Production of Rhamnolipids by Locally Isolated *Pseudomonas aeruginosa* using Sunflower Oil as Carbon Source

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**INTRODUCTION**

The chemically synthesized surfactants are not environmentally friendly and public awareness on the need to protect the environment has influenced to the production of biosurfactants from microorganisms. Biosurfactants can be produced extracellularly by several bacteria [1] and yeast [2] from renewable resources [3]. Apama *et al.* [4] reported maximum biosurfactant can be produced using *Pseudomonas* sp. grown and cultivated at 30°C. Biosurfactant-containing media could emulsify n-hexa-decane, hexane, olive oil, crude oil, diesel and coconut oil which are highly immiscible in water [5]. Silva *et al.*, [6] reported that biosurfactants are quite stable at high incubation temperatures and thus indicates their usefulness in industrial applications.

Rhamnolipids (RLs) are the best known biosurfactants which produced by gram-negative bacterium *Pseudomonas aeruginosa* [7]. Ali *et al.* [8] and Wei *et al.*, [9] reported RLs were produced after the bacterial growth had reached stationary phase as it is a secondary metabolite. When cultivated in liquid medium, *Pseudomonas* excretes mainly two types of RLs such as mono-rhamnolipid and di-rhamnolipid in which the rhamnosyl moiety and the fatty acid moiety are produced by de novo synthesis [10]. In order to get pure surface active agents, it is typically involved multiple consecutive steps in downstream processing that lead to high total production expenditure. Besides, the yield of RLs produced is usually low.

Carbon sources are one of the physicochemical factors that influence its production. In addition to usual water-soluble substrates such as glucose, there are variety of infrequent insoluble substrates have been used to enhance the growth as well as the RLs production. The incorporation of glucose and vegetable oils might potentially increase the yield of RLs [4]. Sunflower oil is one of the examples that can be used as carbon
source for the production of biosurfactants as it contains mixture of monounsaturated and polyunsaturated fats with low saturated fat levels [11].

Other conditions that favor higher level of RLs biosurfactant include high carbon to nitrogen ratio, exhaustion of nitrogen source, stress conditions and high cell densities [12]. In cosmetic industry, biosurfactant is used as emulsifiers, foaming agents, solubilizers, wetting agents, cleaners, antimicrobial agents, mediators of enzyme action, in insect repellents, antacids, bath products, acne pads, antidiandrit products, contact lens solutions, baby products, mascara, lipsticks, toothpaste, dentine cleaners to mention but a few [13]. The purpose of the study is to investigate the effectiveness of sunflower oil in the production of RLs by Pseudomonas aeruginosa in shake flask fermentation. This study was also discussed on comparison of the production of RLs by Pseudomonas aeruginosa under single substrate system (sunflower oil as the sole carbon source) and dual substrate system (sunflower oil and glucose as carbon source).

MATERIALS AND METHODS

Microorganism Preparation

The RLs producing microorganism Pseudomonas aeruginosa was obtained from the study on isolation and screening of high efficiency biosurfactant-producing bacteria conducted by Nordin et al., [14]. The strain was streaked on Nutrient agar (NA) plates and incubated at 30°C for 24 h for fresh growth [15]. For long term storage of P. aeruginosa, the cultures were stored in 30% (V/V) glycerol at -20°C. The cultures were recovered from the frozen stocks by transferring a loopfull to media with 1% w/v glucose as sole carbon source. The culture broth was maintained at 30°C (180 rpm, 7 days). A set of control (without inoculums) was run in parallel for comparison. The sampling processes were aseptically carried out for every 24 h and analysed for cell growth, rhamnolipids concentration, Emulsification index, surface activity and thermal stability. All treatments were conducted in triplicate [15].

Determination of Bacterial Growth

Bacterial growth was monitored by measurement of absorbance at wavelength of 600 nm by using spectrophotometer (Secomam/UvLine 9400). Samples of 1 ml were pipetted into microcentrifuge tube and centrifuged (miniSpin) at 10,000 rpm for 20 min. The cell pellet was resuspended in 1 ml of distilled water and the tube was shaken using vortex followed by optical reading [18].

