Biorefining palm oil agricultural refinery waste for added value rhamnolipid production via fermentation

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Abstract

Rhamnolipids (RL) production by Pseudomonas aeruginosa PAO1 is a potentially attractive route to add value to palm oil refinery agricultural by-products; palm fatty acid distillate (PFAD) and fatty acid methyl ester (FAME). The results showed maximum RL concentration of 3.4 and 2.5 g L\(^{-1}\) when using 10 g L\(^{-1}\) PFAD and FAME respectively; while using 20 g L\(^{-1}\) PFAD and FAME, the RL concentrations achieved were 3.2 and 3.1 g L\(^{-1}\), respectively. The predominant congener produced was identified as dirhamnolipid, Rha-Rha-C10-C10. The RL produced reduced surface tension to 29-32 mN m\(^{-1}\) with a CMC value of 19 mg L\(^{-1}\). A high emulsion index with kerosene, 40 % and sunflower oil, 46 % were measured. This work demonstrates the potential for the utilisation of palm oil refinery agricultural by-products, PFAD and FAME, as low cost and renewable substrates for RL production in integrated palm oil biorefinery systems.
Keywords

*Pseudomonas aeruginosa* PAO1, fermentation, rhamnolipid, palm fatty acid distillate (PFAD), fatty acid methyl ester (FAME), biorefinery
1. Introduction

Biosurfactants are extracellular microbial surfactants that are amphipathic and have high emulsifying activity and surface activity (Ji et al., 2016). Biosurfactants have several advantages compared to synthetic surfactants, such as biodegradability, low toxicity, stability and effectiveness at extreme pH, salinity and temperature and their potential to be produced from renewable substrates (George and Jayachandran, 2013). These features give biosurfactants potential for use as green alternatives to chemical surfactants that can be applied in environmental remediation, as antimicrobial agents, as emulsifiers and stabilisers in the food industry, in enhanced oil recovery processes and medical applications (Elshikh et al., 2016; Moya Ramírez et al., 2015).

Chemical structure plays a major role in biosurfactant classification into glycolipids, lipopeptides, phospholipids, fatty acids and polymeric compounds (Lourith and Kanlayavattanakul, 2009). Rhamnolipids are one of the low molecular mass glycolipid type biosurfactants, being produced mainly from *Pseudomonas aeruginosa* strains that are being intensively studied by researchers (Irorere et al., 2017). There are two main types of rhamnolipids with one (monorhamnolipid) and two (dirhamnolipid) rhamnose sugar moieties, attached to one or two β-hydroxy fatty acid chains (Gudiña et al., 2016). To date, several companies have been producing rhamnolipids in relatively large quantities such as Rhamnolipid Incorporated (http://rhamnolipid.com/), Rhamnolipid Holdings (http://www.rhamnolipidholdings.com/) and AGAE Technologies (http://www.agaetech.com/), thus showing rhamnolipids have enormous potential for commercialisation into various products and application areas.

There are several factors that affect rhamnolipid production which include the carbon source, nitrogen source and environmental factors. *Pseudomonas aeruginosa* is capable of using water immiscible free fatty acid containing substrates such as sunflower oil, olive oil
and soybean oil to produce rhamnolipid. Glucose, glycerol, ethanol and mannitol are commonly used hydrophilic carbon sources, with results showing that hydrophobic substrates give better rhamnolipid production compared to hydrophilic substrates (Nitschke et al., 2011). Nitrogen sources also play an important role in rhamnolipid production. It has been reported that when the nitrogen became limiting there was overproduction of rhamnolipid and cell growth become stationary (Reis et al., 2011). Furthermore, environmental factors such as pH, temperature, agitation speed, strain type and age, oxygen availability and culture media affect rhamnolipid production and cell growth (Banat, 1997).

Despite intensive research efforts into the development of large scale biosurfactant production processes, there still exists the main challenge of high production costs that limit the wider use of biosurfactants (Dobler et al., 2016). The high cost of rhamnolipids production mainly arises from the expensive substrate and downstream processes, making the use of rhamnolipids costly, especially for low added value applications such as environmental remediation (Banat et al., 2014). One of the ways to reduce the production cost is the utilization of some industrial/agro-industrial waste or by-product which could provide a low cost fermentation substrate and an opportunity to generate added value, as well as making use of wastes that can potentially have a harmful impact on the environment (Moya Ramírez et al., 2015).

The Malaysian palm oil industry is one of the most important contributors to the Malaysian economy with substantial export earnings of Malaysian Ringgit (RM) $60 \times 10^9$ and high employment rate. Globally, 34 % of palm oil production is obtained from Malaysia, where in 2015 the planted area was $5.64 \times 10^6$ hectares (MPOB, 2016). Palm oil industry made Malaysia the second largest producer in the world with crude palm oil (CPO) production in 2016 of $17.3 \times 10^6$ tonnes (MPOB, 2017). The refining process of CPO produces a by-product called palm fatty acid distillate (PFAD) that is low value, renewable
and abundant waste substrate. It is estimated that $6.2 \times 10^5$ tonnes of PFAD are produced annually, which accounts for 3.6% of the total CPO processed. Recently, PFAD has been used as a feedstock for the oleochemical industry, animal feed and, as a source of vitamin E in the cosmetics industry and has also been extensively studied for the production of biodiesel (Abdul Kapor et al., 2017; Hosseini et al., 2015).

