Best Medium for Production and the Activity of Biosurfactant from Local Strain of Pseudomonas

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Abstract. A local strain of Pseudomonas has been isolated from Belawan, a big port in Medan, North Sumatera. This isolate was already tested on its ability to break down carbofuran, an active compound of widely used pesticide. The ability of bacteria to degrade carbofuran is closely related to the ability of bacteria to secrete biosurfactant. This compound promotes bacteria on carbofuran metabolism. This study aimed to find out the best medium for the local strain of Pseudomonas to produce biosurfactant and to characterize the compound. The isolate was cultured in several media with different combination of three different carbon sources which are glycerol, glucose, and olive oil; two types of nitrogen sources: sodium nitrate and ammonium sulfate at C/N ratio 20 and 60, two different time of incubation, 24 and 72 hours. The parameters observed were the bacterial growth, the concentration and the activities of biosurfactant. The results showed that during 24 hours of incubation the best medium for the production of biosurfactant was the combination of glucose and ammonium sulfate with C/N ratio was 60. The bacterial population was 2.230 at OD 621 nm, the highest biosurfactant activity, the Index of emulsion was 0.42 and the surface tension was 36.3 mN/m and the concentration of biosurfactant was 75.530 g/L. Meanwhile during 72 hours of incubation the best medium was shown by the combination of glucose and ammonium sulfate with C/N ratio was 20. The bacterial population was 2.252 at OD 621 nm, the Index of emulsion was 0.25 and surface tension was 30.0 mN/m and the biosurfactant concentration was 76.494 g/L.

1. Introduction
Surfactants are surface-active compounds that lower the surface and interface tension between phases. Surfactants have wide application in industries as well as in the environment. Many studies are reported to construct gene modification of bacterium to promote the production of biosurfactant. Other reports tried to modify medium to find out the best carbon and nitrogen sources for high production of biosurfactant. There are many different types of biosuractant such as rhamnolipid. Among them, rhamnolipid (RML) are very important group of compound. This type of biosurfactant shows potential application in a variety of industrial sectors such as the pharmaceutical, cosmetic, food, and oil industry [1-3]. Pseudomonas aeruginosa is the most widely used microorganism for RML production [1]. A local strain of P. aeruginosa has been isolated from Belawansea and has been tested on its ability to degrade carbofuran and other hydrocarbon compounds. It showed a very good potential with the ability to degrade carbofuran up to 96 % during 21 days of incubation. That isolate has been immobilize in sodium alginate as well as in polyurethane. The ability of the immobilized isolate to...
degrade carbofuran did not differ from the ability of its free cell making it a good candidate as the agent of bioremediation in the future.

In order to metabolize carbofuran, bacteria secrete biosurfactant. This compound lower surface tension between hydrocarbon compounds and water causes the compounds are available for the bacteria. The ability of bacteria to secrete biosurfactants are closely related to the ability of the microorganism to degrade hydrocarbon compounds and use them as the carbon sources [4]. Many studies reported the use of various carbon and nitrogen compounds to find out the best medium for the production of biosurfactant. This study explored the production of biosurfactant by a local strain of \textit{P. aeruginosa} under several combinations of carbon and nitrogen sources at different ration C/N and incubation time.

2. Materials and Methods

2.1. Bacterial strain and culture conditions

The local strain of \textit{P. aeruginosa} was subcultured in nutrient slant-nutrient agar before used. The bacterium was grown in minimal salt medium (MSM) with composition: 7.0 g/L K2HPO4; 3.0 g/L KH2PO4; 0.2 g/L MgSO4 \textsubscript{7}H2O; 5 g/L soy pepton; and 5 g/L yeast extract with three different variations of carbon and two of nitrogen as shown by the following table:

| Carbon Sources | Nitrogen Source: NaNO3 (g) | Nitrogen Source: (NH4)2SO4 (g) |
|----------------|--------------------------|-------------------------------|
|                | 3. C/N ratio 20          | 4. C/N ratio 60               |
| Glycerol (ml)  | 30 / 4.46                | 30 / 1.49                     |
| Glucose (g)    | 30 / 5.65                | 30 / 1.88                     |
| Olive Oil (ml) | 30 / 5.65                | 30 / 1.88                     |

Duration of incubation were 24 and 72 hours. All cultures were incubated in shaking incubator at 30\textdegree C at 150 rpm with two replicates [5].

