ENHANCED RHAMNOLIPID PRODUCTION FROM WASTE COOKING OIL BY
Pseudomonas aeruginosa USM-AR2

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UNIVERSITI SAINS MALAYSIA

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ENHANCED RHAMNOLIPID PRODUCTION FROM WASTE COOKING OIL BY
Pseudomonas aeruginosa USM-AR2

by

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<tr>
<td>a</td>
<td>specific surface area</td>
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<tr>
<td>$\alpha, \beta$</td>
<td>Luedeking-Piret constant</td>
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<tr>
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<td>WCO</td>
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PENINGKATAN PENGHASILAN RHAMNOLIPID DARIPADA MINYAK MASAK TERPAKAI OLEH *Pseudomonas aeruginosa* USM-AR2

ABSTRAK

Rhamnolipid adalah salah satu daripada biosurfaktan jenis glikolipid yang paling kerap dikaji. Permasalahan kajian ini adalah minyak masak terpakai yang digunakan sebagai sumber karbon utama tidak larut di dalam fasa akues dan boleh merencat pertumbuhan mikroorganisma yang dikaji iaitu *Pseudomonas aeruginosa* USM–AR2 serta rhamnolipid yang dihasilkan akan berkurangan. Objektif utama kajian adalah untuk meningkatkan penghasilan rhamnolipid secara mikrob pada skala makmal. Oleh itu strategi suapan untuk teknik kelompok suapan yang bersesuaian perlu ditentukan bagi meningkatkan kebolehdapatan dan seterusnya pengambilan sumber karbon oleh mikroorganisma tersebut. Objektif-objektif kajian adalah untuk 1) menilai dan memilih formulasi media dari kajian terdahulu, 2) menentukan kesan keadaan-keadaan pengoperasian terhadap penghasilan rhamnolipid dan tingkah laku pemindahan oksigen di dalam sistem fermentasi secara kelompok, 3) menganalisa kinetik penghasilan rhamnolipid secara kelompok dan 4) menentukan strategi suapan yang terbaik untuk penghasilan rhamnolipid secara kelompok suapan. Hasil kajian menunjukkan bahawa formulasi media yang telah diubahsuai seperti berikut dapat membantu meningkatkan penghasilan rhamnolipid iaitu: $\text{NO}_3^-$, $\text{Mg}^+$, $\text{K}^+$, $\text{PO}_4^{3-}$, unsur surih dan minyak masak terpakai dengan nisbah C/N bersamaan dengan 18. Penambahana surfaktan komersial iaitu Tween 80, tidak menunjukkan sebarang kesan yang ketara kepada peningkatan penghasilan rhamnolipid. Manakala, penghasilan rhamnolipid di dalam bioreaktor berskala makmal adalah di pengaruhi oleh kelajuan ujung pengaduk, di mana penghasilan rhamnolipid pada kelajuan ujung pengaduk
yang rendah (1.13 m/s) adalah 1.5 kali lebih tinggi daripada penghasilan rhamnolipid pada kelajuan pengaduk yang tinggi (1.41 m/s). Penghasilan rhamnolipid yang maksimum iaitu 4.85 g/L dengan daya pengeluaran keseluruhan 0.041 g/L.h dapat dicapai apabila pH media dikawal pada 6.85. Berdasarkan graf perhubungan di antara \( q_p \) dan \( \mu \) dapat ditentukan bahawa rhamnolipid adalah produk daripada kategori bukan pertumbuhan. Sementara itu didapati bahawa nilai \( k_{La} \) tidak terkesan dengan peningkatan kepekatan minyak masak terpakai. Manakala \( k_{La} \) meningkat secara linear apabila kepekatan rhamnolipid meningkat dan nilai \( k_{La} \) akan menurun apabila kepekatan rhamnolipid melebihi 1.0 g/L. Hasil kajian juga menunjukkan strategi suapan kelompok berdasarkan kadar pengambilan substrat maksimum secara automatik adalah berpotensi untuk meningkatkan penghasilan rhamnolipid. Rhamnolipid dapat dihasilkan pada kepekatan yang maksimum iaitu 8.54 g/L dengan daya pengeluaran keseluruhan 0.045 g/L.h melalui strategi tersebut. Rhamnolipid yang dihasilkan melalui kajian ini adalah sebanding dengan penghasilan rhamnolipid oleh *Pseudomonas aeruginosa* ATCC 9027 iaitu 8.5 g/L. Maka, dapat dibuktikan bahawa penghasilan rhamnolipid melalui kaedah kelompok suapan telah meningkat sebanyak 76.4% lebih tinggi daripada penghasilan secara kelompok.
ENHANCED RHAMNOLIPID PRODUCTION FROM WASTE COOKING OIL BY Pseudomonas aeruginosa USM-AR2

ABSTRACT

Rhamnolipid, a glycolipid type of biosurfactant is the most investigated glycolipid biosurfactant. The problem of this study was the waste cooking oil used as a major carbon source is immiscible in aqueous phase and inhibited the growth of the microorganisms studied which is Pseudomonas aeruginosa USM-AR2. The ultimate aim is to enhance microbial production of rhamnolipid on a lab-scale. Thus, the appropriate feeding strategy for fed-batch culture needs to be determined to increase the availability and subsequent intake of the carbon source by the microorganisms. Several objectives have to be met to ensure this strategy is achievable, which include: 1) to evaluate and select different medium formulation from literature.; 2) to determine the effect of operational conditions on rhamnolipid production and the behaviour of oxygen transfer in batch culture; 3) to analyse the kinetics of rhamnolipid production in batch culture; and 4) to identify the best feeding strategy to improve rhamnolipid production in fed-batch culture. Results showed that the modified medium composition to support rhamnolipid production contained the following: NO₃⁻, Mg⁺, K⁺, PO₄³⁻, trace elements and waste cooking oil with C/N equivalent to 18. The addition of Tween 80, a commercial surfactant, into the medium showed no significant impact on rhamnolipid production. In a bench-top bioreactor, the agitator tip speed affected rhamnolipid production. Rhamnolipid production at a lower tip speed (1.131 m/s) was 1.5-fold higher than production at a higher tip speed (1.414 m/s). Rhamnolipid production achieved the maximum concentration of 4.86 g/L (0.041 g/L.h of the overall productivity) when the production medium was controlled at pH 6.85. Based
on a correlation plot between $q_p$ and $\mu$ it was determined that rhamnolipid was a non-growth associated product. The waste cooking oil within the range studied did not affect the $k_{L,a}$. The $k_{L,a}$ increased linearly with rhamnolipid concentration and it started to decrease when the concentration was more than 1.0 g/L. An automatic maximum substrate uptake rate (MSUR) feeding strategy for fed-batch production is a potential feeding strategy to improve rhamnolipid production. The highest rhamnolipid produced in fed-batch culture with MSUR feeding strategy was 8.58 g/L with 0.045 g/L.h of the overall productivity. The rhamnolipid produced by this study are comparable to the production of rhamnolipid by *Pseudomonas aeruginosa* ATCC 9027 which is 8.5 g/L. Thus, rhamnolipid production in fed-batch culture was 76.4% enhanced compared to batch culture.
CHAPTER 1 INTRODUCTION

1.1 Research background

Microbial surfactants or biosurfactants are amphiphilic molecules produced by various microorganisms. These molecules contain both hydrophilic and hydrophobic moieties that partition preferentially at the interface of fluid phases with different polarity, e.g.: oil and water, or air and water interfaces. These compounds can be roughly divided into two main classes (Neu, 1996): low-molecular-weight compounds called biosurfactants, such as lipopeptides, glycolipids, proteins and high-molecular-weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteins that are collectively called bioemulsans (Rosenberg and Ron, 1997) or bioemulsifiers (Smyth et al., 2010b). The former group includes molecules which can efficiently reduce surface and interfacial tension, while the latter are amphiphilic and polyphilic polymers that are usually more efficient in stabilising emulsions of oil-in-water but do not lower the surface tension as much (Smyth et al., 2010a).

The substance may function as detergents, wetting agents, emulsifiers, foaming and antifoaming agents, and dispersants (Deleu and Paquot, 2004). Such properties play a significant role in various fields such as bioremediation, biodegradation, oil recovery, food, pharmaceutics, and many other applications in different industrial sectors.

The most commonly isolated and widely studied group of surfactants produced by the microorganism is glycolipids (Chrzanowski et al., 2012). Among the glycolipids are rhamnolipid, trehalolipids, sophorolipids and mannosylerythritol lipids (MELs). Rhamnolipid are also the most investigated glycolipids biosurfactant based on a high number of listed publications (>900) in ISI Web of ScienceSM and related patents (~100) from European Patent Office (Müller et al., 2012).
Compared to biosurfactants, the commercial production of synthetic surfactants (petrochemical-based surfactants) started in Germany in early twentieth century (Stalmans et al., 2007). Although synthetic surfactants are essential substances utilised in products such as household detergents, healthcare products, cosmetics and pharmaceuticals, some are not biodegradable, able to accumulate and some of the petroleum-based products are toxic to the environment (Banat et al., 2014). In addition, the decrease availability of petrochemical supply may increase the difficulty in accessing the feedstocks and would cause environmental damage (Hayes, 2012).

Thus, bio-based surfactants from oleochemicals such as alkyl polyglycoside (APG) were introduced. Still, the production involved chemicals and harsh conditions (Hayes, 2012). Therefore, the production of microbial surfactants through fermentation processes could be promising option for enhancing sustainability such as lower energy utilisation and the absence of solvents.

The world market demand for bio-based surfactants increased from 344,068 tonnes in 2013 and is expected to reach 461,992 tonnes by 2020. Glycolipid biosurfactant, specifically rhamnolipid had a relatively small market in 2013. However, it is anticipated to register the highest growth at an estimated Compound Annual Growth Rate (CAGR) of 5.4% from 2014 to 2020, owing to its development through bioprocessing technology (www.grandviewresearch.com).

Biosurfactants possess remarkable eco-friendly properties, which are able to meet the biodegradable criteria and test methods for aerobic biodegradability by the European Surfactant Directive Regulation EC No.: 648/2004 (Randhawa and Rahman, 2014). This regulation is set forth to achieve the free movement of detergents and surfactants for detergents in the European market and at the same time, ensure a high degree of protection of the environment and human health.
However, a major downside for commercialization of biosurfactant, especially rhamnolipids, is the high production cost due to the use of high-priced substrates, relatively low product yields, and expensive downstream processing. The current market price of rhamnolipids (R-95, 95%) is USD 20 per mg (AGAE Technologies, USA) compared to only USD 1-3 per kg for alkyl polyglycosides (Henkel et al., 2012). Several factors might contribute to the low cost of alkyl polyglycosides as compared to rhamnolipids, such as low cost substrate, simple production process and high yield (Eskuchen and Nitsche, 1996). The production cost of rhamnolipid should be lowered to USD 4.21 per kg to make it more competitive (Randhawa and Rahman, 2014), but, it is a challenging task to achieve. Moreover, limited companies are known to produce rhamnolipids on a commercial scale, and the manufacturing yield is only in the range of 10 to 20 g/L (Marchant and Banat, 2012b). Thus, research in biosurfactant production especially rhamnolipids is relevant and a suitable choice to pursue.

1.2 Problem statements and objectives

In this study, palm oil derived waste cooking oil will be utilized as the sole carbon source. According to (Henkel et al., 2014), the use of waste cooking oil at a certain concentration may inhibit the growth of the microorganism and consequently affect rhamnolipid production. It is well known that the oil is immiscible with an aqueous solution, thus it is essential to facilitate the oil uptake by the cell. Current knowledge has shown that high rhamnolipid production can be achieved through fed-batch production with immiscible substrate such as sunflower oil and soybean oil (Giani et al., 1997; Zhu et al., 2012). Unfortunately, the strategy was not fully developed for rhamnolipid production. Hence, an effective feeding strategy needs to be designed to avoid inhibition effect of the oil and ensure maximum uptake and thus
consumption of the substrate by the cell, since it is immiscible with an aqueous solution.

Thus, the objectives of the study are:

1. To evaluate and select different medium formulation from literature.
2. To determine the effect of operational conditions on rhamnolipid production and the behaviour of oxygen transfer in batch culture.
3. To analyse the kinetics of rhamnolipid production in batch cultures.
4. To identify the best feeding strategy to improve rhamnolipid production in fed-batch culture.

1.3 Rationale and scope of the project

The ultimate aim of this research is to increase rhamnolipid production using the indigenous isolate Pseudomonas aeruginosa USM-AR2. It was proven that the isolate was a potential producer for the high rhamnolipid production. Current rhamnolipid production by the isolate was 28 g/L with diesel as a carbon source and the fed-batch feeding strategy employed was maximum substrate uptake rate (Noh et al., 2014). However, the primary usage of diesel as transportation fuels may cause a prohibitively high cost to the process. Therefore, waste cooking oil was chosen with justification (as discussed in Section 2.4.2) in place of diesel.

The study started with shake flasks experiments to screen for the suitable medium formulation to support maximum rhamnolipid production. The medium formulation was selected based on their capability to support highest rhamnolipid production reported by Muller et al., (2010), Zhu et al., (2012) and Nur Ashifia et al., (2012). The research proceeded with batch culture study using selected medium from the previous shake flasks experiment. Several criteria were investigated such as
agitation and aeration speed, dissolved oxygen and pH control, medium at similar total carbon and similar carbon to nitrogen ratio. Based on the data obtained, the kinetics of rhamnolipid production in batch culture would be determined. The last part of this research was on the investigation of different feeding strategies for fed-batch culture to achieve the final aim of this research.
CHAPTER 2 LITERATURE REVIEW

2.1 Introduction to biosurfactant

Biosurfactants are an amphiphilic molecule that is made of a hydrophilic head and the hydrophobic tail. The polar or hydrophilic part consists of functional groups containing heteroatoms and shows a strong affinity for polar solvents, particularly water. The apolar or hydrophobic part comprises, in general, one or more linear or branched alkyl chains and shows an affinity for non-polar solutes.

Due to their amphiphilic structure, surfactant molecules exhibit two fundamental properties. One is their tendency to adsorb to surfaces or interfaces in an oriented fashion (Zhang and Somasundaran, 2006). For example, when dissolved in water, surfactant molecules tend to adsorb at the air/water surface and arrange themselves with their hydrophilic groups in the water phase and the hydrophobic groups oriented toward the air. The driving force is to lower the free energy of the system since the presence of the hydrophobic components in the water causes both the water molecules in the hydration shell and the hydrophobic parts to lose some freedom of motion. Thus, removing hydrophobic groups from the water phase maximises entropy. The adsorption of surfactant molecules at the water/air surface reduces the dissimilarity of these two phases, resulting in a lowering of surface tension.

The other fundamental property exhibited by surfactants is that surfactant monomers in solution tend to form dynamic aggregates called micelles above a certain concentration that is known as the critical micelle concentration (CMC) (Cheng and Sabatini, 2007). At the CMC, the number of surfactant monomers in bulk reaches a maximum, and at this maximum, micelles begin to form (Figure 2.1). In aqueous solution, micelles are formed by the aggregation of the hydrophobic tail groups in the interior of the micelle while the hydrophilic head groups are in contact with the water
and form a shell around the tail groups that prevents them from direct contact with the water phase.

**Figure 2.1** Formation of biosurfactant structures at a concentration above the critical micelle concentration (Herman and Maier, 2002)

### 2.1.1 Rhamnolipids, a glycolipid microbial surfactant

Based on molecular structure, biosurfactants can be classified as glycolipids (e.g.: rhamnolipids and sophorolipids), lipopeptides (e.g.: surfactin), polymeric biosurfactants (e.g.: emulsan and alasan), fatty acids (e.g.: 3-(3-hydroxyalkanoyloxy)) alkanoic acids (HAAs)), and phospholipids (e.g.: phosphatidylethanolamine) (Desai and Banat, 1997). Among the glycolipid biosurfactants, rhamnolipids are widely
investigated because they can be obtained at high yields and are considered safe for use in food products, cosmetics and pharmaceuticals.

An enormous diversity of rhamnolipid congeners and homologs are produced by different *P. aeruginosa* strains under many different culture conditions, type of carbon source utilised and also from other bacterial species (Abdel-Mawgoud *et al.*, 2010). Thus, in general, rhamnolipids are glycosides composed of rhamnose moieties (glycon part) and lipid moieties (aglycon part) that are linked through an O-glycosidic linkage (Figure 2.2). The glycon part is composed of one (mono-RLs) or two (di-RLs) rhamnose moieties connected to each other through a α-1,2-glycosidic linkage. The aglycon part, however, is mainly one or two (in few cases three) β-hydroxy fatty acid chains (saturated, mono-, or poly-unsaturated and of chain length varying from C8 to C16) linked to each other through an ester bond formed between the β-hydroxyl group of the distal (relative to the glycosidic bond) chain with the carboxyl group of the proximal chain (Abdel-Mawgoud *et al.*, 2011).

Rhamnolipids displays competitive properties compared to other biosurfactants. It reduces the surface tension of water from 72 to 31 mN/m. At concentrations above the critical micelle concentration (CMC), rhamnolipids form micelles, vesicles, or lamella depending on the pH of the solution, the concentration, and the presence of electrolytes (Figure 2.1). The CMC for rhamnolipids depends on the chemical composition of the various species and their chemical environment and has been reported to range from 5 to 200 mg/L (Nitschke *et al.*, 2011). A low CMC value characterises an effective surfactant.
Figure 2.2 Molecular structure of four different types of rhamnolipids (Leitermann et al., 2010)

For example, the CMC of rhamnolipid 1 (RL1) and rhamnolipid 3 (RL3) is about 20 mg/L in water. Expressed in molar concentrations, this is $3.96 \times 10^5$ mol/L for RL1 and $3.07 \times 10^5$ mol/L for RL3. The CMC of sodium dodecyl sulphate is much higher, i.e. $8.39 \times 10^3$ mol/L (Walter et al., 2010). Rhamnolipids were almost entirely degraded compared to Triton-X-100 and linear alkylbenzene sulphonates (LAS) that were only partially degraded. Also, the aquatoxicity of rhamnolipids, according to their EC$_{50}$ values was 20–77 mg/L, about 12-times lower than synthetic surfactants (Henkel et al., 2012). A crude biosurfactant produced by *P. aeruginosa* SP4 was shown to be heat- and pH-stable (Pornsunthorntawee et al., 2008). The crude biosurfactant could
remain its surface activity after being exposed to a high temperature of 120°C for 15 min at pH range of 3 to 11. Rhamnolipids are also stable to salinity, able to withstand their emulsification activity when exposed to a range of 16 to 40% salinity (Agwu et al., 2012).

2.1.2 Mechanism and role of rhamnolipids in the uptake and biodegradation of immiscible substrates

Rhamnolipids play different roles in microbial cells, but in general, the main function is to permit microorganism to grow on water-immiscible substrates (Nitschke et al., 2005). Research had focused on the uptake of alkanes as a model of immiscible substrate and there are three specific substrate uptake mechanisms for alkanes had been proposed by (Hommel, 1990). They are; uptake of monodispersed dissolved alkanes, direct contact of cells with large oil drops, and contact with fine oil droplets (pseudosolubilised alkanes).

Beal and Betts (2000) explain that the first mechanism involves direct uptake of the alkane dissolved in the aqueous phase. This is naturally a very low amount due to the low solubility of most alkanes; however, this mechanism is thought to operate for the uptake of small chain types. The second mechanism proposes that alkanes are transported into the cell by direct contact of alkane droplets with the microbial cell. In this mechanism, microbial cells attach to droplets that are much bigger than the cells, and substrate uptake is thought to take place through diffusion or active transport. In this hypothesis, biosurfactants would act to increase emulsification, thereby increasing the surface area available for micro-organisms to adhere to the alkane droplets. The third mechanism proposes the uptake of alkanes in a pseudo solubilised form. This mechanism is explain as at low concentration, biosurfactants occur as monomers at the
interface between the aqueous and hydrocarbon phases. When the concentration increases and the space available decreases, biosurfactants tend to arrange into aggregates up to a point called the critical micelle concentration at which micelles are formed trapping the hydrocarbons into their hydrophobic core. Once dispersed, hydrocarbons become more available to uptake by the cells (Perfumo et al., 2010).

In view of alkane uptake by microbial cells, it occurred through direct contact with larger alkane droplets and by pseudo solubilisation. Also, it appears that both mechanisms occur simultaneously (Beal and Betts, 2000). For rhamnolipid-producing microorganism such as *P. aeruginosa*, the uptake mechanism is energy-dependent (Noordman and Janssen, 2002) and that the dispersion of oil is affected by pH and shaking speed (Zhang and Miller, 1992).

