Study on Potential of Biosurfactant Rhamno NR22 on Demulsification of Industrial Emulsion

Farah Nasyitah Esa, Dr. Nik Raikhan Nik Him

Faculty of Chemical Engineering, Universiti Teknologi Mara

Abstract— Chemical synthesized demulsifier in oil industry and illegal dumping of waste motor oil from workshop industry have caused environmental issues. Rhamnolipid biosurfactant produced from Pseudomonas aeruginosa NR22 (Ps. NR. 22) has unique properties to demulsify the unwanted emulsion by lowering the interfacial tension of the emulsion. The ability to demulsify and separate the water-oil emulsion in waste motor oil from local workshop by running several demulsification test was studied. The utilization of Kay's and PPGAS minimal with glucose as carbon substrate has enhanced the production of biosurfactant. 410 ml of foam that lasted for 5 hours long were observed on the biosurfactant which was 51% of total volume implied the stability of the biosurfactant. Characterization from FTIR technique showed the chemical bond presented and provided similar result compared to the commercial biosurfactant. From the bottle test, 35% of demulsification percentage was calculated which demonstrated the separation of oil and water phase. A maximum of 9.4cm diameter of clear zone was obtained after performing oil drop area test within 4 hours of production. A positive result shown from oil drop collapse test proved the interfacial activity. All these results showed Rhamno NR22 biosurfactant has a great potential to be applied as a high efficiency and eco-friendly demulsifier for unwanted emulsion in various industries.

Keywords— Biosurfactant, demulsification, rhamnolipid, Pseudomonas aeuruginosa.

I. INTRODUCTION

To this date, crude oil industry has been growing tremendously and tons of crude oil has been produced. Crude oil emulsions which usually take place during production, transportation as well as processing are very stable and they are undesirable [1]. These emulsion also occur in used motor oil that has collected as waste in workshop industries. These waste will usually been dumped without proper treatment and caused environmentally issues. Emulsion which defined as two immiscible liquid (mainly oil and water) causes several problems associated with corrosion, scale formation, sludge formation, sludge accumulation in storage tanks and reduced distillation efficiency [2, 3]. Therefore, demulsification is crucial as it breaks the emulsion and separate them into two separate phase of oil and water [4]. Only then, the oil phase can be directly used for further process while the water phase can be safely discharged to the treatment plant [5]. Chemical, mechanical and electrical demuslifier are the types of demulsifier to promote separating the oil and water phase. Currently, the most utilized type of demulsifier is chemical synthetic demulsifier. Despite of its effectiveness, it has numbers of disadvantages include large investment as well as ecological issues as it does not degrade readily [6]. Therefore, an initiative of replacing these synthetic demulsifier with an organic demulsifier is done.

Biosurfactant is defined as biological surface-active compounds which are mainly produced by aerobic microorganism in an aqueous media with the presence of carbon source feedstock [7]. The properties of biosurfactant are defined unique as it is biodegradable, has unique functional group, low toxicity, high tolerance towards various environmental factors (temperature and pH), as well as its ability to reduce the surface and interfacial tension of liquid [8, 9]. Other than that, its properties also include foaming power, detergency and lubricating [6]. As in oil industry, biosurfactants have been proven of its applications such as microbial enhanced oil recovery (MEOR), oil storage tank cleaning, enhancing oil transportation and extraction [10].

Biosurfactant is an amphiphilic compounds consist both hydrophilic and hydrophobic moieties that are referring to as head and tail, respectively as shown in Figure 1. As solubility of hydrophilic molecules in biosurfactant increases, it has the ability to reduce the interfacial activity and thus, lower down the surface tension [11]. According to Uzoigwe et. al, the ability of the biosurfactant to reduce the surface and interfacial tension is corresponded with the potential to demulsify and break the stability of the emulsion [12]. Hence, a better efficiency in demulsification is occurred when the surface tension is low as it increases the oil mobility [11].

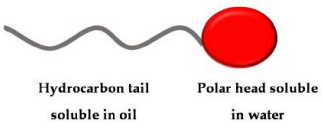

Figure 1 : The hydrophobic moiety represent the tail part which soluble in oil while the hydrophilic is the head part that solutes in water.

