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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 (E_{24}), 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 (E_{24}) was observed using diesel with a maximum value of 57% at room temperature. Good stability to high temperature (120 ºC) 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.

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 (Ss). 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-C_{10}-C_{10}) and di-rhamnolipid (Rha-Rha-C_{10}-C_{10}) [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 oil which is rich in the medium chain as well as saturated fatty acids and the influence contributed by selected physical factors such as pH, temperature and reaction condition.

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 ºC 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 ºC and recovered by transferring a loop full to a flask containing 25 ml of nutrient broth (NB). The culture was then incubated at 30ºC 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 ºC for 15 min. The warm solutions were poured into plates and allowed to cool at room temperature (25 ºC) 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 121ºC and 15 psi for 15 min. The warm solution was poured into 100 ml flask and allowed to cool at room temperature (25 ºC) 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 ºC, 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 ºC 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 ºC 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 (E_{24}), 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, H_2SO_4 (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 H_2SO_4 were added. In the preparation of the sample to be analyzed for RLs concentration, 900 µL of orcinol reagent was added to 100 µL of sample.

Blanks of 100 µL of phosphate buffer were also treated with orcinol reagent. The standard solutions of rhamnose between 0 and 100 mg/l were prepared using phosphate buffer. The sample tubes for all samples, blank and standards were immersed in a water bath o 100 ºC for 20 min. The tubes were then left to cool at room temperature in a dark cupboard for 30 min. Before measuring the Rh concentration in the sample, a zero point was established with blanks (absorbance 421 nm).

Preparation of standard curve
A standard curve was plotted by measuring the optical density of the L-rhamnose standards followed by measuring the Rh concentration in the samples accordingly. The curve was constructed for each batch of samples as freshly prepared orcinol reagent was needed for each batch of analyses [10]. The RLs concentrations were calculated and expressed as rhamnose equivalents, RE (g/L) by multiplying Rh values with a coefficient of 3.0 for RLs [13].

Quantitative emulsification index (E_{24})
The ability of the RLSs to emulsify liquid hydrocarbons such as diesel oil, kerosene, and coconut oil was determined. Cell-broth (2.0 ml) containing RLs was added into each test tube containing 2.0 ml of hydrophobic substances. The mixture was shaken vigorously using vortex for two min. The emulsion was left to equilibrate at room temperature for 24 h. The emulsion index (E_{24}) was determined as the height of the emulsion layer divided by the total height and multiplied by 100 [14, 15].

E_{24} (%) = (*Height of emulsion layer, cm* / *Total height, cm*) x 100%

Oil spreading test
Distilled water (30.0 ml) was filled into a petri dish with a diameter of 25.0 cm. Diesel (50 µl) was dropped onto the water surface to form oil film. The free supernatant (5µL cell supernatant diluted by 10 times with water) was dropped in the midst of the oil film. The diameter of the clear zone expelling circles formed was measured. Distilled water was used as negative control and Triton X-100 as a positive control [12]. The diameter of the clear zone obtained from the cell-free supernatant was compared with the control.

Temperature stability
The effects of different temperatures on the performance and thermal stability of RLs were evaluated. The cell-free broth 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 E_{24} [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.

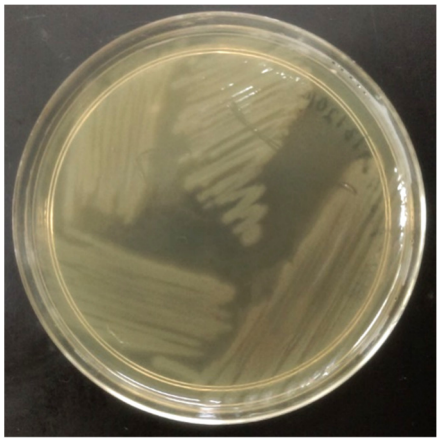

Fig. 1. *Pseudomonas aeruginosa* culture on nutrient agar.

Culture and medium conditions
P. aeruginosa was supplemented with phosphate from KH_2PO_4 and K_2HPO_4, 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 ºC 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.

