Palm kernel fatty acid distillate as substrate for rhamnolipids production using *Pseudomonas* sp. LM19

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Biosurfactants have been used in various industries due to their high surface activity, high biodegradability and low toxicity. However, the applications of biosurfactants are still limited due to their production and cost. This work aimed to determine the viability of palm kernel fatty acid distillate (PKFAD) as an alternative substrate for biosurfactant production using locally isolated *Pseudomonas* sp. LM19. Optimum conditions for biosurfactant production and characteristics of produced biosurfactants were studied. The results showed a 3.2-fold increase in biosurfactant concentration under optimum conditions: 2% (v/v) of PKFAD, pH 7.5, 170 rpm and 192 h incubation time, producing 1.6 g/L of biosurfactant. The produced biosurfactant was capable of reducing the surface tension to 27.7 mN/m with a critical micelle concentration (CMC) of 28 mg/L. Thin-layer chromatography showed presence of both lipid and sugar moieties, thus indicating the produced biosurfactant was glycolipid. Gas chromatography-mass spectrometry recorded the presence of rhamnolipid precursors, thereby revealing that the glycolipid produced was rhamnolipid. High-performance liquid chromatography determined the rhamnolipids produced was a combination of mono-rhamnolipids and di-rhamnolipids. The lipid moiety of both rhamnolipids produced boasted C10-C10 lipid chain majority. To conclude, PKFAD could be a potential substrate for biosurfactant production and add value to the industry.
1. Introduction

The global production of chemical surfactants is valued at around 15 million tons per year (1). The worldwide demand for surfactants used to be met by synthetic chemical surfactants that are usually non-biodegradable and toxic, leading to environmental issues. With a global preference for renewable products, the development of biosurfactants has accelerated. Biosurfactants are biologically synthesized by microorganisms such as bacteria, fungi and yeasts. Biosurfactant has better biodegradability, and is less toxic to the environment, while exhibiting similar physical properties and surface activity as chemical surfactants (2, 3). The usage of biosurfactants with high activity is well-timed and essential to reduce the harmful effects of synthetic surfactants on the environment (4). Despite the aforementioned benefits, the main stumbling blocks for the further application of biosurfactant were its higher production cost and low production yield (5). The production cost of synthetic surfactants was around $1–3/kg, whereas biosurfactants such as rhamnolipids would cost up to $20–25/kg depending on the volumetric productivity of biosurfactant fermentation (6, 7). Because of the challenges encountered in biosurfactant synthesis, there is a need for research into cost-effective biosurfactant production to address these challenges.

Recently, researchers have attempted to discover potential substrates with some feasible yet more economical and environmentally friendly options, as well as optimize its process to enhance the production yield (7, 8). Water-soluble and water-insoluble carbon sources are substrates used for biosurfactant biosynthesis. Water-soluble substrates include glucose, ethanol, glycerol and mannitol, while prime examples of water-insoluble substrates are sunflower oil, coconut oil, soybean oil, olive oil, and n-alkanes. Some studies have reported that biosurfactant production using water-insoluble substrates exhibited a higher production compared to water-soluble substrates. The hydrophobicity of the water-insoluble substrate allows the substrate to be readily consumed by Pseudomonas sp. and thus produce rhamnolipid (9). In addition, water-insoluble substrates are rich in carbon content that can be hydrolyzed into long-chain fatty acids, thus providing precursors for rhamnolipid production (10, 11). Vegetable oil and its derivatives are rich in carbon content with free fatty acid, which favors biosurfactant biosynthesis. However, due to the food competition and high cost of refined vegetable oils, it is more practical to exploit by-products from the vegetable oil industry, e.g. oil refinery by-products (11, 12), used cooking oil (13) and soap stock (14), as a substrate for biosurfactant production.