The most common type of biosurfactant is glycolipids which is a long chain of aliphatic acid or hydroxyl aliphatic acid. It is a composition of carbohydrates linked by ether or ester bonds with long-chain of aliphatic acids or hydroxyaliphatic acids. The general and most used types of glycolipids are treholipids, sophorolipids and rhamnolipids [8]. These three types of glycolipids are usually produced by different microorganisms as stated in Table 1 [13].

Table 1 : Types of biosurfactant with its respective microorganism producers

Type of glycolipid biosurfactant	Microorganism
Rhamnolipids	*Pseudomonas aeruginosa*
Sophorolipids	*Torulopsis bombicol*
Trehalolipids	*Rhodococcus erythropolis*

Rhamnolipid, specifically, consist of one or two rhamnose molecules (monorhamnolipids or di-rhamnolipids) linked to one or two fatty acids alkyl chains [14]. Rhamnolipids generally produced from *Pseudomonas aeruginosa* species are well known of its ability to reduce the interfacial activity as it is a hydrophilic substance [15, 16]. Due to its commercially availability, rhamnolipids have become a significant biosurfactant. It posses extensively high surface activity compared to other types of biosurfactant [5].

The selection of the carbon source that are used in the medium of producing biosurfactant is also one of a significant parameter to ensure a maximum production of biosurfactant. Mainly, the carbon source used to produced rhamnolipid biosurfactant are glycerol, glucose, mannitol, molasses and hexadecane [17]. *Pseudomonas* genus has the capability to apply different substrates as carbon sources [13]. Figure 2 shows the metabolic routes for rhamnolipids production from *Pseudomonas aeruginosa* with applying glucose as carbon source and thus, producing mono-rhamnolipids and di-rhamnolipids as biosurfactant [18]. In this paper, the potential of the rhamnolipids produced mainly from *Pseudomonas aeuruginosa* NR22 to demulsify and destablize the stability of waste motor oil emulsion was studied.

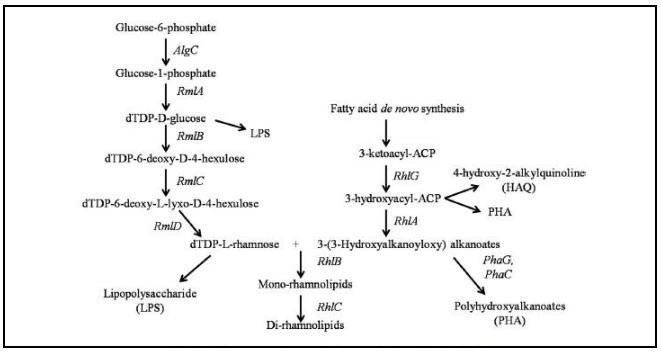

Figure 2 : *Pseudomonas aeruginosa* metabolic routes using glucose for rhamnolipid production

II. METHODOLOGY

A. Materials

Waste motor oil (collected from local workshop located in Klang), *Pseudomonas aeruginosa* NR22 (Ps. NR. 22) bacteria (isolated from lake in Shah Alam), *Pseudomonas* agar powder (Microbiology), distilled water (at pH 7), ammonium dihydrogen phosphate (V90004, Vetec™ reagent grade, 99% $NH_4H_2PO_4$), potassium sulphate (K_2SO_4), glucose, magnesium sulphate heptahydrate ($MgSO_4$), ammonium sulphate ($(NH_4)_2SO_4$), potassium chloride (KCl), tris-hydrocholic acid, proteose peptone, olive oil (extra virgin, Bertolli, Italy).

B. Production of biosurfactant Rhamno NR22

Pseudomonas agar

A pseudomonas agar plate was prepared by boiling 32.5g of pseudomonas powder with 1000ml of distilled water before autoclave at 121°C for 2 hours. The prepared agar was then poured into a petri dish after has been cooled down to 50°C. The petri dish was then sealed using paraffin film and stored at -2°C for overnight before turned into solid phase.

Kay's minimal preparation

A mixture of chemical was prepared inside a 200ml conical flask. It includes 0.3g of ammonia dihydrogen phosphate, 0.2g of potassium sulphate, 0.2g of glucose and 0.1g of magnesium sulphate heptahydrate with 100ml of distilled water. After proper mixing, the pH was adjusted to pH6 by using sodium hydroxide. The minimal was then autoclaved for 2 hours before stored at -2°C. This method was modified from Nik Raikhan, using glucose as carbon source [19].