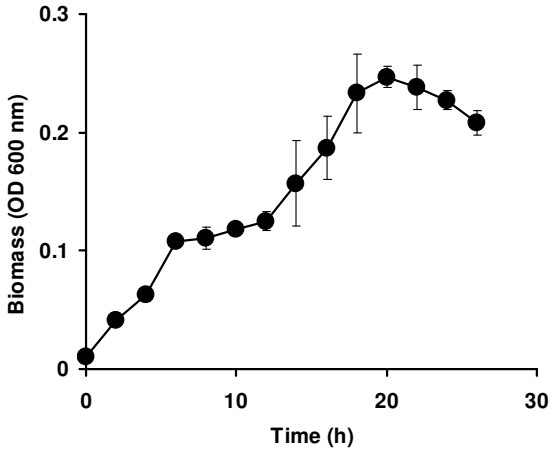

Fig. 2. Standard growth curve of *Pseudomonas aeruginosa* in nutrient broth (NB).

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 decreased 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.

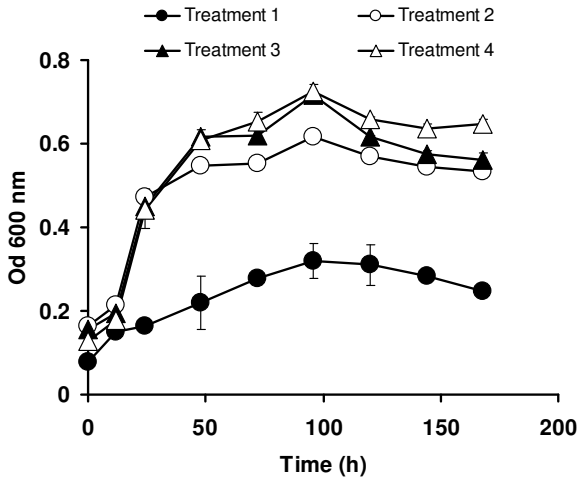

Fig. 3. Kinetic growth of *P. aeruginosa* in four different treatments: (◊), Treatment 1-1% glucose; (□), Treatment 2-1% coconut oil; (Δ), Treatment 3-1% glucose and 1% coconut oil (at 0 h); (x), Treatment 4-1% glucose (at 0 h) and 1% coconut oil (at 8 h).

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 indicating to the approach of the death phase [23].

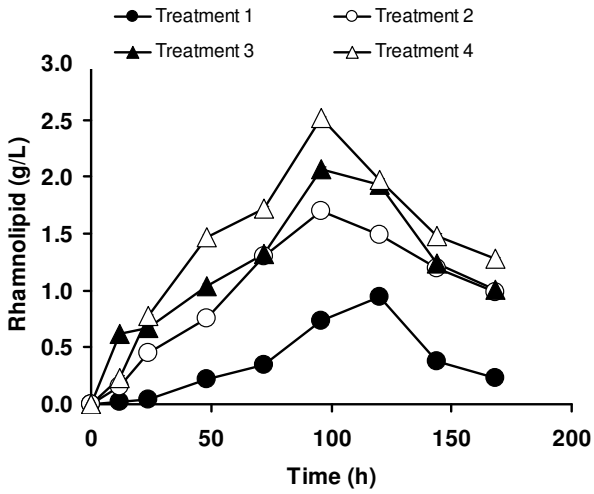

Fig. 4. Rhamnolipids production (g/L) by *P. aeruginosa*: (◊), *Treatment* 1: 1% glucose; (□), *Treatment* 2: 1% coconut oil; (Δ), *Treatment* 3: 1% glucose and 1% coconut oil (both at 0 h); (x), *Treatment* 4: 1% glucose (at 0 h) and 1% coconut oil (at 8 h).

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***.**

Treatment	Substrate	Rhamnolipids Concentration (g/L)
1	Glucose only	0.944±0.04
2	Coconut oil only	1.699±0.02
3	Glucose & coconut oil, 0 h	2.069±0.03
4	Glucose, 0 h; coconut oil, 8 h	2.513±0.03

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].