In 2020, Malaysia’s palm kernel production amounted to 4.7 million tons, while crude palm kernel oil production reached 2.2 million tons (15, 16). The growing production of oil palm products has led to an increase in by-products from the refinery process. One example of such by-products is the free fatty acid-rich palm kernel fatty acid distillate (PKFAD), which originates from the crude palm kernel oil refining process. PKFAD has a low commercial value, allowing it to be a potential substrate for biosurfactant biosynthesis. The biosurfactant biosynthesis conditions are also important for efficient production to ensure the competitiveness of biosurfactants in the market. Optimizing the biosynthesis parameters could ensure a high production while simultaneously reducing the production costs. A statistical optimization method based on the response surface methodology (RSM) can be used to ease the optimization process with increased efficiency. Bertrand et al. (17) reviewed that several studies had been successfully reduce the cost and time consumed to optimize biosurfactant production by statistical method. Various factors that affect biosurfactant biosynthesis have been reported (18), wherein biosynthesis conditions and carbon sources of biosurfactant production are the leading influences (19).

The objectives of this study are: (1) to determine the feasibility of using PKFAD as a substrate in biosurfactant biosynthesis using the indigenous Pseudomonas sp. LM19, (2) optimize the biosynthesis conditions using RSM, and subsequently (3) characterize the produced biosurfactant. The novelty of this study is its utilization of PKFAD as a substrate for biosurfactant production by locally isolated Pseudomonas sp. LM19, to the best of our knowledge this is the first report of its kind. This study has provided fundamental information on the exploration of new feedstock, i.e. low commercial value by-products, for biosurfactant production. The data obtained can also contribute to designing economic strategies for value creation in the oil palm refinery industry.

2. Materials and methods

2.1. Palm kernel fatty acid distillate (PKFAD)

The palm refinery by-product PKFAD was provided by the Malaysian Palm Oil Board, Bandar Baru Bangi, Selangor, Malaysia (2°9669’ N, 101°7407’ E) as a gift for research purposes. PKFAD is a by-product of the crude palm kernel oil from the refining process. It was utilized as a substrate for biosurfactant production. PKFAD is a water-insoluble compound containing mainly free fatty acids. The fatty acid composition of PKFAD is shown in Table 1.
2.2. Microorganism

Pseudomonas sp. LM19 was isolated from a chicken feather processing plant, located at Linggi Pedas, Negeri Sembilan, Malaysia (2.5646° N, 102.0450° E). Isolate LM19 showed 99% sequence similarity with Pseudomonas genus using 16s rRNA sequencing test from the GenBank database of the National Centre for Biotechnology Information. Isolated LM19 was deposited as strain UPMC 1111 in the Microbial Culture Collection Unit, Institute of Bioscience, Universiti Putra Malaysia (20). The culture was maintained in nutrient agar at 4°C and stored in nutrient broth with 40% (v/v) glycerol at −80°C.

2.3. Media and culture conditions

Pseudomonas sp. LM19 was cultured on nutrient agar for 24 h. One loop full of culture was transferred into 50 mL of nutrient broth in a 250 mL conical flask to prepare inoculum. The cultivation condition was 37°C, 180 rpm, and 16–18 h of incubation time. The production medium is composed of (g/L): 2.5 g NaNO₃, 4.0 g K₂HPO₄, 4.0 g KH₂PO₄, 0.1 g CaCl₂, 0.2 g MgSO₄·7H₂O, 1.0 g NaCl, 1.0 g KCl, 1.0 g yeast extract and supplemented with PKFAD as substrate. A 1% (v/v) PKFAD concentration was used and the pH of the medium was non-adjusted for initial studies. All media were autoclaved at 121°C for 15 min to remove bacterial cells. The biosurfactant produced was quantified by an orcinol test.