2.2 Production of biosurfactant

Production of biosurfactant through fermentation process could be a promising option for improving sustainability such as lower energy utilisation and the absence of solvents. Major drawbacks in the production of chemically synthesized surfactants whether they are petrochemical-based or oleochemical-based are related to environmental issues and availability of the petrochemical supply. For example, a highly produced synthetic surfactants; alkyl benzene sulfonate (ABS) in the 1940s was mainly used for the household application. It was not sufficiently removed by sewage treatment owing to its poor biodegradable properties. The remaining surfactant started to accumulate and initiated an excessive foaming when entering rivers and streams (Stalmans et al., 2007). This incident caught the attention of the public and prompted the industry and regulators to scrutinise the environmental properties of the synthetic surfactant.
The long-term availability of petrochemical supply added to the community concerns about synthetic surfactant usage. Several events arise as a consequence of the feedstocks shortage, such as petrochemical price instability, environmental damage and release of greenhouse gases. In 2008, the petrochemical price reached up to $140/barrel (www.macrotrends.net), then down to around $50/barrel in 2015 and will increase to around $103/barrel in 2025 (http://knoema.com). In 2010, the “Deepwater Horizon” off-shore oil well, 5600 m below sea level in the Gulf of Mexico was leaked. The leakage caused significant damage to the environment, and it was the largest environmental disaster in the United States history (Hayes, 2012). Meanwhile, the production of oleochemical-based surfactants involves chemicals at extreme condition. For example, an industrial-scale production of monoacylglycerols (MAGs) is carried out through glycerolysis of triacylglycerol (TAG) or fatty acid methyl esters (FAME) at 220–250°C. However, the product yield is only between 30–40%, with the formation of undesirable by-products. An extra purification step such as molecular distillation is necessary to ensure high purity of MAGs produced (Kaewthong et al., 2005). Another example is the production of Span® of which its preparation involves two steps. First, acid-catalyzed dehydration of sorbitol to form sorbitan, and followed by alkali-catalysed (e.g., NaOCH₃⁻) transesterification between FAME and sorbitan at 200–250°C. In addition, in the preparation of sucrose–fatty acid esters through transesterification of FAME, the reaction was performed at elevated temperatures of more than 100°C and reduced pressure for several h in the presence of toxic solvents such as dimethylformamide (DMF) or dimethylsulfoxide (DMSO). On the other hand, alkyl polyglycolides (APGs) are already produced under solvent-free and mild reaction temperatures. However, it still requires molecular distillation, an energy-intensive method to remove excess reactant (fatty alcohol) (Hayes, 2012). Thus, the
production of biosurfactant through fermentation process offers an environmental friendly alternative since hazardous chemicals are avoided and the process is usually performed under mild condition. The commercial production of glycolipids such as sophorolipids is more common than rhamnolipids (Table 2.1). Common hurdles associated with rhamnolipid production at a larger scales are low yield, high production cost and too many downstream processing units (Marchant and Banat, 2012a).

Accordingly, three primary strategies were suggested to improve the production of biosurfactants to be more cost-competitive (Mukherjee et al., 2006; Walter et al., 2010):

i. Screening of bacterial strains for overproducing wild nonpathogenic type, mutant or recombinant strains,

ii. The use of cheaper substrates from waste to lower the raw material costs involved in the process and

iii. The development of more efficient bioprocesses including optimisation of culture conditions, as well as cost-effective separation processes for maximum biosurfactant recovery.

Therefore, in the next section, these approaches and strategies for rhamnolipid production concerning the above primary strategies are reviewed.
Table 2.1 List of biosurfactant manufacturers around the world. Six companies produce rhamnolipids and others mostly sophorolipid is their product (Randhawa and Rahman, 2014)

<table>
<thead>
<tr>
<th>No</th>
<th>Company</th>
<th>Location</th>
<th>Product(s)</th>
<th>Focus on</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TeeGene Biotech</td>
<td>UK</td>
<td>Rhamnolipids and Lipopeptides</td>
<td>Pharmaceuticals, Cosmetics, antimicrobials and anti-cancer ingredients</td>
</tr>
<tr>
<td>2</td>
<td>AGAE Technologies LLC</td>
<td>USA</td>
<td>Rhamnolipids (R95, an HPLC/MS grade rhamnolipids)</td>
<td>Pharmaceutical, cosmeceutical, cosmetics, personal care, bioremediation (in situ &amp; ex situ), Enhanced oil recovery (EOR)</td>
</tr>
<tr>
<td>3</td>
<td>Jeneil Biosurfactant Co. LLC</td>
<td>USA</td>
<td>Rhamnolipids (ZONIX, a bio-fungicide and RECO, a rhamnolipids used in cleaning and recovering oil from storage tanks)</td>
<td>Cleaning products, EOR</td>
</tr>
<tr>
<td>4</td>
<td>Paradigm Biomedical Inc.</td>
<td>USA</td>
<td>Rhamnolipids</td>
<td>Pharmaceutical applications</td>
</tr>
<tr>
<td>5</td>
<td>Rhamnolipids Companies, Inc.</td>
<td>USA</td>
<td>Rhamnolipids</td>
<td>Agriculture, cosmetics, EOR, bioremediation, food products, pharmaceuticals</td>
</tr>
<tr>
<td>6</td>
<td>Fraunhofer IGB</td>
<td>Germany</td>
<td>Glycolipids, Cellobiose lipids, MELs</td>
<td>Cleansing products, shower gels, shampoos, washing-up liquids, pharmaceutical (bioactive properties)</td>
</tr>
<tr>
<td>7</td>
<td>Saraya Co. Ltd.</td>
<td>Japan</td>
<td>Sophorolipids (Sophoron, a low-foam dishwasher detergent)</td>
<td>Cleaning products, hygiene products</td>
</tr>
<tr>
<td>8</td>
<td>Ecover Belgium</td>
<td>Belgium</td>
<td>Sophorolipids</td>
<td>Cleaning products, cosmetics, bioremediation, pest control, pharmaceuticals</td>
</tr>
<tr>
<td>9</td>
<td>Groupe Soliance</td>
<td>France</td>
<td>Sophorolipids</td>
<td>Cosmetics</td>
</tr>
<tr>
<td>10</td>
<td>MG Intobio Co. Ltd</td>
<td>South Korea</td>
<td>Sophorolipids (Sopholine—functional soap with Sophorolipids secreted by yeasts)</td>
<td>Beauty and personal care, bath supplies, e.g., soaps with new functions</td>
</tr>
<tr>
<td>11</td>
<td>Synthezyme LLC</td>
<td>USA</td>
<td>Sophorolipids</td>
<td>Cleaning products, cosmetics, food products, fungicides, crude oil emulsification</td>
</tr>
<tr>
<td>12</td>
<td>Allied Carbon Solutions (ACS) Ltd</td>
<td>Japan</td>
<td>Sophorolipids (ACS-Sophor-first bio-based surfactant from Indian mahua oil)</td>
<td>Agricultural products, ecological research</td>
</tr>
<tr>
<td>13</td>
<td>Henkel</td>
<td>Germany</td>
<td>Sophorolipids, Rhamnolipids, Mammoslyerthritol lipids</td>
<td>Glass cleaning products, laundry, beauty products</td>
</tr>
<tr>
<td>14</td>
<td>Kaneka Co.</td>
<td>Japan</td>
<td>Sophorose lipids</td>
<td>Cosmetics and toiletry products</td>
</tr>
</tbody>
</table>
2.3 Rhamnolipid-producing bacteria

The majority of strains reported to produce rhamnolipids belongs to the genus *Pseudomonas* and most of them have been identified as *P. aeruginosa*. Other *Pseudomonas* species have also been reported to produce rhamnolipids (Table 2.2).

**Table 2.2** *Pseudomonas* species producing rhamnolipids (Nitschke *et al*., 2011)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Surface tension (mN/m)</th>
<th>CMC (mg/L)</th>
<th>Rhamnolipids (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. putida</em></td>
<td>31.2</td>
<td>91</td>
<td>4.1</td>
</tr>
<tr>
<td><em>P. alcaligenes</em></td>
<td>28</td>
<td>30</td>
<td>2.3</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>35</td>
<td>20</td>
<td>2.0</td>
</tr>
<tr>
<td><em>P. chlororaphis</em></td>
<td>25-30</td>
<td>n.d.</td>
<td>1.0</td>
</tr>
<tr>
<td><em>P. stutzeri</em></td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.5</td>
</tr>
<tr>
<td><em>P. luteola</em></td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.38</td>
</tr>
<tr>
<td><em>P. aeruginosa</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.9</td>
<td>9</td>
<td>12.5</td>
</tr>
<tr>
<td><em>P. aeruginosa</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.3</td>
<td>13.9</td>
<td>3.9</td>
</tr>
<tr>
<td><em>P. aeruginosa</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.3</td>
<td>46.8</td>
<td>46</td>
</tr>
</tbody>
</table>

n.d. not determined, CMC Critical micelle concentration  
<sup>a</sup> Mutant strain,  
<sup>b</sup> Solid state fermentation

A non-*Pseudomonas* species such as *Burkholderia plantarii* DSM 9509<sup>T</sup> was also reported to produce rhamnolipids with excellent surfactant properties but with a different structure from rhamnolipids produced by *P. aeruginosa*. Production of the *Burkholderia* rhamnolipids can lead to applications in detergents, pharmaceuticals, and other industries providing new products in the biosurfactant market (Hörmann *et al*., 2010). *Burkholderia kururiensis* KP23<sup>T</sup> was also reported as a natural rhamnolipid producer. It was identified that *B. kururiensis* KP23<sup>T</sup> produced 23 rhamnolipid congeners and the majority of the rhamnolipid population produced composed of dirhamnolipid (88.70%) (Tavares *et al*., 2013). *Burkholderia thailandensis* is another type of bacterium able to produce rhamnolipids. The proportion of dirhamnolipid to monorhamnolipid produced by *B. thailandensis* was much larger, approximately 13, whereas only a factor of four of dirhamnolipid to monorhamnolipid proportion was observed in *P. aeruginosa* (Dubeau *et al*., 2009). Other non-*Pseudomonas* species
reported were *Burkholderia pseudomallei* (pathogenic bacterium), an Antarctic isolate of *Pantoea* sp., *Pantoea stewartii*, *Acinetobacter calcoaceticus*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Nocardioides* sp. and *Pseudoxanthomonas* sp. (Nitschke *et al*., 2011).

It is well known that *P. aeruginosa* is a pathogenic bacterium, and has been implicated in infecting immune compromised individuals and specific infections related to lung infections associated with cystic fibrosis, corneal disease, burns wounds, urinary tract, hot tub rash, ears, and other organs. But, it is important to remark that only in rare cases that bacteria belonging to other *Pseudomonas* sp. produce rhamnolipids, while all *P. aeruginosa* isolates produce these surfactants (Toribio *et al*., 2010).

Furthermore, rhamnolipid production by other *Pseudomonas* species might be genetically unstable. This is because it was found that the genes encoding the enzymes, which participate in the synthesis of this biosurfactants are very much likely encoded in mobile genetic elements (Toribio *et al*., 2010).

Even though *P. aeruginosa* is known as an opportunistic pathogen, the strain is the most utilised strain for industrial scale production of rhamnolipids. One of the example is a company named Agae Technologies, based in the USA, whose technology was first licensed from Oregon State University using *P. aeruginosa* NY3 to manufacture novel rhamnolipids since 2011 (Houtman, 2011; Stauth, 2010). The company produces various qualities of rhamnolipids which can be applied in various industries as summarised in Figure 2.3.
Figure 2.3 Some of the potential application of rhamnolipid in various industries produced (www.agaetech.com)

Another example of rhamnolipid produced industrially using *P. aeruginosa* sp. is by Rhamnolipid Companies Inc., a company based in St. Petersburg, Florida, USA (DeSanto, 2011). The rhamnolipids produced are used in a topical formulations such as cream and ointments. Jeneil Biosurfactant Company also produces rhamnolipids from *P. aeruginosa* (EPA, 2004) which are marketed as EPA-approved bio fungicide by a trade name ZONIX Biofungicide. Also, its RECO product line is used to clean and recover oils from storage tanks (Jogdand, 2014). The microorganism utilised in this study is an indigineous isolate known as *P. aeruginosa* USM-AR2 (Nur Asshifa, 2009). This microorganism has been shown to be a high producer of rhamnolipid using fuel oil (Noh *et al.*, 2014) as the carbon source.
2.4 Cheap carbon source for biosurfactant production

Raw materials such as carbon and nitrogen sources could cost up to 50% of total production cost. The yield of rhamnolipid reported was low (yield of product over the substrate, $Y_{p/s}$ is around 0.1-0.62 g/g in batch culture) (Henkel et al., 2012) which implied that more substrate was consumed rather than being converted to rhamnolipid. Therefore, besides increasing the yield, the use of cheaper raw materials could significantly affect the production cost.

Various groups of carbon sources have been utilized for rhamnolipid production such as hydrocarbons (Jeong et al., 2004; Santa Anna et al., 2002), sugars (Wu et al., 2008), vegetable oils (Wei et al., 2005), and petrochemical-based oil (Obayori et al., 2009). Cheap substrates such as fermented distillery waste (Dubey et al., 2005), acidic waterwaste and soapstock from sunflower oil refining (Benincasa and Accorsini, 2008), cassava wastewater added with waste cooking oil (Costa et al., 2009), biodiesel waste or bioglycerol (Kumar et al., 2012), waste frying oil (Luo et al., 2013), soyabean oil soapstock (Partovi et al., 2013) were also studied for their potential to support biosurfactant production.

Currently, the highest reported rhamnolipid production by P. aeruginosa was from plant oils. It was reported by Zhu et al., (2012) that a maximum of 70 g/L of rhamnolipid were produced from soybean oil with a productivity of 0.588 g/L.h. However, the use of edible plant oils will be in direct competition with their use in food products. Also, when compared to other substrates, plant oils are rather expensive. The highest theoretical yield of rhamnolipid produced from cheap substrates containing fatty acids was shown to be 1.25±0.01 g rhamnolipid/g substrate compared to 0.51-0.59 g rhamnolipid/g substrate using other wastes containing
sucrose, cellulose, hemicellulose, lignocellulose or glycerol rhamnolipid (Henkel et al., 2012).

Therefore, waste cooking oil may be a suitable candidate as a cheap carbon source for rhamnolipid production. The oil is obtained after edible plant oils (palm, coconut, sunflower or corn) have been used several times for frying and they differ in their properties due to the high heating temperatures during the frying process. Typical fatty acids content of waste cooking palm oil as compared to fresh palm oil is shown in Table 2.3.

<table>
<thead>
<tr>
<th>Table 2.3 Range of fatty acids in waste and fresh cooking oils</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Range of fatty acids (%)</strong></td>
</tr>
<tr>
<td>Waste cooking oil</td>
</tr>
<tr>
<td>Fresh cooking oil</td>
</tr>
</tbody>
</table>

(Muhamad Ghazali et al., 2014; Taufiqurrahmi et al., 2011; Chuah et al., 2016; Lam et al., 2016)

* Malaysian Palm Oil Board

Waste cooking oil is abundantly available around the world as shown in Table 2.4. However, the awareness on proper disposal of waste cooking oil among communities, especially in Malaysia, is considerably low (Hanisah et al., 2013). Malaysia produces approximately 0.5 million tonnes of waste cooking oil annually. Utilising the waste as feedstock for biosurfactant production could offer a better solution for an economical and environmentally friendly disposal method thus simultaneously turning waste into valuable products.
Table 2.4 Estimated amounts of waste cooking oil generated in selected countries and the oil sources (Yaakob et al., 2013)

<table>
<thead>
<tr>
<th>Country</th>
<th>Quantity (million tonnes/year)</th>
<th>Source of oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>United States</td>
<td>10</td>
<td>Soybean oil</td>
</tr>
<tr>
<td>China</td>
<td>4.5</td>
<td>Salad oil, animal fat</td>
</tr>
<tr>
<td>European</td>
<td>0.7 - 10</td>
<td>Rapeseed oil, sunflower oil</td>
</tr>
<tr>
<td>Japan</td>
<td>0.45 - 0.57</td>
<td>Soybean oil, palm oil, animal fat</td>
</tr>
<tr>
<td>Taiwan</td>
<td>0.07</td>
<td>Soybean oil, palm oil, beef oil, lard oil</td>
</tr>
<tr>
<td>Malaysia</td>
<td>0.5</td>
<td>Palm oil</td>
</tr>
<tr>
<td>Canada</td>
<td>0.12</td>
<td>Animal fat, canola oil</td>
</tr>
<tr>
<td>England</td>
<td>1.6</td>
<td>Soybean oil, canola oil</td>
</tr>
<tr>
<td>Ireland</td>
<td>0.153</td>
<td>Rapeseed oil</td>
</tr>
</tbody>
</table>

The discharge of waste cooking oil can cause sewer system blockages and overflow that will increase water treatment and waste management cost. Furthermore, it can also decrease oxygen dissolution in water thus increasing the chemical oxygen demand (COD) and contaminate the water system. Consequently, aquatic lives absorb toxic compounds from the polluted water and later return to human through the food chain (Kulkarni and Dalai, 2006).

Waste cooking oil has been used not only for rhamnolipid production, but it has also been successfully exploited for other glycolipid biosurfactants production (Table 2.5). Up to now, the highest rhamnolipid production with waste cooking oil as substrate was 20 g/L by a mutant strain of *P. aeruginosa* (Zhu et al., 2007). Hence, due to the increase environmental pressure in producing biosurfactants using low-cost waste products, waste cooking oil could be a promising sole carbon source for industrial scale production of rhamnolipid.

However, studies on rhamnolipid production using waste cooking oil are still limited (Table 2.5). Waste cooking oil has the potential to replace edible plant oil as a carbon source in rhamnolipid production, since it is cheaper and may at the same time resolve environmental issues related to waste cooking oil disposal.
Table 2.5 Examples of glycolipid biosurfactants production using waste cooking oil as a cheap substrate

<table>
<thead>
<tr>
<th>Biosurfactant</th>
<th>Producer</th>
<th>Maximum Production (g/L)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular glycolipids</td>
<td><em>Rhodococcus erythropolis</em> 16 LM.USTHB</td>
<td>n.a</td>
<td>Sadouk et al., 2008</td>
</tr>
<tr>
<td>Surfactin</td>
<td><em>Bacillus subtilis</em> MTCC 2423</td>
<td>0.45</td>
<td>Vedaraman and Venkatesh, 2011</td>
</tr>
<tr>
<td>Sophorolipid</td>
<td><em>Candida bombicola</em></td>
<td>50</td>
<td>Fleurackers, 2006</td>
</tr>
<tr>
<td>Rhamnolipid</td>
<td><em>P. aeruginosa</em> ATCC 9027</td>
<td>8.5</td>
<td>Luo et al., 2013</td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em> zju1.m</td>
<td>20</td>
<td>Zhu et al., 2007</td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em> ATCC 10145</td>
<td>7.5</td>
<td>Wadekar et al., 2012</td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em> mutant EBN-8</td>
<td>9.3</td>
<td>Raza et al., 2006</td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em> D</td>
<td>2.26</td>
<td>George and Jayachandran, 2013</td>
</tr>
</tbody>
</table>

*n.a.: not available

2.5 Bioprocessing approaches for rhamnolipid production

2.5.1 Medium components for rhamnolipid production

The components for fermentation medium composed of carbon and nitrogen sources, and traces of other elements such as salts and vitamins. Nitrate has been shown as the best nitrogen source in promoting high rhamnolipid production as compared to other inorganic nitrogen sources such as ammonium sulphate, ammonium chloride and ammonium nitrate (Moussa et al., 2014; Saikia et al., 2013; Wu et al., 2008). The use of organic nitrogen sources such as urea and yeast extract led to a reduced yield of rhamnolipid, but support better growth yield (Guerra-Santos, 1984; Wu et al., 2008).

It is also important to note that carbon to nitrogen (C/N) ratio also influences rhamnolipid production. High C/N ratio, i.e. reduced level of nitrogen limits bacterial growth and favour the cellular metabolism towards the production of metabolites. On the other hand, an excess of nitrogen source directs the substrate to the synthesis of
cellular material and thus limiting the accumulation of products (Silva et al., 2010). Different values of C/N ratio have been reported for enhanced rhamnolipid production, for example; C/N of 23 (Lovaglio et al., 2010), 55 (Li et al., 2011), 27 (Marsudi et al., 2008), 15 (Kumar et al., 2012), 20 (Raza et al., 2014) and 8 (Benincasa and Accorsini, 2008).

As mentioned previously in section 2.4, plant oil is a potential carbon source for high rhamnolipid production. Therefore, it is important to ensure that the carbon source added in the medium formulation could be utilised by the rhamnolipid-producing bacteria since it is immiscible in water. Addition of surfactants can assist in solubilising the immiscible carbon source in order to increase bacterial accessibility and hence, improve the rhamnolipid production. However this has not been fully examined.