To achieve different C/N ratio

Glycerol.
The density of glycerol : 1.26 g/cm3 means 1.26 g/ml.  
It implies that 1 ml of glycerol \(\approx\) 1.26 g of glycerol. 
Glycerol’s formula: C3H8O3, molecular weight: \(3 \times (12) + 8 \times (1) + 3 \times (16) = 92\) g/mol 
Percentage of C in glycerol = 36/92 x 100% = 39.1\% 
Therefore the C content in 1 g of glycerol = \(\frac{39.1 \times 1.26}{100} = 0.49\) g

If 3\% (V/V) of glycerol is used in all media, in 1 L of medium means 30 ml of glycerol is added 969 ml of distilled water + 1 ml of trace element. If 1 ml of glycerol contains 0.49 g of Carbon, 30 ml of glycerol contains 30 x 0.49 g = 14.7 g, so the C content is 14.7 g/L

For NaNO3 
Molecular weight = \(23 + 14 + 3 \times (16) = 85\) g/mol 
Molecular weight of N in NaNO3 = 14 g/mol 
For C/N = 20, in 1L of medium = 14.7 g/N = 20 so N is 0.735 g 
There are 14 g of N in 85 g of NaNO3, therefore 0.735 g of N will be present in \(\frac{85 \text{ g} \times 0.735 \text{ g}}{14 \text{ g}} = 4.46\) g of NaNO3

Thus 4.46 g of NaNO3 will be added to 1L of medium containing 3\% (v/v) of glycerin to obtain C/N ratio of 20.
The parameters observed were:

2.1.1 **Cell growth.** The cell population was observed based on measurement of absorbance at 600 nm and standard plate count (SPC) method. The measurement was done at 24 and 72 hours.

2.1.2 **Determination of rhamnolipid concentration.** The quantification of rhamnolipid was assessed by the indirect quantification of rhamnose. The cultures of each sample was filtered with Whatman filter paper no 1, centrifugated at 5,000 x g for 20 min. One ml of supernatant was added with 100 µl of a 10 M sulfuric acid solution and was heated at 100°C for four hours to hydrolyzerhamnolipid into rhamnose and fatty acid. The mixture was neutralized with 10 M NaOH, filtered with 0.22 µm filter and analyzed using high performance liquid chromatography (HPLC). An animex HPX-87H column was used for the HPLC assay, using 5mM sulfuric acid as the mobile phase at a rate of 0.6 mL/min and oven temperature of 42°C to assess rhamnose. The quantification of rhamnolipid was done at 24 and 72 hours.

2.2 **Determination of biosurfactant activity**

The activity of biosurfactant was measured based on the ability of biosurfactant to emulsify hydrophobic compound of hexadecane and to lower the surface tension of the two phases. Two ml of supernatant was mixed vigorously with 2 ml of n-hexadecane for 2 min. the mixture was left to stand and the volume of emulsion which was formed was measured after 24 hours. The lowering of surface tension was observed according to DuNouy method using DuNouy Tensiometer at room temperature.