PFAD is a by-product of palm oil refining, produced in agricultural palm oil refinery mills. At room temperature, PFAD is a yellow solid, which turns to a dark brown liquid at temperatures above the melting point of $\approx 40 \degree C$ and FAME (fatty acid methyl ester) is the biodiesel that can be derived from PFAD via the esterification process (Chabukswar et al., 2013). The esterification of PFAD to produce FAME, an existing valorisation route, significantly affects its physical properties, changing from solid to liquid at room temperature. PFAD and FAME are mainly composed of fatty acids such as oleic acid, pentadecanoic acid, tridecyl acid, palmitic acid, stearic acid, palmitic acid and others with carbon chain lengths from $C_{11}$ to $C_{18}$ (Nazren Radzuan et al., 2016). There is much interest in biodiesel (FAME) produced from PFAD because of its comparable properties to the diesel fuel produced from petroleum and the increasing demand for biofuels by both developing and developed countries (Yadav et al., 2010). The high production cost of biodiesel, however, is a big challenge that inhibits its industrial growth (Lokman et al., 2014).

There are many studies in which free fatty acids derived from waste or low-value by-products, such as olive mill waste, waste frying oil, glycerol and soap stock, have been used for rhamnolipid production (Lovaglio et al., 2015). De Faria et al., (2011) used raw glycerol produced from biodiesel production as a sole carbon sources for rhamnolipid production which yield 1.36 g L$^{-1}$ meanwhile Moya Ramirez et al., (2015) used olive mill waste produce much lower rhamnolipid production of 0.56 g L$^{-1}$. George and Jayachandran, (2013) present a study that produce 1.97 g L$^{-1}$ of rhamnolipid from used coconut oil meanwhile soap stock
from refining vegetable oil process was used as substrate for rhamnolipid production by
Benincasa et al., (2004) producing 15.8 g L\(^{-1}\). All the studies mentioned used different types
of P. aeruginosa strain thus it shows its ability to used fatty acid as main substrate for
rhamnolipid production, and the production from water-immiscible substrate such as fatty
acid has better production than water-soluble substrate like glucose and glycerol (Banat,
1997).

In this present research, the high fatty acid content of PFAD and FAME have been
investigated as sole carbon sources for rhamnolipid (RL) production using Pseudomonas
aeruginosa PA01 in a minimal culture medium. Cell growth, RL production, RL yields and
RL characteristics were determined and compared to other reports of value added
biosurfactant production routes using agricultural wastes. The novelty of this study is the
production of significantly increased amounts of RL when using minimal media, compared to
our previous study, using PFAD as the sole carbon source, demonstrating the technical
feasibility of producing meaningful amounts of RL from palm oil refinery mill by-product. In
a broader biorefining strategy, it is advantageous to have alternative valorisation routes for
PFAD and FAME which can be utilised based on the prevailing market conditions. One
option is to use the FAME as a substrate for RL production, thus providing an alternative
valorisation route that could be employed as part of a wider palm oil biorefinery industry.
This investigation expands our knowledge and understanding of the usage of palm oil
refinery agricultural waste for RL production and its potential to be transformed into a
valuable product that can be used in various applications.
2. Materials and Methods

2.1 Esterification of PFAD

Palm fatty acid distillate (PFAD) was converted into fatty acid methyl ester (FAME) by esterification. The FAME produced was subsequently used as a substrate to produce rhamnolipids by fermentation. PFAD was obtained from Sime Darby-Jamolina and The Cucurbit Company Sdn. Bhd., Malaysia and dried in a drying oven at 70°C for one day. The esterification was then carried out using a 10:1 ratio of methanol to PFAD, with the addition of sulfuric acid (2.5% weight of PFAD) as a catalyst. The reaction was then carried out at 100°C for 1 hour, using a reflux condenser. The product was then cooled, transferred to a separating funnel and washed with hot distilled water until the bottom water layer becomes evident and the pH reached 7.

2.2 Microorganism

*Pseudomonas aeruginosa* PAO1 was supplied by the School of Biomedical Sciences, Ulster University from their culture collection. The strain was stored at −80°C as master stock and working culture was preserved at 4°C on nutrient agar plates.

2.3 Media and Culture Condition

To prepare cultures *P. aeruginosa* PAO1 was first spread onto a nutrient agar petri dish and incubated at 37°C for 24 h. There were two types culture medium used in this fermentation. The first culture medium used for seed culture was the Protease Peptone Glucose Ammonium Salt (PPGas) medium which consisted of 0.5 g L\(^{-1}\) MgSO\(_4\).7H\(_2\)O, 10 g L\(^{-1}\) peptone, 19 g L\(^{-1}\) Tris-HCl, 1.5 g L\(^{-1}\) KCl, 1 g L\(^{-1}\) NH\(_4\)Cl and 1% glucose. The second culture medium used was minimal medium (MM) which consisted of 0.5 g L\(^{-1}\) MgSO\(_4\).7H\(_2\)O, 1.0 g L\(^{-1}\) KCl, 0.3 g L\(^{-1}\) K\(_2\)HPO\(_4\), 1.0 g L\(^{-1}\) NaNO\(_3\). Trace elements for MM
were 2 g L$^{-1}$ C$_6$H$_5$Na$_3$O$_7$·2H$_2$O, 0.28 g L$^{-1}$ FeCl$_3$·6H$_2$O, 1.4 g L$^{-1}$ ZnSO$_4$·7H$_2$O, 1.2 g L$^{-1}$ CoCl$_2$·6H$_2$O, 1.2 g L$^{-1}$ CuSO$_4$·5H$_2$O and 0.8 g L$^{-1}$ MnSO$_4$·H$_2$O.  

