Physical Characterization of Biosurfactant Rhamno NR22 Produced by *Pseudomonas aeruginosa* (Ps.NR.22)

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***Abstract-Pseudomonas aeruginosa* (Ps.NR.22) is an opportunistic pathogen that have ability to produce glycolipids. Rhamno NR22 is essentially class of glycolipid that consist of one or mostly two 3-hydroxy fatty acids with a different chain of lengths that have a connection with one or two rhamno molecules. Rhamno NR22 was successfully produced by using Phosphate-limited proteose peptone-glucose-ammonium salt (PPPGAS) medium. The extraction of Rhamno NR22 was run by centrifuge at 10000 rpm and 4°C for 15 minutes before it went through freeze dryer to powderized the Rhamno NR22. Thermogravimetric analysis (TGA) and nuclear magnetic resonance (NMR) analysis are applicable as a tools to understand the characteristics of Rhamno NR22. TGA reveal the mass lose in a Rhamno NR22 sample as a function of temperature and time. At 900°C, the Rhamno NR22 still remains at 5 mg in 90 minutes. It remarks the endurance of Rhamno NR22. ^{1}H-NMR spectrum was recorded due to the purity of Rhamno NR22. The obtained of multiplicity represent the assignment (-CH_3-) and (-CH-OH-) on rhamnose moiety while several assignment such as (-CH(O)-CH_2COO) and (-(CH_2)-CH(O)-CH_2COO-) on β-hydroxyfatty acids. The fuctional group, chemical bond and chemical structure study from NMR produced dirhamnolipid which classified as hydrophobic. Hence, the result indicate the physical characterization of rhamno NR22.**

keyword - Rhamno NR22, Ps.NR.22 , TGA, NMR

1) INTRODUCTION

Biosurfactants are one of the fundamental substances of chemical products that have a wide array of applications across various sectors such as agriculture, industry, households and health. Biosurfactants are the most effective replacement since biosurfactants exhibit biodegradability, non-toxicity and cost-effective characteristics compare to chemical surfactant [1]. Moreover, both hydrophobic and hydrophilic molecules appear in amphiphilic compounds which are known as biosurfactants for the purpose to help to reduce surface strains and interfacial tensions between individual molecules respectively. Thus, these properties help biosurfactants to form micro-emulsion; where hydrocarbons are soluble in water or water are soluble in hydrocarbons [2]. However, growing interest in biosurfactant has led to the trend shifted due to intense research towards the exploitation of biological processes for industrial due to the genetic manipulation of microorganism which is cost-effective, awareness of environmentally friendly and biodegradable nature [3].

Hence, the focus has been fixated on the substitution green process for the production of biosurfactants derived from microorganisms. Unique physical features that found in biosurfactants are not impaired by an environmental factor such as pH and temperature [4]. Biosurfactants are considered to be more potent and highly effective compared to chemical surfactants due to the difference in term of Critical Micelle Concentration (CMC) [5]. It has been identified that biosurfactants have the capability to decomposed by bacteria or other living organisms and thereby avoiding pollution. Beside, microbial-derived compounds can be easily destroyed. As an example, synthetic chemical surfactants impose ecological issues. Thus, biodegradable biosurfactants from marine microorganisms were concerned for the biosorption of ineffectively solvent polycyclic sweet-smelling hydrocarbon, phenanthrene contaminated in aquatic surfaces [6]. Furthermore, biosurfactants can act as emulsifiers or de-emulsifiers. Kind of emulsions exist are oil suspended in water (o/w) or water suspended in oil emulsions (w/o) [7]. O/w emulsions are encompassing of oil droplets that defer in an aqueous phase, while w/o emulsions are comprised of water droplets are suspended in a continuous oil phase. Added substances such as protein oxidation and lipid oxidation are used to balance out the stability in emulsification processes [8]. Different types of solid biosurfactants give distinct wetting properties. Solids can be distinguished into three groups with different characteristic namely bipolar, apolar and monopolar. The surface tension of monopolar and bipolar solids can result from the Lifshitz-van der Waals and acid-base intermolecular interactions. Whereas apolar solids can interact with the adherent medium also by the acid-base forces only [9].

Biosurfactants can be classified into four groups; namely glycolipids, surfactin, phospholipids and polymeric. Glycolipids distinguished into several types of glycolipids such as sophorolipids, threhalolipids, and rhamnolipids. Basically, glycolipids are carbohydrate-attached lipids, which are associated with cell membranes. It plays important roles in providing energy and serves as markers for cellular recognition. Glycolipids are comprised of sugar with a long chain of aliphatic acids by methods for either ether or ester gathering. There are plenty of microorganisms involved to produce specific kind glycolipids such as *Mycobacterium sp, Rhodococcus erythopolis. T. apicola and Pseudomonas aeruginosa* [10]. While surfactin is a popular lipopeptide that incorporated by various strains of the gram-positive, endospore-producing and microorganism. Lactone linkage method is used in order to join a seven amino-corrosive ring structure to an unsaturated fat chain. Furthermore, it decreases the surface tension of water Chemical structures of surfactin from 72 to 27 mN/m. Seven amino acids that have sequences to the carboxyl and hydroxy groups on long chain fatty acids are recognized as lilopeptides. [11]. Phospholipids, neutral lipids and some fatty acids are components of cell structures and have surface activity. Some examples of this type of biosurfactants are gramicidin and poly-mixin. Alasan, Liposan and Biodespersan are among the best-contemplated polymeric biosurfactants comes from different microbial origin such as *Acinetobacter calcoaceticus, Candida lipolytica* and *Acinetobacter calcoaceticus*. Emulsan is a powerful emulsifying specialist for hydrocarbons in water, even at a fixation as low as 0.001 to 0.01% [12].