3. **Result and Discussion**

3.1 **Cell Growth**

The ability of local strain of *Pseudomonas aeruginosa* to grow in medium containing different carbon and nitrogen sources at different ratio C/N as measured with spectrophotometer at 600 nm is shown in the following table:

| No. | Variation of carbon and nitrogen sources | Ratio C/N | Cell Population at λ 600 nm |
|-----|-----------------------------------------|-----------|-----------------------------|
|     |                                         | 24 hours  | 72 hours                    |
| 1.  | Glycerol / NaNO₃                        | 20        | 0.679                       | 1.053                       |
| 2.  |                                          | 60        | 1.635                       | 1.469                       |
| 3.  | Glycerol / (NH₄)₂SO₄                    | 20        | 1.721                       | 0.984                       |
| 4.  |                                          | 60        | 0.808                       | 1.992                       |
| 5.  | Glucose/NaNO₃                           | 20        | 1.749                       | 0.727                       |
| 6.  |                                          | 60        | 1.024                       | 1.438                       |
| 7.  | Glucose/(NH₄)₂SO₄                       | 20        | 1.608                       | 2.252                       |
| 8.  |                                          | 60        | 2.230                       | 2.098                       |
| 9.  | Olive oil/NaNO₃                         | 20        | 1.497                       | 1.737                       |
| 10. |                                          | 60        | 1.941                       | 1.745                       |
| 11. | Olive oil/(NH₄)₂SO₄                     | 20        | 1.335                       | 1.749                       |
| 12. |                                          | 60        | 1.281                       | 1.505                       |
During 24 hours of incubation the isolate grew best in medium with glucose and ammonium sulfate as carbon and nitrogen source respectively with ratio C/N was 60. The absorbance of the culture reached 2.230 while the lowest one was shown by medium with glycerol and sodium nitrate at ratio C/N was 20. Among twelve variations of media, the isolate mostly continue to grow up to 72 hours of incubation. As in 24 hours, the best medium for isolate to grow was shown by combination of glucose and ammonium sulfate with C/N ratio of 20 with absorbance: 2.252. It is assumed that glucose was easier to be metabolized by the local strain of *P. aeruginosa* than glycerol and olive oil and ammonium sulfate provided nitrogen better than sodium nitrate. Different result was reported by Abouseoud using *P. fluorescent*. The bacteria grew best in medium with olive oil and ammonium nitrate as the source of carbon and nitrogen respectively with a C/N ratio of 10 [6]. Another study using *P. aeruginosa* showed that the bacteria reached the highest density when grown in glycerol at 144 hours of incubation with a dry weight of 605 mg. Meanwhile, when the bacteria used glucose as carbon source, it showed the lowest growth with a dry weight of 350 mg at 96 hours of incubation [7]. It is hard to determine which carbon and nitrogen source is best for the growth of bacteria, even the same genus.

3.2 Biosurfactant Activities

The surface-active properties of biosurfactant depend mainly on its ability to lower surface and interface tension, CMC (Critical Micelle Concentration) value, and formation of stable emulsion. The ability to reduce surface tension depends on the specific concentration of surface-active compound. The CMC is defined as the minimum concentration of biosurfactant required to give maximum surface tension reduction of water and initiate micelle formation [8]. The lower the CMC value the more efficient the biosurfactant. The measurement of Emulsion index and the surface tension of biosurfactant produced by local strain of *P. aeruginosa* are shown in table 2 below:

**Table 2.** Biosurfactant activities of a local strain of *P. aeruginosa* base on emulsion index (%) and surface tension