Fermentation experiments were conducted in 5 L shake flasks in triplicate, using 1L of MM supplemented with the desired amount of glucose, PFAD or FAME and were carried out at 37°C over three days. Inocula were prepared in two stages; in stage 1 50 mL of PPGas medium containing 1% glucose in a 250 mL flask was inoculated with one loop of bacteria into and grown at 37°C for 24 h. In stage 2, 40 mL of stage 1 culture were transferred to 400 mL PPGas media with 1% of glucose in a 2 L flask and grown for 24 h. For the preparation of the final inoculum, 100 mL of stage 2 culture was centrifuged for 10 min and the cell pellet resuspended in 100 mL of sterile distilled water. This was then used to inoculate the 1 L MM in 5 L shake flask. The initial concentration of the fermentation was maintain at 0.2 g L$^{-1}$.  

2.4 Growth Measurement  

Cell growth was quantified by measuring optical density (OD) from which dry cell weight (DCW) was calculated using a linear correlation $DCW = 0.4639(OD) + 0.0276$ with $R^2 = 0.87$ for glucose and $DCW = 0.5871(OD) + 0.1014$ with $R^2 = 0.92$ for fatty acid substrate. PFAD and FAME were removed from samples by adding 0.5 mL of n-hexane to 1 mL fermentation broth and centrifuging at 13000 g using a Minispin Centrifuge (Eppendorf). Cell biomass was re-suspended in 0.7% sodium chloride solution (physiological saline) and the OD determined by measuring absorption at 600 nm using a UVmini-1240 Spectrophotometer (Shimadzu, USA).  

2.5 Rhamnolipid Extraction  

Rhamnolipids were extracted from 10 mL samples of fermentation broth. Firstly samples were centrifuged for 10 min and the supernatant taken and acidified with 1 M
hydrochloric acid to form a precipitate at pH 3. The acidified supernatant was then mixed
with an equal volume of ethyl acetate and shaken vigorously. The ethyl acetate washing
process was repeated three times, and lastly, traces of water present in the RL containing
ethyl acetate layer were removed by using 0.5 g of magnesium sulphate per 100 mL. Finally,
the samples were filtered, and the solvent evaporated using a rotary evaporator (Cole-Parmer
Ltd, model RE300) at 70 °C to give a crude rhamnolipid biosurfactant extract. The RL
concentration was then determined gravimetrically.

2.6 Biosurfactant Identification

Mass Spectrometry-Electrospray Ionization (MS-ESI) was used for biosurfactant
identification. An Agilent 6510 Q-TOF LC/MS equipped with Agilent 1200 Liquid
Chromatography (LC) was used with 5 µL of crude rhamnolipids extract, diluted in
methanol, injected using 50% ACN with 0.1% formic acid as an eluent with electrospray
(ESI) in negative mode (Smyth et al., 2016).

2.7 Biosurfactant Characterization

A Krüss K11 Tensiometer equipped with a De Nöuy ring was used to measure
equilibrium surface tension and determine the critical micelle concentration (Smyth et al.,
2016). The crude rhamnolipid extract was diluted with 0.1 M Tris-HCl pH 8.0 solution, from
an initial concentration of 1000 mg L\(^{-1}\), and the equilibrium surface tension determined. The
emulsion index was measured over 24 h and calculated as the percent of the height of the
emulsified layer relative to the total height of the liquid. A solution of 3 mL of dissolved
crude rhamnolipids, initial concentration 1000 mg L\(^{-1}\) in 0.1 M Tris-HCl pH 8.0, was mixed
with 3 mL of sunflower oil or kerosene and shaken vigorously for 1 min to obtain maximum
emulsification.
2.8 Gas chromatography analysis

To characterise the PFAD and FAME, 0.2 g of the PFAD, FAME and emulsified PFAD after fermentation respectively were vortex mixed with hexane and 0.1 mL methanolic potassium hydroxide for 30 s and the mixture was centrifuged for 10 min. After centrifuging, 0.2 mL of the top layer was mixed with 2 mL of hexane and 0.1 μL was injected into GC-MS for analysis. A BPX 70 capillary column (SGE, length: 60 cm, ID: 0.22 mm and film thickness: 0.25 μm) was used for separation of FAME compounds. The GC injection port was set at 155°C, and the detector temperature was 220°C. The GC oven was programmed with a temperature ramp from 155°C to 180°C at 2°C/min and then from 180°C to 220°C at 4°C/min.