PPGAS minimal preparation

The preparation method of PPGAS (Phosphate-limited peptone-glucose-ammonia salt) minimal was modified by method by Aparna et al. with glucose as carbon source [20]. 3g of ammonium sulphate, 4.5g of potassium chloride, 51g of tris-HCl, 15g of glucose, 3g of proteose peptone and 1.2g of magnesium sulphate heptahydrate were mixed with 600ml of distilled water in a 1000ml conical flask. After proper mixing using stirrer, the mixture was adjusted until pH6 and was innoculated at 121°C for 2 hours before storage at -2°C .

Production of Rhamno NR22 biosurfactant

Streaking method was used to transfer the isolated *Ps. NR. 22* bacteria into the prepared centrimide agar as shown in Figure 3. This method was modified by the method from El-Sheshtawy [21]. For sterilization purpose, a metal loop was first been heated using Bunsen burner until it turned to hot red colour. After it was cooled down, a loopful of bacteria was streaked from the pure bacteria into the new agar using the metal loop. After observing the healthy growth of the transferred culture, the new cultured *Ps.* NR. 22 agar was then transferred via metal loop, and inoculated aseptically into the nutrient broth inside the test tube and incubated at 200rpm and 37°C for another 24 hours.

Figure 3 : Streaking method of bacteria on centrimide agar

The content of nutrient broth was modified using method by Nik Raikhan which contain olive oil, potassium dihydrogen phosphate (KH_2PO_4) and ammonium chloride (NH_4Cl) [19]. Next, 100ml of Kay's minimal was inoculated with 1% of nutrient broth (1ml). The incubation was done using incubator shaker for 48 hours at 300rpm and 37°C. After 48 hours, the Kay's minimal which has turned darker in colour, was then taken at 6% (36ml) and mixed into the 600ml of PPGAS minimal. After incubation at 300rpm and 37°C for 3 days, the production of biosurfactant was then been observed by its foaming stability by shaking vigorously. The foaming percentage was calculated by using this Equation 1 :

$$Foaming = \frac{Foam\ volume}{Total\ volume}\ x\ 100\% \quad \text{(Eq. 1)}$$

C. Extraction of biosurfactant Rhamno NR22

The extraction of biosurfactant was done by using centrifuge method by modified the method by Aparna et al. [20]. After the biosurfactant has been produced, the sample was then put into a conical centrifuge tube. The extraction process was done by centrifuged the tube at 9,000 rpm at 15°C for 15 minutes to remove *Ps. NR. 22* cells and obtain the supernatant. Then, the pH was adjusted to pH 6 using 1.0M sodium hydroxide resulting in honey-coloured biosurfctant product.

D. Characterizationof biosurfactant Rhamno NR22

The chemical component and structures presented in Rhamno NR22 biosurfactant was charaterized by using FTIR (Fourier-transform infrared spectroscopy) technique. This method needs the absorption of infrared light wavelength from the biosurfactant. FTIR was equipped with attenuated total reflectance (ATR) to analyse the components in the biosurfactant. The IR spectra of the purified rhamno NR22 biosurfactant was recorded on Perkin Almer Spectrophotometer using KBr disc. The functional group as well as bond types were obtained. The analysis was carried out at ATR mode in a range of 500 to 4000cm^{-1}. The FTIR analysis of commercial biosurfactant was also obtained to compare the result.

E. Demulsification test

Bottle test

This method was modified from Long et. al. [5]. 6ml of motor oil was measured into the conical centrifuge tube with 3ml of Rhamno NR22 biosurfactant that has been produced. For better mixing, the sample was then shaken using vortex shaker for 2 minutes at 2000rpm to thoroughly mix the biosurfactant and the waste motor oil emulsion. The tube was then incubated for 24 hours at 50 ºC under static condition in an incubator shaker. The separation was observed after 24 hours by calculating the demulsification percentage equation of the triplicate experiments.

$$\text{Demulsification (\%)} = \left(1 - \frac{\text{remaining emulsion volume}}{\text{total emulsion volume} + \text{added sample volume}}\right) \times 100 \quad \text{(Eq. 2)}$$