Rhamnolipids (RLs) Measurement

The orcinol assay was used for amount measurement of RLs in the extracted sample [16]. The reagent was prepared by adding concentrated sulphuric acid (98% w/w) and 0.19% orcinol (3, 5-dihydroxytoluene) to distilled water. Distilled water (9.4 ml) were added to concentrated sulphuric acid (10.6 ml) as orcinol reagent. Orcinol reagent (900 μl) was added to 100 μl of sample and placed in 100 °C water bath for 20 min and left at room temperature in dark room for 35 min. Measurement was carried out spectrophotometrically at 421 nm. Blank and standard solution (L-rhamnose, 0-0.1 g/L) were also prepared using similar method.

Standard Curve Preparation

Standard curve was plotted as optical density (OD) versus concentration (g/L) of rhamnose [19]. The RLs concentrations were calculated from the standard curves prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (g/L) by multiplying rhamnose values by a coefficient of 3.0 for RLs.

Emulsification Index (E24%)

The ability of RLs to emulsify diesel oil, kerosene and sunflower oil was determined as reported by Abbasi et al. [7]. The emulsion index (E24) was determined as below [4].

$$E_{24} = \frac{\text{Height of emulsion layer (cm)}}{\text{Total height (cm)}} \times 100\%$$
Oil Spreading Test
In this test, distilled water was used as negative control and Triton X-100 was used as positive control as reported by Zhang et al., [15].

Temperature Stability
The cell-free broth was maintained at a constant temperature range of 4, 28, 70 and 120 °C for 60 min and cooled down at room temperature. The E24(%) of the treated RLs were measured against diesel oil [6].

Statistical Analysis
The data represents averages of at least three replicates. The results were represented as mean value ± standard deviation (MS office Excel 2010). The error bars on the graph were indicated as standard deviations [20].

Results and Discussion

Growth of Pseudomonas aeruginosa

*Pseudomonas aeruginosa* was used due to its rapid growth and exponential phase at 8-10 h in batch seed culture [21]. Base on Fig. 1, treatment III and IV shows higher growth rate compared to treatment I and II with maximum at 96 h. The short stationary hour (2.210 g/L) may be due to double substrate used. In treatment IV (SFO as second feeding at 8 h), the production of RLs increased sharply until 3.180 g/L at 120 h. More RLs was needed in treatment IV as the presence of hydrophobic substrate, SFO in the media during log phase.

RLs reduce surface tension of phase boundary by binding of hydrophilic head to cell surface and hydrophobic tail to the oil that makes the cell surface to become more hydrophobic. As a result, the substrates associate more easily to the cell [23]. The RLs production was observed as a growth associated since all treatments recorded the highest RLs production at 120 h even though this time (120 h) is not the time for maximum cell growth. This is because RLs are secondary metabolites as reported by Costa *et al.*, [24]. The higher concentration of RLs even after the decrease in growth of *P. aeruginosa* may be due to the release of cell bound RLs at the early stationary phase (120 h), which leads to the rise in extracellular RLs production in the medium [25].

![Fig 1. Growth of Pseudomonas aeruginosa for seven days. (◊), System I with 1% glucose; (□), System II with 1% SFO; (△), System III with both 1% glucose + 1% SFO (at 0 hour); (x), System IV with 1% glucose (at 0 hour) and 1% SFO (at 8 h).](image)

![Fig 2. Rhamnolipids production (g/L): (◊), System I with 1% glucose; (□), System II with 1% SFO; (△), System III with both 1% glucose + 1% SFO (at 0 hour); (x), System IV with 1% glucose (at 0 hour) and 1% SFO (at 8 h).](image)