Among the highest rhamnolipid production, as reported from the in the literature for past ten years, was through using medium containing edible plant oil as a carbon source and sodium nitrate as a nitrogen source (Müller et al., 2010; Zhu et al., 2012). Noh et al., (2014) reported a significantly high production of rhamnolipid from a medium that contained fuel oil as the carbon source and yeast extract as the nitrogen source by an indigenous P. aeruginosa USM-AR2. However, detailed investigation has not been previously reported before for the production of rhamnolipid from a medium containing a non-edible plant oil and inorganic nitrogen such as waste cooking oil and sodium nitrate. Furthermore, the use of organic nitrogen source such as yeast extract could reduce the rhamnolipid production (Guerra-Santos, 1984; Wu et al., 2008).

In addition to the optimum medium formulation, the production strategies such as batch or fed-batch culture are also important to enhance rhamnolipid production.
Hence, the following sections focus on the strategies and factors that affect their performances.

2.5.2 Batch culture production of rhamnolipid in a bench-top bioreactor

2.5.2(a) Factors affecting rhamnolipid production in batch culture

Among the factors influencing rhamnolipid production in a bench-top bioreactor are pH and dissolved oxygen. It was reported that pH within the natural range, i.e. 6.5 to 7.0 was favourable compared to the acidic or alkaline region (Chen et al., 2007; de Sousa et al., 2011; Guerra-Santos, 1986; Lee et al., 2004). However, Arutchelvi et al., (2011) identified that the pH value was slightly higher than the previous study, which was 7.7. It is important to control the pH at a desired value as when the pH is below or over the predetermined value, it will affect the rhamnolipid production (Chen et al., 2007) but no explanation was given for this observation.

The bioreactor operating conditions such as agitation speed, aeration rate, and dissolved oxygen are among the factors that affect rhamnolipid production. The primary objective of aeration and agitation is to supply the necessary oxygen to the microorganisms to achieve the proper metabolic activities. A secondary function is to keep the microorganism in suspension (Lee et al., 2004). Therefore, selection of agitation speed and aeration rate should compromise between efficient oxygen transfer rate, minimising cell damage, and maximising the effect of mixing.

Chen and et al., (2007) exhibited that rhamnolipid productivity increased with an increase in agitation speed. An agitation speed of 250 rpm was found to be the optimum agitation rate, but when increased to 500 rpm caused 74% reduction in rhamnolipid productivity. Rhamnolipid production was enhanced when agitation speed was increased from 100 rpm to 200 rpm, after which, the production was
declined (Lee et al., 2004). A similar trend i.e. the increase in rhamnolipid production as the agitation speed increased was also reported elsewhere (Table 2.6).

**Table 2.6 Effect of agitation speed on rhamnolipid production**

<table>
<thead>
<tr>
<th>Agitation (rpm)</th>
<th>Aeration (vvm)</th>
<th>RL produced (g/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>800</td>
<td>1.0</td>
<td>7.60</td>
<td>Lovaglio et al., 2010</td>
</tr>
<tr>
<td>550</td>
<td>0.5</td>
<td>3.30</td>
<td>de Lima et al., 2009</td>
</tr>
<tr>
<td>600</td>
<td>1.2</td>
<td>5.37</td>
<td>Borges et al., 2015</td>
</tr>
</tbody>
</table>

RL: Rhamnolipid

Aeration rate is another factor which affects rhamnolipid production. Aeration rate has been shown to have the greatest influence on the production of rhamnolipid (de Lima et al., 2009). Rhamnolipid production by P. aeruginosa and P. aeruginosa BYK-2KCTC reached maximum values when aerated at 0.5 vvm (de Lima et al., 2009) and 0.67 vvm (Lee et al., 2004) respectively. Meanwhile, a significant rise in rhamnolipid yield up to 120 and 220% were reported when incubated at conditions tabulated in Table 2.7.

**Table 2.7 Effect of aeration rate on rhamnolipid production**

<table>
<thead>
<tr>
<th>Agitation (rpm)</th>
<th>Aeration (vvm)</th>
<th>RL yield (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>1.0</td>
<td>4.1</td>
<td>Lovaglio et al., 2010</td>
</tr>
<tr>
<td>500</td>
<td>2.0</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>1.0</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>2.0</td>
<td>16.9</td>
<td></td>
</tr>
</tbody>
</table>

Despite that, there was also a report showing that aeration rate had no significant effect on rhamnolipid production (Salleh et al., 2011). On the other hand, rhamnolipid production increased with increasing agitation speed. Moreover, intense aeration rate and agitation speed lead to the formation of heavy foaming. A severe foam formation could cause broth medium to overflow and might contribute to a reduction in rhamnolipid production (de Lima et al., 2009; Salleh et al., 2011).

Maintaining a dissolved oxygen level during the production of rhamnolipid is another factor to be considered. Adequate oxygen supply into the fermentation is
important since it is an essential nutrient for microbial growth as well as for cellular maintenance (Kronemberger et al., 2008). Nonetheless, the information on controlling the dissolved oxygen level is still insufficient. It is crucial to determine the desired value of dissolved oxygen level to avoid from reaching the critical limit of dissolved level. A reduction in rhamnolipid production and substrate consumption was observed when the production process was controlled at a low oxygen level (Kronemberger et al., 2008). However, each bacteria have their own oxygen requirement to grow and the value reported in literature is specific to the said condition only (Sabra et al., 2002). Therefore, it is crucial to determine the effect of dissolved oxygen and oxygen requirement specifically for the microorganism used in this study.

2.5.2(b) Oxygen transfers in rhamnolipid production

A study on oxygen mass transfer is crucial in aerobic production of rhamnolipid. The volumetric mass transfer rate, k_{La}, is manipulated by the rate of aeration and agitation. Increasing the aeration rates lead to the increase in k_{La} regardless of the agitation rates used. Meanwhile, at a fixed aeration rate, k_{La} also increases gradually when the agitation rate increases (Yeh et al., 2006). It was also demonstrated that the production of another glycolipid biosurfactant such as surfactin was affected by k_{La}. Surfactin production rate was mostly increased with increasing k_{La} values and the maximum value for surfactin production was 0.0132 s^{-1} (Yeh et al., 2006).

For rhamnolipid production, it was revealed that high rhamnolipid production was achieved at a higher k_{La} value (Benincasa et al., 2002). But the effect of k_{La} was only compared with two different values that correspond to different agitation speed
with constant aeration rate. Hence, de Lima et al., (2009) proved that $k_{L_\alpha}$ values were related neither to the cell growth nor biosurfactant synthesis.

The relationship of volumetric mass transfer rate, $k_{L_\alpha}$ with surfactant concentration at agitation is largely unknown (Martinov et al., 2008). It was demonstrated that with increasing biosurfactant concentration up to concentration close to CMC and decreasing surface tension, $\sigma$, $k_{L_\alpha}$ decreased only slightly (typically up to 25 %). So, it has been inferred that at biosurfactant concentration close to CMC, no significant concentration dependence of $k_{L_\alpha}$ occurs which would appear to be favourable for the biosurfactant production (Martinov et al., 2008). It is realised that higher concentration of biosurfactant (i.e. higher than CMC) is always achieved in biosurfactant production. However, since surface tension remains unchanged at biosurfactant concentration higher than CMC, it is deduced that higher biosurfactant concentrations should not have additional effects on $k_{L_\alpha}$ (Martinov et al., 2008).

In addition to biosurfactant concentration, the impact of biomass on $k_{L_\alpha}$ remains misunderstood and needs to be better evaluated. Earlier, both biosurfactant (i.e. rhamnolipid) and biomass were shown to give an insignificant affect the $k_{L_\alpha}$ (Sabra et al., 2002) during the growth of P. aeruginosa PAO1 under controlled air saturation of 5%. However, in another finding, it was demonstrated that an optimal concentration existed for the biomass and the biosurfactant extract, leading to an optimum value of $k_{L_\alpha}$. The combination of biomass and biosurfactant extract was found to induce a negative synergism on $k_{L_\alpha}$ (Aldric et al., 2009).

The oxygen supply problem is further accentuated in rhamnolipid fermentations that commonly require water-immiscible substrates such as vegetable oils and fatty acids. These substrates can be essential for inducing the production of rhamnolipid, but coating the gas–liquid interface with a layer of oil reduces the
volumetric mass transfer coefficient, $k_{La}$. For example, adding palm oil to an aqueous broth has been found to reduce the oxygen mass transfer coefficient (Suhaila and Murthy, 2010). Nevertheless, the effect of rhamnolipid (the product) and immiscible substrate such as waste cooking oil as a carbon source on $k_{La}$ has not been studied in detail before. Hence, in this research, the impact of rhamnolipid and waste cooking oil concentrations on $k_{La}$ is examined.

2.5.3 Fed-batch Production Strategies

Different cultivation strategies have been implemented for rhamnolipid production (Table 2.8). The batch culture is usually performed in fermentation process due to its simplicity compared to fed-batch and continuous culture. Nevertheless, fed-batch cultivations are the most popular process strategy for high productivity (Table 2.8). Current information on rhamnolipid production process demonstrates that this approach has yet to be adequately adopted yet.

Fed-batch culture is a system that may be considered to be intermediate between batch and continuous processes. The term fed-batch is used to describe batch cultures that are fed continuously, or sequentially, with fresh medium without the removal of culture fluid. Thus, the volume of a fed-batch culture increases with time.
### Table 2.8 Production strategies adopted for rhamnolipid production for the last ten years

<table>
<thead>
<tr>
<th>Production process</th>
<th>P. aeruginosa strain</th>
<th>Carbon source</th>
<th>Productivity (g/L·h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>BYK-2 KCTC 18092P</td>
<td>Fish oil</td>
<td>0.078</td>
<td>Lee et al., 2004</td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>Glucose</td>
<td>0.10</td>
<td>Chen et al., 2007</td>
</tr>
<tr>
<td></td>
<td>zju.1M (mutant)</td>
<td>Waste cooking oil</td>
<td>0.233</td>
<td>Zhu et al., 2007</td>
</tr>
<tr>
<td></td>
<td>IFO 3924</td>
<td>Glycerol</td>
<td>0.02</td>
<td>Marsudi et al., 2008</td>
</tr>
<tr>
<td></td>
<td>PACL</td>
<td>Waste frying soybean oil</td>
<td>0.034</td>
<td>de Lima et al., 2009</td>
</tr>
<tr>
<td></td>
<td>ATCC 9027</td>
<td>Glucose</td>
<td>0.013</td>
<td>Clarke et al., 2010</td>
</tr>
<tr>
<td></td>
<td>LB 1</td>
<td>Soapstock</td>
<td>0.148</td>
<td>Lovaglio et al., 2010</td>
</tr>
<tr>
<td></td>
<td>BS-161R</td>
<td>Bio glycerol</td>
<td>0.025</td>
<td>Kumar et al., 2012</td>
</tr>
<tr>
<td></td>
<td>WJ-1</td>
<td>Waste frying oil</td>
<td>0.523</td>
<td>Xia et al., 2012</td>
</tr>
<tr>
<td></td>
<td>USM-AR2</td>
<td>Diesel</td>
<td>0.033</td>
<td>Nur Asshifa et al., 2012</td>
</tr>
<tr>
<td></td>
<td>O-2-2</td>
<td>Soybean oil</td>
<td>0.3</td>
<td>Zhu et al., 2012</td>
</tr>
<tr>
<td>Batch feed with trace elements</td>
<td>PAO1</td>
<td>Sunflower oil</td>
<td>0.43</td>
<td>Müller et al., 2010</td>
</tr>
<tr>
<td></td>
<td>DSM 7108</td>
<td>Sunflower oil</td>
<td>0.26</td>
<td>Müller et al., 2011</td>
</tr>
<tr>
<td></td>
<td>DSM 2874</td>
<td>Sunflower oil</td>
<td>0.35</td>
<td>Müller et al., 2011</td>
</tr>
<tr>
<td>Fed-batch</td>
<td>BYK-2 KCTC 18092P</td>
<td>Fish oil</td>
<td>0.086</td>
<td>Lee et al., 2004</td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>Glucose</td>
<td>0.019</td>
<td>Chen et al., 2007</td>
</tr>
<tr>
<td></td>
<td>USM-AR2</td>
<td>Diesel</td>
<td>0.047</td>
<td>Salwa et al., 2009</td>
</tr>
<tr>
<td></td>
<td>USM-AR2</td>
<td>Diesel</td>
<td>0.054</td>
<td>Nur Asshifa et al., 2012</td>
</tr>
<tr>
<td></td>
<td>O-2-2</td>
<td>Soybean oil</td>
<td>0.588</td>
<td>Zhu et al., 2012</td>
</tr>
<tr>
<td></td>
<td>ATCC 9027</td>
<td>Waste frying oil</td>
<td>0.072</td>
<td>Luo et al., 2013</td>
</tr>
<tr>
<td></td>
<td>USM-AR2</td>
<td>Diesel</td>
<td>0.076</td>
<td>Noh et al., 2014</td>
</tr>
<tr>
<td>Continuous with immobilised cell</td>
<td>DSM 2874</td>
<td>Glycerol</td>
<td>17.5 g/cycle</td>
<td>Heyd et al., 2011</td>
</tr>
<tr>
<td></td>
<td>BYK-2 KCTC 18092P</td>
<td>Fish Oil</td>
<td>0.031</td>
<td>Jeong et al., 2004</td>
</tr>
<tr>
<td>Continuous with immobilized cells under denitrification condition</td>
<td>E03-40</td>
<td>Glycerol</td>
<td>0.017 g/g cell/h</td>
<td>Pinzon et al., 2013</td>
</tr>
</tbody>
</table>
The design of the feeding strategy and its control are most influential in determining the rhamnolipid productivity. A variety of feeding strategies has been developed (Table 2.9) for biosurfactants production especially rhamnolipid. The feeding strategy may be modified during a fed-batch fermentation to achieve different outcomes. It may be controlled in response to online measured fermentation parameters. The feeding methods are classified into two main types: those without any feedback control and those relying on some form of feedback control mechanism.

2.5.3(a) Feeding without feedback control

Constant rate feeding. In constant rate feeding, concentrated nutrients are fed at some pre-determined rate. As the cell population increases, the specific growth rate declines because the feeding rate is fixed but the rate of consumption increases with the increasing population. Constant rate feeding is of course extremely easy to implement. The final biomass concentration is lower in a constant rate feeding operation compared with the final biomass concentration in a specific growth rate controlled operation of the same duration.

2.5.3(b) Feedback control feeding

In feedback controlled feeding, an online measured fermentation property is used to control the rate of feeding. The feedback control strategy is said to be indirect if the variable used to control the feeding is anything other than the measured concentration of the substrate in the bioreactor. Direct feedback control relies on the online measured concentration of the substrate in the bioreactor to control the feeding.
### Table 2.9 Different feeding strategy for fed-batch production of rhamnolipid and another biosurfactant

<table>
<thead>
<tr>
<th>Biosurfactant</th>
<th>Fed-batch feeding strategy</th>
<th>Carbon source</th>
<th>Productivity (g/L.h)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnolipid</td>
<td>Intermittent feeding strategy</td>
<td>Fish oil</td>
<td>0.078</td>
<td>Lee et al., 2004</td>
</tr>
<tr>
<td></td>
<td>pH-stat</td>
<td>Glucose</td>
<td>0.172</td>
<td>Chen et al., 2007</td>
</tr>
<tr>
<td></td>
<td>A combination of pH-stat and fill and draw</td>
<td>Glucose</td>
<td>0.026</td>
<td>Chen et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Pulse-feed</td>
<td>Diesel</td>
<td>0.033</td>
<td>Nur Asshifa, 2012</td>
</tr>
<tr>
<td></td>
<td>Pulse with different pH control</td>
<td>Soybean oil</td>
<td>0.735</td>
<td>Zhu et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Pulse</td>
<td>Waste frying oil</td>
<td>0.072</td>
<td>Luo et al., 2013</td>
</tr>
<tr>
<td></td>
<td>Maximum substrate uptake rate</td>
<td>Diesel</td>
<td>0.076</td>
<td>Noh et al., 2014</td>
</tr>
<tr>
<td></td>
<td>Model-based feeding</td>
<td>Sunflower oil</td>
<td>0.444</td>
<td>Henkel, 2014</td>
</tr>
<tr>
<td>Mannosyl-erythritol lipid</td>
<td>Pulse feed</td>
<td>Soybean oil</td>
<td>0.475</td>
<td>Kim et al., 2006</td>
</tr>
<tr>
<td></td>
<td>Foam-stat</td>
<td>Soybean oil</td>
<td>0.200</td>
<td>Kim et al., 2006</td>
</tr>
<tr>
<td>Sophorolipid</td>
<td>pH-stat</td>
<td>Rapeseed oil</td>
<td>1.901</td>
<td>Kim et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Constant feed rate</td>
<td>Coconut oil and glucose</td>
<td>0.231</td>
<td>Morya et al., 2013</td>
</tr>
<tr>
<td></td>
<td>Step feeding/Pulse</td>
<td>Waste cooking oil</td>
<td>0.232</td>
<td>Maddikeri et al., 2015</td>
</tr>
<tr>
<td></td>
<td>Exponential feeding with foam overflow</td>
<td>Glucose</td>
<td>0.007</td>
<td>Chenikher et al., 2010</td>
</tr>
<tr>
<td>Mycosubtilin</td>
<td>Constant feed rate</td>
<td>Crude glycerol</td>
<td>0.163</td>
<td>Liu et al., 2011</td>
</tr>
<tr>
<td>Surfactin</td>
<td>Exponential feeding</td>
<td>Maldex-15</td>
<td>2.58</td>
<td>Amin, 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(industrial byproduct high fructose syrup manufacturing from corn starch)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For the indirect feedback control, the feed rate is controlled by some online measured property of the fermentation other than the concentration of the substrate. Feeding may be monitored by the measured concentration of dissolved oxygen (DO), the pH, the CER, the OUR, the respiratory quotient (RQ), the biomass concentration and metabolic heat generation rate. The feeding scheme is based on a DO-stat operation.
that relies on the online measured concentration of dissolved oxygen (DO) in the fermentation broth. As the substrate is depleted to a low level, the metabolic activity decreases, and the consumption of oxygen reduces. Substrate depletion results in a rapid rise in the concentration of dissolved oxygen. This signal is used to control the feeding so that the dissolved oxygen concentration remains at some pre-set value. A pH-stat operation that is similar in principle to the DO-stat can be used to control feeding of some fermentation processes.

Estimation of maximum substrate uptake rate is based on dissolved oxygen (DO). The control scheme depends on real-time measurements of DO following a process perturbation: the feeding is interrupted momentarily, and the instance of complete depletion of the substrate is detected by the rise in DO. Immediately, a known amount of substrate is pulsed into the bioreactor. The DO level declines but soon rises (the ‘second’ rise) as the added pulse is exhausted. The interval between the instances of decline and the ‘second’ rise of DO and the known quantity of pulse are used to compute the MSUR. The feeding then resumes at the calculated MSUR (Oh et al., 1998).

Although there have been different strategies being implemented for fed-batch production of biosurfactants, only pulse feeding strategy has been tested when waste cooking oil was utilised as a carbon source (refer Table 2.1). Thus, it is the intention of this research to study different feeding strategy that involves feedback control and without feedback control for fed-batch production of rhamnolipid using waste cooking oils as a sole carbon source.
2.5.4 Kinetics of microbial growth, product formation, and substrate consumption in batch culture

Most of the literatures on kinetics cover parameters such as yield of product and productivity (Wu et al., 2008; Santa Anna et al., 2002; Benincasa and Accorsini, 2008), specific growth rate and specific product synthesis rate (Raza et al., 2006; Müller et al., 2010; Müller et al., 2011), microbial growth, product synthesis and substrate consumption kinetics models, as well as Luedeking-Piret constant (Zhu et al., 2012). Currently Sana et al., (2017) have shown a relationship between specific production rate and specific growth rate for rhamnolipid production using fish fat as a carbon source. Although there have been various studies related to kinetics, none has specifically determined the kinetics for rhamnolipid production on waste cooking oil except for Raza et al., (2006). It is important to note that Raza et al., (2006) did not establish the relationship between specific product synthesis rate as well as the Luedeking-Piret constant. Thus, in the following paragraphs, the theories of batch culture kinetics are reviewed.

The microorganisms undergo several phases in batch culture i.e. the lag phase, exponential growth phase, stationary phase and death phase. A balanced growth occurs during exponential growth where all components of a cell grow at the same rate. The cell growth at exponential phase can be described as follows (Stanbury et al., 1995):

\[
\frac{dx}{dt} = \mu x, \quad x = 0 \text{ at } t = 0
\]  

(2.1)

where \(x\) and \(x_0\) are cell concentrations at time \(t\) and \(t = 0\) and \(\mu\) is the specific growth rate (h\(^{-1}\)).

