Emulsification Characteristics of Rhamnolipids by *Pseudomonas aeruginosa* Using Coconut Oil as Carbon Source

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ABSTRACT  
Rhamnolipids (RLs) production using coconut oil as a carbon source by the bacterium *P. aeruginosa* is studied. This bacterium was grown in media containing 1% carbon source (glucose/coconut oil). The RLs were characterized by emulsification index (E₂₄), thermal stability and oil spreading test. Further RLs quantification was carried out by the orcinol assay with L-rhamnose as the standard. The result showed that the highest production of RLs occurred in the presence of both coconut oil and glucose at 96 h (2.51 g/L). A stable emulsification index (E₂₄) was observed using diesel with a maximum value of 57% at room temperature. Good stability to high temperature (120 °C) was observed when exposed at 55%. Oil displacement activity showed the presence of RLs with the highest value was at the highest RLs production. This study shows *P. aeruginosa* is able to produce RLs using coconut oil as the substrate and may potentially become a good source of biosurfactant for industry in the future.

KEYWORDS  
rhamnolipids  
biosurfactants  
Pseudomonas aeruginosa  
rhamnose  
coconut oil

INTRODUCTION  
Rhamnolipids (RLs) are unique secondary metabolites with hydrophilic and hydrophobic properties in the similar compound. In industries, RLs surfactants are used as emulsifier, foaming agent, wetting agent, adhesive, lubricant and penetrant [1]. For consumer’s final products, they have been used in detergent, shampoos and toothpaste. The RLs have found to be a niche in personal care market because of their moisturizing properties and skin compatibility. RLs are excreted extracellularly by diverse groups of microorganisms such as bacteria, fungi and yeast leading to their diverse structure and surface properties.

Currently, they are produced mainly by bacteria such as *Pseudomonas aeruginosa*. The chemical structures of RLs depend on the treatment given during bacterial growth and the media culture composition such as carbon, nitrogen, phosphate, reaction temperature, pH media and rate of oxygen supply. As compared to synthetic surfactants, RLs possess several advantages including high biodegradability, low toxicity, low irritancy and good compatibility with human skin [2]. Due to these superior characteristics, RLs have also shown the potential application in environmental management and petroleum-related industries such as cleaning oil spills, enhance oil recovery and crude oil removal from oil sludge. Rhamnolipids (RLs) are a type of Biosurfactants (BSs) with a head contains monosaccharide, disaccharides or polysaccharides while the tail contains saturated or unsaturated fatty acids with unique nature properties to reduce the surface and interfacial tension [3]. They tend to accumulate at the interface between two immiscible fluids (oil and water) due to their amphiphilic nature, and, as a result, the two immiscible fluids can be well blended [4]. Nowadays, the concern about environmental protection has considered RLs as alternatives to synthetic surfactants (SSs). The RLs are anions above pH 4.0 since
one of the carboxylic acids is free. *P. aeruginosa* is considered the primary producing RLs of the *Pseudomonas* species [5]. *Pseudomonas* strains produce two major types of RLs in a liquid medium: mono-rhamnolipid (Rha-C10-C10) and di-rhamnolipid (Rha-Rha-C10-C10) [6]. It has been proposed as a promising strain for a large-scale production of biosurfactants [7]. In a study for carbon source substrate for RLs production, one of the important parameters is the type of fatty acids of oils used such saturation level and the length of the fatty acids molecules in the triglycerides.

These properties influence the production of different lipid precursors that require the RLs synthesis [8]. Base on the above facts, this study was conducted to evaluate the effect of coconut precursors that require the RLs synthesis [8].

**MATERIALS AND METHODS**

Rhamnolipids (RLs) producing bacteria (*P. aeruginosa*)

*P. aeruginosa* was obtained from Nordin et al. [9]. The isolated potential bacteria producing RLs was identified as *Pseudomonas aeruginosa* with the probability of 99% upon analysis using Biolog Gen III microplate. The strain was streaked on nutrient agar (NA) plates and incubated at 30 °C for 24 h.

**Storage of *P. aeruginosa* culture**
The strain of *P. aeruginosa* was maintained based on the method used by Ballot (2009) [10] with slight modification. The culture was stored in 30% (v/v) glycerol at -20 °C and recovered by transferring a loop full to a flask containing 25 ml of nutrient broth (NB). The culture was then incubated at 30°C for 48 h. The plates were prepared from the broth, and the subcultures were made for two to three passages. Finally, the cultures were transferred to nutrient slant agar, which was then stored in a refrigerator for up to a month.

