of Rhamnolipid Biosurfactant

Concentrations of rhamnolipid are resulting from various types of carbon sources have different concentrations. The greatest concentration of rhamnolipid was produced from waste cooking oil, which was 85 mg/L. While the rhamnolipid concentration of carbon source glucose, banana peel, molasses and glycerol have a range that is quite similar, each concentration was: 74; 63; 64 and 66

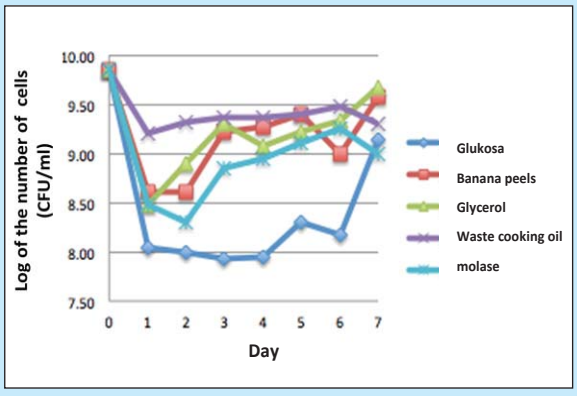


Figure 1
The growth curve of pseudomonas aeruginosa.

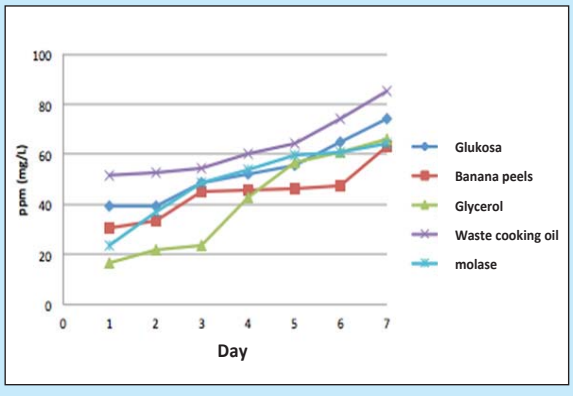


Figure 2
Concentration of biosurfactant from any carbon sources.

mg/L respectively. Here is a picture of concentration differences rhamnolipid produced, among others:

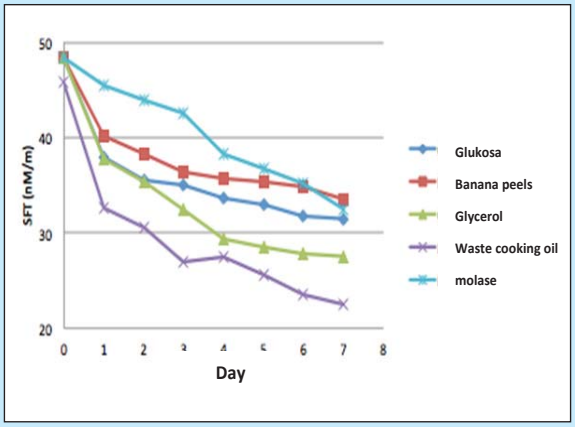
Rhamnolipid production from waste cooking oil has the greatest concentration which is caused by an increase in the pseudomonas population, that much produced many secondary metabolites. In addition, free fatty acids contained in waste cooking oil can stimulate the formation of biosurfactant. The process of rhamnolipid production of glycerol under goes gluconeogenesis to form glucose to be synthesized by bacteria into rhmanose group which will later become rhamnolipid. So, on the first day there was little biosurfactant produced. However, after 7 days of fermentation, the concentration of rhamnolipid produced by glycerol was no different from that produced by banana peel and molasses. The rhamnolipid produced using glucose substrate has a higher value than banana peel, molasses, and glycerol, because as the concentration of glucose in much greater in the glucose substrat than in banana peel, molasses or glycerol. To see the conversion of the substrate/carbon source into the product rhamnolipid, researchers compared the number of rhamnolipid concentrations generated by the number of substrates used in the fermentation culture (Y_{PS}) with each using 10 g (w/v) carbon source. Yield of products resulting from this study of glycerol, banana peel, molasses, glucose and waste cooking oil are 0.66%; 0.63%; 0.64%; 0.74% and 0.85% respectively.

C. Surface Tension

The surface tension can be used as a parameter for predicting biosurfactant produced by bacteria during growth (Morikawa et al. 2000). The value of the surface tension correlates with high biosurfactant produced by the cultivation media. Surface tension is the effort required to expand the surface of the liquid per unit area. Surface tension is a force that arises along the surface of a liquid to tighten, so that it is surface is covered by a layer which is elastic. Depending on the nature of the liquid, the substance is able to hold small objects on the surface.