Integration of equation 2.1 gives

\[
\ln \frac{x_t}{x_0} = \mu t \quad \text{or} \quad x_t = x_0 e^{\mu t}
\]  

(2.2)

A linear form of equation 2.2 yields
\[ \ln x_t = \ln x_0 + \mu t \]  

(2.3)

Plotting of \( \ln x_t \) versus \( t \) gives a straight line with a slope equal to \( \mu \). During the exponential phases, nutrients are in excess and the organism is growing at its maximum specific growth rate, \( \mu_{\text{max}} \).

Meanwhile, the product formation rate is given by the Leudeking-Piret equation (Bailey and Ollis, 1986):

\[ \frac{dp}{dt} = \alpha \frac{dx}{dt} + \beta x \]  

(2.4)

where \( P \) is product concentration; \( \alpha \) is growth associated constant for product formation and \( \beta \) is non-growth associated constant for product formation. Microbial products can be classified in three major categories based on the relationship between production kinetics and cell growth. They are growth associated, non-growth associated and partially-growth associated (Figure 2.4).

![Figure 2.4](image)

**Figure 2.4** Kinetics patterns of growth and product formation in batch fermentation: (a) growth associated product formation (if \( \beta = 0 \)), (b) mixed-growth associated product formation and (c) non-growth associated product formation (if \( \alpha = 0 \)) (Shuler and Kargi 2002).

Equation 2.4 can be written as follows and knowing that \( \frac{dx}{dt} = \mu x \):

\[ \frac{dp}{dt} = \alpha \mu x + \beta x \]  

(2.5)
Dividing equation 2.5 with \( x \) gives the specific rate of product formation (g/g.h) as follows (Shuler and Kargi, 2002):

\[ q_p = \alpha \mu + \beta \]  

(2.6)

For substrate utilisation kinetics, the following equation is given, which considers substrate conversion to cell mass, product and substrate consumption for maintenance (Ollis, 1983):

\[
\frac{dS}{dt} = -\frac{1}{Y_{x/S}} \frac{dx}{dt} - \frac{1}{Y_{P/S}} \frac{dP}{dt} - k_e x
\]

(2.7)

where \( S \) is substrate concentration (g/L); \( k_e \) is maintenance coefficient (h\(^{-1}\)); \( Y_{x/S} \) is cell yield coefficient on substrate; \( Y_{P/S} \) is product yield coefficient on substrate. Then the term \( \frac{dP}{dt} \) in equation 2.7 is replaced by equation 2.4, and it yields:

\[
\frac{dS}{dt} = -\frac{1}{Y_{x/S}} \frac{dx}{dt} - \frac{1}{Y_{P/S}} \left( \alpha \frac{dx}{dt} + \beta x \right) - k_e x
\]

Rearranging the above equation:

\[
\frac{dS}{dt} = -\left( \frac{1}{Y_{x/S}} + \frac{\alpha}{Y_{P/S}} \right) \frac{dx}{dt} - \left( \frac{\beta Y_{P/S}}{Y_{x/S}} + k_e \right) x
\]

and alternatively, rewritten as

\[
\frac{dS}{dt} = -\eta x - \gamma \frac{dx}{dt}
\]

(2.8)

Where \( \eta = \frac{\beta}{Y_{P/S}} + k_e \) and \( \gamma = \frac{1}{Y_{x/S}} + \frac{\alpha}{Y_{P/S}} \)

When equation 2.8 is divided by \(-x\), the specific rate of substrate utilization, \( q_S \) (h\(^{-1}\)), is obtained as:

\[
q_S = \frac{\mu}{Y_{x/S}} + \frac{q_p}{Y_{P/S}} + k_e
\]

(2.9)

The yield coefficients i.e. the biomass yield coefficient (\( Y_{x/S} \)) on substrate, product yield coefficient on substrate (\( Y_{P/S} \)) and product yield coefficient on biomass (\( Y_{P/x} \)) are defined based on the amount of consumption of another material. The general form of yield coefficients is given as (Doran, 2013):
\[ Y_{JK} = \frac{\Delta J}{\Delta K} \]  

(2.10)

where \( Y_{JK} \) is the yield factor, \( J \) and \( K \) are substance involved in metabolism, \( \Delta J \) is the mass or moles of \( J \) produced, and \( \Delta K \) is the mass or moles of \( K \) consumed. The negative sign is required in equation 2.14 because \( \Delta K \) for a consumed substance is negative in value and yield is calculated as a positive quantity. In batch culture, \( \Delta J \) and \( \Delta K \) are calculated as overall yield i.e. the difference between initial and final states. Yields can vary during culture, so instantaneous yields at a particular point in time are commonly evaluated. If \( r_J \) and \( r_K \) are the volumetric rates of production and consumption of \( J \) and \( K \) respectively, in a closed, constant-volume reactor, the instantaneous yield can be calculated as follows:

\[
Y_{JK} = \lim_{\Delta K \to 0} \frac{\Delta J}{\Delta K} = \frac{dJ}{dK} = \frac{dJ}{dt} \frac{r_J}{r_K} \]

(2.11)

The time or period to which the yields refer to should be stated when reporting the yield coefficients.

### 2.5.5 Theoretical determination of volumetric gas flow rate (\( F_g \))

Correlations have been developed for predicting the operating conditions under which impeller loading and complete gas dispersion are achieved using Rushton turbines. These relationships are expressed using two dimensionless variables, the gas flow number \( F_{lg} \):

\[
F_{lg} = \frac{F_g}{N_iD_i^2} \]

(2.12)

And the Froude number \( Fr \):

\[
Fr = \frac{N_i^2D_i}{g} \]

(2.13)
where \( F_g \) is the volumetric gas flow rate, \( N_i \) is stirrer speed, \( D_i \) is impeller diameter and \( g \) is gravitational acceleration. Conditions at the flooding-loading transition for Rushton turbine are represented by the following equation:

\[
F_{Lg} = 30 \left( \frac{D_i}{D_T} \right)^{3.5} Fr
\]  \hspace{1cm} (2.14)

The conditions for complete dispersion of gas by Rushton turbines are represented by another equation:

\[
F_{Lg} = 0.2 \left( \frac{D_i}{D_T} \right)^{0.5} Fr^{0.5}
\]  \hspace{1cm} (2.15)

where \( D_T \) is tank diameter. Thus, \( F_g \) is calculated by rearrangement of equation 2.12 as follows:

\[
F_g = F_{Lg} N_i D_i^3
\]  \hspace{1cm} (2.16)

The value calculated from equation 2.16 is compared with the operating flow rate. If the calculated value is greater than the operating value than it can be concluded the air is completely dispersed and the impeller is loaded or flooded.

2.6 Concluding remarks and research gaps

Overall, the review in this section suggests that, it is viable to study the enhancement of rhamnolipid production by \textit{P.aeruginosa} although it is known as opportunistic pathogen. This is based on:

i. \textit{P. aeruginosa} is the known species to produce rhamnolipid while other \textit{Pseudomonas} sp rarely produces rhamnolipid.

ii. other non-pathogenic bacteria are genetically not stable for rhamnolipid production.

iii. \textit{P. aeruginosa} is the only microbial producer being used for rhamnolipid production at industrial scale.
Therefore, the strain used in this research is *P. aeruginosa* USM-AR2.

Meanwhile waste cooking oil is chosen as a potential and cheap carbon source in this study. It is because:

i. waste cooking oil is not directly competitive with food.

ii. may reduce environmental threat due to improper waste disposal.

iii. contains fatty acid, which is shown as the substrate that supports the highest theoretical yield of rhamnolipid.

Based on reported studies on rhamnolipid production the following research gaps are identified, which provide the basis of initiating this research. The gaps are outline in Table 2.10. As such, the methods of the research are designed to provide answers based on research gaps and questions outlined in the Table 2.10.
<table>
<thead>
<tr>
<th>Research gaps</th>
<th>Research questions</th>
<th>Objective</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selection of medium formulation</td>
<td>Which medium formulation could support high rhamnolipid production?</td>
<td>To evaluate different medium formulation from literature and select the medium that support highest possible rhamnolipid production from waste cooking oil</td>
</tr>
<tr>
<td></td>
<td>What C/N ratio that could support high rhamnolipid production in the selected medium?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Does addition of surfactant into medium formulation could enhance the production?</td>
<td></td>
</tr>
<tr>
<td>Factors affecting rhamnolipid production in batch</td>
<td>What are the effects of pH, dissolve oxygen and agitation speed on rhamnolipid production?</td>
<td>To determine the effect of operational conditions on rhamnolipid production in batch culture as well as the oxygen transfer behaviour in the fermentation system</td>
</tr>
<tr>
<td></td>
<td>Oxygen transfer in rhamnolipid production:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- What are the effects of agitation and aeration on oxygen transfer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- What are the effects of rhamnolipid and waste cooking oil on oxygen transfer</td>
<td></td>
</tr>
<tr>
<td>The kinetics of rhamnolipid production with waste cooking oil as a carbon source in batch and fed-batch culture</td>
<td>Growth kinetics - specific growth rate</td>
<td>To analyse the kinetics of rhamnolipid production in batch and fed-batch cultures.</td>
</tr>
<tr>
<td></td>
<td>Product synthesis - specific product synthesis rate, Luedeking-Piret constant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Relationship between specific growth rate and specific product synthesis rate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yield of product and yield of biomass</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall Productivity</td>
<td></td>
</tr>
<tr>
<td>Effective feeding strategy for fed-batch cultures</td>
<td>Which feeding strategy is the best for fed-batch culture?</td>
<td>To identify the best feeding strategy for improving rhamnolipid production in fed-batch culture</td>
</tr>
<tr>
<td></td>
<td>Does rhamnolipid production improves in fed-batch culture compared to batch culture?</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 3 MATERIALS AND METHODS

3.1 The microorganism

An indigenous isolate *P. aeruginosa* USM-AR2 (Nur Asshifa, 2009) was used throughout the research. A 40% (v/v) of glycerol stock was prepared and stored at -20°C for a long-term storage of the bacterium.

3.2 Preparation of bacterial stock in 20% (v/v) of glycerol

The organism was first of all inoculated into 50 mL of nutrient broth in 250 mL Ehlenmeyer flask and grown for 24 h at 25°C on an orbital shaker (Certomat® R, B. Braun, Germany) agitated at 200 rpm. The 40% (v/v) glycerol stock was prepared by mixing 40 mL of glycerol with 60 mL of distilled and sterilised by autoclaving for 15 min at 121°C. To prepare a 1.0 mL of 20% (v/v) glycerol stock, 0.5 mL of the bacterial culture was added to 0.5 mL of 40% (v/v) glycerol in a sterile 1.5 mL microcentrifuge tube and was mixed evenly. The glycerol stock was kept at -20°C and revived for every three months to maintain the performance of the bacteria.

3.3 Inoculum preparation

The inoculum was prepared by culturing a 1 mL of bacterial glycerol stock, in 100 mL shake flask containing 20 mL nutrient broth. The culture was incubated at 25°C and agitated at 200 rpm on a shaker (Certomat® R, B. Braun, Germany) for 24 h. A loopful of the culture was then streaked on nutrient agar (HiMedia) and incubated at 25°C for 24 h. A single colony was transferred into 50 mL nutrient broth to serve as the seed culture and incubated at the same condition. When the seed culture reached an optical density (OD$_{540nm}$) of 2.0, a 2% (v/v) of the culture was used to inoculate the production medium.
3.3.1 Nutrient broth preparation

The ingredients for the preparation of nutrient broth are as listed in Table 3.1. All ingredients were weighed (ED224S, Sartorius, Germany) and dissolved in distilled water. The amount of the solution was brought to 1.0 L total volume. The solution was dispensed to the required amount into beakers and autoclaved at 121°C for 15 min (Hirayama Hiclave™, HV-85, Korea).

Table 3.1 Medium composition for nutrient broth

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount (g/L)</th>
<th>Chemicals brand name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Lab-lemco</td>
<td>1.0</td>
<td>Oxoid</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0</td>
<td>R&amp;M Chemicals</td>
</tr>
</tbody>
</table>

3.4 Production Medium Formulation

Three production media were prepared according to the formulations by Müller et al., (2010), Zhu et al., (2012) and Nur Asshifa et al., (2012). In this study, waste cooking oil from a local cafe was used as a sole carbon source in all the formulations instead of sunflower oil, soybean oil and diesel that were used in the original formulations of Medium A, B and C respectively. The compositions of each media are as tabulated in Table 3.2. The chemicals were dissolved in distilled water to the desired volume (depending on shake flasks or bioreactor studies). The solution was sterilised at 121°C for 15 min in an autoclave (Hirayama Hiclave™, HV-85, Korea). K2HPO4 was autoclaved separately and added to the sterile medium aseptically.
Table 3.2 Compositions of production media

<table>
<thead>
<tr>
<th>Medium compositions</th>
<th>Medium A</th>
<th>Medium B</th>
<th>Medium C</th>
<th>Chemicals brand name</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃ (g/L)</td>
<td>15.0</td>
<td>3.0</td>
<td>-</td>
<td>Systerm®ChemAR®</td>
</tr>
<tr>
<td>MgSO₄·7H₂O (g/L)</td>
<td>0.5</td>
<td>0.2</td>
<td>0.5</td>
<td>Bendosen</td>
</tr>
<tr>
<td>KCl (g/L)</td>
<td>1.0</td>
<td>1.1</td>
<td>-</td>
<td>QRëC™</td>
</tr>
<tr>
<td>K₂HPO₄ (g/L)</td>
<td>0.3</td>
<td>6.0</td>
<td>-</td>
<td>QRëC™</td>
</tr>
<tr>
<td>KH₂PO₄ (g/L)</td>
<td>-</td>
<td>4.0</td>
<td>-</td>
<td>Fischer Scientific</td>
</tr>
<tr>
<td>NaCl (g/L)</td>
<td>-</td>
<td>1.1</td>
<td>-</td>
<td>R&amp;M Chemicals, UK</td>
</tr>
<tr>
<td>Anhydrous CaCl₂ (g/L)</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>Riedel-de Haën</td>
</tr>
<tr>
<td>Yeast extract (g/L)</td>
<td>-</td>
<td>-</td>
<td>6.0</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Tween 80 (mL)</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>Systerm® ChemPur</td>
</tr>
<tr>
<td>Waste cooking oil (g/L)</td>
<td>250</td>
<td>80</td>
<td>1.2</td>
<td>Local cafe of Universiti Sains Malaysia</td>
</tr>
<tr>
<td>Trace elements (mL)</td>
<td>1.0</td>
<td>5.0</td>
<td>-</td>
<td>Reference Müller et al., 2010 Zhu et al., 2012 Nur Asshifa et al., 2012</td>
</tr>
</tbody>
</table>

3.4.1 Trace elements preparation

The composition of the trace elements are tabulated in Table 3.3. Every ingredient was dissolved in distilled water and filter-sterilised with a sterile 0.20 μm nylon filter (NY, Sartorius, Germany). The stock solution was maintained in a sterile Schott Duran bottle at 4°C in the refrigerator for further use.

Table 3.3 Trace elements composition

<table>
<thead>
<tr>
<th>Trace elements compositions</th>
<th>Medium A</th>
<th>Medium B</th>
<th>Chemicals brand name</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₃H₂N₅O₇·2H₂O (g/L)</td>
<td>2.0</td>
<td>-</td>
<td>R&amp;M Chemicals</td>
</tr>
<tr>
<td>FeCl₃·6H₂O (g/L)</td>
<td>0.28</td>
<td>-</td>
<td>Fluka</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O (g/L)</td>
<td>1.4</td>
<td>3.0</td>
<td>Fluka</td>
</tr>
<tr>
<td>CoCl₂·6 H₂O (g/L)</td>
<td>1.2</td>
<td>-</td>
<td>HmbG Chemicals</td>
</tr>
<tr>
<td>CuSO₄·5H₂O (g/L)</td>
<td>1.2</td>
<td>-</td>
<td>R&amp;M Chemicals</td>
</tr>
<tr>
<td>MnSO₄·2H₂O (g/L)</td>
<td>0.8</td>
<td>3.0</td>
<td>BDH Analar</td>
</tr>
<tr>
<td>FeSO₄·7H₂O (g/L)</td>
<td>-</td>
<td>12</td>
<td>Riedel-de Haën</td>
</tr>
<tr>
<td>CoSO₄·7H₂O (g/L)</td>
<td>-</td>
<td>1.0</td>
<td>Merck</td>
</tr>
<tr>
<td>Reference</td>
<td>Müller et al., 2010 Zhu et al., 2012</td>
<td>Nur Asshifa et al., 2012</td>
<td></td>
</tr>
</tbody>
</table>
3.5 Shake flasks study

3.5.1 Media evaluation for rhamnolipid production

Various experiments were conducted based on the media formulations as listed in Table 3.4. Experiments 1 and 2 were performed in triplicates in 500 mL shake flasks containing 100 mL of media. The flasks were incubated at 25°C and agitated at 200 rpm (Certomat® R, B. Braun, German) for five days. A 1 mL of sample was withdrawn from each flask twice a day. Experiments 3 and 4 were separately conducted in a 3.6 L bench-top bioreactor (Labfors 4, Infors HT®, Switzerland). The bioreactor was agitated at 400 rpm, 0.3 vvm aeration rate at a temperature of 28°C.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition of medium (g/L)</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NaNO₃ 15.0</td>
<td>15.0</td>
<td>5.5</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MgSO₄·7H₂O 0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>KCl 1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>K₂HPO₄ 0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Trace elements (mL) 1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>WCO 250.0</td>
<td>80.0</td>
<td>18.4</td>
<td>18.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tween 80 (% v/v)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>KH₂PO₄ 4.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K₂HPO₄ 6.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaNO₃ 3.0</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>NaCl 1.1</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>KCl 1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MgSO₄·7H₂O 0.2</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Anhydrous CaCl₂ 0.2</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Trace elements (mL) 5.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WCO 80.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Yeast extract 6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>MgSO₄·7H₂O 0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Tween 80 (% v/v) 0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>WCO 1.2</td>
<td>80.0</td>
<td>1.62</td>
<td>24.1</td>
<td></td>
</tr>
</tbody>
</table>

Experiment 1: Evaluation of different formulation for rhamnolipid production
Experiment 2: Evaluation of different formulation at similar carbon source concentration
Experiment 3: Evaluation of media A and C at similar C/N ratio
Experiment 4: Evaluation of media A and C at similar total carbon

No changes were made to the composition of trace elements for medium A and B as specified in section 3.2.1
3.5.2 Effect of C/N ratio on rhamnolipid production

The effect of C/N ratio on rhamnolipid production was examined using medium A under two different conditions, which were at constant concentrations of nitrogen source, or constant carbon source. In this study, the media were formulated to give C/N ratios of 9, 18 and 27. Details of the formulated media were shown in Table 3.5. Incubation conditions applied in this experiment were similar to experiments 1 and 2 as stated in Section 3.5.1.

Table 3.5 Composition of medium at different C/N ratio

<table>
<thead>
<tr>
<th>Composition of medium (g/L)</th>
<th>Constant carbon source (g/L)</th>
<th>Constant nitrogen source (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C/N = 9</td>
<td>C/N = 18</td>
</tr>
<tr>
<td></td>
<td>C/N = 9</td>
<td>C/N = 18</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>15.0</td>
<td>7.5</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>KCl</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Trace elements (mL)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>WCO</td>
<td>25.6</td>
<td>25.6</td>
</tr>
<tr>
<td>Tween 80 (% v/v)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

3.5.3 Effect of chemical surfactant addition on rhamnolipid production

In this study, medium A was used. Rhamnolipid production was examined in the medium with or without surfactant addition. The medium composition for this experiment is shown in Table 3.6. The incubation conditions applied in this experiment were similar to experiments 1 and 2 as stated in section 3.5.1.

Table 3.6 Composition of medium A with and without surfactant

<table>
<thead>
<tr>
<th>Composition of medium (g/L)</th>
<th>With surfactant</th>
<th>Without surfactant</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>KCl</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Trace elements (mL)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>WCO</td>
<td>25.6</td>
<td>25.6</td>
</tr>
<tr>
<td>Tween 80 (% v/v)</td>
<td>0.1</td>
<td>-</td>
</tr>
</tbody>
</table>
3.6 Batch culture study in a bench-top bioreactor

Various parameters were investigated for rhamnolipid production in batch culture using a 3.6 L bench-top bioreactor with a working volume of 1.5 L (Labfors 4, Infors HT®, Switzerland). The bioreactor was operated and controlled at 28°C, agitation speed of 400 rpm and 0.3 vvm aeration without pH control, unless otherwise stated. 20 mL of sample was withdrawn periodically for analysis.