3. Results and Discussion

3.1 Esterification of PFAD

Biodiesel or fatty acid methyl ester (FAME) has attracted much research and industrial interest due to depletion of petroleum reserves, an increasing awareness of environmental issues and drove towards sustainability (Hosseini et al., 2015). FAME can be produced from renewable resources that contain a high percentage of free fatty acids, such as PFAD (Lokman et al., 2014). One of the methods to derive FAME from renewable resources is esterification, using methanol in the presence of an acid as a catalyst (Yadav et al., 2010). In this work, esterification using methanol and sulfuric acid was used to convert PFAD into FAME. The esterification reaction significantly transforms the physical properties of PFAD from solid to liquid FAME at room temperature. Due to this change, it was expected that the availability of FAME in culture medium for fermentation process would be higher than that of solid PFAD, thus promoting improved rhamnolipid production by *P. aeruginosa* PAO1.
In order to assess the availability of the substrates to the microorganism, three different substrate types; PFAD, FAME and emulsified PFAD after fermentation were analysed using GC-MS to determine any significant differences in fatty acid composition between the substrates. Table 1 showed the main fatty acid components of PFAD, FAME and emulsified PFAD after fermentation. As expected, PFAD and FAME contain the same fatty acid components, as the methyl esters are derived from the fatty acids present in the PFAD. The main elements of PFAD/FAME are stearic acid 50.18%, pentadecanoic acid 16.98% and palmitic acid 16.91%. Meanwhile, the emulsified fraction of PFAD after fermentation was 42.9% stearic acid and 32.4% palmitic acid, which is consistent with Gapor Md Top (2010). This leads to the understanding that for PFAD substrate, primarily stearic palmitic acid was available to \textit{P. aeruginosa} PAO1 for growth and rhamnolipid production, with both fatty acids emulsified in the culture medium. Other fatty acids might have formed sticky solids clumps during the fermentation process, the formation of solid lumps which accumulated on the surface of the culture media was observed during shake flask experiments. Meanwhile for FAME, being liquid at room temperature may help to increase the availability of the free fatty acid content to the microbial culture for growth and rhamnolipid production, mitigating issues with poor emulsification of the PFAD substrate.

PFAD and FAME have the potential to be utilised as renewable substrates for strain growth and rhamnolipid production as they contain various types of free fatty acids at high concentrations (Nazren Radzuan et al., 2016). In 2016, several researches reported the use of fatty acid containing substrates for rhamnolipid production with \textit{P. aeruginosa} strains. For example Reddy et al. (2016) used mango kernel waste, Ji et al. (2016) used olive oil, Gudiña et al. (2016) and Moya Ramirez et al. (2016) used olive oil mill wastewater and Lotfabad et al. (2016) used soybean oil waste as the sole carbon sources for rhamnolipid production. This high number of publications highlighted using fatty acid waste as substrates shows the
potential in promoting rhamnolipid production, through utilising low-cost substrates and transforming fatty acid rich wastes into added value products.

3.2 Effect of type and concentration of carbon source on rhamnolipid production

3.2.1 General observations

A series of 5 L shake flask fermentation experiments were carried out using glucose, PFAD and FAME as the sole carbon sources, to determine and compare the rhamnolipid production and fermentation kinetics. The fermentation broth showed a significant change in colour from colourless to green in all experiments. Pyocyanin pigment has been reported to be responsible for the green colour change in the culture medium, and this pigment also has a positive correlation to the growth of this strain (El-Fouly et al., 2015). At the end of fermentations, the foam was observed accumulating on the top of the culture medium which is caused by the production of extracellular rhamnolipids, the surface-active target product (Junker, 2007). The occurrence of foaming during rhamnolipid production has also been observed by other researchers such as Funston et al. (2016), Diaz De Rienzo et al. (2016) and Lotfabad et al. (2016).

3.2.2 Glucose as the carbon source

Rhamnolipid production using 10 g L\(^{-1}\) and 20 g L\(^{-1}\) of glucose as the sole carbon source under batch fermentation conditions is presented in Figure 1(a). This experiment acts as a baseline control to allow for the major rhamnolipid congeners produced to be identified and to compare growth, production, and yields to those obtained when using fatty acids from PFAD and FAME (Zhang et al., 2012). Steady growth and rhamnolipid production were observed during the experiment to 72 h and 84 h at initial glucose concentrations of 10 g L\(^{-1}\) and 20 g L\(^{-1}\) respectively, after which time the growth rate decreased until the end of fermentation. In detail, using 20 g L\(^{-1}\) of glucose the final dry cell weight (DCW) was
1.2 g L$^{-1}$, and the final rhamnolipid (RL) concentration was 2.0 g L$^{-1}$. While with 10 g L$^{-1}$ of glucose, lower DCW and RL of 1.0 g L$^{-1}$ and 1.34 g L$^{-1}$ were reached respectively. Tiso et al. (2016) and Varjani and Upasani (2016) reported that when using 10 g L$^{-1}$ of glucose as, *P. putida* KT2440 and *P. aeruginosa* NCIM 5514 were able to produce 3.0 g L$^{-1}$ of RL while Moya Ramirez et al. (2016) showed that using *P. aeruginosa* PAO1, a lower RL production of 0.045 g L$^{-1}$ was achieved when using 2 g L$^{-1}$ of glucose. This result also suggests that by using *P. aeruginosa* PAO1 in batch fermentation, up to a point, the higher the concentration of glucose the better the cell growth and RL production, which is consistent with Clien et al. (2007). Clien et al. (2007) also reported that *P. aeruginosa* S2 could significantly increase its RL production from 0.5 g L$^{-1}$ to 2.5 g L$^{-1}$ when doubling the initial glucose concentration from 20 g L$^{-1}$ and 40 g L$^{-1}$. However, Varjani and Upasani (2016) observed the inverse pattern for RL production by *P. aeruginosa* NCIM 5514 which decreased from 3.0 g L$^{-1}$ to 1.0 g L$^{-1}$ when increasing the initial glucose concentration from 10 g L$^{-1}$ to 50 g L$^{-1}$. The differences in RL production with varying initial glucose concentrations may be caused by the varying characteristic of the different strains and the influence of culture conditions, which potentially affect the growth kinetics and yields.