This paper will be focusing on glycolipids, specifically Rhamno NR22 produced by *Pseudomonas aeruginosa* (Ps.NR.22). Rhamnolipids is a type of biosurfactant descend from glycolipids biosurfactants, in which one or two molecules of rhamnose are connected to one or two molecules of hydroxyfatty acids [13]. Mainly, rhamnolipids yield two forms of rhamnolipids,

monorhamnolipid and dirhamnolipid. Rhamnolipids biosynthesis takes place in three enzymatic reactions respectively involving *RhlA*. *RhlB* and *RhlC* [14]. First, it involves *RhlA* synthesizes 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs). Then, to embody the monorhamnolipids, association between HAA molecule with dTDP-L-rhamnose obtained from glucose-6-phosphate by *RhlB*. The resulting monorhamnolipids are converted to dirhamnolipids in a third reaction catalyzed by the *RhlC* . Figure 1(1) shows stratification of rhamno NR22 production while figure 1(2) shows chemical structure of monorhamnilipids and dirhamnolipids.

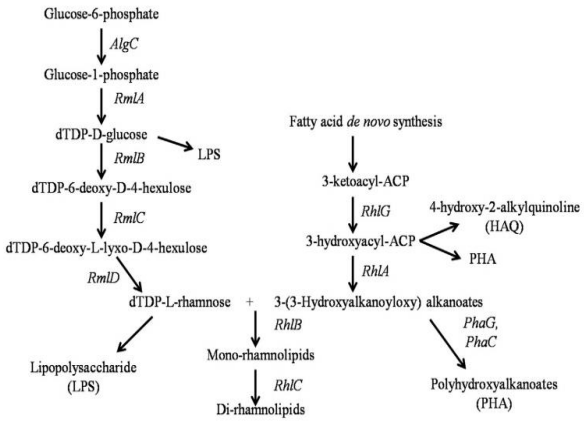

Figure 1(1): Ps.NR.22 metabolic routes for Rhamno NR22 production.

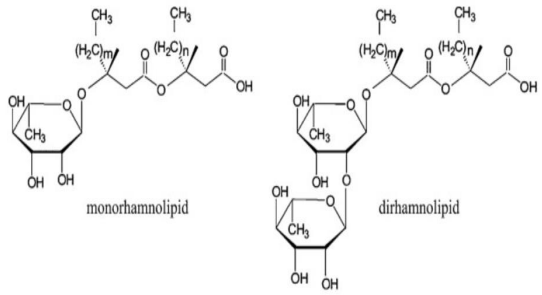

Figure 1(2): Chemical structure monorhamnolipid and dirhamnolipids.

The application of biosurfactants is very huge. In oi indusrry, Correlation between biosurfactant and bio-emulsifiers atoms bring to the most effective result that advanced microbial innovation can offer in fields. For instance, biosurfactants have the ability to remove crude oil from contaminated soil [15]. Beside, biosurfactants can be utulize into agriculture in the removel of organic insoluble pollutant from soil [16]. In comestic industry, Rhamnolipid is found to act as an active ingredient that is exceptional attributes to several skin treatments such as wound healing with reduced fibrosis and cure of burn shock. Biosurfactants also applicable in pharmaceuticals, therapeutics, commercial laundry and detergent.

This study highlights the TGA and NMR as the techniques used to observe the characterization of biosurfactant Rhamno NR22 produced by Ps.NR.22. TGA analysis is to determines the decomposition temperature of Rhamno NR22. It also measures the amount of weight change of each rhamnolipids, either as a function of increasing temperature or isothermally as a function of time [17]. Thus, enabling the structure of Rhamno NR22 to be studied. While NMR spectroscopy is an analytical chemistry technique used to determine ^{1}H-NMR by identifying the functional groups and chemical bond of the biosurfactant [6].

2) METHODOLOGY

The methods and the conditions to obtain Rhamno NR22 is design in order to achieve the experiment objective. Thus, The experimental work is carried out to produced Rhamno NR22. All the chemicals and materials used in this experiment are listed in Table 1 and Table 2 underneath.

Table 1: List of Chemical

Chemical	Chemical formula	Purity	The molecular weight ($gmol^{-1}$)
Pseudomonas agar	-	-	-
Nutrient agar	-	-	-
Nutrient broth	-	-	-
Glucose	$C_6H_{12}O_6$	Ultrapure	180.156
Peptone	-	-	-
Sodium Chloride	NaCl	≥997%	58.44
Ammonium Dihydrogen Phosphate	$NH_4H_2PO_4$	98%	115.03
Potassium Dihydrogen Phosphate	KH_2PO_4	99%	136.086
Iron Sulphate	$FeSO_4$	≥99%	151.908
Magnesium Sulphate Heptahydrate	$MgSO_4.7H_2O$	≥99%	120.366
Ammonium Chloride	NH_4Cl	>99.5%	53.491
Potassium Chloride	KCl	99%	74.548
Tris-HCl	$C_4H_{11}NO_3$	>99%	121.14

Table 2: Instrument used in experimentation

No.	Name
1	Autoclave
2	Incubator
3	Sterilizer
4	Centrifuge
5	Freeze Dryer

32.5 g of *Pseudomonas aeruginosa* isolation agar based and 1000 ml of distilled water were used to produce a medium for *Pseudomonas* agar plate. The medium requires the autoclave for 2 hours at temperature 121°C. After the medium start to cool down to 50°C, it poured into Petri dishes and turn to the solid phase (agar). Then, the petri dish was wrapped using paraffin film. After 24 hours, the nutrient broth prepared was inoculated with bacteria using a metal loop. Bunsen burner was used to flame the metal inoculum loop until it reached red hot and allowed to cool for a few seconds.

The loop was used to collect one loopful of bacteria aseptically from pure culture and transfer to sterilized nutrient broth medium. The medium was then incubated in an incubator at 37°C and 200 rpm for 24 hours to set the bacteria in stationary phase [18].

Figure 2.1(1): Ps.NR.22 on Centrimide agar

After the incubation process, the inoculum was used for the next medium which was Kay's minimal medium. Kay's minimal medium was prepared by adding 0.3 g ammonium dihydrogen phosphate, 0.2 g potassium sulphate, 0.2 g glucose and 0.1 g magnesium sulphate heptahydrate into 250 ml shake flask and the volume brought up to 100 ml by using distilled water. Then, the medium was placed in the autoclave machine at 121°C for two hours. After the sterilization process, the medium temperature was cool down until it reached room temperature before utilization. Figure 2.1 (2) shows the kay's minimal medium composition.