Oil drop collapsing test

Oil drop collapsing test has been found to be positively related with the surface tension reduction according to Bodour et. al. By using petri dish, 40ml of distilled water was poured. Then, a thin layer of 200µL of waste motor oil was added on the surface of the water. After the layer merged, by using micro-pipette, 10µL of biosurfactant was dropped on the centre of the surface of the emulsion on the petri dish. The demulsification process was observed by the ability of the biosurfactant to collapse the oil to the side of the petri dish. If the biosurfactant able to collapse and push the oil to the side, it was labeled as positive and negative if vice versa [9, 22]. The video of the collapsing process was recorded to observe the activity and to obtain the time taken for the oil to fully collapse.

Oil displacement test

This test was similar with oil drop collapsing test but the diameter was calculated after the biosurfactant was dropped on the oil-water interface emulsion. The diameter of the clear zone was then measured under bright light and clear atmosphere. The diameter of the clear zone was measured for consecutive 5 hours to evaluate its stability [22].

III. RESULTS AND DISCUSSION

A. Production of biosurfactant Rhamno NR22

After 24 hours of incubation of streaked *Pseudomonas* agar, the result was shown in Figure 4 which displayed the fluorescent green colour on the streaks as well as the agar. Sánchez reported that *Pseuodomonas* bacteria was a Gram negative rod-shaped bacteria bacteria. The green colour indicated that the bacteria was growing healthily [13, 23]. It is important to ensure the growth of *Ps. aeruginosa* NR22 to obtain rhamnolipid type of biosurfactant.

Figure 5 depicted the changes appearance of nutrient broth after incubation. The translucent appearance of nutrient broth turned cloudy. This phenomena indicated the growing activity of bacteria. The cloudiness of nutrient broth proved that the bacteria consumed the nutrients that present inside the broth and released enzymes within particles. The nutrient broth provided an adequate amount of food source for the *Ps. NR. 22* to live and grow. According to Campos, olive oil as the broth that supplied the nutrient was generally used for rhamnolipid biosurfactant from Pseudomonas aeruginosa. It was studied that the selection of the substrate must complied with a balanced nutrients for the growth of the bacteria, as well as a high yield of the biosurfactant produced which has high carbohydrate and lipid content [24].

Figure 4 : The fluorescent green depicted positive growth of *Ps. NR. 22*

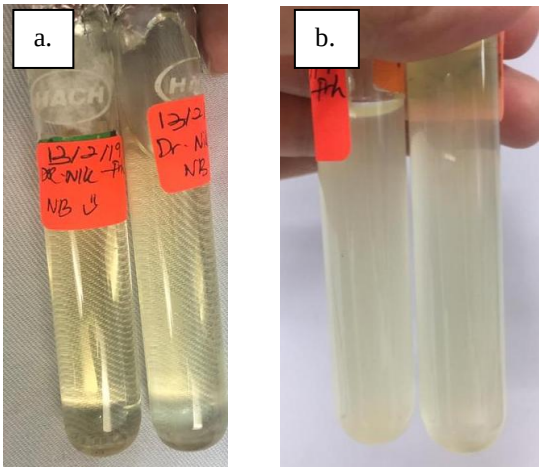

Figure 5 : (a) The translucent appearance of nutrient broth before contacting with cultured *Ps. NR. 22* and (b) cloudy appearance of nutrient broth after 24 hours of streaking the bacteria.

By referring to Figure 6, the biosurfactant has already been preliminary produced before the extraction process. The brownish colour as well as the presence of foam indicated the success of the production of biosurfactant [21]. This can also be supported with the study made by Rocha e Silva [6]. It was stated by Sarubbo that the minimal medium containing both carbon and nitrogen sources enhanced the production of biosurfactant and thus, the result [25]. In this study, Kay's and PPGAS medium both played an important role to the production of Rhamno NR22 biosurfactant as they assist the *Ps. NR. 22* to grow and produce the biosurfactant. The presence of glucose in the medium was as the carbon substrate while peptone acted as nitrogen source [26]. This can be supported by the study made by Aparna et al, when comparing between peptone and yeast extract, peptone produced more of biosurfactant when coupled with glucose as carbon substrate [26].