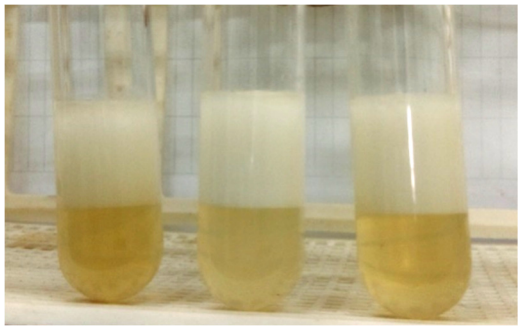

Fig. 5. Emulsification index test (E_{24}) of *Treatment* 4. From left: diesel, coconut oil and kerosene

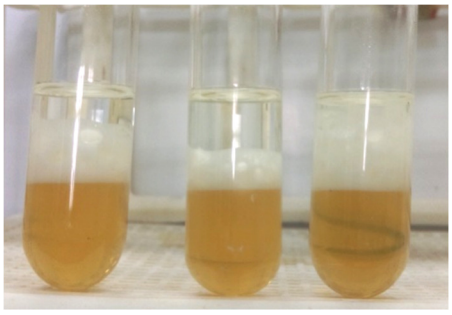

Fig. 6. Emulsification index test (E_{24}) of *Treatment* 4. From left: diesel, coconut oil and kerosene after 7 days of incubation at room temperature

E_{24} on diesel was the highest followed by kerosene and coconut oil (**Table 2**). This result shows that RLs produced by *P. aeruginosa* able to emulsify hydrocarbon and the best can be observed in diesel and kerosene. The E_{24} for coconut oil was 38-45% suggesting that low emulsification index may be due to the inability of RLs to stabilize the microscopic droplets inside the emulsification system [27]. Abbasi et al. [14] reported that E_{24} of biosurfactants are generally less efficient to emulsify vegetable oils such as corn and coconut oil as compared to hydrocarbons which vary from 55.8 to 100%.

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

Treatment	E_{24} (%)		
	Diesel	Coconut oil	Kerosene
Treatment 1 (Glucose, 0 h)	54.08±1.66	38.89±1.92	51.04±1.80
Treatment 2 (Coconut oil, 0 h)	54.55±3.03	43.09±1.57	54.44±3.85
Treatment 3 (Glucose & coconut oil, 0 h)	56.67±0.00	44.22±0.81	56.00±0.00
Treatment 4 (Glucose, 0 h; coconut oil, 8 h)	57.56±0.00	44.69±0.81	56.29±0.64
1% Triton-X 100 (positive control)	64.52±0.00	64.52±0.00	64.52±0.00

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 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.

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

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 ^{o}C to 120 ^{o}C), the emulsification ability decreased. The highest percentage of emulsifying activity (E_{24}) was observed to be 57.56% at 28 ^{o}C. The emulsification activity of the cell-free broth containing RLs against diesel was considered stable and heat tolerance at high temperatures (120 ^{o}C) and unstable at low temperature (28 ^{o}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_{24} (%) of the rhamnolipids from *Pseudomonas aeruginosa* in different temperature storage.

Treatment	E_{24} (%)			
	4 °C	28 °C	70 °C	120 °C
Treatment 1 (Glucose, 0 h)	23.33±1.66	54.08±1.66	50.47±1.81	0.00±0.00
Treatment 2 (Coconut oil, 0 h)	30.11±1.80	54.55±3.03	52.22±3.85	0.00±0.00
Treatment 3 (Glucose & coconut oil, 0 h)	41.04±1.80	56.67±0.00	56.00±0.00	51.04±1.80
Treatment 4 (Glucose, 0 h; coconut oil, 8 h)	44.44±3.85	57.56±0.00	56.00±0.00	56.00±1.81
1% Triton-X 100 (positive control)	64.52±0.00	64.52±0.00	64.52±0.00	64.52±0.00

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 study. 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.

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