**Effect of Different Fermentation Treatments on RLs Production**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RLs Production (g/L)</th>
<th>RLs Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment I</td>
<td>0.55</td>
<td>55</td>
</tr>
<tr>
<td>Treatment II</td>
<td>1.25</td>
<td>125</td>
</tr>
<tr>
<td>Treatment III</td>
<td>1.75</td>
<td>175</td>
</tr>
<tr>
<td>Treatment IV</td>
<td>3.18</td>
<td>318</td>
</tr>
</tbody>
</table>

Table 1 shows production of RLs (at 120 h) by *P. aeruginosa* using different substrates formulation. Treatment IV recorded the highest RLs concentration, 3.18 g/L. In comparison single substrate treatment, treatment II (SFO) produced higher amount of RLs compared to treatment I (glucose). It can be concluded that SFO was more effective to be used for RLs production compared to glucose indicating that RLs was produced for a function to solubilise SFO so that the substrate can be associated more easily for better bacterial consumption [Wei *et al.*, 2005; [26]. In addition, Mata-Sandoval *et al.* [27] found that hydrophobic substrates can assist in the production of greater amounts of RLs than hydrophilic substrates.

This study was supported by Thaniyavarn *et al.*, [28] that found linoleic acid (C18:2) can assist to the high yield of RLs. Treatment IV recorded the highest RLs production due to utilization and addition of sunflower oil at 8 h as during this stage number of cell were increased exponentially and the
requirement of carbon source were at high level. The strategy used in this treatment trigger the RLs and induced RLs production if compared to single substrate treatments.

**Table 1: Effect of different fermentation treatments on RLs production by Pseudomonas aeruginosa (at 120 h of fermentation time).**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rhamnolipids concentration (g/L) ± standard deviation (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.95 ± 0.04</td>
</tr>
<tr>
<td>II</td>
<td>1.71 ± 0.05</td>
</tr>
<tr>
<td>III</td>
<td>2.21 ± 0.05</td>
</tr>
<tr>
<td>IV</td>
<td>3.18 ± 0.03</td>
</tr>
</tbody>
</table>

**Emulsification Index (E24%) of Rhamnolipids (RLs)**

Fig. 3 shows emulsification index test of treatment IV against sunflower oil, diesel and kerosene after 24 h of fermentation. RLs produced from *P. aeruginosa* have emulsifying activity against all tested hydrophobic substrates. The emulsification can be achieved through the interaction of hydrophobic tail of RLs with hydrocarbons and the interaction of hydrophilic head with the water molecules. The high emulsion layer (white colour) indicates the greater emulsification activity.

**Table 2: Emulsification index (E24%) after 24 h of fermentation.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sunflower oil</th>
<th>Diesel</th>
<th>Kerosene</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>38.89 ± 1.92</td>
<td>55.56 ± 1.75</td>
<td>56.00 ± 0.00</td>
</tr>
<tr>
<td>II</td>
<td>42.22 ± 7.67</td>
<td>55.56 ± 5.09</td>
<td>54.44 ± 3.85</td>
</tr>
<tr>
<td>III</td>
<td>44.81 ± 1.70</td>
<td>56.67 ± 0.00</td>
<td>56.29 ± 0.64</td>
</tr>
<tr>
<td>IV</td>
<td>46.67 ± 0.00</td>
<td>60.00 ± 0.00</td>
<td>56.67 ± 0.00</td>
</tr>
<tr>
<td>Control</td>
<td>64.52 ± 0.00</td>
<td>64.52 ± 0.00</td>
<td>64.52 ± 0.00</td>
</tr>
</tbody>
</table>

**Fig. 4, Emulsification index (E24%) for treatment IV against, from left sunflower oil, diesel, and kerosene after 7 d of fermentation at room temperature.**