3.2.3 Palm fatty acid distillate (PFAD) and Fatty acid methyl ester (FAME) as sole carbon sources

PFAD and FAME have similar free fatty acid content but have significant differences regarding physical characteristics, as discussed in Section 3.1. PFAD is the raw, solid form of the by-product from palm oil agricultural refinery mill. Meanwhile FAME is biodiesel derived from PFAD via esterification process. Both substrates have been used as sole carbon sources in this experiment to understand their suitability for use as substrates and the determine differences in cell growth, RL production and characterisation. PFAD was also
used as the sole carbon source for RL production in minimal media, leading to increased
production in comparison to earlier reports (Nazren Radzuan et al., 2016).

PFAD was used as the sole carbon source at initial concentrations of 10 g L$^{-1}$ and
20 g L$^{-1}$. Figure 1 (b) presents the fermentation kinetics observed, in which better growth and
RL production were observed at 10 g L$^{-1}$ initial PFAD compared to the case with 20 g L$^{-1}$
PFAD. Growth and RL production increased to a maximum level at 72 and 84 h for 10 g L$^{-1}$
and 20 g L$^{-1}$ respectively. The maximum DCW and RL concentration for 10 g L$^{-1}$ of PFAD
were 3.3 g L$^{-1}$ and 3.4 g L$^{-1}$ while at 20 g L$^{-1}$ of PFAD 2.6 g L$^{-1}$ and 3.1 g L$^{-1}$ of DCW and
RL were produced respectively.

FAME produced from the esterification of PFAD was used as the sole carbon source
with initial concentrations of 10 and 20 g L$^{-1}$. Figure 1 (c) shows growth and more RL
production at higher FAME concentration. With 10 g L$^{-1}$ of FAME, the DCW and RL
concentrations were 3.1 g L$^{-1}$ and 2.5 g L$^{-1}$, reaching the maximum level at 72 h, while with
20 g L$^{-1}$ of FAME the DCW and RL concentration was 2.8 g L$^{-1}$ and 3.0 g L$^{-1}$, respectively,
which was the maximum level reached after 84 h.

*P. aeruginosa* PAO1 can grow and produce biosurfactant in minimal culture medium
using PFAD and FAME as sole carbon sources with a maximum crude RL concentration in
the range 3-3.5 g L$^{-1}$, which is in the middle range of reported RL production based on Table
2. The different effects of varying substrate concentration on RL production were seen in
Figure 1, which were mainly due to the different physical characteristics of PFAD, being
solid at the fermentation temperatures compared with FAME which was in liquid form. This
plays an important role in the availability of the substrate to the strain and also how the
carbon sources are converted into RL.
3.2.4 Comparison of Yields

Table 2 shows the comparison of results obtained from this study to those reported by other researchers, in terms of maximum biomass produced (DCW$_{\text{max}}$), maximum rhamnolipid produced (RL$_{\text{max}}$), biomass formed related to initial substrate (*$Y_{X/S}$, g g$^{-1}$), product yield related to initial substrate (*$Y_{P/S}$, g g$^{-1}$) and volumetric productivity (PR$_L$, g L$^{-1}$ h$^{-1}$) at different concentrations of glucose, PFAD, FAME and other substrates used. In this study, maximum RL production and the time taken to reach this point for each substrate concentration were the criteria used to determine the *$Y_{X/S}$, *$Y_{P/S}$, $Y_{P/X}$ and PR$_L$. Generally, the *$Y_{X/S}$, *$Y_{P/S}$, and PR$_L$ observed for glucose were significantly lower compared to PFAD and FAME because using glucose shows lower growth and RL production throughout the fermentation process. PFAD can be potentially used as low-cost renewable substrate for rhamnolipid production using *P. aeruginosa* PAO1 as it shows better growth, substrate conversion into the product and high productivity.