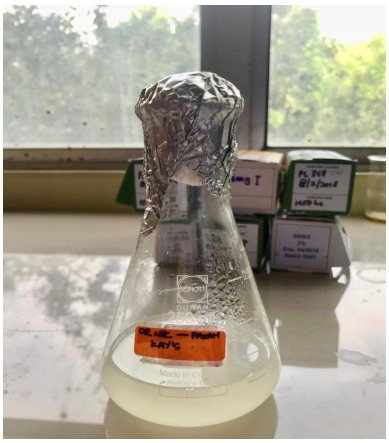

Figure 2.1(2): Kay's Minimal Medium

1 ml of the inoculum was transferred aseptically by using a micropipette into Kay's minimal medium. The medium then incubated at 37°C and 200 rpm for 24 hours. After 24 hours, the medium was used for the phospate-limited proteose peptone-glucose-ammonium salt (PPPGAS) medium preparation. The PPPGAS medium was prepared by adding 1.0 g ammonium chloride, 1.5 g potassium chloride, 19 g Tris-HCL, 5 g glucose, 1 g bacteriological peptone and 0.4 magnesium sulphate heptahydrate into 1 L shake flask. The volume brought up to 200 ml by using distilled water. The pH of the mixture was balanced to 7.0 by using 0.1 M sodium hydroxide. The medium placed into autoclave machine for sterilization process at 121°C for two hours. Then, the medium was allowed to cool until room temperature before utilization. Figure 2.1 (3) shows the PPPGAS medium.

Figure 2.1 (3): Phosphate-limited proteose peptone-glucose-ammonium salt (PPPGAS) medium.

12 ml of Kay's minimal medium was transferred aseptically by using micropipette into the PPGAS medium. Then, the medium was incubated for at 37°C and 200 rpm for 72 days. Then, PPGAS medium went through a centrifugation process to discard unwanted sludge from the PPGAS medium. The final solution underwent a freeze-dry process to powderized Rhamno NR22 for analysis.

2.2 Thermo Gravimetric Analysis (TGA)

TGA was used to determine chemical components inside by heating the Rhamno NR22. As a function of temperature, the chemicals can be indicated by weight loss of the biomass during the heating process. TGA was conducted by using nitrogen gas. TGA run used 20mg powder of Rhamno NR22 and the temperature range from 27°C to 900°C.

2.3 Nuclear Magnetic Resonance (NMR)

NMR was adopted to decide ^{1}H-NMR or ^{13}C-NMR chemical shift due to the purity of Rhamno NR22. Heavy water is known as deuterium oxide (D_2O) used as a solvent in NMR. The deuterium oxide samples were left to equilibrate for at east 10 min before the data were collected.

3) RESULTS AND DISCUSSION

3.1 Extraction and Purification of Rhamno NR22

Presence of foam in the shake flask is a good indication of Rhamno NR22 produced during the incubation process. The higher the thickness foam displayed a good production of Rhamno NR22 solution. The foam produced as shown in figure 3.1 (1) is about 35% from the solution and capable to withstand in 4 to 5 hours.

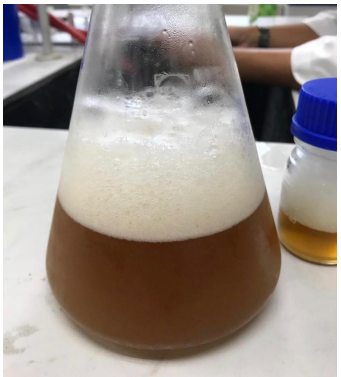

Figure 3.1(1): Foam produced from PPPGAS solution.

The production broth was centrifuged at 10000 rpm and 4°C for 15 minutes in order to remove all bacteria cells. After the centrifugation process, the supernatant was collected in a beaker and the precipitate was discarded [19]. The cells precipitated at the bottom of the centrifuge bottle leaving the product in the supernatant. The supernatant is also known as the cell-free product.

Figure 3.1 (2): Bacteria cells precipitated after centrifugation process.

After the precipitate was discarded, the supernatant underwent a freeze-dry process to turn the liquid into powder form for analysis. 40 ml of Rhamno NR22 in liquid form was freezed until -80°C before it placed in freeze drying machine. It took 10 days to the Rhamno NR22 liquid to dry completely. The colour of Rhamno NR22 in powder was light brown as shown in figure 3.1 (3) underneath.

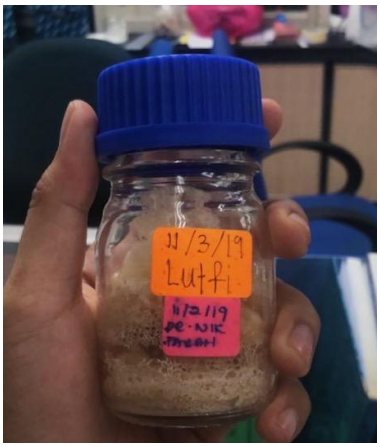

Figure 3.1 (3):Rhamno NR22 in powder form.

3.2 Determination of Temperature Decomposition of Rhamno NR22

The analysis was done using nitrogen gas with the flow rate was 50 L/min at temperature 27°C to 900°C. Figure 3.2 is depicting the correlation of rhamno NR22 sample weight with temperature and time. The actual sample was 20 mg. According to [20], supposedly at first 110°C, only 1.2% degradation of sample weight would occur and after temperature reaches at 400°C, the degradation would start. However, the uptake of sample weight decreased dramatically at the first five minutes. Reduction recorded for sample weight as much as 10%. Hence, the sample weight drops to 18 mg. These events are related to the loss of water and degradation of Rhamno NR22 [21] as well as changes of structure from dirhamnolipids to monorhamnolipids due to the fragility.