A qualitative indication of production of biosurfactant can be obtained by its foaming stability. The produced biosurfactant was first vigorously hand-shaken for 2 minutes to observe and qualify the formation of foam. El-Sheshtawy reported that a good microbial biosurfactant has a high stability of foaming power [21]. As shown in Figure 7, the foam was observed of its duration of stability and the volume of the foam was measured. The maximum result obtained for the foaming formation percentage was 51% which was 410ml of foam from a total volume of 800ml. The duration of the foam to last was up to 5 hours which implied a good stability of biosurfactant production. This measurement of stability of the foam was also supported by Müller that showed the biosurfactant properties. The biosurfactant was usually implied as high concentration when it is in foaming fraction and the stability of this foam was resulting from its interfacial activity [27]. According to El-Sheshtawy's study, the resulting foam of *Pseudomonas* bacteria species can go up to 70% [21].

Figure 6 : PPGAS medium after 3 days of incubation showed the production of biosurfactant

The positive result in the foaming production also corresponded with the type of carbon source. The type of carbon source played an important role in producing high concentration of biosurfactant [28]. As in producing biosurfactant for this study, the content of glucose inside both mediums were used as its carbon source. Glucose was proven to be one of the best carbon source for the production of rhamnolipid [29]. Based on Varjani, in order to produce rhamnolipids, one of the method is by the biosynthesis process of glucose into mono-rhamnolipid and di-rhamnolipid [18].

Sánchez has studied that Pseudomonas has a flexibility in utilizing some organic compound as food source which promotes its capability to colonize within the ecological where carbon

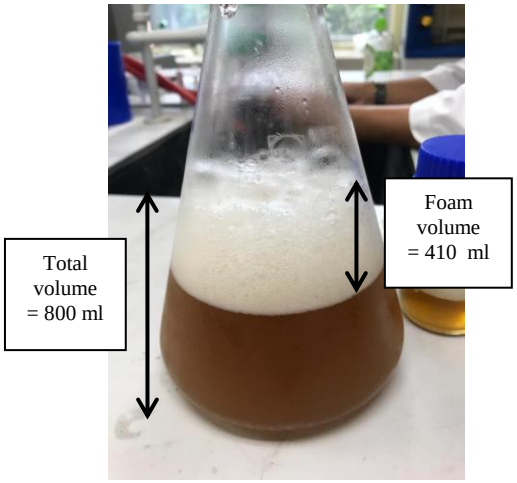

Figure 7 : Foam on top of the biosurfactant production

sources were limited [23]. It was believed that the potential of biosurfactant to demulsify wasslightly higher when the bacterial strain was supplied with glucose in the medium and incubated at 30°C [30]. Both of the medium containing carbon and nitrogen source enhanced the production of biosurfactant. Unlike the nutrient broth, these minimals provide a very limited food source to the bacteria. Therefore, once *Pseudomonas* cell detected this struggle, it produces biosurfactant as a back-up. According to Campos, the biosurfacant was secreted into the medium. to facilitate the growth of the bacteria. The production of biosurfactant assist the translocation of insulable substrates along the membranes of the cells [24]. Due to this foaming properties, biosurfactant was able to apply in microbial enhanced oil recovery (MEOR) with glucose as its carbon substrate.

For further purification of biosurfactant, extraction was made by using centrifugal method to obtain the supernatant. Upon extraction by using centrifuge method, the cell was sedimented at the bottom of the centrifugal tube as shown in Figure 8. The extracted biosurfactant was taken as the supernatant obtained from each centrifugal tubes. It was roughly 600ml of biosurfactant obtained from 800ml of cultured medium.