**Nutrient agar (NA) preparation**
The NA powder (20 g) was suspended in distilled water (1 L) and stirred as reported by Ali et al. [11]. The initial pH was adjusted to 7.0 using sodium hydroxide (NaOH) or hydrochloric acid (HCl). The NA was autoclaved at 121 °C for 15 min. The warm solution was poured into plates and allowed to cool at room temperature (25 °C) before streaking.

**Nutrient broth (NB) preparation**
The NB powder (8 g) was suspended in distilled water (1 L) and stirred until the solutions mixed well [9]. The initial pH was adjusted to 7.0 after titration using NaOH or HCl before sterilization at 121°C and 15 psi for 15 min. The warm solution was poured into 100 ml flask and allowed to cool at room temperature (25 °C) before inoculation.

**Media preparation**
The composition (g/L) of basal mineral salt (BMS) was prepared according to Zhang et al. [12]. Initial pH was adjusted to 7.0 using NaOH or HCl. The media was autoclaved at 121 °C, 15 psi for 20 min and left to cool at room temperature.

**Carbon source preparation**
Glucose (1% w/v) and coconut oil (1% v/v) were used for carbon source preparation. Coconut oil was autoclaved separately prior addition to the production media (with glucose).

**Inoculum development and operating conditions**
A loop full of the *P. aeruginosa* culture was inoculated into 20 ml of NB as seed media in a flask, incubated at 30 °C and stirred in rotary shaker (LabCompanion/IS-971R) at 180 rpm/min for 10 h [9, 12].

**Operating conditions for the production of RLs**
Different types of fermentation treatments were carried out to evaluate the production of RLs which also consists of a single substrate (glucose or coconut oil) and dual substrates (glucose and coconut oil) as carbon sources. The cultures were maintained at 30 °C in a rotary shaker at 180 rpm/min and left for seven days as below:

**Treatment 1**: Inoculums 5% (v/v) was transferred from NB into 500 ml flask containing 250 ml media formulated with 1% (w/v) glucose at 0 h as a sole carbon source.

**Treatment 2**: Inoculums 5% (v/v) was transferred from NB into 500 ml flask containing 250 ml media formulated with 1% (v/v) coconut oil at 0 h as a sole carbon source.

**Treatment 3**: Inoculums 5% (v/v) was transferred from NB into a flask containing 250 ml media formulated with 1% (w/v) glucose and 1% (v/v) coconut oil at 0 h as mix-substrate.

**Treatment 4**: Inoculums 5% (v/v) was transferred from NB into 500 ml flask containing 250 ml media formulated with 1% (w/v) glucose as first substrate feeding. After eight h of incubation, 1% (v/v) coconut oil was added into media as second substrate feeding.

A flask containing only production media (without inoculums) was incubated along and used as a parallel control throughout the experiment. The sampling processes were carried out at appropriate time intervals (every 24 h). After the fermentation period was over (seven days), the whole flasks were removed from the shaker.

The samples were aseptically taken from the production media in laminar air flow (CFM 4/ ERLA). The samples were analyzed and monitored for cell growth, and the RLs production was measured by orcinol assay, quantitative emulsification index (E43), oil spreading test and temperature stability characterization. The experiment was conducted in triplicate, and the average reading was obtained and recorded [12].

**Determination of cell growth profile**
Spectrophotometry or Optical density (OD) was used as an indirect measurement of cell concentration. The cell growth profiles of *P. aeruginosa* culture in NB and in production media for four types of treatments were measured as reported by Nordin et al. [9].

**Spectrophotometry of culture in production media**
Optical density was used to measure the cell concentration in each fermentation treatments. The growth of *P. aeruginosa* was monitored at a wavelength of 600 nm. The samples were taken at regular intervals (24 h). A volume of 1.0 ml sample was pipetted into micro centrifuge tube and centrifuged at 10,000 rpm for 20 minutes. The cell pellet was suspended in 1.0 ml of distilled water, and the mixture was blended using a vortex. The optical readings were taken in a glass cuvette [9].
Extraction of RLs
Crude RLs were obtained using solvent extraction method for indirect quantification of RLs. The supernatant (400 μl) was mixed with 750 μl diethyl ether in micro centrifuge tube. The mixture was mixed using vortex for 3 min. The organic phase (top layer) was taken gently using micro pipette and transferred to a new tube without removing any of the aqueous phase (bottom layer).

The solvent addition and extraction were repeated twice, and the ether fractions were pooled and evaporated to dryness for 8 h in a fume hood. A phosphate buffer of pH 8 was used to dissolve the precipitate left in the tubes [10].