For the next 10 minutes, another reduction occurs to sample weight. However, it took only 1.0 mg diminution which is 5% degradation from 18 mg to 17 mg. A weak endothermic peak was found in this stage as shown in Figure 3.2 (B), which was mainly because of a degradation process of depolymerization [22]. In Figure 3.2 (B), the first 15 minutes show the deflation curve. In other words, the activity was greatly correlated with the amount of degraded sample weight which confirmed the interaction between temperature and weight loss of the Rhamno NR22.

Base on Figure 3.2 (A), the huge degradation occurs at 190°C to 265°C with 20% of losing weight recorded. Hence, the sample weight decreased from 17 mg to 13 mg. It took 8 minutes from 16 minutes to 24 minutes for the degradation to occur. This is the crucial part during analysis as the graph is shown dependencies between sample weight and temperature during a particular period. At this moment, this is the most not stable hydrophobic due to the graph slope is shown in Figure 3.2 (B). Hence, the weakest bonds hydroxide, consist of an oxygen and hydrogen atom held together by covalent bond broke down lead to the loss of sample weight. Another reduction of sample weight occurs at 25 minutes until 38 minutes. Within the period, 50% from actual weight degraded. Thus, the remaining Rhamno NR22 sample was 10 mg.

Research by Abbas et al [23] has been done by conducting TGA experiment to investigate the thermostability of rhamnolipid. However, the reported rhamnolipid produced by *P. aeruginosa* exhibited less thermostable compared with the present study, which showed almost 80% mass loss when the temperature reached around 300 °C and degraded continuously as the temperature rose. For the present study, only 60% degradation recorded at temperature 300%.

According to Worakitsiri et al [24], the study has shown rhamnolipid started to degrade at 400°C. Comparing to Figure 3.2, the sample started to stable at 400°C. The graph shows the minor rate of decreasing of weight sample. Hence, the declination still occurs from time to time. However, it took longer time to degrade. Figure 3.2 (A) shows the graph decline steadily after 38 minutes the analysis was run until the end. The percentage of sample weight reduced gradually in parallel with temperature rise. Although at 900°C, the sample still remains at 5 mg after 90 minutes. This scenario was caused by the intensity of Rhamno NR22 after went through break the intermolecular forces. This remarks the endurance of Rhamno NR22 toward high temperature.

3.3 Chemical bond, functional group and structure of Rhamno NR22

The analysis of NMR used heavy water is known as Deuterium Oxide (D_2O) as solvent decided ^{1}H-NMR chemical shift due to the purity of Rhamno NR22. The^{1}H-NMR spectrum consists of 18 signals [25]. Biosurfactant was characterized as a mixture of dirhamnolipid and monorhamnolipid by NMR technique. Slight variations in chemical shifts of these compounds and of cutting agents were observed on comparing standard and sample solutions or sample solutions together [26] The analysis was run for not pure Rhamno NR22 which was produced from Ps.NR.22 and pure rhamno bought from international company (Merck).

Figure 3.3 (A) shows a typical ^{1}H-NMR spectrum of not pure Rhamno NR22 which is produced from Ps.NR.22 that examplifies what was normally observed. It was found that region 1.192 ppm was in multiplete group. This is due to the stabilization of the variation. Hence, it has assignment of (-CH_3) on rhamnose moiety. It was observed the chemical shift, region 1.8-2.4 ppm is an interval where the hydrogen signals are more distinguishable, allowing for the identification of aromatic and olefinic hydrogen atom of Rhamno NR22 [27]. The type of hydrogen known in this region is allylic. Base on Figure 3.3 (A), there are singlet and multiplet at 1.821 ppm and in between 2.118 ppm to 2.135 ppm.

In contrast, the region between 3.0-3.8 ppm had a huge signal to express an information. For instance, at 3.626 ppm, is has singlet multiplicity with assignment of (-CH-OH-) on rhamnose moiety that attached to aromatic ring which may be part of lactone ring [28]. The absorption frequencies data of ^{1}H-shifts for the rhamno NR22 by NMR analysis are shown in Tables 3.

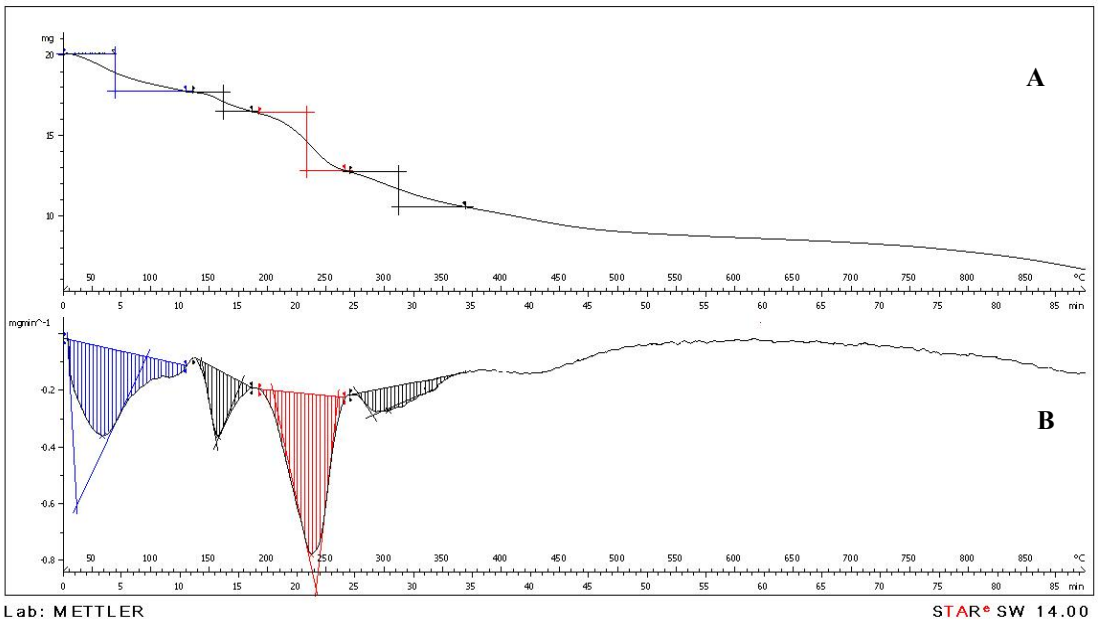

Figure 3.2 Rhamno NR22 Weight Degradation (mg) vs Temperature (°C) and Time (Minutes)

Table 3: Absorption frequencies data of ¹H-NMR chemical shifts for the Rhamno NR22 (not pure) by NMR analysis [29].