3.6.1 Effect of tip speeds

Two different tip speeds were investigated to determine the best speed for rhamnolipid production. The chosen tip speeds were 1.13 m/s and 1.41 m/s, which corresponded to 400 rpm and 500 rpm respectively. Medium C with the following formulation was used in this particular study:

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount (g/L)</th>
<th>Chemicals brand name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>6.0</td>
<td>Oxoid</td>
</tr>
<tr>
<td>MgSO₄•7H₂O</td>
<td>0.5</td>
<td>Bendosen</td>
</tr>
<tr>
<td>Tween 80 (mL)</td>
<td>0.5</td>
<td>Systerm® ChemPur</td>
</tr>
<tr>
<td>Waste cooking oil (mL)</td>
<td>60.0</td>
<td>Local café, USM</td>
</tr>
</tbody>
</table>

3.6.2 Effect of controlling pH and dissolved oxygen

The effect of pH and dissolved oxygen level on rhamnolipid production were studied using medium A with the following variations:

a. The pH of medium A was controlled at pH [6.5 ± 0.5] (Müller et al., 2010) with 1 M HCl and 1 M NaOH but dissolved oxygen level was not controlled.

b. For the control of dissolved oxygen level, a cascade loop between dissolved oxygen probe and the impeller was set up to maintain the level at [20% ± 5%]. The pH was not controlled.

c. pH and dissolved oxygen levels were controlled at values given in (a) and (b).

d. pH and dissolved oxygen levels were not controlled at values in (a) and (b).
3.7 Feeding strategies for fed-batch culture

The fed-batch culture was implemented as a strategy to increase the yield of rhamnolipid production. The cultivation was started as a batch culture followed by feeding of fresh carbon source upon the depletion of the initial carbon source, as indicated by a sharp increase in the dissolved oxygen level. The feeding was carried out through a calibrated peristaltic pump. Different feeding strategies, controlled by a sequence (Appendix B) written in IRIS Version 5.2 (Infors HT®, Switzerland) and later updated to Version 6.0 (Infors HT®, Switzerland), on the production of rhamnolipid were studied. The feeding strategies implemented were as follows:

a. Pulse feeding - substrate was fed in pulses every 24 h, and the amount being fed was determined based on consumption rate from previous pulse feed.

b. Constant feeding - the feed pump was set to deliver a predetermined flow rate (3.96 mL/h) of substrate continuously.

c. DO-stat with constant feeding - the same setting of feed pump as constant feeding (b) was used and the pump started to feed when DO level was more than 50% and to stop when DO level was less than 30%.

d. Automatic MSUR feeding strategy – substrate was fed when DO level increased which indicated the depletion of the substrate. Immediately the DO level dropped as the substrate being fed. The substrate was consumed by the microorganism and once it exhausted, the DO level increase again and the feeding cycle with was restarted.
3.7.1 Calculation of feed rate based on maximum substrate uptake rate

The feeding strategy was based on the dissolved oxygen (DO) response due to microbial oxidation of a substrate (waste cooking oil). Substrate consumption was shown by a drop in dissolved oxygen level when the substrate was fed into the culture and remained at low level until depletion of the substrate. A rapid rise in the dissolved oxygen level was observed upon depletion of the substrate. The maximum substrate uptake rate (MSUR) was then estimated as (Noh et al., 2014):

\[
MSUR = \frac{S}{t_2 - t_1}
\]

(3.5)

where \( t_1 \) is the time (h) at which a known amount of carbon substrate is fed, following the first rise in DO, \( t_2 \) is the time (h) at which the second rise in DO occurs and \( S \) is the amount of substrate fed (waste cooking oil in mL).

The feeding of substrate was resumed with the calculated flow rate (i.e., the MSUR) as soon as the second DO rise was spotted. The next MSUR was estimated following the same procedure when a declined in DO level was again detected.

3.8 Critical oxygen level measurement

At the end of fermentation, aeration was stopped, and agitation was slowed down to 50 rpm and the decline in dissolved oxygen level was continuously recorded with DO probe (InPro6820, Mettler Toledo, USA). The critical oxygen level, \( C_{\text{crit}} \) (Figure 3.1) was determined at the point which the decline in oxygen concentration deviates from a linear relationship with time (Stanbury et al., 1995):
3.9 Determination of oxygen uptake rate (OUR) and volumetric mass transfer coefficient (k_{L}a).

The dynamic gassing out method was implemented in a fermentative system. In this method, when the air supply to the fermentation was stopped, and dissolved oxygen level dropped due to the cellular respiration. Before reaching the C_{crit}, the aeration was resumed, and dissolved oxygen level increased until it reached a constant oxygen concentration (Figure 3.2). The decrease and increase in dissolved oxygen level were recorded by a DO probe (InPro6820, Mettler Toledo, USA), and data was captured by IRIS Software, Version 5 (Infors HT®, Switzerland).

The uptake of oxygen by the respiring culture was expressed as:

\[
\frac{dC_L}{dt} = k_{L}a(C^* - C_L) - q_{O_2}x
\]  \hspace{1cm} (3.1)

where \( \frac{dC_L}{dt} \) is the accumulation of oxygen in the liquid phase, the first term of the equation is oxygen transfer rate (OTR) and the second term is oxygen uptake rate (OUR). When aeration was switched off, equation 3.1 could be simplified into:

\[
\frac{dC_L}{dt} = -q_{O_2}x
\]  \hspace{1cm} (3.2)

Thus, OUR can be estimated from the slope of the plot of dissolved oxygen concentration (after stopping air flow) against time (Figure 3.2).
Rearrangement of equation 3.1 leads to a linear correlation as given below:

\[ C_L = -\frac{1}{k_{L,a}} \left( \frac{dC_L}{dt} + q_{O_2} \right) + C^* \]  

(3.3)

To determine \( \frac{dC_L}{dt} \) values, difference method approached was applied by assuming that:

\[ \frac{dC_L}{dt} \approx \frac{\Delta C_L}{\Delta t} \]

Therefore,

\[ \frac{\Delta C_L}{\Delta t} = \frac{(C_{L1}-C_{L0})}{(t_1-t_0)} + \frac{(C_{L2}-C_{L1})}{(t_2-t_1)} + \cdots + \frac{(C_{Ln}-C_{Ln-1})}{(t_n-t_{n-1})} \]

Figure 3.2 (a) Schematic illustration of dynamic gassing out a technique for the estimation of OUR and \( k_{L,a} \) (b) Estimation of \( k_{L,a} \) (Garcia-Ochoa et al., 2010; Stanbury et al., 1995)
And $C_L$ would be $C_{L(ave)}$

$$C_L = \frac{C_{L1} + C_{L0}}{2}, \frac{C_{L1} + C_{L0}}{2}, \ldots, \frac{C_{Ln} + C_{Ln-1}}{2}$$

So, equation 3.3 can be expressed as;

$$C_{L(ave)} = -\frac{1}{k_{l,a}} \left( \frac{\Delta C_L}{\Delta t} + q_{O_2} x \right) + C^*$$  \hspace{1cm} (3.4)

A plot of $C_L$ versus $\left( \frac{\Delta C_L}{\Delta t} + q_{O_2} x \right)$ would yield a straight line and the $k_{l,a}$ value could be estimated from the slope given by $-\frac{1}{k_{l,a}}$.

### 3.10 Volumetric mass transfer coefficient ($k_{l,a}$) studies by static gassing out technique

The $k_{l,a}$ was determined from a non-fermentative system by static gassing out method (Stanbury et al., 1995). The increase in DO (%) was assessed using a polarographic O₂ sensor (InPro6820, Mettler Toledo, USA) and the data was captured by IRIS Software, Version 5.2. For all experiments, the temperature was maintained at 28°C with 1.5 L working volume in distilled water system.

In this technique, oxygen was gassing out from the medium by gassing in nitrogen gas until the dissolved oxygen level reached almost zero. Then oxygen was flowing into the medium at constant aeration rate, and the increase in dissolved oxygen (DO) level was monitored with a dissolved oxygen probe. The mass balance of the system was given by equation 3.1, in section 3.6.5. The OUR is zero since no microorganism was present in the system. Thus, Equation 3.1 was reduced into;

$$\frac{dC_k}{dt} = k_{l,a}(C^* - C_L)$$ \hspace{1cm} (3.6)

By integration, equation 3.2 can be expressed as;

$$\ln(C^* - C_L) = -(k_{l,a})t$$ \hspace{1cm} (3.7)

Thus, $k_{l,a}$ can be estimated from the slope of $\ln(C^* - C_L)$ versus t plot.
3.10.1 Effect of agitation speed and aeration rate on \( k_{L_a} \)

The effect of agitation speed on the \( k_{L_a} \) was studied between 200 to 1000 rpm agitation with aeration maintained at 1.0 vvm. For the effect of aeration, the investigation was conducted within the range of 0.33 to 2.5 vvm with the agitation speed maintained at 500 rpm.

3.10.2 Effect of rhamnolipid and waste cooking oil on the \( k_{L_a} \) value

The cell-free broth from a batch fermentation containing 3.0 g/L of rhamnolipid was used for this study. To 1 L of broth, 500 mL of distilled water was added, to produce 1.5 L of broth with a final concentration of 2.0 g/L of rhamnolipid. To study the effect of various concentrations of rhamnolipid on \( k_{L_a} \), the medium was diluted to give final concentrations ranging from 0.031 to 2.0 g/L.

The effect of used cooking oil on \( k_{L_a} \) was studied at a range of concentrations from 0.5 to 4.0% (v/v) of oil in distilled water. The \( k_{L_a} \) measurements were carried out in a bioreactor with a working volume of 1.5 L, agitated at 400 rpm and aerated at 0.3 vvm, which was similar to the actual fermentation conditions.

3.11 Analytical Methods

3.11.1 Biomass determination

Cell biomass was determined based on the turbidity of the fermentation broth measured with a UV-visible spectrophotometer (Genesys 20, Model 4001-04, USA) at 540 nm. The fermentation broth was centrifuged (Centrifuge 5424, Eppendorf, Germany) at 10,000×g for 5 min. The pellet was washed with acetone followed by distilled water while the supernatant was used for rhamnolipid measurement. Cell
3.11.2 Quantification of rhamnolipid through orcinol method

Rhamnolipid production was quantified based on rhamnose (Acros) concentration using orcinol-sulphuric acid method (Koch et al., 1991). First, 0.19% (w/v) of orcinol (Acros) was dissolved in 53% (v/v) of sulphuric acid (97%, AR grade, QRëC™). Then, 0.3 mL cell-free extract was mixed with 2.7 mL of orcinol-sulphuric acid solution and heated in a waterbath (Memmert, Germany) at 80°C for 30 min. After the solution was allowed to cool to room temperature, the absorbance was read at 421 nm using a spectrophotometer (Genesys 20, Model 4001-04, USA). The standard curve was constructed using rhamnose (Acros) within the range of 0.0 to 0.1 g/L as a standard and treated the same way as the cell-free extract samples. Rhamnolipid concentration was expressed as rhamnose equivalent as determined from the standard curve (Appendix A).

3.11.3 Leftover oil measurement

Leftover oil was determined gravimetrically as adapted from Müller et al., (2010). The cell-free extract was mixed with hexane (AR grade, QRëC™) at a ratio of (1:1; v/v). The mixture was then centrifuged at 10,000×g (Sigma® 3-18 K Sartorius, Germany) for 10 min. The upper phase was transferred to a preweighed centrifuge tube, and hexane was left to evaporate in an oven at 71°C (boiling point of hexane is 68°C) for 24 h. The tube with leftover oil was weighed again after cooling to room temperature and the weight of oil in g/L was calculated as:
Weight of leftover oil (g/L) = $\frac{\text{Weight of tube with leftover oil} - \text{weight of empty tube (g)}}{\text{Volume of sample (L)}}$

3.4

3.11.4 Fatty acids analysis of leftover oil

Fatty acid content of the leftover oil was analysed using high-performance liquid chromatography as described by Guarrasi et al., (2010).

3.11.4(a) Fatty acid standards preparation

Two fatty acid standards, palmitic acid (Merck) and oleic acid (R&M Chemicals) were prepared. A 0.345 g of palmitic acid and 12.5 µL of oleic acid were dissolved in 10 mL of methanol (HPLC grade, QRëC™) separately. The standards were filtered through a 0.45 µm nylon filter (Captiva Econofilters, Agilent, USA) into a pre-rinsed 1.5 mL amber vial (Agilent, USA) before HPLC injection.

3.11.4(b) Fatty acids sample preparation

A known weight of dried leftover oil (in g, from section 3.9) was hydrolyzed with 0.5 M NaOH at 1:10 ratio (w/v) at 100°C for 1 h. After cooling, the dispersion was extracted twice with 50 mL of petroleum ether (QRëC™), and the upper layer was discarded to remove the unsaponifiable fraction. The remaining dispersion was acidified with 1 M HCl to pH 2.0, then free fatty acids were extracted twice with 50 mL petroleum ether by pooling the upper layer solution. The organic solvent was removed at 40°C with a rotary evaporator (N-100, Eyela, Tokyo). Finally, the acids were recovered with 4 mL of methanol and filtered through 0.45 µm Nylon filters (Captiva Econofilters, Agilent, USA) before HPLC injection.
3.11.4(c) HPLC system condition

The HPLC system was an Agilent 1260 Infinity Series equipped with a UV–vis photodiode array detector (G4212B), an autosampler (G1329B), a quaternary pump VL (G1311C) and a 20-μL sample loop. A Zorbax reversed phase SB-C18 (250 mm×4.6 mm i.d., particle size 5 μm) column (Agilent, USA) was used. The separation was done isocratically at a ratio of 90:8:2 (v/v/v) of a mobile phase that contained acetonitrile, methanol, and n-hexane (all of HPLC grade, QRëC®). A 10 μL sample was injected at a flowrate of 1.0 mL/min and the acids were monitored at 208 nm. All mobile phase solvents were filtered with a 0.45 μm nylon membrane filter (47 mm diameter, Whatman, UK) and degassed (Sonicor, USA) prior to use.

3.11.5 Residual nitrate quantification

Residual nitrate was measured using a nitrate test kit AQUANAL®-plus Nitrat (NO₃) (Fluka) for a concentration range of 1-50 mg/L. The cell-free supernatant was diluted before the testing. A 5 mL of diluted supernatant was taken for the reaction with reagents following the instructions available in the test kit. Once the reaction was completed, the colour of the mixture was compared with the colour chart to determine the nitrate concentration.

3.11.6 Critical micelle concentration (CMC) measurement

A serial dilution of cell-free containing rhamnolipid broth was prepared using distilled water to bring the biosurfactant concentration in the range of 9 mg to 48 mg/L. The surface tension was measured by the Ring Method using the CSC-DuNoüy Tensiometer (CSC Scientific Company, Inc). The measurement was performed in triplicate for reproducibility of the outcomes. The tensiometer was calibrated against
distilled water. Before each measurement, the probe was immersed several times in 70% ethanol and wiped with a Kimwipe tissue. CMC was determined from a graph of the surface tension versus biosurfactant concentration. The value where the deflection of the curve occurs was estimated as CMC (Abbasi et al., 2012).

3.12 Kinetic models analysis

All calculations of the kinetic parameters were conducted using Microsoft Excel® 2007. The following equations are listed for estimation of parameters as follows:

Specific growth rate, \((h^{-1}) = \frac{\ln\left(\frac{x_{n+1}}{x_n}\right)}{t_{n+1} - t_n}\), where \(n\) is 1, 2, 3, 4……..\(n+1\)

\(x\) is the cell concentration (g/L) and \(t\) is incubation time (h)

Specific product synthesis rate,

\[ q_p = \frac{\Delta P_{n+1,n}}{\Delta t_{n+1,n} \times \bar{x}_{n+1,n}}, \] (where \(n\) = 1, 2, 3, 4……..\(n+1\))

\(\Delta P\) is produced rhamnolipid between \(t_{n+1}\) and \(t_n\) (g/L), \(\Delta t\) is incubation time difference (h) and \(\bar{x}\) is mean concentration of cell between \(t_{n+1}\) and \(t_n\) (g/L).

Yield of product over substrate, \(Y_{PS}(g/g) = \frac{P_{produced}}{S_{consumed}}\)

Yield of product over biomass, \(Y_{P/x}(g/g) = \frac{P_{produced}}{x_{produced}}\)

Yield of biomass over substrate, \(Y_{x/S}(g/g) = \frac{x_{produced}}{S_{consumed}}\)

Overall productivity \((g/L.h) = \frac{P_{max}}{t_{max}}\)

\(P_{max}\) is maximum rhamnolipid produced (g/L) and \(t_{max}\) is time at \(P_{max}\) (h).
Figure 3.3 Research flowchart

Pseudomonas aeruginosa
USM-AR2
+ Waste cooking oil
= Rhamnolipids

Research flow

Shake flasks

Medium evaluation:
- at initial oil concentration
- similar oil concentration
- similar C/N ratio
- similar total carbon

Different C/N ratio:
- C/N = 9
- C/N = 18
- C/N = 27

Effect of chemical surfactant addition (Tween 80)

Kₕₐ determination (non-fermentative system):
- Effect of agitation
- Effect of aeration
- Effect of WCO and RL

Effect of chemical surfactant addition (Tween 80)

Oxygen transfer behaviour

Batch culture

Effect of operational conditions on RL

Kinetics study

Fed-batch culture

Feeding strategy

Manual feeding

DO-stat

MSUR

Constant feeding

Analysis

Biomass

Rhamnolipid

Oil leftover

Nitrogen leftover

OUR and OTR determination in fermentative system

Effect of agitation:
- 400 rpm
- 500 rpm

pH and DO control:
- pH control only
- DO control only
- pH and DO control
- no control
CHAPTER 4 RESULTS

4.1 Shake flasks study

In the first stage of the research, studies were conducted to determine the medium formulation suitable for high rhamnolipid production. The experiments were designed to evaluate medium which could support high rhamnolipid production with waste cooking oil as a sole carbon source at several conditions i.e.: i) Media examined at the original composition as stated in the literature (experiment 1); ii) media formulated at a similar carbon source concentration (experiment 2); iii) media formulated at similar carbon to nitrogen ratio (experiment 3); and lastly 4) media tested at similar total carbon. Results of the experiment are described in the following section.

4.1.1 Medium formulation evaluation

The high rhamnolipid production was reported from previous works by Müller et al., (2010), Zhu et al., (2012) and Noh et al., (2014). It was noted that different media formulations with different carbon sources (sunflower oil, soybean oil and diesel) were utilised in their works for rhamnolipid production. The oil used was either edible or fuel type. However, none of the previously stated works used waste cooking oil as the carbon source in their formulations. Therefore, in this particular work, investigations were conducted to identify which media formulations combined with waste cooking oil as the carbon source could support high rhamnolipid production by indigenous isolate P. aeruginosa USM-AR2. Experiments conducted are as tabulated in Table 3.1 where medium A, B and C were adapted from Müller et al., (2010), Zhu et al., (2012) and Noh et al., (2014) respectively.

In the first experiment, it was found that rhamnolipid production in medium (A) increased rapidly starting from 32 h onwards until incubation was terminated. The
highest product measured at the end of the incubation time (at 120 h) was 4.49 g/L. However, for media B and C, an increased in production was observed from 48 to 56 h, and afterwards, the production was constant until the end of incubation time (Figure 4.1a). In medium B, the highest rhamnolipid achieved was 1.19 g/L at 72 h of incubation, but slightly reduced to 1.13 g/L after 120 h of incubation period. For medium C, the highest rhamnolipid obtained was 1.25 g/L at the end of incubation time.

![Figure 4.1](image_url)

**Figure 4.1** Concentration of (a) rhamnolipid and (b) cell dry weight from three formulations as measured after 120 h incubation
A different trend was observed for growth as shown in Figure 4.1b. The highest amount of biomass produced were 9.41 g/L and 7.55 g/L for media C and A, respectively, after 48 h of incubation for both media. Medium B produced the lowest amount of biomass at 6.28 g/L after 96 h of incubation. It was observed that at 56 h of incubation, there was a sudden drop in biomass in media A and B. This could be due to the low volume of sample taken at that incubation time and furthermore the samples were not well mixed, thus these values were not considered in the trend of biomass production by these media.

Measurement of residual waste cooking oil concentration showed that the substrate consumption was low. Only 31%, 29% and 33% of substrate were utilized from media A, B and C respectively (Figure 4.2). Note that, more oil was consumed in medium A as compared to media B and C since in the initial oil concentration in medium A was also high (refer Table 3.2). This fact could explain why high rhamnolipid could be produced from medium A. Meanwhile, the consumption of oil was higher in medium B than that of medium C, but the production of rhamnolipid and biomass was fewer than medium C. Therefore, the second experiment was carried out with media A and C only.