| No. | Variation of carbon and nitrogen sources | Ratio C/N | Emulsion Index (E<sub>24</sub>) | Surface Tension (mN/m) |
|-----|------------------------------------------|-----------|---------------------------------|------------------------|
|     |                                          |           | 24 hours | 72 hours | 24 hours | 72 hours |
| 1.  | Glycerol / NaNO₃                         | 20        | 0.22     | 0.21     | 43.3    | 45.7     |
| 2.  |                                          | 60        | 0.23     | 0.13     | 46.0    | 43.0     |
| 3.  | Glycerol / (NH₄)₂SO₄                     | 20        | 0.29     | 0.16     | 44.3    | 45.0     |
| 4.  |                                          | 60        | 0.28     | 0.16     | 43.0    | 41.7     |
| 5.  | Glucose / NaNO₃                          | 20        | 0.23     | 0.25     | 46.0    | 41.7     |
| 6.  |                                          | 60        | 0.25     | 0.22     | 42.7    | 45.0     |
| 7.  | Glucose / (NH₄)₂SO₄                      | 20        | 0.3      | 0.12     | 39.7    | 43.3     |
| 8.  |                                          | 60        | 0.42     | 0.09     | 40.7    | 39.7     |
| 9.  | Olive oil / NaNO₃                        | 20        | -        | -        | 36.3    | 32.0     |
| 10. |                                          | 60        | 0.09     | 0.04     | 43.0    | 33.7     |
| 11. | Olive oil / (NH₄)₂SO₄                    | 20        | 0.15     | -        | 36.3    | 30.3     |
| 12. |                                          | 60        | 0.16     | 0.04     | 40.0    | 30.0     |

The best combination medium for the highest emulsion index was a combination between glucose and ammonium sulfate with a C/N ratio of 60. The value of E<sub>24</sub> was 0.42 which was much higher than others. The same medium but C/N ratio showed the best second which was 0.3. Surprisingly, the bacteria that grew in olive oil as the carbon source had extremely low activity of emulsion or even had no activity of emulsion at all as shown by isolate with combination of olive oil and sodium nitrate with a C/N ratio of 20 (test tube no. 9) of below figure.
Figure 1. Emulsion activity of local strain of P. aeruginosa at different carbon and nitrogen sources and at different ratio C/N

The different result was shown by the ability of isolate to reduce the surface and interface tension. For this parameter, the best medium with the lowest surface tension was shown by olive oil as carbon source. When olive oil was combined with sodium nitrate as well as ammonium sulfate at two different C/N ratios, all resulted in significantly low surface tension 30.0 – 33.7 mN m$^{-1}$. Meanwhile the biosurfactant activity to reduce the surface tension produced by both glycerol and glucose as the carbon sources showed the value which are about the same, 43 – 45 mN m$^{-1}$. It is likely that the data from E24 did not confirm with the data from the surface tension. The one that had no ability or extremely low ability to form stable emulsion on the contrary showed very good capability of reducing the surface and interfacial tensions. Many studies are reported that the ability of biosurfactant to form stable emulsion confirm with its ability to reduce the surface tension such as: biosurfactant from P. aeruginosa PAO1 which was able to fully emulsify n-hexadecane was able to reduce the surface tension of water (75 mN/m) to 29.33 mN/m [9].

3.3 Biosurfactant Concentration
Biosurfactant produced by the local isolate which was analyzed with HPLC at incubation time of 24 hours and 72 hours is shown by the following table:
Table 3. Concentration of biosurfactant from a local strain of *P. aeruginosa* at different source of carbon and nitrogen, ratio C/N, and time of incubation.

| No. | Variation of carbon and nitrogen sources | Ratio C/N | Biosurfactant Concentration (g/L) |
|-----|----------------------------------------|-----------|-----------------------------------|
|     |                                        |           | 24 hours                          |
| 1.  | Glycerin / NaNO3                       | 20        | 75.530                            |
| 2.  |                                        | 60        | 61.766                            |
| 3.  | Glycerol / (NH4)2SO4                   | 20        | 56.867                            |
| 4.  |                                        | 60        | 47.221                            |
| 5.  | Glucose/NaNO3                          | 20        | 19.898                            |
| 6.  |                                        | 60        | 18.148                            |
| 7.  | Glucose/(NH4)2SO4                      | 20        | 13.467                            |
| 8.  |                                        | 60        | 11.661                            |
| 9.  | Olive oil/NaNO3                        | 20        | Not detected                      |
| 10. |                                        | 60        | Not detected                      |
| 11. | Olive oil/(NH4)2SO4                    | 20        | Not detected                      |
| 12. |                                        | 60        | Not detected                      |