Orcinol assay
The orcinol assay was used for the direct assay of the number of glycolipids presents in the sample [10]. Extracellular glycolipids concentration was evaluated in triplicate by measuring the concentration of Rh. The orcinol reagent was prepared by adding concentrated sulphuric acid, H₂SO₄ (98% w/w) and 0.19% orcinol (3,5-dihydroxytoluene) to distilled water. The final concentration of acid was 53% w/w. For a total of 20 ml orcinol reagent, 0.038 g orcinol, 9.4 ml of distilled water and 10.6 ml of concentrated acid was maintained at a constant temperature of 4, 28, 70 and 120 °C for 60 min., and finally was left to cool at room temperature. Diesel oil was used for the measurement of E₂₄ [16].

Statistical analysis
The data represent the arithmetical averages of triplicates for each treatment. The results were represented as mean value ± standard deviation (SD). The statistical analysis was performed using MS office Excel 2007 for calculation of mean, standard deviation and standard error. The error bars on the graph were indicated the standard deviations as suggested by Saravanan and Vijayakumar [17].

RESULTS AND DISCUSSION
Preparation of Pseudomonas aeruginosa culture
Fig. 1 shows the culture of P. aeruginosa which was grown using the streak plate technique. P. aeruginosa was selected due to rapid growth and the ability to reach exponential phase at 8-10 h especially in batch culture. Base on a previous study, high production yields of RLs was reported by Zhang et al. [12] using similar microorganism and consequently, a good potential for commercial exploitation can be obtained.

![Fig. 1. Pseudomonas aeruginosa culture on nutrient agar.](image)

Culture and medium conditions
P. aeruginosa was supplemented with phosphate from KH₂PO₄ and K₂HPO₄, while the source of potassium was KCl, as suggested by Zhang and Dequan, (2013) [18]. The agitation speed was 180 rpm to inhibit an anaerobic condition. Nitrate was used as final electrons acceptors for cellular respiration. The temperature, 30 °C was used to decrease the viscosity and increase the solubility of media [8] that can assist in the increase of the biodegradation and diffusion rate of the hydrophobic substrate.

Kinetic growth profile of Pseudomonas aeruginosa in nutrient broth (NB)
Fig. 2 shows the growth curve of P. aeruginosa after 32 h incubation during inoculum preparation. From 2 h to 8 h, the OD value increased slightly and constant until 14 h. After this period the OD increased for a second time from 14h to 22 h before...
decreased slightly to 28 h. The trend line obtained was similar to
the growth profile obtained by El-Amine Bendaha et al. [19].
Based on the previous report, the best culture time (exponential
phase) for inoculum preparation was at 8 h [9]. Our study showed
that the exponential phase could be reached after 10 h. Thus, the
preferred culture time for the P. aeruginosa strain in this study
was considered at 10 h.

Fig. 2. Standard growth curve of Pseudomonas aeruginosa in nutrient
broth (NB).

**Kinetic growth profile of Pseudomonas Aeruginosa in
production media**

Fig. 3 shows the growth of P. aeruginosa in a different treatments
condition. For Treatments 1 to 3, the OD value increased rapidly
from 12 h to the maximum 96 h and decreased slightly after 96 h.
The triglycerides of coconut oil are a less polar compound
(insoluble), and the glucose (soluble) is highly polar. Thus longer
time was needed for the consumption of the less polar substrate
if compared to the more polar substrate. In the present of
insoluble substrates in the media, better production of the RLs
can be observed similar to the finding by Desai and Ibrahim [20].

The growth rate of P. aeruginosa in Treatment 2 (single
substrate) was slightly lower than that in Treatment 3 and 4 (dual
substrates). Overall higher growth rate can be observed in
Treatment 4 may be due to the double addition time level of
carbon sources (at 0 and 8 h) (Fig. 2). Perhaps this is the reason
why higher growth rate indicating to the rapid cell increase can
be observed as suggested by Ali et al. [21] (from 12 to 48 h,
during exponential phase).

For Treatments 2, 3 and 4, the stationary phase was reached
between 48 to 72 h may be due to the production of secondary
metabolites (RLs) causing the division of the cell to increase
the cell density. In Treatment 4, after 96 h the growth was less
decided until the end of the incubation period if compared to
other treatment indicating to the utilization of second feeding
carbon source (coconut oil).