![Figure 4.2 Amount of waste cooking oil consumed and leftover oil after 120 h of fermentation](image-url)
In the first experiment, it was indicated that the low production of rhamnolipid could be due to the oil concentration in medium C being extremely low. Thus, in the second experiment, the carbon source was added at the same concentration of 80 g/L in both media A and C. The concentration added was based on the maximum waste cooking oil utilized in the previous experiment using medium A, with 77.9 g/L. Results obtained from this investigation showed a marked difference in rhamnolipid production from medium A and medium C (Figure 4.3a). The maximum rhamnolipid achieved in medium A was 3.78 g/L with 0.032 g/L.h of overall productivity. The overall productivity and production of rhamnolipid from medium A increased two-fold than the overall productivity and production of rhamnolipid from medium C. It was determined that 1.89 g/L of rhamnolipid was produced with 0.016 g/L of the overall productivity from medium C.

Medium C was found to produce higher biomass compared to medium A (Figure 4.3b). The maximum biomass concentration attained for medium C was 11.68 g/L at 48 h of incubation. The concentration was almost two times higher than the maximum biomass achieved in medium A. Nevertheless, the biomass slightly reduced to 10.45 g/L at the end of incubation time. Whereas for medium A, a maximum of 6.84 g/L of biomass was produced at the end of incubation period. Waste cooking oil utilization was rather low. The amount of waste cooking oil consumed was only 31.9% and 42.1%, which corresponded to 25.6 g/L and 33.6 g/L for both media A and C respectively.

In the second experiment, the initial oil concentration in both media A and C were the same. However, medium C contained yeast extract, which acted not only as a nitrogen source but also as the carbon source. Therefore, medium C contained more carbon source.
Figure 4.3 (a) Rhamnolipid and (b) biomass concentrations from medium A and C at similar substrate concentration.

Thus, to make a fair comparison between both media, further investigation was conducted by formulating the media at the same carbon to nitrogen ratio of 18. Results from this investigation indicate that medium A did support higher rhamnolipid production than medium C. The maximum rhamnolipid obtained were 2.31 g/L. Rhamnolipid produced were only 0.39 g/L from medium C (Figure 4.4a). In contrast, maximum production of biomass could be seen from medium C with the highest concentration being 3.36 g/L at the end of the incubation time. The concentration was 1.4-fold higher than the maximum biomass achieved in medium A.
Due to an extremely low oil concentration in medium C at similar C/N ratio of 18, the incubation ceased after 48 h of incubation since the cell growth was continuously declining. Further experiment was conducted where medium C was formulated to contain total carbon similar to medium A due to a very low oil concentration at similar C/N ratio. In this formulation, the initial oil concentration in medium C was 24.1 g/L, higher than previously at similar C/N ratio, which was 1.62 g/L. Even when tested at similar total carbon, the highest rhamnolipid produced from medium C was only 1.74 g/L at 84 h of incubation, and afterward, it dropped to 1.43...

Figure 4.4 Rhamnolipid and biomass produced from medium A and C at similar C/N and similar total carbon
g/L at 120 h of incubation. The highest rhamnolipid produced in medium C was 33% less than maximum rhamnolipid produced in medium A. On the other hand, high biomass could be produced when medium C was utilised. The highest biomass obtained was 7.64 g/L at 84 h of incubation period. In both media, total waste cooking oil consumed after 120 h of incubation was 95.5% for medium C (at similar total carbon condition) and 73.8% for medium A (Figure 4.5).

![Waste cooking oil leftover in medium A and C at similar total carbon](image)

**Figure 4.5** Waste cooking oil leftover in medium A and C at similar total carbon

The performance of media A, B and C in rhamnolipid production could be clearly differentiated based on the specific product yield. It was shown that high rhamnolipid were produced from medium A as shown by the specific product yield ($Y_{P/X}$) for each medium (Figure 4.6).
Figure 4.6 Specific product yield from different media in each experiment

Even when tested at similar carbon source concentration (experiment 2), similar C/N ratio (experiment 3) and similar total carbon (experiment 4), rhamnolipid production and specific product yield in medium A were always higher than medium C as depicted in Figures 4.3(a) and 4.4(a); whereas, medium C supported higher biomass production compared to media A and B in all experiments conducted. Even though biomass was greater in medium C, it did not support high rhamnolipid production.

It has been demonstrated that medium A supported relatively high rhamnolipid production compared to medium B and C using waste cooking oil as a sole carbon source. The specific yield of product for medium A was 0.95 g/g. This yield value was higher compared to 0.22 for medium C (from experiment 4). On the other hand, medium C supported high biomass production but not rhamnolipid. The lowest production of rhamnolipid and biomass were shown in medium B. Thus, medium A was used in all subsequent experiments of this research.
4.1.2 Rhamnolipid production with medium A at different carbon to nitrogen (mol C/mol N) ratios

Medium A has been demonstrated to produce relatively high rhamnolipid production compared to media B and C. Another factor that was investigated was the effect of C/N ratios on rhamnolipid production. Various optimum C/N ratios had been reported to improve rhamnolipid production (refer to section 2.5.1), which means that the values were unique under the conditions being studied. Xia et al., (2012) reported a C/N ratio of 8 to be optimum for rhamnolipid production using waste sunflower oil. Meanwhile, Marsudi et al., (2008) showed that a C/N ratio of 27 was optimum for rhamnolipid production with edible palm oil as a carbon source. No report on C/N ratio from waste cooking oil (that was derived from palm oil) has been reported so far. Thus, experiments were conducted to examine the effect of C/N ratio on rhamnolipid production from medium A with waste cooking oil as a sole carbon source. Three ratios; 9, 18 and 27 of mol C/mol N were tested based on the optimum value of C/N ratio reported by Xia et al., (2012) as well as Marsudi et al., (2008).

The effect of C/N ratio was first tested with a constant carbon source value, which was 25.6 g/L and nitrogen source was varied from 5.5 g/L, to 7.5 g/L and 15.0 g/L for C/N = 9, 18 and 27 respectively. Results indicated that at C/N = 18, rhamnolipid production was higher than the other two ratios (Figure 4.7). The maximum rhamnolipid produced was 5.95 g/L at C/N = 18, slightly higher than the other two ratios at constant carbon source condition. Compared to C/N = 9 and C/N = 27, the maximum rhamnolipid attained was 4.45 g/L and 4.83 g/L respectively. The biomass produced were not significantly different at the different C/N ratios.
The maximum biomass produced was in the range of 5.38 g/L to 5.63 g/L at all C/N ratios. Noted that the optimum biomass production could be achieved even at the minimum nitrogen source utilized. Thus, the nitrogen source at the concentration of 5.5 g/L was employed in the subsequent experiments.

Investigation was continued by varying the amount of carbon source, but at a constant value of nitrogen source which was at 5.5 g/L to give the same C/N ratio as previously conducted. At a constant nitrogen source condition with the C/N ratio of 18, the highest rhamnolipid produced was 4.74 g/L. This result confirmed that C/N = 18 was a suitable ratio to improve rhamnolipid production since the product concentration was four times greater than rhamnolipid produced at C/N = 9. At C/N = 27, the amount of rhamnolipid produced was 4.01 g/L, which was slightly lower than the maximum rhamnolipid produced at C/N = 18. However, the biomass concentration was lower at constant carbon source condition compared to biomass concentration at constant nitrogen source condition (Figure 4.8). The maximum biomass attained for all C/N ratios was in the range of 4.47 g/L to 4.71 g/L.

**Figure 4.7** Effect of varying C/N ratio on rhamnolipid production at constant carbon source condition and constant nitrogen source conditions
The product yields were plotted to show the effect of biomass, nitrogen, and waste cooking oil on rhamnolipid production at different C/N ratio. Overall, the product yields were the highest at C/N ratio = 18 (Figure 4.9). The specific product yield (YP\textsubscript{X}) was equivalent to 1.05 and 1.00 at constant carbon source and constant nitrogen conditions respectively. The Y\textsubscript{PN} was 5.19 at constant nitrogen condition and slightly low at constant carbon condition which was 4.78.

Meanwhile, the yield of product over carbon source, Y\textsubscript{PS} was 0.24 at constant carbon condition and 0.30 at constant nitrogen condition. Generally, the lowest product yields were recorded for C/N = 9. For instance, the Y\textsubscript{PX} was 0.24 for C/N = 9 at constant nitrogen condition. Even though the Y\textsubscript{PX} value was high at constant carbon condition which was 0.83, still it was 27% less than the Y\textsubscript{PX} of C/N = 18 under the same condition. Nevertheless, the Y\textsubscript{PN} was 1.82 and 1.21 at constant carbon and constant nitrogen conditions respectively. The value for the Y\textsubscript{PS} was 0.19 at constant carbon condition and 0.13 at constant nitrogen condition.
Figure 4.9 The product yields at different carbon to nitrogen ratio at both constant carbon source and constant nitrogen source conditions
Thus, based on the data of C/N ratio, it was demonstrated that the rhamnolipid production could be improved at C/N = 18. Elevated C/N ratios promote the production (Soberon-Chavez et al., 2005), since this ratio reflects the nitrogen-limiting condition, which was claimed to be the preferred condition for enhanced rhamnolipid production (Kumar et al., 2012). In this particular investigation, the nitrogen source was limited to 5.5 g/L from the initial concentration of 7.5 g/L at C/N ratio = 18. This limitation caused the $Y_{\text{P/N}}$ to improve by 8.6% and the $Y_{\text{P/S}}$ by 27.2%.

However, there was a slight difference in rhamnolipid production at constant carbon and constant nitrogen conditions. Rhamnolipid production at constant carbon source was 25% higher than at constant nitrogen source. This is simply because more oil has been consumed compared to the medium at the constant nitrogen condition (Figure 4.10).

![Figure 4.10](image_url) Amount of waste cooking oil consumed at different C/N ratio

Different C/N ratios had been reported in literature for the optimum production of rhamnolipid (Table 4.1), this was due to the fact that the optimal C/N ratio varied with strains and carbon sources (Li et al., 2011; Wu et al., 2008). If compared to the other optimal C/N ratio, it was noted that the finding in the current study was consistent with the range of optimal C/N reported by other researchers. Results in this investigation demonstrated that C/N ratio did affect the rhamnolipid production. The
C/N ratio equivalent to 18 was optimum for relatively high rhamnolipid production compared to C/N=9 and C/N=27. Therefore, the C/N = 18 was used for formulation of medium A in the later stage of this research.

**Table 4.1** Optimum carbon to nitrogen ratio for rhamnolipid production reported in the literature

<table>
<thead>
<tr>
<th>Carbon/Nitrogen source</th>
<th>Optimum C/N</th>
<th>RL\textsubscript{max} (g/L)</th>
<th>Volumetric productivity (g/L.h)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol/NaNO\textsubscript{3}</td>
<td>12.5</td>
<td>5.6</td>
<td>0.0583</td>
<td>(Saikia et al., 2013)</td>
</tr>
<tr>
<td>Soapstock/NaNO\textsubscript{3}</td>
<td>8</td>
<td>7.3</td>
<td>0.1014</td>
<td>(Benincasa and Accorsini, 2008)</td>
</tr>
<tr>
<td>Deodorizer distillate/NaNO\textsubscript{3}</td>
<td>12.5</td>
<td>38.93</td>
<td>0.1622</td>
<td>(Partovi et al., 2013)</td>
</tr>
<tr>
<td>Glycerol/NaNO\textsubscript{3}</td>
<td>52</td>
<td>7.5</td>
<td>0.0446</td>
<td>(Wu et al., 2008)</td>
</tr>
<tr>
<td>Glucose/NaNO\textsubscript{3}</td>
<td>26</td>
<td>6.8</td>
<td>0.0404</td>
<td>(Wu et al., 2008)</td>
</tr>
<tr>
<td>Soybean oil/NaNO\textsubscript{3}</td>
<td>100</td>
<td>1.42</td>
<td>0.0147</td>
<td>(Prieto et al., 2008)</td>
</tr>
<tr>
<td>Bioglycerol/NaNO\textsubscript{3}</td>
<td>15</td>
<td>3.312</td>
<td>0.0345</td>
<td>(Kumar et al., 2012)</td>
</tr>
<tr>
<td>Palm oil/NaNO\textsubscript{3}</td>
<td>55</td>
<td>30.4</td>
<td>0.2533</td>
<td>(Li et al., 2011)</td>
</tr>
<tr>
<td>Blackstrap molasses/NaNO\textsubscript{3}</td>
<td>20</td>
<td>1.5</td>
<td>0.0208</td>
<td>(Raza et al., 2014)</td>
</tr>
<tr>
<td><strong>Waste cooking oil/NaNO\textsubscript{3}</strong></td>
<td><strong>18</strong></td>
<td><strong>4.7</strong></td>
<td><strong>0.0395</strong></td>
<td><strong>Current work</strong></td>
</tr>
</tbody>
</table>

**4.1.3 Effect of Tween 80 on rhamnolipid production**

Waste cooking oil used in this research as a sole carbon source is immiscible with water. An addition of surfactant into the production medium may improve miscibility of the oily substrate through interfacial tension reduction, hence increasing the accessibility of the substrate by the cells. It was expected that the cell growth rate and product synthesis rate would be accelerated by increasing the substrate availability to the cells. Thus, in this study, the effect of commercial surfactant, Tween 80 on rhamnolipid synthesis rate was studied.

Tween 80 is a non-ionic surfactant, which has the hydrophilic-lipophilic balance (HLB) value of 15 (HLB systems, 2018). At this HLB value, the Tween 80 has the
characteristic as oil in water emulsifier (Dow Answer Center, 2016). Thus, it makes Tween 80 suitable for emulsifying the waste cooking oil to increase its miscibility with water. The concentration of Tween 80 added was 0.1% (v/v). After five days of incubation, the maximum rhamnolipid produced were 4.57 g/L (Figure 4.11) from the medium supplied with a commercial surfactant. A low rhamnolipid production was detected in the medium without addition of surfactant. The maximum rhamnolipid production achieved was 3.53 g/L after five days of incubation. Similarly, no visible difference in biomass production was observed for both media. The maximum biomass attained was 5.45 g/L for a medium with surfactant and 5.13 g/L for a medium without biosurfactant at 96 h of incubation. At the end of incubation period, biomass dropped to 4.60 g/L for both media. However, the substrate uptake in medium with surfactant improved slightly by 12.7% over the medium without surfactant.

It was shown that the trend of rhamnolipid and biomass productions in medium with and without surfactant overlapped with each other (Figure 4.11). The maximum specific growth rate in both media was achieved within eight h of incubation and the rate was 0.289 h⁻¹ for medium with commercial surfactant. This rate was slightly higher than the specific growth rate for medium without commercial surfactant which was 0.200 h⁻¹ (Figure 4.12a).
Figure 4.11 (a) Rhamnolipid and (b) biomass production in the medium with or without commercial surfactant.
Meanwhile, the specific product synthesis rate decreased from 0.007 g/g.h at 24 h to 0.004 g/g.h at 32 h of incubation in the medium with commercial surfactant. After that it started to increase until the maximum rate was achieved which was 0.008 g/g.h. On the other hand, the specific product synthesis rate was increased at 24 h of incubation until it reached the maximum value of 0.008 g/g.h at 48 h of incubation in the medium without surfactant. After 48 h, the rate dropped to 0.006 g/g.h at the end of incubation time. The results showed that the addition of external commercial surfactant only acted to enhance the oil accessibility to the cells and was not required.
for the process to take place under the condition that was studied in this research. This fact is due to the nature of the microorganisms which themselves produce the biosurfactant for the uptake of oily substrate. Thus, the medium used in the subsequent experiment was without Tween 80.

4.2 Batch culture study in a bench-top bioreactor

Studies in shake flasks demonstrated that rhamnolipid production was potentially higher in medium A compared to media B and C. In the second part of the research, further studies were performed at a larger scale using a 3.6 L bench-top bioreactor, Labfors 4 HT, Switzerland to improve the yield of rhamnolipid production at a larger laboratory scale. All results are described and discussed in the following sections.

4.2.1 Oxygen transfer in a bench-top bioreactor for rhamnolipid production

Generally, the production of rhamnolipid by P. aeruginosa occur under aerobic condition (Kronemberger et al., 2008). The study of oxygen supply and oxygen transfer in the culture medium would be important criteria to be determined to ensure sufficient oxygen is supplied to the microbial population in an aerobic culture. The OTR value depends on the air flow rate, the stirrer speed, mixing, etc. (Bandaiphet and Prasertsan, 2006) and volumetric mass transfer coefficient (kLa) is the parameter usually studied to determine effective oxygen transfer rate in an aerobic fermentation. The addition of an inert water-immiscible substrate in which oxygen has a higher solubility such as hydrocarbon or perfluorocarbon had been reported as an alternative method of improving the OTR (Clarke and Correia, 2008; Correia et al., 2010). The
effect of agitation, aeration as well as concentration of waste cooking oil on the $k_{L,a}$ during rhamnolipid production were studied. Any potential occurrence of oxygen limitation was also observed in this study.

4.2.1(a) Effect of agitation and aeration on volumetric mass transfer coefficient

The effect of agitation and aeration rate on volumetric mass transfer rate ($k_{L,a}$) in a non-fermentative system were studied at different impeller speeds and aeration rate. The effect of impeller speed was examined at the range of 200 to 1000 rpm with constant aeration at 1.0 vvm. A sharp increase of the $k_{L,a}$ was observed at the range of 200 to 300 rpm of impeller speed (Figure 4.13). The $k_{L,a}$ was 19.8 h$^{-1}$ at 200 rpm and enhanced by 36.3% to 27.0 h$^{-1}$ at 300 rpm. Then, the $k_{L,a}$ improved slightly with a maximum value of 28.8 h$^{-1}$ at the impeller speed of 400 rpm, after which the $k_{L,a}$ value leveled off. Aeration rate also showed a significant effect on the $k_{L,a}$. It was demonstrated that $k_{L,a}$ increased linearly within the range of aeration rate studied (Figure 4.14). The lowest $k_{L,a}$ was 10.8 h$^{-1}$ at 0.3 vvm with fix agitation speed of 500 rpm and reached the maximum value of 72.0 h$^{-1}$ as the aeration rate increased to 2.5 vvm.

![Figure 4.13](image-url) Effect of agitation speed on $k_{L,a}$ at a constant aeration rate
The increase in agitation speed increased the area for oxygen transfer by dispersing the air into small bubbles and thus reducing the liquid-film thickness at the gas-liquid interface. In this case, the air flow rate ($F_g$) that can be completely dispersed by the impeller was $0.0019 \text{ m}^3\text{min}^{-1}$ achieved at 400 rpm and above since $F_g$ was greater than the operating flow rate ($0.0015 \text{ m}^3\text{min}^{-1}$) (Table 4.2). The dispersed air with increasing area for oxygen transfer might contribute to the increase in $k_La$. However, the $k_La$ levelled off at 500 rpm onwards. Meanwhile, aeration rate showed a positive influence on $k_La$. Based on the calculation of $F_g$, the air bubbles were fully dispersed at all aeration rate tested, which increased the area for oxygen transfer. This occasion most probably contributed to the linearly increased of $k_La$ due to the aeration rate.
Table 4.2 Air flow rate at different agitation speed with constant operating flow rate of 1.5 Lmin\(^{-1}\)

<table>
<thead>
<tr>
<th>Agitation speed (rpm)</th>
<th>Air flow rate, F(_g) (m(^3)min(^{-1}))</th>
<th>Air dispersion*</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>0.0009</td>
<td>Not dispersed</td>
</tr>
<tr>
<td>300</td>
<td>0.0014</td>
<td>Not dispersed</td>
</tr>
<tr>
<td>400</td>
<td>0.0019</td>
<td>Dispersed</td>
</tr>
<tr>
<td>500</td>
<td>0.0023</td>
<td>Dispersed</td>
</tr>
<tr>
<td>600</td>
<td>0.0028</td>
<td>Dispersed</td>
</tr>
<tr>
<td>800</td>
<td>0.0037</td>
<td>Dispersed</td>
</tr>
<tr>
<td>1000</td>
<td>0.0047</td>
<td>Dispersed</td>
</tr>
</tbody>
</table>

*The air is fully dispersed when F\(_g\) is higher than operating flow rate of 0.0015 m\(^3\)min\(^{-1}\) (1.5 Lmin\(^{-1}\))

4.2.1(b) Effect of rhamnolipid and waste cooking oil on k\(_L\)a

The investigation of agitation and aeration rate was carried out in distilled water without the presence of a surface-active agent. In the fermentation process, the k\(_L\)a could be affected by the rhamnolipid produced and waste cooking oil used as the substrate. Both the oil and rhamnolipid have been shown to have a surface-active properties (Esteban et al., 2012; Lan et al., 2015). As both oil and rhamnolipid were present in the fermentation studies carried out, it is therefore expected similar effect will be observed.