Surface tension resulting from the biosurfactant by Pseudomonas aeruginosa in a wide range of carbon sources can be seen in Figure 3. Surface tension in this study tended to decrease during the specified period. In the sample biosurfactant with the carbon source waste cooking oil, the surface tension value is the smallest, amounting to 22.46 mN/m with a decrease in the surface tension of 51.1%. This is due to high concentration of biosurfactant, that is dissolved in the culture fermentation, which reduces the value of the surface tension of liquid layers in that culture. In contrast, glucose has its highest a surface tension of 33.55 mN/m with a decrease in surface tension of 30.6%. To decrease the surface tension on a banana peel, glycerol, molasses row is 32.51; 27.55; and 31.49 mN/m and the presentation of the decline can be seen in Figure 3.

The decrease is due to the culture medium *Pseudomonas* which begins to produce extracellular compounds having surface-active properties (biosurfactant). Biosurfactant is able to produce an expanding pressure against a tendency to shrink the surface (forming hollows). The larger the surface tension value for a given biosurfactant the more it will lead to further diminution of the value of surface tension after forming a monolayer, or the greater the ability of surfactants to lower the surface tension.



Gambar 3
Decrasing surface tension from any carbon sources.

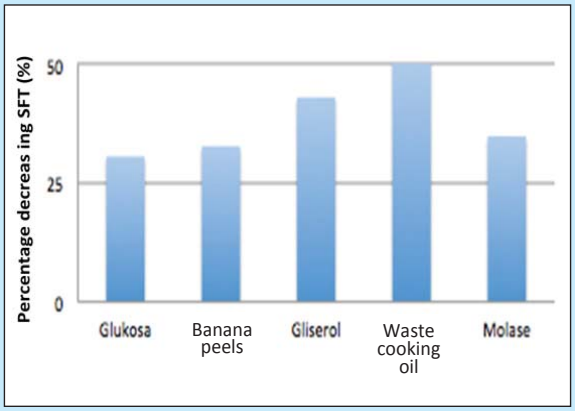


Figure 4
Precentage of decrasing surface tension from any carbon sources.

D. Interfacial Tension

The resulting interfacial tension decreased significantly as shown in Figure 5. The smallest interfacial tension is 0:44 mN / m for waste cooking oil with a 97.6% decline. As for the value of the interfacial tension glucose, banana peels, glycerol, and molasses are: 15.20; 8.15; 13.78; 0.44; and 11.2 mN/m respectively. The interfacial tension value of waste cooking oil and glycerol is greater than the reduction in the interfaciak tension of molasses, banana peel and glucose. Tis is because the waste cooking oil in composed fatty acid compounds which are hydrophobic. This is also the case for glycerol, which has triglycerides compounds which are hydrophobic (Muller et al. 2012).

The drop in interfacial voltage was caused by the composition of the surfactant, namely the hydrophobic and hydrophilic groups. These two groups have different levels of polarity. This result in formation of a film at the interface of two different fluid phases. This cause a drop in the voltage of the record surface. (Long et al. 2013). Voltage drop interface also influenced the occurrence of the bacterial adhesion mechanism at the interface layer. A hydrophobic portion on the outer layer of the cell surface may bind biosurfactant. Cells will interact with the hydrophilic interface and do not interact with the hydrophobic interface. Biosurfactant also may be oriented in other ways, for example by exposing the hydrophilic portion to the outer layer so that in this case the cells can only interact with the hydrophobic interface (Hidayati et al. 2011) biosurfactants by *Pseudomonas aeruginosa* deliberately secreted into the medium to increase the availability of substrate hydrophobic. With the biosurfactant, the substrate in the form of liquid will be emulsified into micelles and pass it to the bacterial cell surface and solid substrates are cleaved by the biosurfactant making for easier entry into the cell. (Izzah 2013).

E. Biosurfactant Characterization

These characteristics are used to determine the functional groups of biosurfactant produced by using FTIR. The spectrum in Figure 7 is the standard spectra of mono-rhamnolipid with a concentration of 200 ppm which was dissolved in sodium bicarbonate. From the IR spectra it can be observed that there is a functional group on the standard mono-rhamnolipid. In the region of 3344.57 cm^{-1} and 3354.21 cm^{-1} shows the hydroxyl group hydrogen bonds (-OH), which describes the predominant water molecules in this sample. The presence of the carbonyl group (C = O) is found at 1635.64 cm^{-1} regions while the ester group (CO) is located at the peak of 1211.30 cm^{-1} to 1288.45 cm^{-1}. For alkane group / methyl (CH) is shown to be in the peak range 1338.60 to 1471.69 cm^{-1} cm^{-1}. According to Frank (2013). In the fingerprint region of the FTIR spectra (1200-1460 cm^{-1}) shows the typical deformation of CH and OH rhamnosa carbohydrate molecules.