The standard curve of rhamnose was done using a pure rhamnose with a series concentration of 25, 50, 75, and 100 ppm. The best medium for production of biosurfactant was found in medium with glycerol and sodium nitrate with a C/N ratio of 20 both at 24 hours as well as 72 hours with the value of 75.530 g/L and 76.594 g/L respectively. The use of glucose as the carbon source combined with sodium nitrate or ammonium sulfate resulted in quite low concentration of biosurfactants compare to glycerol. The same result was reported by Dobler using *P. aeruginosa* EstA in which higher concentration of biosurfactant was obtained when glycerol and sodium nitrate was used compare to glucose [5]. Many more studies reported that the use of glucose as the source of carbon for biosurfactant production yielded low concentration of glucose, only 1.5 g/L [9]. The more extreme result was shown by media with olive oil as the carbon source. Regardless of the source of nitrogen, the ratio of C/N and time of incubation, no biosurfactant was detected.

4. Conclusion
The results showed that during 24 hours of incubation the best medium for the production of biosurfactant was the combination of glucose and ammonium sulfate with C/N ratio was 60. The bacterial population was 2.230 at OD 621 nm, the highest biosurfactant activity, the Index of Emulsion was 0.42 and the surface tension was 36.3 mN/m and the concentration of biosurfactant was 75.530 g/L. Meanwhile during 72 hours of incubation the best medium was shown by the combination of glucose and ammonium sulfate with C/N ratio was 20. The bacterial population was 2.252 at OD 621 nm, the Index of Emulsion was 0.25 and surface tension was 30.0 mN/m and the biosurfactant concentration was 76.494 g/L. The source of nitrogen, the ratio of C/N and time of incubation, no biosurfactant was detected.

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References
[1] Sekhon KK and Rahman PKSM. 2014. Rhamnolipid biosurfactant- Past, Present, and Future scenario of global market. Front Microbiol. 5: 1 - 7
[2] Ricalovic MG, Vivic MM, and Karadzic IM. 2015. Rhamnolipid biosurfactant from Pseudomonas aeruginosa: From discovery to application in contemporary technology. J. Serbian Chem Soc 80:279 – 304

[3] Dobler L, Viela LF, Almeida RV, and Neves BC. 2016. Rhamnolipid in perspective: gene regulatory pathways, metabolic engineering, production and technological forecasting. N. Biotechnol 33: 123 – 135

[4] Guera-Santos L, Kappeli O, Fiechter A. 1984. Pseudomonas aeruginosa biosurfactant production in continuous culture with glucose as carbon source. Appl. Environ. Microbiol. 48(2):301 – 305

[5] Dobler L, Bruna RC, Wilber SA, Bianca CN, Denise MG, and Rodrigo VA. 2017. Enhance rhamnolipid production by Pseudomonas aeruginosa over expressing estA in a simple medium. PLoS ONE 12(8):e0183857

[6] Abouseout, M, R. Maachi, A. Amran, S. Boudergua, and A. Nabi. 2008. Evaluation of different carbon and nitrogen sources for the production of biosurfactant by Pseudomonas fluorescent. Desalination 223 (1-3): 143 – 151

[7] Ehinmitola, EO. Elizabeth, FA, and Oluwaseyi, PA. 2018. Comparative study of various carbon sources on rhamnolipid production. South African Journal of Chemical Engineering (26): 42 – 48

[8] Rufino RD, Juliana M de L, Galba M de CK, and Leonie AS. 2014. Charccterization and properties of biosurfactant produced by Candida lypolitica UCP 0988. Electric Journal of Biotechnology 17: 34 – 38

[9] Wittgens A, Tiso T, Amat TT, WenkP, Hemmerich J, and Muller C. 2011. Growth independent rhamnolipid production from glucose using non pathogenic P. putida KT2240. Microb Cell Fact. Biomed Central Ltd 10:80