**Table 3: Relationship between RLs concentration (g/L) and diameter of clear zone.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of RLs (g/L)</th>
<th>Diameter Clear Zone (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.95 ± 0.04</td>
<td>2.33 ± 0.15</td>
</tr>
<tr>
<td>II</td>
<td>1.76 ± 0.05</td>
<td>4.57 ± 0.12</td>
</tr>
<tr>
<td>III</td>
<td>2.21 ± 0.05</td>
<td>4.90 ± 0.30</td>
</tr>
<tr>
<td>IV</td>
<td>3.18 ± 0.03</td>
<td>6.06 ± 0.12</td>
</tr>
</tbody>
</table>

**Effect of Temperature on Emulsification Activity**

Emulsification activity of all treatments was reduced due to instability of RLs when temperature increases, and it also affected by long-term storage (Table 4). Based on temperature factor alone, the emulsification activity of the RLs against diesel oil was quite stable from 4 to 120°C. This indicates the usefulness of biosurfactants reported in this study for the application in industries such as to enhanced oil recovery and it has great potential for biomedical applications due to its robust heat tolerance even after being submitted to autoclave sterilization [33]. This study was in accordance to Silva et al., [6] which also reported that activity of biosurfactants were not affected by extremes temperature.
Table 4: Temperature influence on emulsifying index (E24%) activity for RLs on diesel in 60 min.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4°C</th>
<th>28°C</th>
<th>70°C</th>
<th>120°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3.4±0.00</td>
<td>55.6±0.00</td>
<td>50.5±1.81</td>
<td>0.0±0.00</td>
</tr>
<tr>
<td>II</td>
<td>55.6±1.92</td>
<td>55.6±5.09</td>
<td>54.4±3.85</td>
<td>52.2±3.85</td>
</tr>
<tr>
<td>III</td>
<td>55.6±1.92</td>
<td>56.7±0.00</td>
<td>52.2±3.84</td>
<td>52.2±3.84</td>
</tr>
<tr>
<td>IV</td>
<td>56.7±0.00</td>
<td>60.0±0.05</td>
<td>56.7±0.00</td>
<td>56.7±0.00</td>
</tr>
<tr>
<td>Control</td>
<td>64.5±0.00</td>
<td>64.5±0.00</td>
<td>64.5±0.00</td>
<td>64.5±0.00</td>
</tr>
</tbody>
</table>

**Conclusion**

Incorporation of sunflower oil in production media enhanced the growth and rhamnolipids production by *Pseudomonas aeruginosa*. As hydrophobic substrate, sunflower oil could trigger the production of rhamnolipids in order to enhance its solubility. Therefore, the substrate can be associated more easily into *P. aeruginosa*. The use of glucose alone resulted in lower growth rate and rhamnolipids production. Glucose was highly soluble in the production medium as low production of rhamnolipids were obtained. Besides, high rhamnolipids production with the use of sunflower oil was due to the composition of sunflower itself. The high percentage of linoleic acid composition (about 60%) in sunflower oil may be responsible for the increase in rhamnolipids production. Dual substrate fermentation showed high growth of *P. aeruginosa* compared to single substrate. At the same time, high growth rate of *P. aeruginosa* also resulted in high rhamnolipids production in dual substrate treatment. The utilization of glucose as co-substrate was important for the growth initiation and addition of sunflower oil as second feeding enhanced the growth of *P. aeruginosa*. The production of rhamnolipids during stationary phase indicated the production of rhamnolipids as secondary metabolites. Based on this study, the rhamnolipids produced have ability to emulsify hydrocarbons such as diesel and kerosene. It also indicates the potential use of rhamnolipids in handling industrial emulsion. From the results, rhamnolipids produced have lower ability to emulsify vegetable oil compared to hydrocarbon due to the inability of the RLs to stabilize the microscopic droplets. However, it can still be suggested to other potential use in cosmetic and pharmaceutical industries. Besides, this study also found that the rhamnolipids were not affected by extremes temperature. Thus, this indicates the potential application in industries such as in microbial enhanced oil recovery as it has great potential for biomedical application due to its robust heat tolerance.

**ACKNOWLEDGEMENT**

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