2.4. Optimization of Biosurfactant Production

A 2⁴ full-factorial central composite design (CCD) was made using Design-expert software. Four variables were selected to determine the optimum conditions for biosurfactant production, namely: 1–5% PKFAD percentage (A), pH 6–8 (B), 160–200 rpm agitation speed (C), and 120–216 h incubation time (D), respectively. The parameters and range were screened and determined based on a preliminary study (unpublished results) and peer studies (22–25). The desirable values of temperature and inoculum size for biosurfactant production were determined before the optimization process via one-factor-at-a-time (OFAT) experiments (unpublished results). All thirty experimental runs were performed at 37°C and inoculum size of 5% (v/v) with various combinations of factors A, B, C, and D, according to the CCD. The rhamnolipid concentration quantified by the orcinol test was chosen as the responding factor in this software-guided optimization. The statistical analysis of the model was performed by way of analysis of variance (ANOVA). The experimental data obtained after performing experiments were analyzed using Design-expert 7.0.0 software and multiple regressions were employed to evaluate the effect of process variables on the biosurfactant concentration.

2.5. Analytical methods

2.5.1. Orcinol test

The orcinol assay was used for a direct assessment of the concentration of glycolipids (25). A 1 mL of cell-free supernatant was extracted twice with a 1 mL mixture of chloroform and methanol (2:1 v/v). The extracted organic layer was evaporated to dryness and then 1 mL of distilled water was added. Extracellular glycolipid concentration was evaluated by measuring the concentration of rhamnose, where 100 μL of each sample was added with 900 μL solution containing 0.19% orcinol in 53% H₂SO₄ and heated for 30 min at 80°C. The mixture was cooled to room temperature, and then the optical density or absorbance was measured at 421 nm (23, 26). The rhamnolipid concentration was determined using a standard curve prepared by the L-rhamnose monohydrate. A fixed concentration of L-rhamnose in the range of 0 mg/mL to 0.18 mg/mL was utilized, and then the absorbance of corresponding concentrations was measured at 421 nm.

2.5.2. Recovery of biosurfactant

The extracted biosurfactant was obtained using a combination of acid precipitation and solvent extraction method (27). The fermentation broth was centrifuged at 6,800 × g for 15 min to remove bacterial cells. The biosurfactant was recovered by acidifying the supernatant to pH 2.0 using 1 M sulfuric acid and kept overnight at 4°C. An acidified mixture containing biosurfactant was then pelleted by centrifugation at 12,000 × g for 20 min, before subsequently extracting three times with equal volume of chloroform: methanol (2:1 v/v) mixture (28). The mixture was mixed evenly, before being allowed to stand until phase separation. The organic phase was collected and dried to remove the solvent. The extracted biosurfactant obtained was used for further analysis.
2.5.3. Surface tension and critical micelle concentration (CMC)

The extracted biosurfactant was used for surface tension and CMC measurement using Kruss K100MK3 surface tensiometer (Krüss, Germany). CMC is the surfactant concentration at which micelles begin to form. A serial dilution of extracted biosurfactant was prepared (0–100 mg/L). Then, the CMC measurement was determined by measuring the surface tension of the extracted biosurfactant in distilled water with the prepared dilutions until a constant value of surface tension is achieved (29).

2.5.4. Thin layer chromatography (TLC)

The extracted sample was dissolved in chloroform and applied onto a TLC silica gel 60 F\textsubscript{254} plate at the point of origin near the bottom of the plate. The plate was then developed in a solvent system of chloroform, i.e. methanol: acetic acid (65:15:2, v/v/v). The developed plate was visualized using various reagents to detect the presence of sugar, lipid and amino groups. TLC was done three times for each sample for it to be examined with different reagents respectively. The anthrone reagent was used to detect the presence of sugar moieties. The reagent was prepared by mixing 63 mL of sulfuric acid, 25 mL of water, and 0.125 g of anthrone under icy conditions. The developed plate was sprayed evenly with the prepared reagent and then placed in an oven at 110°C for 10 min (30). Detection of lipid moieties was possible using iodine vapors. The developed plate was kept in a container saturated with iodine vapors to allow iodine vapors to stain any lipid groups present in the sample (31). The presence of amino acid groups was observed using the ninhydrin reagent. The reagent was prepared by dissolving 0.2 g of ninhydrin into 100 mL of pure acetone. The developed plate was sprayed with ninhydrin reagent and heated to 120°C (32). The movement of the separated components along the plate was described by Eraqi et al. (27) using the \( R_f \) value (Eq. 1), where:

\[
R_f = \frac{\text{distance traveled by the spot centre of sample (cm)}}{\text{distance traveled by the solvent front (cm)}}
\]