The most favourable free fatty acid substrate and concentration found in this study for growth and substrate conversion to RL is 10 g L$^{-1}$ of PFAD with the highest *$Y_{X/S}$ of 0.337 g g$^{-1}$ and *$Y_{P/S}$ of 0.343 g g$^{-1}$, compared to other renewable substrates such as mango kernel oil, crude oil, olive mill waste, olive oil and soybean oil soap stock (Gudiña et al., 2016; Ji et al., 2016; Lotfabad et al., 2016; Reddy et al., 2016; Varjani and Upasani, 2016). However, the PR$_L$ for 10 g L$^{-1}$ of PFAD is 0.048 g L$^{-1}$ h$^{-1}$ is the second highest value compared the PR$_L$ from 40 g L$^{-1}$ of olive oil which is 0.130 g L$^{-1}$ h$^{-1}$, mainly because of higher RL production by *P. aeruginosa* M408 during the fermentation process (Ji et al., 2016).
3.3 Rhamnolipid Identification

The mass spectra (MS) of crude RL produced by *P. aeruginosa* PAO1 using glucose, PFAD and FAME all showed similar peaks with various types of RL congeners being present with molecular weight in the range 400 to 700 m/z and a high abundance of molecular ions at 475, 503, 529, 621, 649 and 647 m/z. As seen in Table 3, the m/z values are consistent with the molecular structure of Rha-C$_8$-C$_{10}$, Rha-C$_{10}$-C$_{10}$, Rha-C$_{12:1}$-C$_{10}$, Rha-Rha-C$_8$-C$_{10}$, Rha-Rha-C$_{10}$-C$_{10}$ and Rha-Rha-C$_8$-C$_{12}$ respectively. Specifically, the monorhamnolipid (Rha-C$_{10}$-C$_{10}$) was present in a significantly lower abundance compared to dirhamnolipid (Rha-Rha-C$_{10}$-C$_{10}$). This result is consistent with other researchers (George and Jayachandran, 2013; Nazren Radzuan et al., 2016). Table 3 shows the ratio of mono- to di- rhamnolipids produced during the fermentation experiments. Whilst dirhamnolipid were more abundant for all substrates used the ratio of Rha-C$_{10}$-C$_{10}$ (mono) to Rha-Rha-C$_{10}$-C$_{10}$ (di) was greater in the case of production from PFAD and FAME compared to glucose. For PFAD and FAME ratios of approximately 1:2 and greater were observed, compared to a ratio of 1:1.3 for glucose. This suggests that the substrate can be selected to favour the production of particular rhamnolipid congeners. It was demonstrated that the strain could produce monorhamnolipid and dirhamnolipid, with dirhamnolipid being the most abundant at the end of the fermentation process. However, the type of congener and overall rhamnolipids mixture composition is typically affected by many factors such as types of strain, type of carbon source, the age of culture and culture conditions (Aparna et al., 2012).

3.4 Rhamnolipid characterization

The crude RL extracted from experiments with glucose, PFAD and FAME were characterised by their capability to reduce the surface tension of a solution, the critical
micelle concentration and the ability to emulsify kerosene and sunflower oil. Figure 2 shows the ability of crude RL product by *P. aeruginosa* PAO1 in this study to reduce the surface tension of Tris-HCl pH 8.0 buffer to values between 29-32 mN m\(^{-1}\). Figure 2 also shows that the crude rhamnolipid extract gave the lowest CMC value of 8 mg L\(^{-1}\) when glucose was used as a substrate. Meanwhile, PFAD and FAME gave the same CMC value of 19 mg L\(^{-1}\).

The comparison of surface tension and CMC values are shown in Table 4, which demonstrates that despite the variability observed, the surface tension values reported in this study are comparable with those of others (Aparna et al., 2012; Lan et al., 2015; Varjani and Upasani, 2016). The CMC value for rhamnolipids produced from PFAD and FAME were lowest when compared to those produced on other substrates such as waste cooking oil, glycerol, molasses and sodium citrate, as shown in Table 4.

The emulsion index (EI) of crude RL extracts at a concentration of 1 g L\(^{-1}\) are in the range of 40-50 % for an emulsion of Tris-HCl pH 8.0 solution with either kerosene or sunflower oil. The percentage of emulsification decreased as the crude rhamnolipids concentration decreased, as shown in Figure 3. When compared to other literature results, Table 4, the EI for this study is lower when compared with rhamnolipids produced from other substrates such as crude oil and waste cooking oil (Varjani and Upasani, 2016).

