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

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ABSTRACT

Biosurfactants are surface active compounds and amphiphatic in nature which consist of hydrophilic head and hydrophobic tail accumulating at the interphase of two immiscible liquid with different polarity. A study was conducted to investigate the effectiveness of sunflower oil in the production of rhamnolipids (RLs) by locally isolated *Pseudomonas aeruginosa* in shake flask fermentation. In this process, four different fermentation treatments were done for seven days at 30°C and 180 rpm. Sampling was carried out in time intervals of 24 h followed by monitoring of cell growth and biosurfactants production. Colorimetric Orcinol analysis was used for determination of RLs concentrations (g/L). The RLs were studied for emulsification activity using emulsification index (E24%) methods. In addition, oil displacement activity and thermal stability were also studied (4-120°C). All treatments allow the growth of *P. aeruginosa* and the utilization of sunflower oil as carbon source and glucose as growth initiator were observed to be the best strategy for maximum RLs production. The maximum RLs production was achieved after 120 h with 3.18 g/L of RLs. Diesel shows the highest emulsification activity among the substrate tested ranging from 55.56% - 60.00%. The oil displacement activity was corresponding to RLs concentration with stability up to 120°C (for 60 min). Therefore, from this research a good potential of RLs that may provide good application for industry were produced.

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 reach 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 varieties 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, cleansers, antimicrobial agents, mediators of enzyme action, in insect repellents, antacids, bath products, acne pads, antidandruff products, contact lens solutions, baby products, mascara, lipsticks, toothpaste, dentine cleansers to mention but a few [13]. The purpose of the study is to investigate the effectiveness 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).

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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 flask of 25 ml nutrient broth and incubated at 30ºC for 48 h followed by subcultured for two passages. Lastly, the cultures were transferred to nutrient slant agar and stored in the refrigerator for up to 3 months [16].

Media Preparation, Inoculum Development and Operating Conditions

Nutrient agar (NA) was used for plating [17] and nutrient broth (NB) (seed media) for inoculums development [14] were prepared at the pH 7.0±0.2, 25ºC. The composition (g/L) of production medium was prepared according to Zhang *et al.* [15]. The pH of medium was adjusted to 7.0 with 1 M sodium hydroxide (NaOH) or 1 M hydrochloric acid (HCl). Glucose (1% w/v) and sunflower oils (1% v/v) were used as carbon sources. Glucose and production media were autoclaved together, and sunflower was autoclaved separately. One loopful of culture was inoculated into 20 ml of NB in 100 ml flask and was incubated at 30°C and stirred in rotary shaker at 180 rpm for 10 h [14, 15].

Biosurfactant Production and Operating Conditions

Four different treatments were conducted for the production of biosurfactants including single and dual substrate treatment by using glucose (1%) and sunflower oil (1%) as carbon sources as followed: System I: Inoculums (5 % v/v) was transferred from NB media into 500 ml flask containing 250 ml of production media with 1% w/v glucose as sole carbon source. The culture was maintained at 30°C in a rotary shaker (180 rpm, 7 days). System II: Sunflower oil (1 % v/v) was added as sole carbon source into 500 ml flask containing 250 ml of production media at 0 h and inoculated with 5 % (v/v) of inoculums. The culture was maintained at 30°C (180 rpm, 7 days). System III: Sunflower oil (1% v/v) was added into 500 ml flask containing 250 ml production media with 1% w/v glucose as co-substrate at 0 h and inoculated with 5% (v/v) of inoculums. The culture was maintained at 30°C (180 rpm, 7 days). System IV: In the first batch, inoculums (5 % v/v) was transferred from NB media into 500 ml flask containing 250 ml of production media formulated with 1% (w/v) of glucose as first feeding (during 0 h). During 8 h of incubation time, sunflower oil (v/v) was added as second feeding. The culture 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 pipette 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) Extraction

Crude RLs was obtained using solvent extraction method. A culture broth (500 µl) was centrifuged (10,000 rpm, 20 min) and 400 µl of supernatant was taken and mixed with 750 µl diethyl ether. The mixture was shaken using vortex for 3 min. The organic top layer was transferred to microcentrifuge tube. The process was repeated twice, and the ether fractions were pooled and evaporated to dryness. Phosphate buffer (pH 8) was used to redissolve the precipitate left. Desired dilution was adjusted by addition of buffer (400 µl of buffer added for an original sample volume of 400 µl would equal to 1x dilution) [16].