2.5.5. Gas chromatography-mass spectrometry (GC-MS)

The fatty acyl moiety in glycolipid was determined using GC-MS (Agilent, USA, 7890A gas chromatography with a model 5975C mass selective detector and a HP-5MS capillary column: 30 m × 0.25 mm × 0.25 µm). Five mg of the extracted samples with 1 mL of 6 M HCl solution were added to a 2 mL ampoule bottle, sealed and hydrolyzed at 120°C for 90 min. The hydrolysate was subsequently extracted with 3 mL chloroform: methanol (2:1) and then esterified with 1 mL 10% H\textsubscript{2}SO\textsubscript{4} in methanol solution at 55°C for 6 h. After esterification, a 5 mL of dH\textsubscript{2}O was added to the reaction mixture, followed by three-time extraction with 3 mL chloroform: methanol (2:1). The solvent was evaporated at room temperature and the sample was dissolved in 1 mL methanol before being analyzed by GC-MS. The column temperature was initially held at 80°C for 3 min and increased to 280°C at a rate of 8°C/min and kept for 10 min. A 1 µL sample was then used with a split ratio of 10:1 (33).

2.5.6. High performance liquid chromatography (HPLC)

HPLC was performed to identify the components present in the sample. From TLC, the separated spots were collected by scraping off the TLC plate and then extracting three times with chloroform: methanol (2:1, v/v). The sample was then vortexed for 1 min, followed by centrifugation for 10 min to settle the silica down. The supernatant was pipetted out and dried to obtain the sample (34). The sample was further analyzed by the Acquity Arc system liquid chromatography, coupled with the Acquity QDa mass detector and evaporative light scattering detector (ELSD) (Waters, Milford, MA) using a Cortecs C18 column (2.7 µm × 4.6 mm × 50 mm). This method was modified according to Haba et al. (13). The LC flow rate was 1 mL/min while the injection volume was 10 µL/min. For the mobile phase, an acetonitrile–water (0.1% formic acid) gradient was used, starting with 40% of acetonitrile and 60% of water for 5 min. Then, an acetonitrile concentration was raised from 40% to 100% across a period of 4 min 30 s. The gradient was maintained at 100% acetonitrile for 1 min, then returned to initial conditions over a period of 2 min. Mass spectrometry (MS) was performed with a QDa mass detector, equipped with ELSD. A range of m/z 100–1000 data was scanned and obtained.

3. Results

3.1. Optimization of Biosurfactant Production

The effect of different variables on biosurfactant synthesis by \textit{Pseudomonas} sp. LM19 was investigated using CCD. The effect of dependent variables on the experimental response can be explained by the quadratic equations (as shown in Eq. 2) generated by the system. X is the concentration of rhamnolipid synthesized, while A, B, C and D are the dependent variables: PKFAD percentage, pH, agitation and incubation.
The results obtained for rhamnolipid concentration response were best fitted to a quadratic polynomial model where such quadratic model was significant and demonstrated a $p$ value of less than 0.05. The model was found to be significant using a rhamnolipid concentration as a responding parameter with an F-value of 18.05. There was only a mere 0.01% chance that a larger model F-value could happen due to noise ($p$ value <0.0001). Independent variables PKFAD percentage (A), pH (B), agitation speed (C), and incubation time (D) were all found to significantly influence the rhamnolipid produced ($p$ <0.01). The interaction between variables AC, AD, BC, BD and CD also portrayed a significant effect on the response, as they all provided $p$-values of less than 0.05. The $p$ value of lack of fit (0.3957) in the models implied that the lack of fit is insignificant ($p > 0.05$) relative to the pure error. The coefficient of determination, $R^2$ of the model (0.9440) indicated that the sample variation of 94.40% for biosurfactant was attributed to the independent variables. The adjusted $R^2$ was a modified version of $R^2$ that has been adjusted for the number of predictors in the model. The adjusted $R^2$ for rhamnolipid concentration was 0.8916. Based on the polynomial equation deduced by CCD, the predictive runs were speculated and calculated. Experimental runs were randomly generated by the software to validate the CCD model. From the test runs, the suggested optimum conditions were set at 2% PKFAD, pH 7.5, 170 rpm, and incubated for 192 h, with a predicted rhamnolipid concentration of 1.5 g/L. The physical experimental result of the conditions mentioned obtained a 1.6 g/L of rhamnolipid, which is a 3.2-fold increase compared to the rhamnolipid concentration yield of 0.5 g/L obtained while biosynthesis conditions were unoptimized. The test runs also showed that the experimental result was similar and in agreement with