During the early stage of fermentation, large oily droplets or
oil layer appeared on the surface of production media and some
of them attached to shake flask wall. They dispersed gradually
into tiny oil droplets and disappeared after several h. The
transformation of oil into tiny droplets has increased the surface
area of the substrate and, therefore, it can be consumed easily by
P. aeruginosa for metabolism and conversion into respected
metabolites. Nordin et al. [21] suggested that the disappearance
of oil droplets indicated to the RLs production in order to
emulsify the coconut oil substrate for better accessibility of the
P. aeruginosa to their carbon source consumption.

Fig. 3. Kinetic growth of P. aeruginosa in four different treatments; (○),
Treatment 1-1% glucose; (□), Treatment 2-1% coconut oil; (△),
Treatment 3-1% glucose and 1% coconut oil (at 0 h); (×), Treatment 4-
1% glucose (at 0 h) and 1% coconut oil (at 8 h).

3.5 **Quantification of rhamnolipids (RLs)**

The concentrations of RLs were determined using the orcinol
method. The patterns of RLs production (from 0 – 168 h) were
close to each other for all treatments indicating to the production
of RLs to enhance the solubility of the substrates through the
reduction of the substrate surface tension. This is due to the
binding of the hydrophilic head to a cell surface and hydrophobic
tail to the oil resulting in the cell surface to become more
hydrophobic. The substrates associate more easily as the result of
increasing the direct contact between the cell and the soluble
substrate [22]. Overall, the RLs production was observed to be
growth associated with P. aeruginosa, and the cell growth kinetic
was seemed to be proportional to the RLs production. According
to Rahman et al. [13], the maximum RLs obtained was within 96
h of the incubation period. After 96 h incubation, the production
decreased in all treatments due to the decrease in cell density
decreasing to the approach of the death phase [23].

![Fig. 4. Rhamnolipids production (g/L) by P. aeruginosa. (○), Treatment
1: 1% glucose; (□), Treatment 2: 1% coconut oil; (△), Treatment 3: 1%
glucose and 1% coconut oil (both at 0 h); (×), Treatment 4: 1% glucose
(at 0 h) and 1% coconut oil (at 8 h).](image-url)
Table 1 shows higher RLs concentration in Treatment 2 if compared to Treatment 1 showing that the use of less soluble substrate (coconut oil) aggressively triggered the production of RLs. In this case, *P. aeruginosa* needs to enhance the solubility of coconut oil for carbon consumption during the active growth phase/ log phase [24]. Treatment 4 showed the highest RLs concentration might be due to the supplementation of coconut oil as second feeding at 8 h gave second-time trigger for the growth of *P. aeruginosa* to produce more RLs [25]. As a result, the dual substrate treatments have higher RLs concentration compared to the single substrate treatments which are in a good agreement with Maier and Sober’on-Ch’avez, (2000) [1] that reported the use of insoluble substrate was more effective for RLs production.

**Table 1. Different fermentation treatments and rhamnolipids (RLs) production by *Pseudomonas aeruginosa*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Substrate Time</th>
<th>Rhamnolipids Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose only</td>
<td>0.944±0.04</td>
</tr>
<tr>
<td>2</td>
<td>Coconut oil only</td>
<td>1.699±0.02</td>
</tr>
<tr>
<td>3</td>
<td>Glucose &amp; coconut oil, 0 h</td>
<td>2.069±0.03</td>
</tr>
<tr>
<td>4</td>
<td>Glucose, 0 h; coconut oil, 8 h</td>
<td>2.513±0.03</td>
</tr>
</tbody>
</table>

**Quantitative emulsification index ($E_{24}$) test**

Fig. 5 shows emulsification ability of the RLs by *P. aeruginosa* on the hydrophobic substances after one day incubation period at room temperature. The compact emulsion layer (white cloudy layer) indicates to the stable emulsification system. Fig. 6 shows the emulsification index ($E_{24}$) for Treatment 4 against diesel, coconut oil and kerosene after 7 days of incubation at room temperature. The stability decreased since low molecular weight RLs were unable to produce stable emulsion compared to other high molecular weight RLs which primarily act as emulsion stabilizer [26].