The results of this study vary when compared with other reported research listed in in Table 4, suggesting that the crude rhamnolipid produced by different *Pseudomonas* strains and substrates are different regarding component and concentration of each congener, and degree of purity thus affecting the surface tension, CMC and emulsion index characteristics. The concentration of the crude rhamnolipid has a significant effect on the functionality of the biosurfactant as an emulsifier.
In this section, we discuss the effect using either PPGas or minimal media on *P. aeruginosa* PAO1 growth, rhamnolipid production and characteristics, yields and the cost of culture media in 5 L shake flask fermentation using 20 g L$^{-1}$ of PFAD as the sole carbon source. A comparison between this study and a previous study by Nazren Radzuan et al., 2016 is noteworthy. Figure 4 (a) shows the dry cell weight for both media were almost the same, 2.52 g L$^{-1}$ using PPGas media and with minimal media, DCW 2.66 g L$^{-1}$. Meanwhile, significant differences were found in rhamnolipid production on minimal media with 3.19 g L$^{-1}$ of rhamnolipids, 8.4 times higher than the rhamnolipid concentration attained using PPGas media. This significant increase in RL production is suggested mainly because of nitrogen limitation. PPGas media contains peptone, a source of amino acids, peptides and protein, meaning the media contains a high amount of nitrogen in excess of the nitrogen present in minimal medium, which contains 1 g L$^{-1}$ of nitrogen from NaNO$_3$. Nitrogen limitation potentially stimulated over production of rhamnolipid and caused the cell growth to enter stationary phase (Banat, 1997). In Figure 4 (b), the $Y_{PS}$ and $P_{RL}$ from using minimal media are 0.160 g g$^{-1}$ and 0.038 g L$^{-1}$ h$^{-1}$, higher when compared to PPGas media, $Y_{PS}$ 0.019 g g$^{-1}$ and $P_{RL}$ 0.0014 g L$^{-1}$ h$^{-1}$. The comparison of rhamnolipid characteristics regarding surface tension, CMC and EI is shown in Figure 4 (c).

The ability of the rhamnolipids produced from PPGas media and minimal media to reduce surface tension were similar, 29.00 mN m$^{-1}$ and 31.73 mN m$^{-1}$. The EI with kerosene and sunflower oil of rhamnolipid produced from minimal media also showed a higher result of 40% and 45%, compared to rhamnolipids produced from PPGas with an EI of 25% and 30% for kerosene and sunflower oil respectively. Lastly, Figure 4 (d) shows the lower media cost of rhamnolipid production using minimal media of £0.07 per litre, compared £2.40 per litre when using PPGas media. This indicates that rhamnolipids produced from minimal
media performed better than those from PPGas media regarding production, yields,
characteristic and cost. The minimal media is the best for scale up in the bioreactor and has
greater potential to be commercialised for rhamnolipid production by *P. aeruginosa* PAO1
using PFAD as the sole carbon source.
4. Conclusion

The use PFAD and FAME as sole carbon sources for rhamnolipid production by *P. aeruginosa* PAO1 using minimal media significantly improved rhamnolipid production from 1 g L\(^{-1}\) to 3 g L\(^{-1}\) with a \(Y_{X/S}\) of 0.3 g g\(^{-1}\), a \(Y_{P/S}\) of 0.3 g g\(^{-1}\) and \(P_{RL}\) of 0.4 g L\(^{-1}\) h\(^{-1}\).

Furthermore, both substrates PFAD and FAME have the potential to be used as renewable substrates in larger scale RL production. Based on the results of this study, using minimal media an estimated of 2 \(\times\) 10\(^5\) tonnes yr\(^{-1}\) of RL can be produced from the PFAD available from palm oil refining, which at current RL prices can generate an estimated £200 million gross income. Since PFAD is a low-cost by-product from palm oil refinery mills, this will significantly decrease RL production cost. In addition, FAME can be further utilised by providing a new value-added route for rhamnolipid production, rather than limiting use to only the production of biofuels for transportation. This would be advantageous in a palm oil biorefinery where the overall production strategy could be tailored to maximise added value, with the flexibility to further convert FAME to biosurfactant in the face of market variation for biodiesel. This flexibility is crucial for future palm oil biorefineries economic feasibility.
Acknowledgements

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acid cosubstrates provide B-oxidation precursors for rhamnolipid biosynthesis in 
pseudomonas aeruginosa, as evidenced by isotope tracing and gene expression assays. 
Figure Captions

Figure 1  Time course profiles of *P. aeruginosa* PAO1 cell growth and rhamnolipid production at 200 rpm and 37 °C by using (a) Glucose, (b) PFAD and (c) FAME as carbon sources. Solid line is 10 g L\(^{-1}\) and dashed line is 20 g L\(^{-1}\) of substrate fed. (■) is dry cell weight and (●) is rhamnolipid production.

Figure 2  Critical micelle concentration (CMC) of rhamnolipid from glucose, PFAD and FAME. (■) Glucose, (●) PFAD and (▲) FAME.

Figure 3  Emulsion Index of rhamnolipids with (a) kerosene and (b) sunflower oil. (■) Glucose, (●) PFAD and (▲) FAME.

Figure 4  Bar chart comparison of (a) Growth and Production, (b) Yields, (c) Rhamnolipid Characteristics and (d) Cost for different culture medium in 5 L shake flask fermentation using 20 g L\(^{-1}\) of PFAD as sole carbon source. (       ) denotes PPGas and (         ) Minimal Media
Table 1 Fatty acid content of PFAD, FAME and emulsified PFAD fraction