The data of experimental and predicted values for rhamnolipid concentration were in good agreement, exhibited a $p$ value of 0.8526, thus indicating there were no significant differences between experimental and predicted data. ANOVA for the quadratic model of the rhamnolipid production obtained by Pseudomonas sp. LM19 is shown in Table 3. Some experimental runs have shown variation between the predicted value and experimental value, which may affect the $p$ value. However, most of the experimental runs have shown results close to the predicted value.

### Table 2. CCD matrix with coded value and their corresponding experimental and predicted rhamnolipid concentration response value.

| Run | A  | B  | C  | D  | Experimental (g/L) | Predicted* |
|-----|----|----|----|----|-------------------|------------|
| 1   | 3  | 7  | 160| 168| 1.11              | 1.20       |
| 2   | 1  | 7  | 180| 168| 0.91              | 0.92       |
| 3   | 4  | 7.5| 170| 192| 0.94              | 0.99       |
| 4   | 3  | 6  | 180| 168| 0.36              | 0.32       |
| 5   | 2  | 7  | 170| 192| 1.59              | 1.48       |
| 6   | 3  | 7  | 180| 168| 0.76              | 0.63       |
| 7   | 4  | 6.5| 170| 192| 0.65              | 0.63       |
| 8   | 3  | 7  | 180| 168| 0.58              | 0.63       |
| 9   | 3  | 8  | 180| 168| 0.46              | 0.57       |
| 10  | 3  | 7  | 200| 168| 0.37              | 0.35       |
| 11  | 2  | 6.5| 190| 192| 0.40              | 0.50       |
| 12  | 3  | 7  | 180| 168| 0.59              | 0.63       |
| 13  | 4  | 7.5| 170| 144| 0.89              | 0.77       |
| 14  | 3  | 7  | 180| 168| 0.61              | 0.63       |
| 15  | 2  | 6.5| 170| 144| 0.59              | 0.63       |
| 16  | 3  | 7  | 180| 216| 0.74              | 0.81       |
| 17  | 2  | 7.5| 170| 144| 0.88              | 0.94       |
| 18  | 3  | 7  | 180| 168| 0.64              | 0.63       |
| 19  | 2  | 7.5| 190| 144| 0.35              | 0.34       |
| 20  | 4  | 6.5| 170| 144| 0.60              | 0.61       |
| 21  | 4  | 6.5| 190| 144| 0.56              | 0.65       |
| 22  | 5  | 7  | 180| 168| 0.57              | 0.63       |
| 23  | 4  | 6.5| 190| 192| 0.41              | 0.38       |
| 24  | 2  | 6.5| 190| 144| 0.46              | 0.44       |
| 25  | 3  | 7  | 180| 168| 0.52              | 0.63       |
| 26  | 4  | 7  | 190| 144| 0.34              | 0.40       |
| 27  | 2  | 6.5| 170| 192| 1.00              | 0.98       |
| 28  | 4  | 7  | 190| 192| 0.39              | 0.32       |
| 29  | 2  | 7  | 190| 192| 0.57              | 0.59       |
| 30  | 3  | 7  | 180| 120| 0.53              | 0.53       |