Figure 8 : Extracted biosurfactant after centrifuge method

B. Characterization of Rhamno NR22 biosurfactant

The functional group and bond types existed inside the rhamno NR22 biosurfactant was analysed by FTIR technique. Both commercial and produced biosurfactant were tested using FTIR to compare its functional group. *Pseudomonas* microorganism producing rhamnolipids has variations in the number of rhamnose molecules, length as well as composition of alkyl chain [20]. A range of wavelength between 500 to 4000cm^{-1} was analysed

a) b)

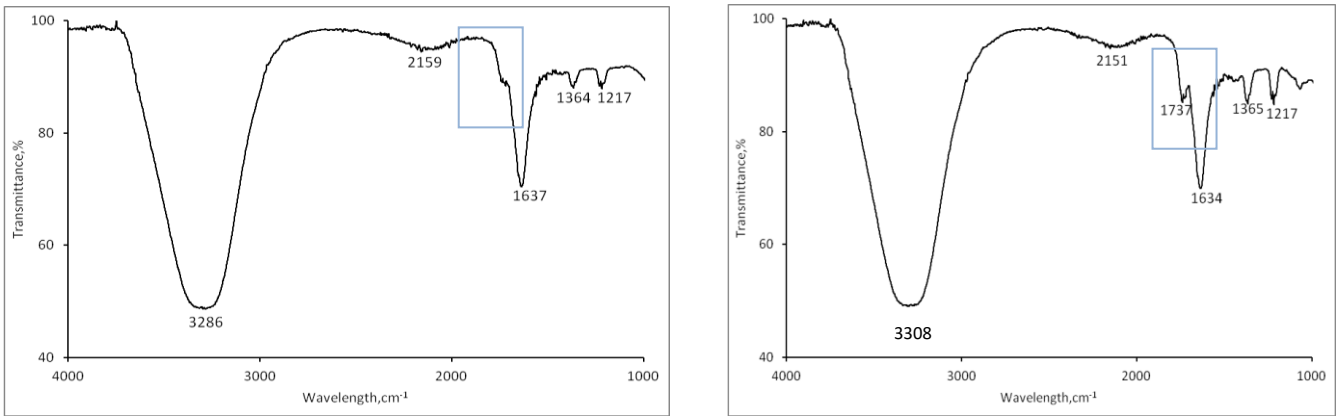

Figure 9 : Analysis of FTIR from (a) commercial and (b) *Pseudomonas*-produced rhamnolipid biosurfactant

but only six significant spectra wavelengths were identified and characterized. From the wavelength obtained from each peaks, the chemical bond presented in both commercial and produce biosurfactant can be identified by its assignment compound class as shown in Figure 9.

Figure 9(a) depicted the result of the IR spectra for commercial biosurfactant which has higher concentration of biosurfactant comparing to the produced one which shown in Figure 9(b). The broad band appearing at 3308cm^{-1} and 3286cm^{-1} for commercialand produced biosurfactant respectively indicated the intermolecular bonded of O-H band as hydroxyl groups existed in the biosurfactant. Absorption at 2151cm-1 for commercial and 2159 cm-1 for produced biosurfactant were assigned to the symmetric stretch of C-C stretching vibrations of alkyl groups of –CH and CH3-. The wavelength of 1637cm-1 for produced biosurfactant and 1634cm-1 for commercial biosurfactant both indicates a strong stretching bond of C=C which in alkene group.

The characteristics displayed at 1217cm^{-1} for both commercial and produced biosurfactant are corresponded to the C-O vibrations which implied the existing of carbon bond with the hydroxyl group. Strong symmetric carboxylic acid group C=O band was at 1365cm^{-1} for commercial biosurfactant which related to the carboxylic acid group, as well as for produced biosurfactant at 1364cm^{-1}. The only difference between these two analysis was the one has been squared based on both Figure 9(a) and 9(b). The produced biosurfactant did not have that particular peak compared to the commercial one. The commercial biosurfactant obtained an absorption at 1737cm^{-1} which indicated the C=O stretching vibration of esters. The absence of this peak might be due to its low concentration of fatty acid produced from the rhamnolipid biosurfactant and hence, low concentration of the ester [19].

Based on Figure 6(b), all absorption at 3286cm, 1637cm^{-1} and 1217cm^{-1} indicates the chemical structure that is similar to rhamnolipids which were composed of rhamnose rings and long hydrocarbon chain [13].

C. Demulsification test

The percentage of the demulsification proved the ability of the biosurfactant to separate the oil and water in the emulsion. Referring to Figure 10, a clear separation of the two phases was demonstrated after incubated for 24 hours. The motor oil was layered at the top of the tube while the water was separated at the bottom part. As depicted in Figure 10, the waste motor oil tend to form a layer of mixture in the range of brown to black colour. These layers represent various composition presented in the waste motor oil including organic compounds, esters, as well as resins as additive [31].