Results showed that the k\(_L\)a was enhanced by a factor of 3.0 times with the presence of 1.0 g/L of rhamnolipid compared to the k\(_L\)a in the rhamnolipid-free medium and the maximum k\(_L\)a achieved was of 31.68 h\(^{-1}\) (Figure 4.15). The k\(_L\)a then started to decrease, reaching the minimum value of 23.28 h\(^{-1}\) as the concentration of the rhamnolipid was increased to 2.0 g/L.
Figure 4.15 The effect of rhamnolipid concentration on volumetric mass transfer rate ($k_La$)

Meanwhile, it seems that waste cooking oil did not show much effect on the $k_La$ since the value remained almost constant (Figure 4.16) throughout the whole range of the oil used.

Figure 4.16 The effect of waste cooking oil fraction on volumetric mass transfer rate ($k_La$)
4.2.1(c) Oxygen transfer in batch fermentation of rhamnolipid production

In the actual batch fermentation for rhamnolipid production, the operating condition was set at 0.3 vvm of aeration rate and agitation speed of 400 rpm. The waste cooking oil utilized was at 4% (v/v) and the final rhamnolipid production in this particular batch fermentation was 2.31 g/L. Batch fermentation was conducted to observe any potential occurrence of oxygen limitation with the established operating conditions. The results indicated that aeration rate influenced the increase in the \( k_{La} \) values more than the agitation speed. Earlier studies showed that the \( k_{La} \) increased almost linearly with aeration rate, achieving about 2.4 times higher values when compared to the maximum obtained at increased agitation. However, increasing the aeration to achieve a higher \( k_{La} \) may lead to high foaming, causing medium overflow hence lower production yield (Lee and Kim, 2004; Salwa et al., 2011). Due to this, the minimum value of aeration i.e. 0.3 vvm was set for batch fermentation so that foaming effect could be minimized. It was demonstrated that under these conditions the OTR was always more than the OUR at all incubation period (Figure 4.17).

![Figure 4.17](image-url) Variation of OUR and OTR in a batch culture. The OUR and OTR was measured through dynamic method of gassing out during the course of fermentation period
An exception was for the final hour of incubation that the OTR dropped to 19.18 mmol/L.h, which was slightly higher than the OUR (15.88 mmol/L.h). Thus, it was shown that oxygen limitation may not occur in batch culture under the predetermined conditions.

The trend showed a reduction in OTR throughout the fermentation period and with the increase in rhamnolipid production. However, in the culture such as fed-batch fermentation where higher rhamnolipid production was expected, a biosurfactant concentration more than CMC should have no additional effect on the $k_{L,a}$ since surface tension remained unchanged (Figure 4.18) (Martinov et al., 2008).

![Critical micelle concentration of rhamnolipid and surface tension reduction at different concentration of rhamnolipid](image)

**Figure 4.18** Critical micelle concentration of rhamnolipid and surface tension reduction at different concentration of rhamnolipid

### 4.2.2 Effect of operating conditions on rhamnolipid production

The operating conditions studied in batch culture for rhamnolipid production were the agitation speed, control of pH and dissolve oxygen levels. It has been shown elsewhere in the literature that aeration rate and $k_{L,a}$ had no impact on rhamnolipid production (de Lima et al., 2009; Salleh et al., 2011). The aeration rate was fixed at 0.3 vvm to minimise foaming; whereas the $k_{L,a}$ was fluctuating due to rhamnolipid production (section 4.2.1(b) and Figure 4.15). Therefore, the effect of aeration and $k_{L,a}$
on rhamnolipid production was not studied. Results of the effect of operating conditions on rhamnolipid production are presented in the subsequent section.

4.2.2(a) Effect of agitation speed on rhamnolipid production

Agitation is important to disperse gases such as air through liquid in the form of small bubbles and to disperse immiscible liquids to form an emulsion or suspension of fine droplets (Doran, 2013). The agitation speeds examined in this study were based on results from section 4.2.1(a) where the $k_{L,a}$ value was constant from 400 rpm onwards. Therefore, investigation needed to be carried out to determine which agitation speed could support high production of rhamnolipid and at the same time sufficient for effective mixing of the cells.

The production was tested at two tip speeds; 1.13 m/s and 1.41 m/s which corresponded to 400 rpm and 500 rpm agitation respectively. Effective mixing was assumed at both agitations since the fluid flow showed turbulent flow regime based on calculated Reynolds number i.e. 21842.69 and 27303.37 at 400 rpm and 500 rpm respectively. At 300 rpm, it was shown that there was a limitation in oxygen availability in the fermentation broth since the level reached 0% (Figure 4.19). But this limitation was not shown at 400 and 500 rpm. Therefore, the rhamnolipid production was not conducted at 300 rpm. Results showed that rhamnolipid production was affected by tip speed. At the tip speed of 1.13 m/s, the maximum rhamnolipid produced was 2.49 g/L at 72 h of incubation. Less rhamnolipid was produced when the bioreactor was operated at a tip speed of 1.41 m/s.
Figure 4.19 DO and stirrer profiles at different agitation speeds in a batch culture
The highest rhamnolipid produced at the high tip speed was 1.67 g/L at the end of incubation time. So, it was shown that the production at low tip speed was 1.5-fold higher than the production at higher tip speed. The overall productivity was 0.0346 g/L.h at 1.13 m/s tip speed and 0.0232 g/L.h at 1.41 m/s tip speed. As observed from Figure 4.20, rhamnolipid started to form at as early as 16 h of the incubation period for both tip speeds. After the incubation period reached 36 h onwards, the trend in rhamnolipid production increased considerably until the maximum concentration was reached at the end of the incubation time.

Figure 4.20 Effect of tip speeds on (a) rhamnolipid and (b) biomass productions
A similar pattern was observed in biomass production. More biomass was produced at a slower tip speed with the maximum concentration of 11.13 g/L attained at 32 h of incubation time. At a higher tip speed, the maximum biomass concentration obtained was 7.85g/L. The biomass produced at a tip speed of 1.13 m/s was 42% higher than that produced at a tip speed of 1.41 m/s. Thus, agitation at 400 rpm was utilized in the succeeding bench-top bioreactor experiments.

4.2.2(b) Effect of pH and dissolved oxygen on rhamnolipid production

During the first attempt of batch fermentation for rhamnolipid production in this study, it was found that the pH of the culture reached a maximum of more than 9 when the pH culture was not controlled (Figure 4.21d). Chen et al., (2007) indicated that the best rhamnolipid production occurred when the pH of the culture was controlled at 6.8. However, when the pH was controlled at lower than 6.5 or higher than 7.5, rhamnolipid production decreased significantly along with a lower cell growth rate. Therefore, based on observation made by Chen et al., (2007), the pH culture in this study was controlled at 6.85 ± 0.5 by pumping in 1 M HCl or 1 M NaOH. The problem at the controlled pH condition was that the DO level was limiting (Figure 4.21a). However, this limitation was not observed when pH was not controlled (Figure 4.21d). The lowest value of DO level was in the range of 45 to 55% (Figure 4.21d) when pH and DO were not controlled. Hence, the batch culture with controlled dissolved oxygen at 20% ± 5% by cascading DO to stirring rate was attempted (Figure 4.21b). Since the pH of the culture at controlled DO was high and it reached more than 8, thus, batch culture with both pH and DO controlled was conducted (Figure 4.21c).
Figure 4.21 pH and dissolved oxygen profile for batch cultures at (a) control pH, (b) control DO, (c) control pH and DO and (d) without control of pH and DO.
In the controlled DO experiment, the level was controlled at a predetermined value of 20% ± 5% by cascading DO with stirring rate (Figure 4.21b). During the first 48 h of fermentation, the DO level was not stable. The culture was intensely agitated to maintain the DO level at the set point. After two days of incubation, the DO level started to settle at the pre-set value and remained constant until the end of the fermentation. When both parameters (pH and DO) were controlled, the agitation was more intense than when only DO was controlled. The maximum stirring rate reached was 1215 rpm compared to 660 rpm of the maximum stirring rate achieved at controlled DO only. The DO level remained almost constant around the set point after three days of fermentation time.

The time course profile for cell growth, product synthesis and substrate consumption of all conditions were plotted. When the pH was controlled, the stationary phase started at 24 h of incubation time and no deceleration phase was observed. It was noticed that rhamnolipids were produced mainly at the stationary phase (Figure 4.22).

**Figure 4.22** Time course profile of bacterial growth, rhamnolipid production and substrate consumption at controlled pH condition
The production of rhamnolipids started after 24 h of incubation, and a significant increase could be observed from 48 h onwards. The rhamnolipid production showed an uncertain trend of production where at 105 h of incubation, the production dropped by 28.9% from 4.04 g/L at 96 h. However, the production recovered and reached a maximum of 4.86 g/L of rhamnolipid at the end of incubation time.

When DO was controlled, the deceleration phase could be detected starting from 24 h and it lasted for 1 day, before stationary phase began at 48 h onwards (Figure 4.23). Rhamnolipid were produced after 24 h of incubation which was at the beginning of deceleration phase. The product concentration continued to rise until it reached 2.99 g/L at the end of incubation time. The production of rhamnolipid at the controlled DO was 38.4% less than rhamnolipid production at controlled pH condition.

**Figure 4.23** Time course profile of bacterial growth, rhamnolipid production and substrate consumption when DO was controlled at 20%

A different growth profile was observed when both pH and DO were controlled. The deceleration phase remained for only about 6 h (Figure 4.24). Rhamnolipid production started at the beginning of the stationary phase, which was at 30 h of incubation time.
Figure 4.24 Time course profile of bacterial growth, rhamnolipid production and substrate consumption when at controlled pH condition and DO were controlled at 20%.

At 72 h of incubation, 2.60 g/L of rhamnolipid was produced. This value was the highest amount of rhamnolipid obtained at this period of incubation as compared to the other conditions. For instance; at the same incubation time, the rhamnolipid produced were 2.05, 2.02 and 1.54 g/L at controlled pH and DO and without controlled conditions respectively. However, after 72 h incubation, the production trend was almost constant. Note that the waste cooking oil was exhausted at this time and this limitation might be responsible for a constant production of rhamnolipid. The maximum production observed was 2.78 g/L at the end of the fermentation.

The culture without control showed the longest deceleration phase starting from 12 h and ended at 57 h of incubation (Figure 4.25). A continuous production was observed during this phase but at the stationary phase, the production slowly increased. The maximum production achieved at the end of incubation was 2.31 g/L. This value was the lowest rhamnolipid production compared to the other control conditions.
Nevertheless, no obvious effect on rhamnolipid production was observed when pH and/or DO were controlled. Even though the highest rhamnolipid production was achieved when pH was controlled, it occurred only at the end of the incubation time (Figure 4.26); whereas, the production from earlier fermentation time overlapped with each other. The overall productivity for all batch cultures were between 0.024 to 0.051 g/L.h where the highest productivity was from batch culture with controlled pH.

**Figure 4.25** Time course profile of bacterial growth, rhamnolipid production and substrate consumption when pH and DO were not controlled

**Figure 4.26** Comparison of rhamnolipid production in batch cultures with different control type
The highest apparent yield ($Y_{P/X}$) was obtained in batch culture with controlled pH and DO level where $Y_{P/X}$ was 2.04 g/g. This yield was slightly higher than the yield obtained in batch culture with controlled pH with $Y_{P/X}$ of 1.72 g/g (Figure 4.27). Biomass production was affected when DO was controlled (Figure 4.28). The highest biomass obtained at the end of incubation time (120 h) was 4.45 g/L at a controlled DO condition. When both DO and pH were controlled, the maximum biomass produced was 3.78 g/L at 57 h of incubation time, 15% less than the maximum biomass obtained at the controlled DO condition. The reduction in biomass after 57 h of incubation when DO and pH were controlled was due to exhaustion of waste cooking oil.

![Graph](image.png)

**Figure 4.27** Apparent yield of product over biomass and productivity for batch cultures
Afterwards, the biomass declined to 1.69 g/L at 72 h of incubation and 1.36 g/L at the end of incubation time. The maximum biomass achieved at controlled pH condition was 3.31 g/L at 72 h of incubation time. The lowest biomass attained was 2.42 g/L when DO and pH were not controlled.

More biomass produced from the substrate consumed when DO was controlled as well as when it was not controlled. This is shown by higher yield of biomass over substrate \( (Y_{x/S}) \) than yield of product over biomass \( (Y_{P/S}) \) (Figure 4.29). The highest \( Y_{x/S} \) is when DO alone was controlled which was 0.28 while the \( Y_{P/S} \) at the same controlled condition was 0.19 only, which meant that \( Y_{x/S} \) was 1.5-fold higher than the \( Y_{P/S} \). In contrast, when pH was controlled, the \( Y_{P/S} \) was 0.27 which was 8% higher than the \( Y_{x/S} \). However, when both DO and pH were controlled, the \( Y_{x/S} \) was 1.5 times higher than the \( Y_{P/S} \) similar to when DO alone was controlled.
At the end of the fermentation time, the amount of waste cooking oil consumed was determined. The highest consumption was 100% when both DO and pH were controlled, followed by 96.1% when pH was controlled. At controlled DO, 80.2% of the total waste cooking oil was consumed, while the lowest substrate utilization was 73.8% when no pH and DO control were applied (Table 4.3).

**Table 4.3** Maximum production of rhamnolipid and biomass at controlled pH and/or dissolved oxygen conditions

<table>
<thead>
<tr>
<th>Controlled parameter(s)</th>
<th>Rhamnolipid_{max} (g/L)</th>
<th>Biomass_{max} (g/L)</th>
<th>Total substrate consumed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH only</td>
<td>4.86</td>
<td>3.31</td>
<td>96.2%</td>
</tr>
<tr>
<td>DO only</td>
<td>2.99</td>
<td>4.45</td>
<td>80.2%</td>
</tr>
<tr>
<td>DO and pH</td>
<td>2.78</td>
<td>3.78</td>
<td>100%</td>
</tr>
<tr>
<td>No control</td>
<td>2.31</td>
<td>2.42</td>
<td>73.8%</td>
</tr>
</tbody>
</table>

Based on HPLC analysis, the highest composition of free fatty acid in the waste cooking oil was 38% oleic acid and 20% palmitic acid. The range of the two fatty acids obtained in this study were similar to those reported in the literature (Table 2.3). Oleic acid and palmitic acid were also the dominant fatty acids in waste cooking oil as was reported by a previous researcher (Table 2.3). In the batch culture with controlled pH,
91% of oleic acid and 70% of palmitic acid were consumed by the end of fermentation time. However, only 25% of oleic acid and 52% of palmitic acid were consumed in batch culture without controlled of pH or DO.

The leftover nitrogen was measured for cultures with pH control and also for cultures where both pH and DO were not controlled (Figure 4.30). It was shown that the nitrogen was depleted after 24 h of incubation time in the culture with pH control. The cell concentration at pH control started to increase after 12 h of incubation and was almost constant after 24 h of fermentation time. On the other hand, it was observed that rhamnolipid production started at 24 h of incubation and the rhamnolipid concentration increased afterwards until it reached maximum level of 4.86 g/L at 120 h. The sudden drop at 105 h of incubation was considered as outliers which due to sample taken was half of samples taken at 96 h and 120 h of incubation. Thus, was not considered as a trend. However, the cell concentration at no control condition was increasing until 72 h of incubation. Then, the cell concentration was almost constant after 72 h of incubation.

Similarly, the nitrogen was exhausted after 24 h in batch culture with no control condition. The cells started to grow after 6 h of incubation until the concentration was 2.20 g/L at 57 h. After that the growth was almost constant with the maximum concentration of 2.40 g/L was attained. Rhamnolipid was produced after 12 h of incubation and increasing steadily until maximum concentration of 2.31 g/l was achieved at the end of incubation time.
4.2.3 Kinetics of batch culture

Rhamnolipids are secondary metabolites but it is not clear whether this biosurfactant is growth-associated, non-growth associated or mixed-growth associated type of product formation. A typical plot of types of product formation is shown in Figure 2.4. A similar plot was constructed using data from this study for all batch production of rhamnolipid as shown in Figure 4.31a - d. From the plot of biomass or rhamnolipid concentration versus time, two different types of product were displayed by *P. aeruginosa* USM-AR2. One was growth-associated type of product when the
dissolved oxygen was not controlled. But at the condition where the pH was controlled (whether only pH was controlled or both DO and pH were controlled), rhamnolipid production showed a non-growth associated type of product. In order to verify the above observation, a time course profile of specific growth rate and specific product formation rate was plotted (Figure 4.32). The plots were divided into two phases where t₁ is when μₘₐₓ was reached and t₂ is when qₚₜₘₐₓ was reached (Figure 4.32). The specific production rate increased only when the specific growth rate ceased. There was no correlation between qₚ and μ at phase 1 while a negative correlation was shown between qₚ and μ at phase 2 (Figure 4.33). This would suggest that the rhamnolipid production in this study was non-growth associated.

A second peak of qₚ was observed in all the plots. This qₚ value might represent different types of rhamnolipid being produced which are mono or dirhamnolipid. A number of researchers have reported that the dirhamnolipid were the predominant molecule compared to monorhamnolipid (Mata-Sandoval et al., 2001; Müller et al., 2011; Perfumo et al., 2006).
Figure 4.31 Kinetic patterns of growth and product formation in batch fermentation of rhamnolipid at different control schemes (a) pH control (b) pH and DO control (c) DO was control (d) pH and DO was not control
Figure 4.32 Time course profile of specific growth rate and specific product formation rate for batch fermentation of rhamnolipid at different control schemes (a) pH control (b) pH and DO control (c) DO was control (d) pH and DO was not control. $t_1$, when $\mu_{\text{max}}$ was reached and $t_2$, when $q_{p_{\text{max}1}}$ was reached.
Figure 4.33 The relationship between $q_p$ and $\mu$. (a) Cultivation time from $t=0$ to $t=1$ when $\mu_{\text{max}}$ was reached and (b) cultivation time from $t=1$ to $t=2$ when $q_{p_{\text{max}}}$ was reached.
However, the type of rhamnolipid produced was not characterised in this study. This was because rhamnolipid production measurement was based on rhamnose value only. Thus, this finding would require further characterisation on the types of rhamnolipid produced and their distribution. Moreover, it offers an opportunity to strategize for production of targeted rhamnolipid for specific application.

4.3 Fed-batch production of rhamnolipid

It has been shown in previous section 4.2.3 that rhamnolipid is a non-growth-associated type product therefore, to improve rhamnolipid production, the strategy of fed-batch is to limit the cell growth of P. aeruginosa. It is postulated that in fed-batch culture, prolonging the stationary phase may lead to higher rhamnolipid production through controlled feeding of waste cooking oil. Few strategies were implemented in this study to set the optimum feeding rate for fed-batch culture. They were manual feeding, constant feed rate, DO-stat, and automatic maximum substrate uptake rate (MSUR) feeding strategies. The performance of these strategies were compared to determine the best feeding strategy for the improvement of rhamnolipid production and yield. All fed-batch cultures in this study were started as batch cultures. During the batch culture phase, the amount of oil added was 8.2 mL. The fed-batch phase was started as soon as a sharp rise in dissolved oxygen level was detected which indicated that the substrate or the carbon source was completely consumed. In all fed-batch cultures, the pH was controlled at (6.5 ± 0.5).

For the manual feeding, a total amount of 8.03 mL of oil was fed by manually pumping in the oil according to the calculated maximum substrate uptake rate (MSUR). The calculation of MSUR was reviewed in Chapter 3, section 3.7.1. The total amount fed was estimated for 24 h of consumption by the bacteria during the batch
culture phase. However, the substrate was finished within 14 h of feeding time (Figure 4.34).

Since the feed pump was manually controlled, it did not automatically activate to pump in the substrate when it was exhausted. This led to starvation phase for more than 10 h before the next feeding. At the second feeding, the total amount fed was doubled from the calculated MSUR to avoid any starvation phase that might happen. Instead, excess oil was detected 24 h after second feeding. The oil feeding was stopped after the third feeding, and the last feeding was initiated after four days from the third feeding. The maximum rhamnolipid produced from this strategy was 4.78 g/L at 176 h of incubation period (Figure 4.35) while the highest biomass gained was 6.69 g/L at the end of the incubation period.