IV. CONCLUSION

It can be concluded that based on the test results of biosurfactant activity, concentration, and the yield of biosurfactant, the optimum carbon source was cooking oil. In terms of cooking oil, the value of surface tension and interfacial tension generated is 22.46 mN/m and 0.44 mN/m with a percentage decrease of 97%. The biosurfactant produced in this study is rhamnolipid.

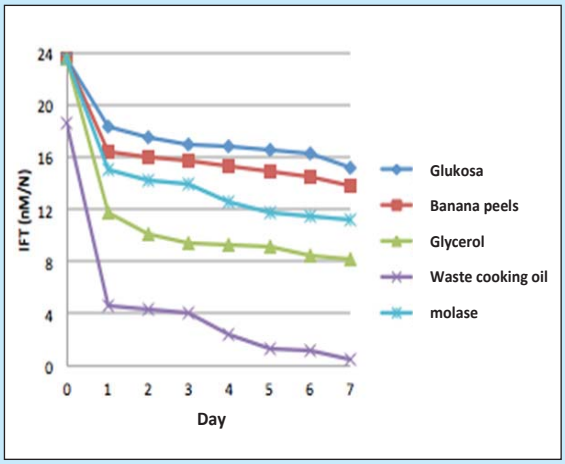


Figure 5
Decreasing Interface Tension from Any Carbon Sources.

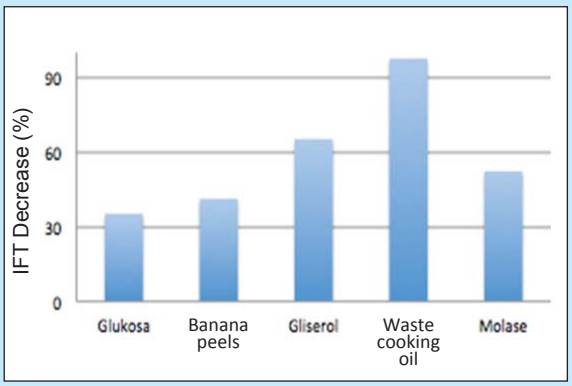
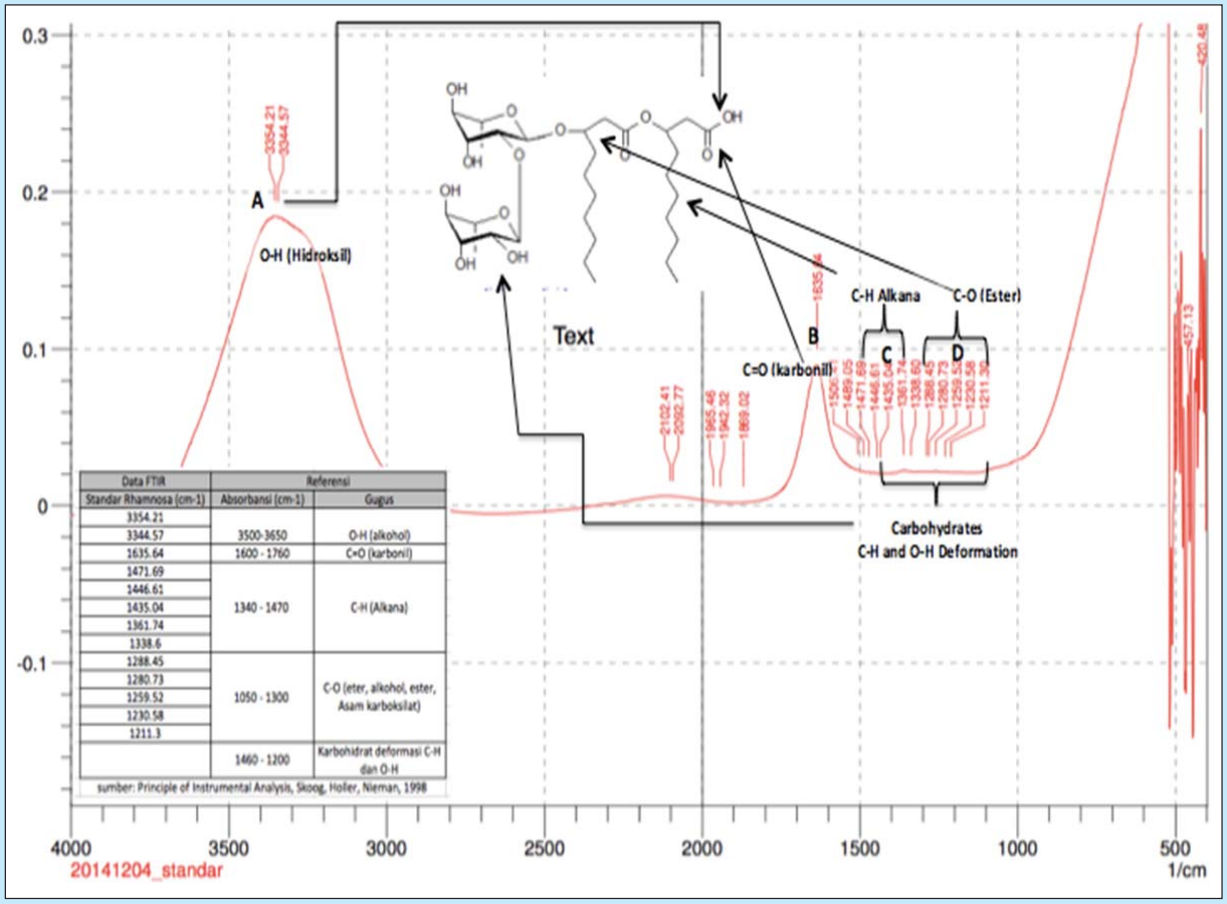