The demulsification percentage using equation 2 resulting in a maximum of 35±0.1%. This proved the ability of the biosurfactant to break the stability of the emulsion occurred in the waste motor oil. The stability of the waste motor oil emulsion were slightly high. As reported by de Cássia, the emulsion stability of waste motor oil was as high as 90% [32]. Therefore, it was asignificant result to obtain 35% percentage of breaking this high stability of emulsion. According to Rocha e Silva, the demulsification percentage was related to the ability to destabilising the emulsion [6]. Due to the its ability to destabilize the high stability of waste motor oil emulsion, it was stated that Rhamno NR22 biosurfactant has a low surface and interfacial tension. The adsorption of biosurfactants at the polar end in the water and hydrocarbon chain in the oil has contributed to the reduction in the interfacial tension in the motor oil [33]. The separation between this two phases also can be utilized in various applications. As in oil industry, the separated oil can be used for further process due to its low content of water. On the other hand, the separated water which now contains low concentration of oil, it can be discharged in the treatment plant [5]. According to Muthusamy, rhamnolipid type of biosurfactant has the ability to act as a demulsifier which generally can be applied in waste treatment and oil recovery [34].

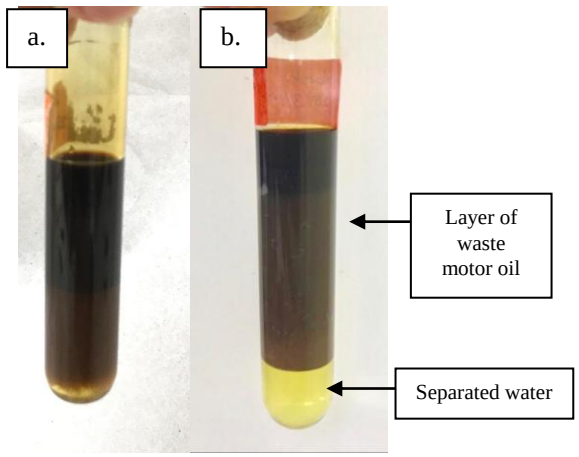

Figure 10: (a) Emulsion of crude oil and (b) Breaking the emulsion by separation of oil and water using biosurfactant rhamno NR22