¹H-NMR Chemical Shift (ppm)	Multiplicity	Assignment
1.192	Multiplet	-CH_3 (on rhamnose moiety)
1.821	Singlet	-(CH_2)-CH(O)-CH_2 COO (on β-hydroxyfatty acids)
2.118	Multiplet	-CH(O)-CH_2 COO (on β-hydroxyfatty acids)
2.135	Multiplet	-CH(O)-CH_2 COO (on β-hydroxyfatty acids)
3.260	Singlet	-(CH_2)-CH(O-Rha)-CH_2 C OO (on β-hydroxyfatty acids)
3.626	Singlet	-CH-OH- (on rhamnose moiety)

The obtained result of ¹H-NMR spectrum of pure Rhamno NR22 which is bought from international company (Merck) are represented in figure 3.3 (B). The presence of the terminal branching in the fatty acid component (-CH_3-) was confirmed at 0.764–0.799 ppm while the terminal branching on rhamnose moiety component (-CH_3-) is ratify at 1.070-1.225 ppm. Moreover, the presence of aliphatic chain (-CH_2-) is displayed at 1.495-1.562 ppm.

The chemical shift at the region 1.1821 ppm and 2.136 ppm yield singlet multiplicity with the assignment of (-(CH_2)-CH(O)-CH_2COO-) and (-CH(O)-CH_2COO). Both the assignment are relay o on fatty acid terminal branching. While the region 2.364-2.404 ppm have different assignment on terminal brancing in fatty acid which was (-CH(O)-CH_2COO) due to the multiplet wave on ¹H-NMR spectrum. However, the similarities is aliphatic chain showed on all asignment at 1.821-2.404 ppm.

Base on Figure 3.3 (B), the region between 3.619-3.781 ppm has multiplet multiplicity with assignment of (-CH-OH-) on rhamnose moiety that attached to aromatic ring. Also, the presence of the ester carbonyl group is indicated at 3.983-4.033 ppm which is part of lactone ring [30] due to the assignment -(CH_2)-CH(-O-C=O)-CH_2COO on the β-hydroxyfatty acids. Therefore, not pure rhamno which is produced from Ps.NR.22 exhibit the features similar to the pure rhamno in term of functional group, chemical bond and chemical structure.

Table 4: Absorption frequencies data of ¹H-NMR chemical shifts for the Rhamno NR22 (pure) by NMR analysis [29].

¹H-NMR Chemical Shift (ppm)	Multiplicity	Assignment
0.764, 0.776, 0.792. 0.799	Multiplet	-CH_3 (on β-hydroxyfatty acids)
1.070, 1.168, 1.178, 1.199, 1.225	Multiplet	-CH_3 (on rhamnose moiety)
1.495, 1.562	Multiplet	-(CH_2)-CH(O)-CH_2 COO (on β-hydroxyfatty acids)
1.821	Singlet	-(CH_2)-CH(O)-CH_2 COO (on β-hydroxyfatty acids)
2.136	Singlet	-CH(O)-CH_2 COO (on β-hydroxyfatty acids)
2.364, 2.404	Multiplet	-CH(O)-CH_2 COO (on β-hydroxyfatty acids)
3.261, 3.293, 3.309, 3.372	Multiplet	-(CH_2)-CH(O-Rha)-CH_2 COO (on β-hydroxyfatty acids)
3.619, 3.715, 3.781	Multiplet	-CH-OH- (on rhamnose moiety)
3.983, 4.033	Multiplet	-(CH_2)-CH(-O-C=O)-CH_2 COO (on β-hydroxyfatty acids)

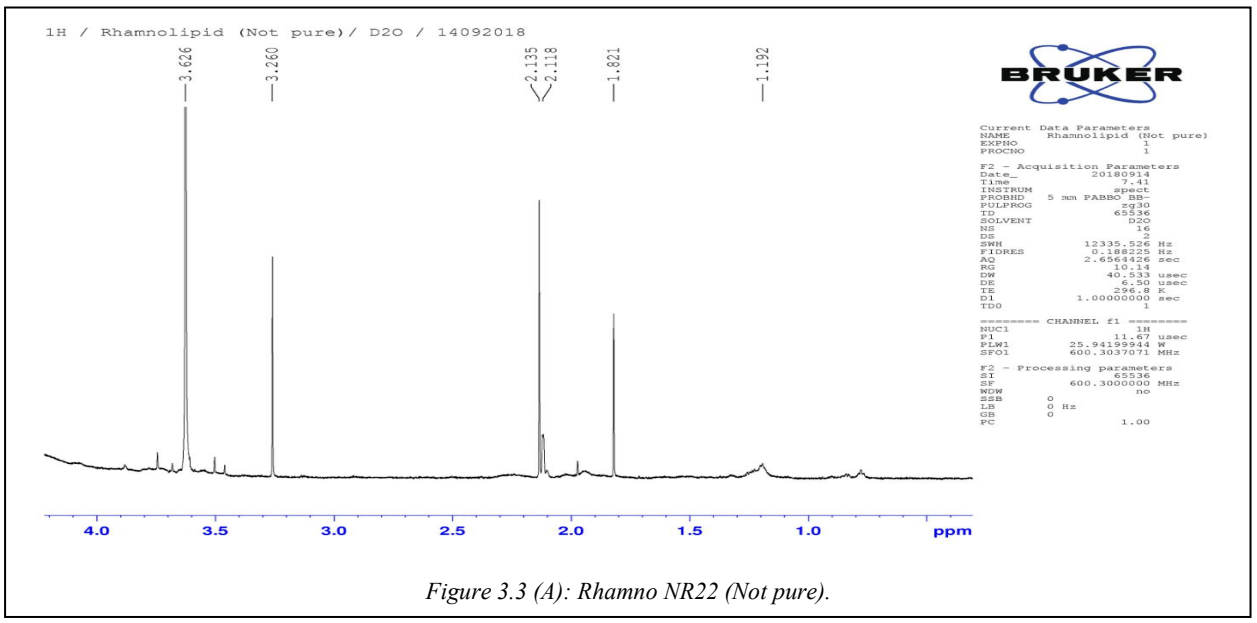

Figure 3.3 (A): Rhamno NR22 (Not pure).