* Predicted rhamnolipid concentration was calculated based on Eq. 2

### Table 3. ANOVA for response surface quadratic model based on CCD for rhamnolipid concentration response.

| Source | F value | p value |
|--------|---------|---------|
| Model  | 18.05   | < 0.0001|
| A (PKFAD %) | 15.36   | 0.0014 |
| B (pH) | 11.58   | 0.0039 |
| C (Agitation) | 134.52  | < 0.0001|
| D (Incubation time) | 15.22   | 0.0014 |
| AB     | 2.76    | 0.1175 |
| AC     | 6.29    | 0.0241 |
| AD     | 12.91   | 0.0027 |
| BC     | 20.66   | 0.0004 |
| BD     | 4.59    | 0.0490 |
| CD     | 10.24   | 0.0060 |
| Lack of Fit | 1.33   | 0.3957 |
| $R^2$  | 0.8940  |         |
| Adjusted $R^2$ | 0.8916 |         |
the predicted value, thereby validating the accuracy of the CCD model.

### 3.2. Characterization of Biosurfactant

The effect of the rhamnolipid concentration on surface tension is shown in **Figure 1**. The surface tension of distilled water was gradually reduced from 72.0 mN/m to 27.7 mN/m, in conjunction with a continual increase in rhamnolipid concentration. Thereafter, the reduction in surface tension stopped and surface tension remained at 27.7 mN/m, even when the rhamnolipid concentration continued to increase after 28 mg/L. The result showed that the CMC of the extracted rhamnolipid is approximately 28 mg/L.

TLC analysis of the extracted sample showed a positive reaction on anthrone reagent and iodine vapor, indicating the presence of sugar moieties and lipid moieties, respectively. Meanwhile, the negative reaction on the ninhydrin reagent (no spots were detected) indicates the absence of amino groups in the sample. Therefore, the TLC showed that the biosurfactant obtained is a glycolipid. Two spots were obtained with *R*<sub>t</sub> values of 0.68 and 0.38.

The GC-MS was used to screen trace amounts of fatty acids to detect the rhamnolipid congeners present. The molecules were negatively ionized in the negative electro-spray ionization process. The produced negative mass spectra model shows the m/z of [M–H]- of selected peaks containing methyl-esterified fatty acids as shown in **Figure 2**(a, b). According to the pseudo-molecular ion and retention time, two different methyl-esterified fatty acids in the form of β-hydroxy fatty acids were elucidated at 12.74 and 15.82 min, as shown in **Figure 2**(a, b), respectively. Both β-hydroxy fatty acids showed a based peak of the pseudo-molecular ion at m/z 103. Based on the mass spectra, elucidation at 12.74 min is expected to be β-hydroxydecanoic acid (C<sub>10</sub>), with fragment ions seen at m/z 43, 61, 71, 103, 153 and 201, as shown in **Figure 2**(a). Meanwhile, elucidation at 15.82 min is expected to be β-hydroxydodecanoic acid (C<sub>12:1</sub>), and similar fragment ions were seen at 43, 61, 71, 103, 138, and 229, as shown in **Figure 2**(b).

HPLC-QDa-ELSD analyzed the molecular component of the sample collected from previous TLC analysis at *R*<sub>t</sub> values of 0.68 and 0.38. The HPLC results identified that *Pseudomonas* sp. LM19 produced different rhamnolipid congeners. The retention time with its corresponding rhamnolipid congeners, relative abundance and m/z ratio value is shown in **Table 4**. The spot with *R*<sub>t</sub> values of 0.68 exhibits mono-rhamnolipid congeners, while the spot with *R*<sub>t</sub> values of 0.38 contains di-rhamnolipid congeners. Both mono- and di-rhamnolipid samples each produced 3 different fractions. The mono-rhamnolipid fractions were expected to be rhamnolipid congeners of Rha-C10-C10, Rha-C10-C12:1/Rha-C12:1-C10, and Rha-C10-C12/Rha-C12-C10. Meanwhile, the di-rhamnolipid fractions were expected to be congeners of Rha-Rha-C10-C10, Rha-Rha-C12:1-C10/Rha-Rha-C10-C12:1, and Rha-Rha-C12-C10/Rha-Rha-C10-C12. Further investigation revealed that the lipid moiety of each of the two rhamnolipid samples produced from *Pseudomonas* sp. LM19 were identical. This means that although mono-rhamnolipids and di-rhamnolipids were collected from two distinctive spots at TLC, yet rhamnolipid congeners with C<sub>10</sub>-C<sub>10</sub> lipid chain dominated based on its relative abundance, where 90.82% of the mono-rhamnolipid sample and 89.58% of the di-rhamnolipid sample demonstrated C<sub>10</sub>-C<sub>10</sub> lipid chain.