Figure 6
Precentage of Decrasing Interface Tension from Any Carbon Sources.



Data FTIR	Referensi	
Standar Rhamnosa (cm-1)	Absorbansi (cm-1)	Gugus
3354.21	3500-3650	O-H (alkohol)
3344.57		
1635.64	1600 - 1760	C=O (karboni)
1471.69	1340 - 1470	C-H (Alkana)
1446.61		
1435.04		
1361.74		
1338.6		
1288.45	1050 - 1300	C-O (eter, alkohol, ester, Asam karboksilat)
1280.73		
1259.52		
1230.58		
1211.3		
	1460 - 1200	Karbohidrat deformasi C-H dan O-H

sumber: Principle of Instrumental Analysis, Skoog, Holler, Nieman, 1998

Figure 7
Spektrum FTIR rhamnose standard.

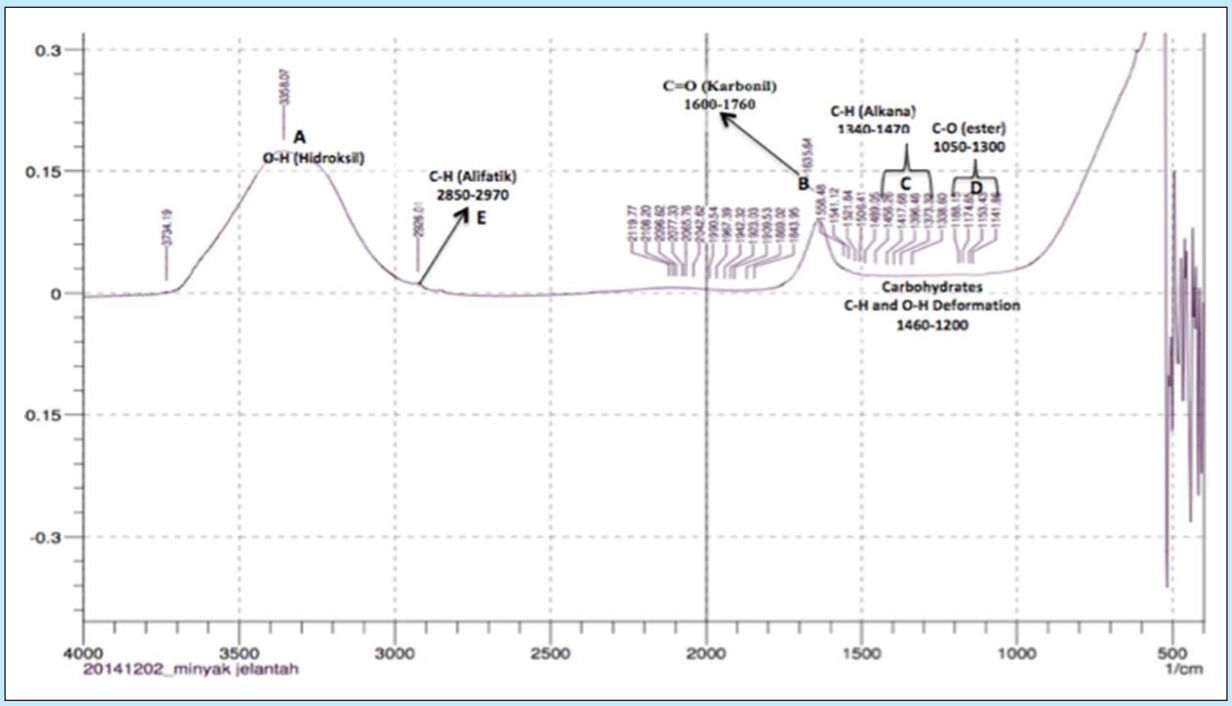


Figure 8
Spektrum FTIR biosurfactant from waste frying oil.

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