- **Table 2. Emulsification index ($E_{24}$) for hydrophobic substrates of cell-free broth containing rhamnolipids (RLs) from *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diesel</th>
<th>Coconut oil</th>
<th>Kerosene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment 1</td>
<td>54.0±1.66</td>
<td>38.9±1.92</td>
<td>51.0±1.80</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>54.5±1.03</td>
<td>43.0±1.57</td>
<td>54.4±3.85</td>
</tr>
<tr>
<td>Treatment 3</td>
<td>56.7±0.00</td>
<td>44.2±0.81</td>
<td>56.0±0.00</td>
</tr>
<tr>
<td>Treatment 4</td>
<td>57.5±0.00</td>
<td>44.6±0.81</td>
<td>56.3±0.64</td>
</tr>
<tr>
<td>1% Triton-X 100 (positive control)</td>
<td>64.5±0.00</td>
<td>64.5±0.00</td>
<td>64.5±0.00</td>
</tr>
</tbody>
</table>

**Oil spreading test**

The results show that the diameter of the clear zone is proportional to the RLs concentration. Even, oil expelling circles ranged from 2.4 cm to 5.4 cm can still be obtained only by using five µl sample (Table 3). The highest oil displacement activity was given by the highest RLs concentration with a diameter of 5.4 cm while the lowest oil displacement activity was given by the lowest RLs concentration with a diameter of 2.4 cm. The results from this study were also in accordance to Morikawa et al. [28] that reported the diameter of the clear zone is directly proportional to the concentration of the RLs.

- **Table 3. The relationship between RLs concentration (g/L) produced by *Pseudomonas aeruginosa* with a diameter of a clear zone measured by the oil spreading technique.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Substrate</th>
<th>Time addition (h.)</th>
<th>concentration of RLs (g/L)</th>
<th>diameter of clear zone (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose</td>
<td>0</td>
<td>0.944±0.04</td>
<td>2.4±0.15</td>
</tr>
<tr>
<td>2</td>
<td>Coconut oil</td>
<td>0</td>
<td>1.699±0.02</td>
<td>4.6±0.12</td>
</tr>
<tr>
<td>3</td>
<td>Glucose &amp; Coconut oil</td>
<td>0</td>
<td>2.069±0.03</td>
<td>5.1±0.15</td>
</tr>
<tr>
<td>4</td>
<td>Glucose &amp; Coconut oil</td>
<td>0 &amp; 8</td>
<td>2.513±0.03</td>
<td>5.4±0.11</td>
</tr>
</tbody>
</table>

**Temperature stability characterization**

Low $E_{24}$ was observed in Treatment 1 (Table 4) indicating to the low RLs activity at low temperature. All treatments showed as the temperature increased (28 °C to 120 °C), the emulsification ability decreased. The highest percentage of emulsifying activity ($E_{24}$) was observed to be 57.56% at 28 °C. The emulsification activity of the cell-free broth containing RLs against diesel was considered stable and heat tolerance at high temperatures (120 °C) and unstable at low temperature (28 °C).

This indicates to the usefulness of the RLs as a robust heat tolerance compound for bioremediation such as for oil recovery problem [29]. This study was in accordance with Silva et al. [16] which also reported that the activity of RLs was not affected by extreme temperature.
Table 4. The emulsifying activity E(2) (%) of the rhamnolipids from *Pseudomonas aeruginosa* in different temperature storage.

<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>Treatment 1</td>
<td>23.33±1.66</td>
<td>54.08±1.66</td>
<td>50.47±1.81</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>(Glucose, 0 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment 2</td>
<td>30.11±1.80</td>
<td>54.55±3.03</td>
<td>52.22±3.85</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>(Coconut oil, 0 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment 3</td>
<td>41.04±1.80</td>
<td>56.67±0.00</td>
<td>56.00±0.00</td>
<td>51.04±1.80</td>
</tr>
<tr>
<td>(Glucose &amp; coconut oil, 0 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment 4</td>
<td>44.44±3.85</td>
<td>57.56±0.00</td>
<td>56.00±0.00</td>
<td>50.00±1.81</td>
</tr>
<tr>
<td>(Glucose, 0 h, coconut oil, 8 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% Triton-X 100 (positive control)</td>
<td>64.52±0.00</td>
<td>64.52±0.00</td>
<td>64.52±0.00</td>
<td>64.52±0.00</td>
</tr>
</tbody>
</table>

CONCLUSION

RLs production was associated with the growth of *Pseudomonas aeruginosa*. The increase of RLs production was obtained with the incorporation of coconut oil in the fermentation media. A dual substrate system, combining glucose and coconut oil would enhance the growth of *Pseudomonas aeruginosa* if compared to a single substrate system (glucose or coconut oil alone) proportional to the increase of carbon source. The dual system with glucose as initiator feeding and coconut oil as second feeding (separate feeding system) enhanced the RLs production which is better than single feeding. The RLs produced have a good ability to emulsify hydrocarbons. The high emulsifying activity of the RLs indicated the potential use in bioremediation application. This study found that the RLs were not affected by extremes of temperature. Due to their robust heat tolerance, they can contribute to a good potential application in industries such as in microbial oil recovery and in bioremediation application.

REFERENCES