### 4. Discussion

#### 4.1. Optimization of Biosurfactant Production

The optimum biosynthesis conditions reported in this study were comparable with peer studies (22, 23, 34, 35). The optimum substrate concentration was found to be 2% for biosurfactant production by *P. aeruginosa* using waste frying coconut oil (34). Nitschke et al. (35) reported that rhamnolipid biosynthesis increased when substrate concentration increased from 1% to 2%, even though biosynthesis remained constant thereafter despite a further increase in substrate concentrations. This showed that an appropriate substrate level is vital to providing the energy required for rhamnolipid biosynthesis. Conversely, the optimum agitation speed obtained in this study was comparable to peer studies where all used a 170 rpm agitation speed to ensure optimum cell growth and rhamnolipid biosynthesis in a shake flask (36–38). The agitation speed manipulates the mass transfer efficiency rate of both media.

![Figure 1](https://example.com/figure1.png)  
**Figure 1.** Effect of rhamnolipid concentration on surface tension.
components and oxygen, which are crucial in the growth and metabolism of aerobic bacteria like *Pseudomonas* sp. strains during biosynthesis (39). The optimum incubation time obtained in this study was 8 d, a result that was comparable to those reported by peers, which were in the range of 7 d to 9 d albeit with different substrates (22, 34, 40). Meanwhile, the optimum pH for rhamnolipid biosynthesis reported in this study was pH 7.5, showing some similarities with El-Housseiny et al. (23), where rhamnolipid biosynthesis by *P. aeruginosa* isolate P6 were reported to be maximum at the slightly basic pH of pH 7.5. The same study also showed rhamnolipid biosynthesis to be reduced gradually with a decreasing pH until pH 4. Nonetheless, the optimum pH for rhamnolipid production varies among different species and strains of microorganisms (27, 41). With the RSM-guided optimized conditions, the rhamnolipid concentration produced was successfully increased by 3.2-fold to 1.6 g/L from the unoptimized rhamnolipid yield of 0.5 g/L. The magnitude of increment was comparable with the results reported by Sharma et al. (24), which also showed a 3.2-fold increase after optimization through CCD design from 0.82 g/L to 2.65 g/L. These results highlighted the importance of the optimization process for enhancing rhamnolipid production.

### 4.2. Characterization of Biosurfactant

The biosurfactant synthesized in this study reduced the surface tension of distilled water from 72.0 mN/m to...
27.7 mN/m, with a CMC of 28 mg/L. This was comparable to the result reported by Singh and Tiwary (42), where the CMC of biosurfactant produced by P. aeruginosa P4 was reported to be 40 mg/L. In other studies, Cheng et al. (26) and Radzuan et al. (43) reported that the CMCs obtained from biosurfactant produced by P. aeruginosa ZS1 and P. aeruginosa PA01 were 120 and 420 mg/L, respectively (26, 43).

Conversely, the synthetic surfactant exhibited a CMC of 1500 mg/L (26). The aforementioned results showed that the rhamnolipid produced by Pseudomonas sp. is more capable of reducing the surface tension at a lower concentration compared to synthetic surfactant. Although most of the aforementioned studies utilized Pseudomonas strains to produce rhamnolipid, the CMCs obtained were different. This could be due to the different congeners of rhamnolipid that were produced as a result of dissimilar biosynthesis conditions and substrates used (29, 33).