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<tr>
<th>Substrates</th>
<th>Component</th>
<th>Chemical formula</th>
<th>Percentage (%)</th>
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<td>Pentadecanoic acid</td>
<td>C₁₅H₃₀O₂</td>
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<td>C₁₆H₃₂O₂</td>
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<td></td>
<td>Linoleic acid</td>
<td>C₂₀H₃₅ClO₂</td>
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<td></td>
<td>Squalene</td>
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<td>2-Nonadecanone</td>
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<td>Emulsified PFAD</td>
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<tr>
<td></td>
<td>Tridecanoic acid</td>
<td>C₁₅H₃₀O₂</td>
<td>3.56</td>
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Table 2 Comparison of maximum biomass produced (DCW\textsubscript{max}), maximum rhamnolipid produced (RL\textsubscript{max}), biomass formed related to initial substrate (*Y\textsubscript{X/S}, g g\textsuperscript{-1}), product yield related to initial substrate (*Y\textsubscript{P/S}, g g\textsuperscript{-1}), product yield related to biomass formed (Y\textsubscript{P/X}, g g\textsuperscript{-1}) and volumetric productivity (P\textsubscript{RL}, g L\textsuperscript{-1} h\textsuperscript{-1}) on different concentration of glucose, PFAD and FAME.

<table>
<thead>
<tr>
<th>Types of fermentation</th>
<th>Microorganism</th>
<th>Substrate</th>
<th>Concentration (g L\textsuperscript{-1})</th>
<th>Time\textsubscript{max} (h)</th>
<th>DCW\textsubscript{max} (g L\textsuperscript{-1})</th>
<th>RL\textsubscript{max} (g L\textsuperscript{-1})</th>
<th>*Y\textsubscript{X/S} (g g\textsuperscript{-1})</th>
<th>*Y\textsubscript{P/S} (g g\textsuperscript{-1})</th>
<th>P\textsubscript{RL} (g L\textsuperscript{-1} h\textsuperscript{-1})</th>
<th>References</th>
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<td>72</td>
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<td>1.340</td>
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<td>0.038</td>
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<td>20</td>
<td>84</td>
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<td>2.537</td>
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<td>250 mL flask</td>
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<td>1.050</td>
<td>0.143</td>
<td>0.105</td>
<td>0.015</td>
<td>(Reddy et al., 2016)</td>
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<td>Palmitic</td>
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<td><strong>P. aeruginosa</strong></td>
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*Y_{PS} and *Y_{XS} are using initial substrate fed, calculated only for this study*
Table 3 Chemical composition and mono- to di-rhamnolipid ratio of rhamnolipid mixture produced by *P. aeruginosa* PAO1 from mass spectrometry analysis.

<table>
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<tr>
<th>Carbon source</th>
<th>Ratio</th>
<th>Rhamnolipid congeners</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>Rhamnolipid congeners</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Pseudomolecular Ion, m/z)</td>
</tr>
<tr>
<td>Glucose</td>
<td>1:1.28</td>
<td>Rha-C&lt;sub&gt;8&lt;/sub&gt;-C&lt;sub&gt;10&lt;/sub&gt; (475)</td>
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<td></td>
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<td>Rha-C&lt;sub&gt;10&lt;/sub&gt;-C&lt;sub&gt;10&lt;/sub&gt; (503)</td>
</tr>
<tr>
<td>PFAD</td>
<td>1:2.52</td>
<td>Rha-Rha-C&lt;sub&gt;8&lt;/sub&gt;-C&lt;sub&gt;10&lt;/sub&gt; (621)</td>
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<td>FAME</td>
<td>1:2.38</td>
<td>Rha-Rha-C&lt;sub&gt;10&lt;/sub&gt;-C&lt;sub&gt;10&lt;/sub&gt; (649)</td>
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<tr>
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<td></td>
<td>Rha-Rha-C&lt;sub&gt;12&lt;/sub&gt;-C&lt;sub&gt;12&lt;/sub&gt; (677)</td>
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Table 4 Comparison of effect of different carbon sources and microorganism on surface tension (mN m$^{-1}$), critical micelle concentration (CMC, g L$^{-1}$) and emulsion index (E24, %) with previous research.

<table>
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<tr>
<th>Microorganism Substrate</th>
<th>Surface tension reduction (mN m$^{-1}$)</th>
<th>CMC (mg L$^{-1}$)</th>
<th>Rhamnolipid Concentration (g L$^{-1}$)</th>
<th>Emulsion Index (%)</th>
<th>References</th>
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<td>5.00</td>
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</table>
Figure 1 Time course profiles of *P. aeruginosa* PAO1 cell growth and rhamnolipid production at 200 rpm and 37 °C by using (a) Glucose, (b) PFAD and (c) FAME as carbon sources. Solid line (—) is 10 g L\(^{-1}\) and dashed line (----) is 20 g L\(^{-1}\) of substrate fed. (■) is dry cell weight and (●) is rhamnolipid.
Figure 2 Critical micelle concentration (CMC) of rhamnolipid from glucose, PFAD and FAME. (■) Glucose, (●) PFAD and (▲) FAME.
Figure 3 Emulsion Index of rhamnolipids with (a) kerosene and (b) sunflower oil. (■) Glucose, (●) PFAD and (▲) FAME.
Figure 4 Bar chart comparison of (a) Growth and Production, (b) Yields, (c) Rhamnolipid Characteristics and (d) Cost for different culture medium in 5 L shake flask fermentation using 20 g L$^{-1}$ of PFAD as sole carbon source. ( ) denotes PPGas and ( ) Minimal Media.