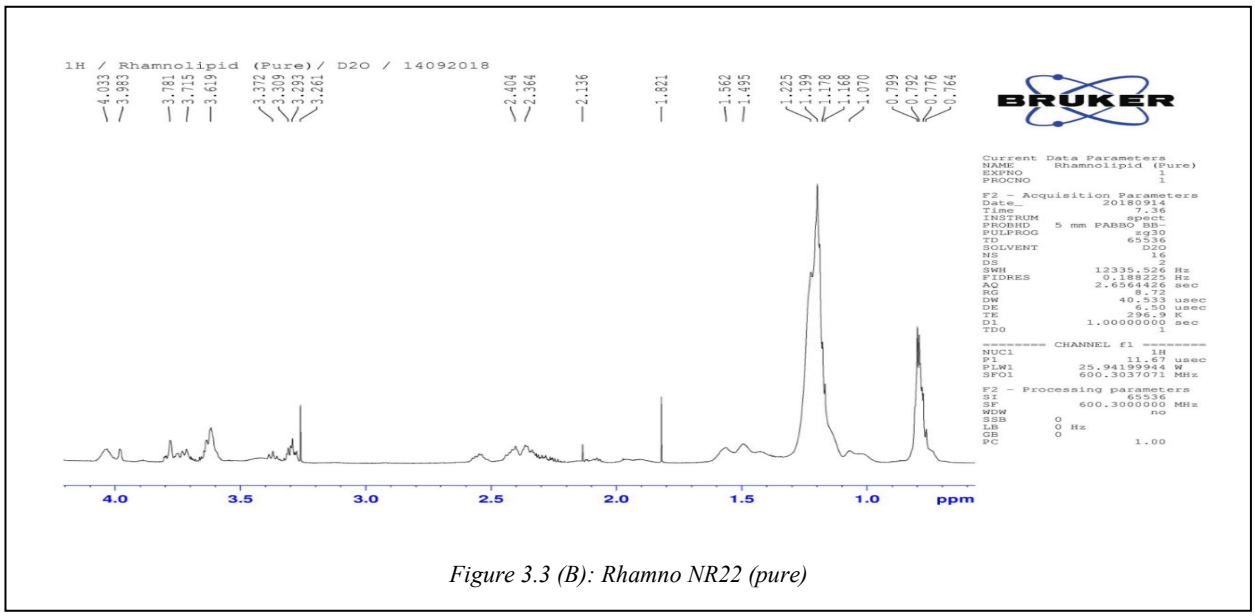

Figure 3.3 (B): Rhamno NR22 (pure)

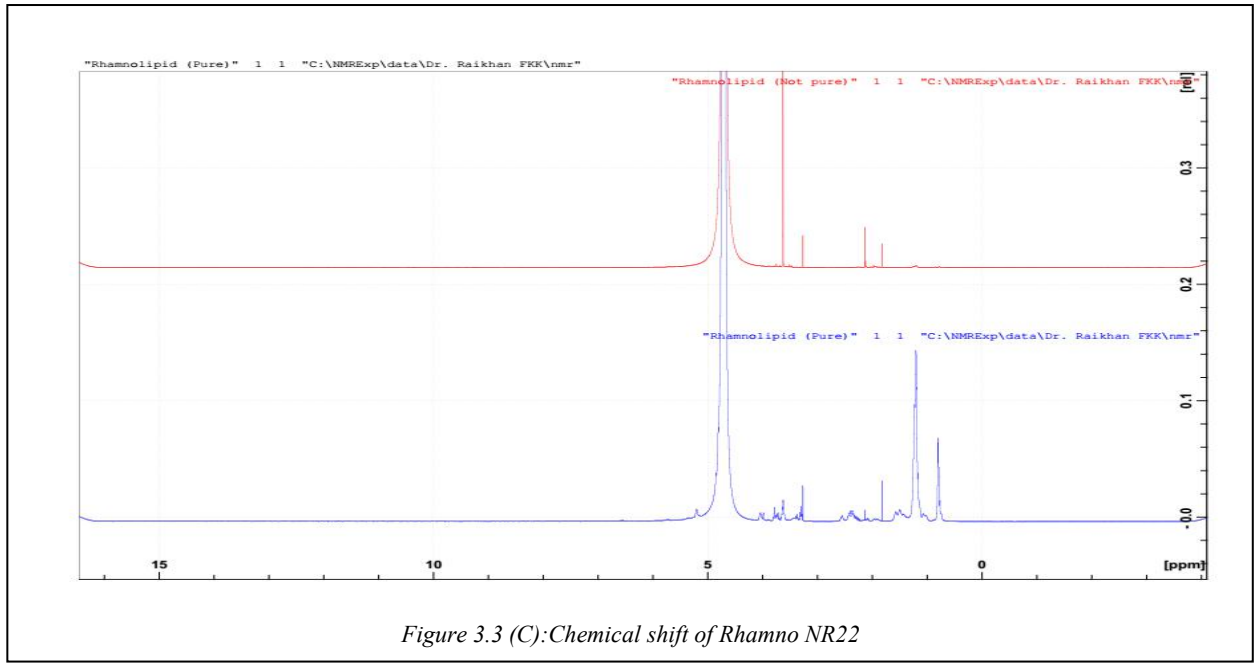

Figure 3.3 (C):Chemical shift of Rhamno NR22

Due to functional group, chemical bond and chemical structure, Rhamno NR22 was classified as hydrophobic. Hydrophobic molecules are molecules that do not have a charge, meaning they are non-polar [31]. Hydrophobic tails arranged in much more ordered structures. The structure of dirhamnolipid was formed with combining TGA and NMR result by referring to structure developed assignment data for not pure Rhamno NR22. According to the Figure 3.3 (D), it can be classified as dirhamnolipid with two rhamnose molecules linked to one or two molecules of hydroxy acid. The head group of two rhamnose moiety occupied with (-CH-OH-) and (-CH_3-) on rhamnose. While the branch two fatty acid tails known as β-hydroxyfatty acids exist at second rhamnose moiety.