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 ºC 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 (E_{24}%)

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].

$$E_{24} = \frac{\text{Height of emulsion layer (cm)}}{\text{Total height (cm)}} \text{X100\%}$$

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 phase showed by treatment I and II was due to the carbon limitation as suggested by Reis *et al.* [12]. After 8 h of fermentation, the *P.* aeruginosa reached exponential phase as it adapted to the environment [15]. For treatment with SFO, the lower growth rate at an early stage of fermentation may be due to the β-oxidation to produce more RLs [22]. During 12 h of fermentation period, growth of *P. aeruginosa* in treatment IV was higher compared to treatment III even though both were supplied with same amount of glucose (1%) and sunflower oil (1%). The strategies in treatment IV which glucose was added at 0 h as growth initiator and sunflower oil was added at 8 h as second feeder was enhanced the growth of bacteria as rapid increase in cell density can be observed [8].

At the early fermentation period, oily droplets were observed on the surface of fermentation media and after a few h they were replaced by tiny droplets as a simple indicator to a presence of RLs. The tiny droplets have increased surface area of substrate and make it easily to be consumed by *P. aeruginosa* and disappearance of oil droplets can be related to the production of RLs [14].

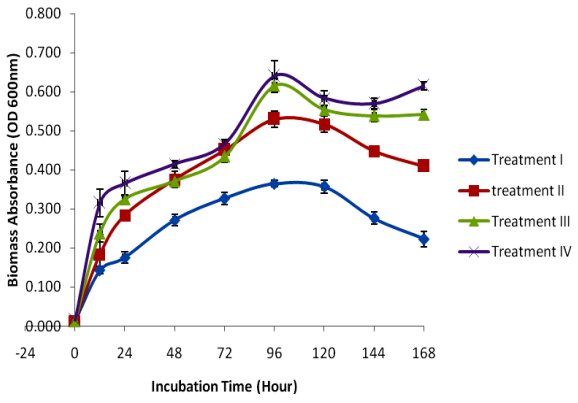

Fig 1. Growth of *Pseudomonas aeruginosa* for seven days. (◊),System I with 1% glucose; (□),System II with 1% SFO; (Δ),System III with both 1% glucose + 1% SFO (at 0 hour); (x), System IV with 1% glucose (at 0 hour) and 1% SFO (at 8 h).

Rhamnolipids (RLs) production by *Pseudomonas aeruginosa*

Fig. 2 shows RLs production by *P. aeruginosa* in seven days of fermentation period. Treatment I (glucose) and (II) (SFO) show the production reached maximum level at 0.944 and 1.710 g/L respectively (120 h). In treatment III (SFO + glucose, added at 0 h), the production of RLs increased at 24 h (1.301 g/L) and 120 h (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].

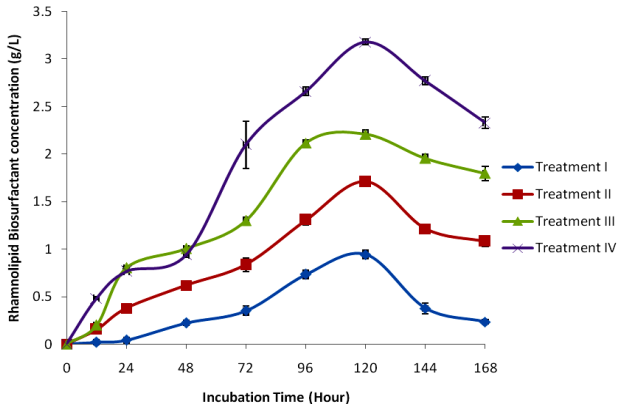

Fig. 2. Rhamnolipids production (g/L): (◊), System I with 1% glucose; (□), System II with 1% SFO; Δ, System III with both 1% glucose + 1% SFO (at 0 hour); (x), System IV with 1% glucose (at 0 hour) and 1% SFO (at 8 h).