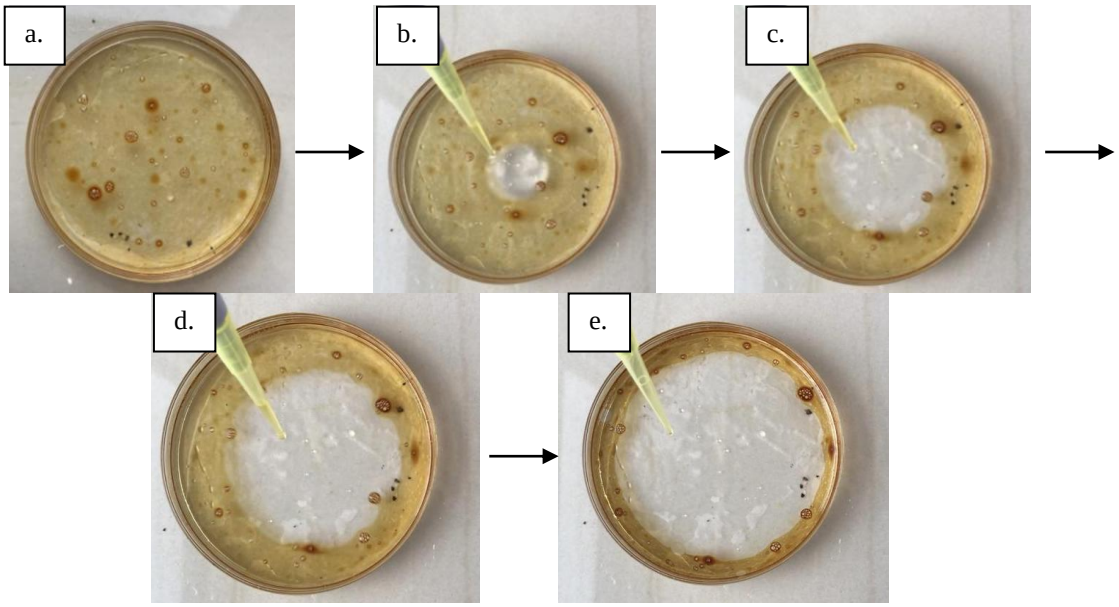

Figure 11 : This process was recorded using video. (a) A thin layer of waste motor oil on 40 ml of distilled water, (b) Produced biosurfactant was dropped on the layer using micropipette, (c) The biosurfactant starting to collapse the oil to the side of the petri dish, (d) The diameter of the clear zone started to expand bigger, (e) Final result observed after 3 seconds and the diameter of the clear zone was calculated.

Oil drop collapsing test and oil displacement test consisted of similar preliminary method. Both methods were rapid and easy to conduct as it does not have required a specific equipment. Figure 11 showed the result of dropping the biosurfactant Rhamno NR22 on top of the water-motor oil layer after 2 minutes and 10 seconds. The process was recorded using video and the observation was captured step by step.

As the biosurfactant was added, the emulsion of oil started to destabilize and tend to separate from the water. The result obtained was labeled as positive as the oil was pushed to the side of the petri dish. This qualitative analysis also proved by Yela and Qinhong Cai [9, 35]. The emulsion occur inside the waste motor oil destabilize and cause an imbalance of intermolecular forces, due to the addition of the biosurfactant [35]. This demonstrated the low interfacial tension between oil and water. The observation made was a qualitative method to prove the low surface tension of biosurfactant that enhanced the demulsification process [11]. The efficiency of the biosurfactant to collapse the oil can be applied in bioremediation of water contaminated with hydrocarbon wastes [36].

The diameter of the clear zone was measured and stated as in Figure 9. Within 5 hours of production of biosurfactant, the test was repeated hourly to measure the diameter of the clear zone. At the first hour, the diameter result was still at 6.5±0.04cm which indicated a low efficiency of destabilizing the emulsion. However, the graph slowly increased within time and stabilized at 4th hour. At the 4th hour, the diameter of the clear zone on the petri dish was at 9.4±0.04cm which was the highest.

This similar result also obtained by Xiangfeng Huang [37] and can be supported by study made by Hua Yin [38]. It was reported that at the time 0 to 48 hours after culture time of production of biosurfactant, the surface tension of the biosurfactant reduces sharply. The hydrophilic and hydrophobic properties of biosurfactant contributed to this result. This is because, when biosurfactant are dropped onto the surface of the thin layer of water-motor oil emulsion, the water phase of the emulsion absorbs the hydrophilic part of the biosurfactant while the oil phase absorbs the hydrophobic part. This resulting in reduction of the interfacial tension between the forces inside the emulsion and destabilize the

molecules [35]. Upon destabilizing, the thin film on the surface of the emulsion is removed which then undergo coalescence, settling of droplets and clarification of the continuous phase which is demulsification [6].

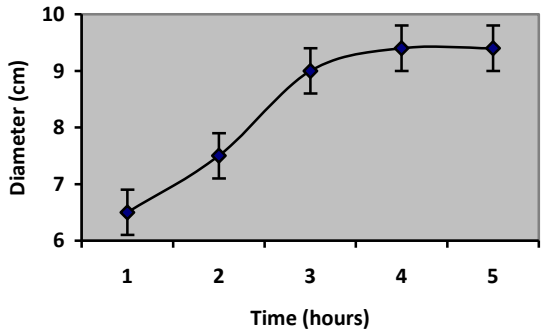

Figure 12 : Diameter of the clear area in oil displacement test within 5 hours

Conclusion

By applying suitable carbon substrate and nitrogen source, the biosurfactant has been enhanced and thus, increase the stability of the biosurfactant. Rhamno NR22-produced biosurfactant has a nearly similar chemical characterization with the commercial biosurfactant which implied the thrive in producing the biosurfactant. The unique properties of biosurfactant contributed to the ability to demulsify and break the stability of the emulsion by separating the oil and water phase. This correlated with the reduction in interfacial activity of the surfactant. From the result obtained, the research was a success and Rhamno NR22 biosurfactant is a good demulsifier that can be applied in various industrial emulsion.

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