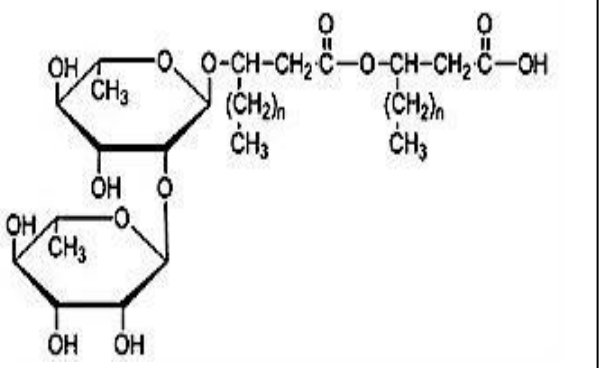

Figure 3.3 (D): Structure of Dirhamnolipid.

4) CONCLUSION

The process to produce Rhamno NR22 itself was very tough. All the procedure needed to be done particularly in order to ensure the bacteria is alive. Dealing with bacteria is very challenging since it is sensitive microorganisms. Thus, tolerance and details work is needed to produce Rhamno NR22. From the TGA analysis, it clearly shows that decomposition of sample weight Rhamno NR22 reduced from time to time while the temperature is increasing. At certain temperature, an aggressive degradation occur. However, the sample weight remain stable once the weakest bond undergo break down.. It means, the structure of Rhamno NR22 has change because of shifted temperature and the structure of Rhamno NR22 remain the same after went through the break of covalent bond. While NMR revealed the Rhamno NR22 produced by Ps.NR.22 have the similar characteristic with pure rhamno due to the functional group and chemical structure even though they have different component. Due to functional group, chemical bond and chemical structure, Rhamno NR22 was classified as hydrophobic and the structure of Rhamno NR22 known as dirhamnolipid was successful discovered..

5) ACKNOWLEDGEMENTS

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REFERENCES

[1] Sharma, R., Singh, J., & Verma, N. (2018). Production, characterization and environmental applications of biosurfactants from Bacillus amyloliquefaciens and Bacillus subtilis. Biocatalysis And Agricultural Biotechnology, 16, 132-139. doi: 10.1016/j.bcab.2018.07.028.

[2] Varjani, S., & Upasani, V. (2017). Critical review on biosurfactant analysis, purification and characterization using rhamnolipid as a model biosurfactant. Bioresource Technology, 232, 389-397. doi: 10.1016/j.biortech.2017.02.047.

[3] Mondal, M., Sarkar, A., Maiti, T., & Saha, B. (2017). Microbial assisted (pseudomonas sp) production of novel bio-surfactant rhamnolipids and its characterization by different spectral studies. Journal Of Molecular Liquids, 242, 873-878. doi: 10.1016/j.molliq.2017.07.089.

[4] Md, F. (2012). Biosurfactant: Production and Application. Journal Of Petroleum & Environmental Biotechnology, 03(04), 2-5. doi: 10.4172/2157-7463.1000124.

[5] Sabturani, N., Latif, J., Radiman, S., & Hamzah, A. (2016). Spectroscopic Analysis of Rhamnolipid Produced by Pseudomonas aeruginosa. Malaysian Journal Of Analytical Science, 20(1), 31-43.

[6] Gharaei-Fa, E. (2011). Biosurfactants in Pharmaceutical Industry (A Mini-Review). American Journal Of Drug Discovery And Development, 1(1), 58-69. doi: 10.3923/ajdd.2011.58.69.

[7] Clark, J. (2013). Emulsions: When Oil and Water Do Mix - IFT.org.

[8] Chen, Y., Wang, C., Liu, H., Liu, Q., & Kong, B. (2018). Enhanced physical and oxidative stability of porcine plasma protein hydrolysates based oil-in-water emulsions by adding oxidized chlorogenic acid. .Colloids And Surfaces A: Physicochemical And Engineering Aspects, 558, 330-337. doi: 10.1016/j.colsurfa.2018.08.067.

[9] Zdziennicka, A., & Jańczuk, B. (2018). Wetting and adhesion properties of rhamnolipid and surfactin. International Journal Of Adhesion And Adhesives. 84, 275-282 doi: 10.1016/j.ijadhadh.2018.04.005.

[10] S.J., G., Banat, I., & Joshi, S. (2018). Biosurfactants: Production and potential applications in microbial enhanced oil recovery (MEOR).Biocatalysis And Agricultural Biotechnology, 14, 23-32. doi: 10.1016/j.bcab.2018.01.010.

[11] Chen, W., Juang, R., & Wei, Y. (2015). Applications of a lipopeptide biosurfactant, produced by microorganisms. Biochemical Engineering Journal, 103, 158-169. doi: 10.1016/j.bej.2015.07.009

[12] Karlapudi, A., Venkateswarulu, T., Tammineedi, J., Kanumuri, L., Ravuru, B., Dirisala, V., & Kodali, V. (2018). Role of biosurfactants in bioremediation of oil pollution-a review. Petroleum. 4(3), 241-249. doi: 10.1016/j.petlm.2018.03.007

[13] Roy, A. (2017). A Review on the Biosurfactants: Properties, Types and its Applications. Journal Of Fundamentals Of Renewable Energy And Applications,08(01), 2. doi: 10.4172/2090-4541.1000248,

[14] Wittgens, A., Kovacic, F., Müller, M., Gerlitzki, M., Santiago-Schübel, B., & Hofmann, D. et al. (2016). Novel insights into biosynthesis and uptake of rhamnolipids and their precursors.Applied Microbiology And Biotechnology, 101(7), 2865-2878. doi: 10.1007/s00253-016-8041-3.