Effect of Different Fermentation Treatments on RLs Production

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).

Treatment	Rhamnolipids concentration (g/L) ± standard deviation (n=3)
I	0.95 ± 0.04
II	1.71 ± 0.05
III	2.21 ± 0.05
IV	3.18 ± 0.03

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.

Fig. 3. Emulsification index (E24%) for treatment IV against, from left, sunflower oil, diesel and kerosene after 24 h of fermentation.

Table 2 shows emulsification index (E24%) of RLs produced by *P. aeruginosa* against sunflower oil, diesel and kerosene. All showed comparable result against Triton-X 100 (control). Treatment IV shows the highest percentage against all substrate tested compared to other treatment as it has the highest RLs concentration (**Table 2**). Generally, the results obtained shows that the RLs produced were more efficient to emulsify diesel and kerosene compared to sunflower oil. Sunflower oil recorded the lowest emulsifying activity (31-47%) and diesel recorded the highest (55.56-60.00%) may be due to the characteristics of RLs which contain more hydrophobic congener. This study in accordance to Abbasi *et al*., [7] and Abouseoud *et al*., [29] which reported that diesel and kerosene were the best substrate of emulsification activity for RLs that produced from *Pseudomonas sp*. From the results, it can be concluded that diesel and kerosene were good substrates for emulsification with emulsification index above 50%.

The ability of the RLs to emulsify diesel and kerosene indicates the potential use to facilitate the treatments of hydrocarbon in environment. Thus, it can be used in handling industrial emulsion and biodegradation as suggested by Kosaric, [30]. As diesel shows the highest emulsification index (E24%), diesel was selected to be the substrate tested for thermal stability test.

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

Treatment	Sunflower oil	Diesel	Kerosene
I	38.89 ± 1.92	55.56 ± 1.75	56.00 ± 0.00
II	42.22 ± 7.67	55.56 ± 5.09	54.44 ± 3.85
III	44.81 ± 1.70	56.67 ± 0.00	56.29 ± 0.64
IV	46.67 ± 0.00	60.00 ± 0.00	56.67 ± 0.00
Control	64.52 ± 0.00	64.52 ± 0.00	64.52 ± 0.00

Fig. 4 shows emulsification activity of treatment IV against sunflower oil, diesel and kerosene at room temperature. The stability of the emulsion formed was reduced after 7 days may be due to low molecular weight of RLs molecule which develops emulsion with low stability [31].

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

Oil Displacement Activity of Rhamnolipids (RLs)

Diameter of clear zone on oil surface was proportional to the concentration of RLs in the solution (**Table 3**). Culture with RLs concentration, 3.18 g/L from treatment IV shows the highest ability to displace diesel (clear zone, 6.06 cm). This study was in accordance to Morikawa *et al.*, [32] as they found the diameter of the clear zone formed by a surfactant-containing solution is directly proportional to the amount of biosurfactants used.

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

Treatment	Concentration RLs (g/L)	Diameter Clear Zone (cm)
I	0.95 ± 0.04	2.33 ± 0.15
II	1.76 ± 0.05	4.57 ± 0.12
III	2.21 ± 0.05	4.90 ± 0.30
IV	3.18 ± 0.03	6.06 ± 0.12

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.

Treatment	4 ºC	28 ºC	70 ºC	120 ºC
I	3.3±0.00	55.6±0.00	50.5±1.81	0.0±0.00
II	55.6±1.92	55.6±5.09	54.4±3.85	52.2±3.85
III	55.6±1.92	56.7±0.00	52.2±3.84	52.2±3.84
IV	56.7±0.00	60.0±0.00	56.7±0.00	56.7±0.00
Control	64.5±0.00	64.5±0.00	64.5±0.00	64.5±0.00

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 it 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 solubile 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 initiator 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 if 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.

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