In TLC analysis, the presence of both lipid and glycosyl moieties inferred that the biosurfactant produced was a glycolipid. The Rf values of the rhamnolipid synthesized were similar to the Rf values of 0.68 and 0.40 obtained using 95% purity rhamnolipid standard with di-rhamnolipid dominant (Sigma-R95DD). Henceforth, this study’s Rf values (0.68 and 0.38) were expected to be spots of mono-rhamnolipids and di-rhamnolipids, respectively. Mono-rhamnolipid exhibits a rhamnose sugar molecule compared to two rhamnose sugar molecules in di-rhamnolipid. As a result, mono-rhamnolipid would be more hydrophobic compared to di-rhamnolipids. This would allow mono-rhamnolipids to travel further up the TLC with a higher Rf value compared to di-rhamnolipids. The TLC results of this study showed a similar separation profile to that of Deepika et al. (44) and Lotfabad et al. (45) which obtained two spots with Rf values of 0.71 and 0.31 and Rf values of 0.73 and 0.31, respectively.

The GC-MS was used to interpret the composition of fatty acids present in the produced rhamnolipids. This was done by converting the extracted rhamnolipid sample into methyl esters before GC-MS to determine the lipid moiety. The selected mass spectra containing methyl-esterified fatty acids with a fragment of β-hydroxy fatty acids at m/z 103 were elucidated at 12.74 min (Figure 2(a)) and 15.82 min (Figure 2(b)). This pseudo-molecular ion was expected to be the precursor for rhamnolipid production, thus confirming that rhamnolipids were present in the sample (33). The prevalence of methyl ester of β-hydroxydecanoic acid and β-hydroxydodecanoic indicated that C_{10} and C_{12:1} were the main fatty acid components in the extracted rhamnolipid. Various studies have also reported a similar prevalence of C_{10} fatty acids in respective GC-MS results of produced rhamnolipid biosurfactants (33, 46).

The HPLC-QDa-ELSD was done to complement the GC-MS results, as GC-MS alone only identifies the lipid moiety and could not affirm the exact rhamnolipid congeners produced. HPLC-QDa-ELSD proved that the biosurfactants produced were indeed a combination of both mono-rhamnolipids and di-rhamnolipids. The result also showed that the lipid moiety of both mono-rhamnolipids and di-rhamnolipids were similar, as both exhibited a majority of rhamnolipid congeners containing the C_{10}-C_{11} lipid chain. This finding concurred with peer studies where rhamnolipid congeners produced by different Pseudomonas strains were mono- or di-rhamnolipid with predominantly C_{10}-C_{11} lipid chain, irrespective of substrates being utilized (29, 33). However, the mono-/di-rhamnolipid ratio of the produced rhamnolipid might be affected by the microorganism strains. Correspondingly, the properties of the rhamnolipid will be influenced by the congeners present in the produced rhamnolipids.

5. Conclusions

In conclusion, Pseudomonas sp. LM19 has successfully synthesized biosurfactants using PKFAD as a substrate. The RSM results showed a 3.2-fold increase in biosurfactant biosynthesis under optimized conditions of pH 7.5, 2% (v/v) PKFAD, 170 rpm, and 192 h incubation time. The biosurfactant produced is capable of reducing the surface tension of distilled water from 72.0 mN/m to 27.7 mN/m whilst exhibiting CMC of 28 mg/L. The TLC analysis showed that the biosurfactant produced was glycolipid, where both lipid and glycosyl moieties were detected. The GC-MS discovered the pseudo-molecular ion of rhamnolipid precursor in the form of β-hydroxy fatty acids, thus confirming that the biosurfactant produced was rhamnolipid. The HPLC confirmed that the two TLC spots were mono-rhamnolipids and di-rhamnolipids, respectively. This study has shown the feasibility of using palm refinery by-product PKFAD as a substrate in rhamnolipid biosynthesis, thereby converting it into a value-added biotechnological product. The results obtained in this study can serve as the fundamental data for further development of a cost-effective biosurfactant biosynthesis process using a palm refinery industry by-product. Further separation and purification processes can be done before characterization to obtain samples with higher purity.
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Disclosure statement
No potential conflict of interest was reported by the author(s).

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Declaration of Interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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