[15] Rahman, K., Rahman, T., Kourkoutas, Y., Petsas, I., Marchant, R., & Banat, I. (2003). Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients.Bioresource Technology, 90(2), 159-168. doi: 10.1016/s0960-8524(03)00114-7.

[16] Sachdev, D., & Cameotra, S. (2013). Biosurfactants in agriculture. Applied Microbiology And Biotechnology, 97(3), 1005-1016. doi: 10.1007/s00253-012-4641-8.

[17] Forensics, G. (2012) How Does Thermogravimetric Analysis Work?.

[18] Nik Him, N. R., Khairul Izwan, A. R., & Ahmad Robert, K. (2017). Novel treatment of heavily oiled wastewater using Pseudomonas aeruginosa Nr.22 producing usable free fatty acids (FFA) (Vol. 8).

[19] de Melo, B., Martins Shimojo, A., Marcelino Perez, A., Duarte Lana, J., & Andrade Santana, M. (2018). Distribution, recovery

and concentration of platelets and leukocytes in L-PRP prepared by centrifugation. *Colloids And Surfaces B: Biointerfaces, 161*, 288-295. doi: 10.1016/j.colsurfb.2017.10.046

[20] Lan, G., Fan, Q., Liu, Y., Chen, C., Li, G., Liu, Y., & Yin, X. (2015). Rhamnolipid production from waste cooking oil using Pseudomonas SWP-4.*Biochemical Engineering Journal*, *101*, 44-54. doi: 10.1016/j.bej.2015.05.001.

[21] Han, Y., Kusunose, T., & Sekino, T. (2009). One-step reverse micelle polymerization of organic dispersible polyaniline nanoparticles. *Synthetic Metals*, *159*(1-2), 123-131. doi: 10.1016/j.synthmet.2008.08.011.

[22] Lotfabad, T., Shourian, M., Roostaazad, R., Najafabadi, A., Adelzadeh, M., & Noghabi, K. (2009).An efficient biosurfactant-producing bacteriumPseudomonas aeruginosa MR01, isolated from oil excavation areas in south of Iran. Colloids And Surfaces B: Biointerfaces, 69(2), 183-193. doi: 10.1016/j.colsurfb.2008.11.018.

[23] Abbasi, H., Hamedi, M., Lotfabad, T., Zahiri, H., Sharafi, H., & Masoomi, F. et al. (2012). Biosurfactant-producing bacterium, Pseudomonas aeruginosa MA01 isolated from spoiled apples: Physicochemical and structural characteristics of isolated biosurfactant. *Journal Of Bioscience And Bioengineering*, *113*(2), 211-219. doi: 10.1016/j.jbiosc.2011.10.002.

[24] Worakitsiri, P., Pornsunthorntawee, O., Thanpitcha, T., Chavadej, S., Weder, C., & Rujiravanit, R. (2011). Synthesis of polyaniline nanofibers and nanotubes via rhamnolipid biosurfactant templating. *Synthetic Metals*, *161*(3-4), 298-306. doi: 0.1016/j.synthmet.2010.11.039.

[25] ROMANI, A., ZUCCACCIA, C., & CLEMENTI, C. (2006). An NMR and UV–visible spectroscopic study of the principal colored component of Stil de grain lake. *Dyes And Pigments*, *71*(3), 218-223. doi: 10.1016/j.dyepig.2005.07.005.

[26] Balayssac, S., Retailleau, E., Bertrand, G., Escot, M., Martino, R., Malet-Martino, M., & Gilard, V. (2014). Characterization of heroin samples by 1H NMR and 2D DOSY 1H NMR. *Forensic Science International*, *234*, 29-38. doi: 10.1016/j.forsciint.2013.10.025.

[27] chemomede A. Leite, J., de Oliveira, M., Conti, R., de S. Borges, W., Rosa, T., & Filgueiras, P. et al. (20 18). Extraction and isolation of cannabinoids from marijuana seizures and characterization by 1H NMR allied to tric tools. *Science & Justice*, *58*(5), 355-365. doi: 10.1016/j.scijus.2018.06.005.

[28] Zou, C., Wang, M., Xing, Y., Lan, G., Ge, T., Yan, X., & Gu, T. (2014). Characterization and optimization of biosurfactants produced by Acinetobacter baylyi ZJ2 isolated from crude oil-contaminated soil sample toward microbial enhanced oil recovery applications. *Biochemical Engineering Journal*, *90*, 49-58. doi: 10.1016/j.bej.2014.05.007

[29] Ali, H., Ismail, D., & El-Gendy, N. (2014). The Biotreatment of Oil-Polluted Seawater by Biosurfactant Producer HalotolerantPseudomonas aeruginosaAsph2. *Energy Sources, Part A: Recovery, Utilization, And Environmental Effects*, 36(13), 1429-1436. doi: 10.1080/15567036.2014.880092.

[30] Ohadi, M., Dehghannoudeh, G., Forootanfar, H., Shakibaie, M., & Rajaee, M. (2018). Investigation of the structural, physicochemical properties, and aggregation behavior of lipopeptide biosurfactant produced by Acinetobacter junii B6. *International Journal Of Biological Macromolecules, 112*, 712-719. doi: 10.1016/j.ijbiomac.2018.01.209.

[31] Santos, D., Rufino, R., Luna, J., Santos, V., & Sarubbo, L. (2016). Biosurfactants: Multifunctional Biomolecules of the 21st Century. *International Journal Of Molecular Sciences*, *17*(3), 401. doi: 10.3390/ijms17030401.