The subsequent feeding strategy was designed to calculate the MSUR automatically and activated the feeding pump. The automatic MSUR feeding strategy was important to ensure enough carbon source was supplied to the microorganism and at the same time to avoid over-feeding of the substrate. A control sequence (Appendix B) was written for automatic feeding of the substrate when a sharp rise in DO value was detected (Figure 4.37).
Figure 4.34 Profile for manual oil feed feeding strategy
In the control sequence, the feeding pump was set to stop feeding the oil when DO reached 30%. Unfortunately, the DO did not always go down to the pre-set value which was 30% as shown in Figure 4.37, but the value was higher than 30%. In addition, the problem was the control sequence was not able to predict the increase variation of the DO over the fermentation period. After 60 h of incubation, no more reduction in DO level was observed. From 60 h of incubation onwards, the
microorganism stopped growing since the value of $\mu$ reached almost zero, indicating oxygen was not used for respiration and thus, no drop in DO level was recorded. Due to that, it caused the feeding pump to continuously feed the oil which led to excessive addition of the oil into the fermentation culture. Nevertheless, the highest productivity obtained was 0.045 g/L.h with MSUR feeding strategy and the maximum rhamnolipid achieved was 8.57 g/L (Figure 4.36).

The next feeding strategy was a DO-stat strategy. Similarly, a control sequence was written for automatic feeding of the substrate based on a sharp rise in DO value (Appendix B). In DO-stat strategy, no calculation of the substrate uptake rate was included. Instead, the feed pump was set at a certain value to deliver the oil at the required rate, which meant that the substrate was pumped into the fermenter at a constant rate. As compared to the automatic MSUR feeding strategy where the feed pump set point was determined automatically according to the MSUR. Similar problem was observed with DO-stat strategy where the pump continuously pumped in the oil for almost 4 days (Figure 4.38). This led to, about 243 mL of oil accumulated in the fermentation broth. Despite that, the maximum production of rhamnolipid were 8.35 g/L with a productivity of 0.044 g/L.h and the maximum biomass achieved was 4.06 g/L (Figure 4.39).
Figure 4.37 Profile of dissolve oxygen level for automatic MSUR feeding strategy
Figure 4.38 Profile for DO-stat feeding strategy
The previous three feeding strategies were based on indirect feedback from a rise in the DO level. These feeding strategies were compared with feeding strategy without the feedback control where the substrate was fed at a predetermined (constant) rate. The feed rate was set at 1.7 mL/h. Through this strategy, maximum rhamnolipid produced was 6.69 g/L at 168 h of incubation time with a productivity of 0.040 g/L.h. The maximum production achieved was 27.3% higher than the maximum rhamnolipid achieved in batch production at controlled pH (Figure 4.40).

The performance for all fed-batch cultures were compared based on maximum concentration of rhamnolipid produced, yield and productivity (Figure 4.41). In terms of rhamnolipid production, the highest production was shown from MSUR feeding strategy (8.58 g/L) which was 79.9% and 36.2% higher than manual and constant feed rate feeding strategy.
Figure 4.40 Time course profile of rhamnolipid and biomass production from fed-batch culture with constant feed rate feeding strategy

Figure 4.41 Comparison of performance for fed-batch feeding strategies in terms of (a) rhamnolipid and biomass concentrations, $Y_{p/x}$ and (b) Productivity and $Y_{x/s}$
But it was only 0.03% higher than the rhamnolipid produced from DO-stat feeding strategy. The highest yield of product over biomass which was 18.29 g/g was obtained from DO-stat feeding strategy, almost doubled the yield from MSUR feeding strategy.

Meanwhile, the highest yield of biomass over substrate was obtained from manual feed feeding strategy, which was 0.16 (g/g). For other feeding strategies, the yield was in the range of 0.02 to 0.03 (g/g). The productivity for all fed-batch cultures was low and it was in the range of 0.028 to 0.049 g/L.h.

Fed-batch culture was performed to improve the production of rhamnolipid production. In order to see the improvement, a comparison was made between the batch production (with pH control and both pH and DO control) and fed-batch production (with automatic MSUR and DO-stat feeding strategies) since the highest rhamnolipid concentration and yield (Y_p/x) could be obtained from these cultures. Rhamnolipid was improved by 76.4% in fed-batch culture (with MSUR feeding strategy) compared to the batch culture (with control pH). An improvement of 9.0 time of yield was shown in fed-batch culture (with DO-stat feeding strategy) from batch culture (with control pH and DO level). However, the productivity in fed-batch and batch cultures was comparable with each other and it was 0.049 and 0.051 g/L.h in fed-fed and batch cultures respectively (Figure 4.42). Current production in fed-batch culture was comparable with the rhamnolipid production reported in literature. Luo et al., (2013) produced a maximum of 8.5 g/L of rhamnolipid from waste cooking oil with pulse feeding after 72 h of incubation. While the highest production in this study using automatic MSUR feeding strategy was 8.57 g/L.
Figure 4.42 Comparison of batch (control pH and/or DO) and fed-batch (MSUR and DO-stat) performances in the rhamnolipid production

However, the productivity in the previous study was about 1.6-fold higher than the productivity of the current production. Despite that, both automatic MSUR and DO-stat feeding strategies were shown as the potential strategies for rhamnolipid production enhancement in terms of final concentration produced and production yield. In the previous study, the pulse-pause (intermittent) feeding to determine the MSUR and the MSUR-based feeding strategies were performed in two different fermentations (Noh et al., 2014). On the contrary, in this study, the two steps which were the pulse-pause feeding to determine the MSUR and the MSUR-based feeding strategies were possible to be performed in a single fermentation with the assistance of control language sequence using IRIS 6 software from INFORS HT, Switzerland. Meanwhile, DO-stat strategy offers simplicity in terms of control implementation, since no calculation on MSUR is required. Still, similar improvement in rhamnolipid production was achieved.
CHAPTER 5 DISCUSSION

5.1 Shake flask study

Nutrient types and their concentrations in the medium play an important role in the production of primary and secondary metabolites as limited supply of an essential nutrient can restrict the growth of microbial cells or product formation. Generally, carbon and nitrogen sources present in the medium can influence the metabolite production and an optimised production medium is required to maximize the metabolite yield (Singh et al., 2017). Due to the facts, one of the approach in this research was to select the best medium composition that could enhance rhamnolipid production by *P. aeruginosa* USM-AR2 using waste cooking oil as the sole carbon source. Three media were selected based on the highest overall productivity of the rhamnolipid to be used in this study as stated in Section 4.1.1.

Results in Section 4.1.1 indicated that medium A supported the highest rhamnolipid production compared to media B and C. In medium A, the rhamnolipids were produced by consuming the excess carbon source after the cell growth had ceased. The carbon source was in excess since 70% oil was still available in medium A (Figure 4.2). However, in both media B and C, the rhamnolipid production increased for a short period of time (6 h) after the growth ceased in experiment 1 (Figure 4.1). Thereafter, the production was constant starting from 56 h until the end of incubation time and the maximum rhamnolipid produced were 27% ± 1% of the rhamnolipid produced in medium A.

Even though the carbon source in media B and C was also in excess (as shown by leftover oil in Figure 4.2), they did not show increases in rhamnolipid production as displayed by medium A. Medium B consisted of several salts as part of its ingredient, for instance, K$_2$HPO$_4$, KH$_2$PO$_4$, NaCl, KCl and CaCl$_2$ (Table 3.1) which
accounted for 1.26% (w/v) of total medium volume. Whereas, less salts components were available in medium A (0.18% w/v) or medium C (0.05% w/v). It was expected that the presence of various salts in the medium would increase the salinity level of the medium, hence the osmotic potential, and consequently inhibit the rhamnolipid production. It has been shown that the growth of *P. aeruginosa* USM-AR2 was inhibited when more than 0.5% of NaCl was added in the medium (Nur Asshifa, 2009), but the effect on rhamnolipid production was not studied. However, it has been shown that an increase in potassium concentration exerted a negative effect on rhamnolipid production by *P. aeruginosa* MSIC02 (de Sousa *et al.*, 2011). Furthermore, rhamnolipid production was terminated when the medium was treated with 0.5M NaCl during the exponential growth of the microorganism (Bazire *et al.*, 2005). This defect was due to lower expression of genes involved in rhamnolipid synthesis which were the *rhlAB* and *rhlC* (Bazire *et al.*, 2009). Thus medium B was not selected for subsequent studies on the production of rhamnolipid.

Medium C produced more biomass than medium A and B but rhamnolipid production was low. A total of 95% of oil was consumed in medium C, and 90% of it was mainly utilized for bacterial growth within 48 h of incubation as shown in Figure 4.5b and 4.5. Another 5% was used for rhamnolipid production (Figure 4.5a). On the other hand, 10% of the total oil consumed in medium A was utilized for rhamnolipid production. This might explain why rhamnolipid production was higher in medium A as compared to medium C. It was shown in Figure 4.4(a) and (b), there was a drastic drop in rhamnolipid and biomass production at 96 h of incubation time. During the fermentation, heavy foaming occurred. The foam was stable and hardly collapsed. Furthermore, no antifoam was used in this study. It was postulated that, the rhamnolipid and biomass were carried over and concentrated within the foam that lead
to the low rhamnolipid and biomass in broth as shown in Figure 4.4(a) and (b). This was found to be the case in studies by Salleh et al. (2011) where high rhamnolipid concentration was found in the foam compared to the fermentation broth. At 56 h, the bacterial growth in Medium A ceased eventhough oil was still in excess as shown in Figure 4.5. It is suggested that the nitrogen source was limited causing the cell growth to cease. Thus, the cells metabolism is directed to rhamnolipid production instead of bacterial growth (Figure 4.4a, b). This fact is well known for a secondary metabolites where they are produced at stationary phase when the bacterial growth stop.

Another factor that might lead to low rhamnolipid in Medium C was the use of yeast extract as the nitrogen source in this medium. It was shown that yeast extract promoted cell growth but was unfavourable to production of glycolipid biosurfactant (Guerra-Santos et al., 1984). Kim et al., (2006) also observed that when an organic nitrogen source was used, the synthesis of biosurfactant was repressed. Similar results were obtained in this study where cell biomass in medium C was higher than medium A but rhamnolipid production was low (Refer Figure 4.1, 4.3, 4.4 and 4.5). On the contrary, in medium A with NaNO₃ as the nitrogen source produced higher rhamnolipid than medium C even though the cell biomass was low. It has been repeatedly demonstrated that NaNO₃ is the best nitrogen source that support maximum production of rhamnolipids by *P. aeruginosa* (Wu et al., 2008; Kumar et al., 2012, Saikia et al., 2011). Indeed, NO₃⁻ elicit higher rhlAB expression than NH₄⁺ (Soberón-Chávez et al., 2005) and rhlAB is the gene that encodes a rhamnosyltransferase.

Other than carbon and nitrogen sources, the factor that is fundamental for rhamnolipid production is the carbon to nitrogen ratio. The C/N ratio was determined at constant nitrogen condition in order to determine the limited nitrogen source concentration that could support good cells growth but at the same produced
significant rhamnolipid yield. In this case, the limited nitrogen source determined was 5.5 g/L. Then, the constant carbon condition was conducted with the limited nitrogen source determined earlier in order to determine the maximum carbon source for rhamnolipid production. In this research, the best C/N ratio for optimum rhamnolipid production was 18 which was in the range of C/N reported in literature (Ma et al., 2016; Guerra et al., 1984). Nevertheless, (Santos et al., 2002) had reported that optimum C/N ratio was different for different type of carbon sources by the same strain of P. aeruginosa. It is well known that rhamnolipid are typical secondary metabolites where they are synthesized under growth-limiting condition (Walter et al., 2010). Therefore, the production strategy is by limiting one of the growth factors such as nitrogen source but not the carbon source (Muller and Hausmann, 2011). It was shown that during the biosynthesis of rhamnolipid, lipid, not sugar, formation is the rate-determining factor, and nitrogen limitation may promote lipid accumulation (Mulligan and Gibbs, 1989). Therefore, the assimilation of nitrate as a nitrogen source is slower, simulating a nitrogen-limiting condition that is favourable to rhamnolipid production (Prieto et al., 2008).

In this investigation, Tween 80 was added to the medium formulation to increase the accessibility of the immiscible substrate by the cells. However, the medium was not added with rhamnolipid. One way of improving the miscibility of the oily substrate is through emulsification, thus, an emulsification test using rhamnolipid and waste cooking oil had been conducted. Results showed that the waste cooking oil was 50% emulsified when tested with 5 g/L of rhamnolipid and no changes on the emulsification capacity of the waste cooking when more than 5 g/L of rhamnolipid was used in the test. The emulsification capacity of the waste cooking oil was similar when tested with 10% (v/v) of Tween 80. Since the emulsification capacity of these
surfactants were comparable so, Tween 80 was utilised in further investigations. Furthermore, Tween 80 is easily available and required no extra preparation before it can be used in the experiments.

It was demonstrated that the addition of a commercial surfactant may seem to accelerate the cell growth. This was shown by the higher maximum specific growth rate in the medium with commercial surfactant by 44.5% than the higher maximum specific growth rate in the medium without commercial surfactant. However, it did not affect the overall rhamnolipid production as much. According to Soberon-Chavez et al., (2008), one of the putative physiological functions of rhamnolipid is solubilisation and uptake of hydrophobic substrates due to the interfacial activity. This means that the bacteria produced the biosurfactants to increase their own access to the oily substrate by enhancing its dispersion into the medium. At the early stage of rhamnolipid production in the medium with surfactant, i.e. between 24 h of incubation, the commercial surfactant was useful for solubilisation of oily substrate for ease of uptake, hence improving the growth and production rates. Thus, along the incubation period when more rhamnolipid were being produced, the oil was solubilised by this biosurfactant for uptake of the substrate. An odd trend was observed in Figure 4.12b where the specific productivity dropped 43% at 32 h of incubation from 0.007 g/g.h at 24 h of incubation in the medium added with Tween 80. It was shown that the production of rhamnolipid was very low during the first 32 h of incubation which affected the accuracy of rhamnolipid measurement. Furthermore, the increase in rhamnolipid from 24 h to 32 h of incubation was 1.5 fold which was less than the increase in cell growth. The cells grew 2 fold within the same period. These scenario might lead to the sudden drop of productivity in the medium supplemented with Tween 80.
Similarly, in the medium without external surfactant the uptake of oily substrate by the microorganism could happen through directly adhering to hydrocarbon droplets (Noordman and Janssen, 2002) during the first 24 h of incubation. In a later hours of the fermentation, the mechanism was via pseudosolubilisation when enough rhamnolipid was produced to solubilise the oily substrate into a smaller droplets (Hua and Wang, 2012). It has been reported previously that these two mechanisms occurred simultaneously during the uptake of alkane by *P. aeruginosa* (Beal and Betts, 2000). It was also reported that *P. aeruginosa* ATCC 9027 and ATCC 27853 were able to grow on hexadecane by adhering to the hydrocarbon droplets. However, the growth rate on hexadecane by the bacteria were lower without addition than with addition of biosurfactant (Al-Tahhan *et al.*, 2000). The adherence of the microorganism to the hydrocarbon was measured in terms of cell surface hydrophobicity (Rosenberg, 1984). Adhesion is particularly important when the hydrocarbons are not emulsified, giving limited interfacial area between the two liquid phases (Abbasnezhad *et al.*, 2011).

*P. aeruginosa* is known to produce lipase, an enzyme which catalyses the hydrolysis of triglycerides to glycerol and free fatty acids over an oil–water interface (Treichel *et al.*, 2010). Thus, once the immiscible substrate which is the waste cooking oil is available for uptake by the cells through direct adherence, it was then metabolised to glycerol and free fatty acids.

Hence, the addition of commercial surfactant was able to accelerate the cell growth by increasing the accessibility of the oil to the cells but not necessarily improve the product yield.
5.2 Batch culture

5.2.1 Oxygen transfer behaviour

In aerobic fermentations, the oxygen transfer which is characterized by the \( k_{L,a} \) is an important parameter to be examined. The rate of oxygen transfer (OTR) from gas to liquid interface is a function of physicochemical properties of the culture medium, the geometrical parameters of the bioreactor, and presence of cells (Garcia-Ochoa et al., 2010). The oxygen supply to the cells in a stirred tank bioreactor is achieved through aeration. Then, agitation disperses the oxygen bubbles and promotes mass transfer of the gas bubbles through the gas-liquid (cell culture medium) interface. Therefore, when the agitation speed increased, the \( k_{L,a} \) increased. The gas bubbles entered the bioreactor through aeration were dispersed into small bubbles which caused a reduction in bubbles diameter and increased surface area. Thus there is a greater opportunity for oxygen to transfer into the cell culture medium and the \( k_{L,a} \) also improved. However, the \( k_{L,a} \) no longer increased at agitation speed above 500 rpm. At higher stirrer speeds the impeller becomes loaded and gas is distributed throughout the upper part of the bioreactor vessel above the stirrer. Further increases in stirrer speed allow complete dispersion of bubbles below as well as above the impeller. According to the results obtained, a complete dispersion might have been achieved at 500 rpm. Thus increasing agitation speed with constant aeration rate did not further increase the \( k_{L,a} \) since complete dispersion had been achieved. Similarly when the aeration was increased, the \( k_{L,a} \) also increased. Increasing the aeration rate means more oxygen was supplied to a bioreactor and more oxygen molecules was available manifesting in increased \( k_{L,a} \).

According to Kawase and Moo-Young (1990), the overall change in \( k_{L,a} \) due to the addition of surface-active materials depended on the relative magnitudes of their
effects on the liquid phase mass transfer coefficient ($k_L$) and specific surface area ($a$). The relative balance between the changes in $k_L$ and $a$ determines whether $k_La$ will increase or decrease. In the case of surfactants, usually the increase in $a$ overcomes the decrease in $k_L$ and as a result, the $k_La$ product increases.

The biosurfactant may form a thin layer on the bubbles surface, reducing the tendency to coalesce thus forming small bubbles (Cents et al., 2005). Consequently, the increase interfacial area would provide more area for oxygen transfer and leading to enhanced the $k_La$ (Jia et al., 2015). The appropriate gas dispersion was furnished by the agitation since the calculated $F_g$ (0.0037 m$^3$min$^{-1}$) was higher than the operating flow rate, $Q$ (0.0005 m$^3$min$^{-1}$) employed in this study. Furthermore, the resistance to molecular diffusion decreased due to a reduction in the thickness of the stagnant fluid layer surrounding the bubble (Clarke and Correia, 2008), which could also enhance the $k_La$.

After 1.0 g/L of rhamnolipid concentration was reached, the $k_La$ started to decrease. One of the possible explanations that led to this situation was the increase in liquid viscosity due to higher fraction of biosurfactant solution (66.7% v/v) available in the aqueous solution since the biosurfactant utilized in this study was in the form of cell-free broth. It has been demonstrated that the viscosity decreased the gas hold-up through enhanced coalescence and reduced turbulence in viscous fluids, which led to the formation of larger bubbles (Correia et al., 2010). The larger bubbles passed more quickly through the fluid with higher bubble rise velocities, resulting in a decreased gas hold up. Consequently, it was likely that at higher biosurfactant concentrations, viscosity would decrease the interfacial area through its impact on the gas hold up, ultimately decreasing $k_La$. Moreover, oil droplets might accumulate at the interface as
the concentrations were increased that led to an increased in the interfacial and liquid film resistance and thus, the $k_{La}$ decreased (Clarke et al., 2006).

The behaviour of $k_{La}$ showed in this study due to waste cooking oil addition was determined as type 3 behaviour where neither increase nor decrease in $k_{La}$ was observed (Clarke and Correia, 2008). This trend was not observed when olive oil was used as a second liquid phase to promote the oxygen transfer rate. It was found that olive oil reduced the $k_{La}$ at all the concentrations studied (0.0 - 0.25% volume fraction) (Amaral et al., 2008). Similarly, the observation made by Suhaila et al., (2010) indicated that the $k_{La}$ decreased when 5% (v/v) of palm oil was added into the medium which in this case was water.

In this study, the operating conditions for rhamnolipid production in bench-top bioreactor were set at 0.3 vvm aeration rate and 400 rpm agitation speed. During the fermentation, the cells, substrate and product concentrations as well as the medium properties such as viscosity and coalescence properties changed. These changes affected the $k_{La}$ along the fermentation time as shown in Figure 4.17. It was expected that the increase in rhamnolipid and biomass concentrations led to decrease in OTR. This could be due to changes in medium viscosity that caused the gas bubbles coalesce and become larger bubbles, consequently reduce the interfacial area and thus reduce the $k_{La}$. Furthermore, it was shown in this study that rhamnolipid did affect the $k_{La}$ where the $k_{La}$ decreased when rhamnolipid concentration reached 1.0 g/L and above (Figure 4.15).

Nevertheless, the OTR in this study was above the OUR throughout the fermentation time which means that the oxygen was not limiting and sufficient for the bacteria to grow.
5.2.2 Effect of operational conditions on rhamnolipid production

The effect of agitation speeds at 400 rpm (1.13 m/s tip speed) and 500 rpm (1.41 m/s) on rhamnolipid production in a batch culture were studied. It was shown that biomass production was higher at low tip speed as compared to a high tip speed. The low biomass production at high tip speed could be due to cell damage caused by higher shear rate at a higher impeller speed. According to Metzner-Otto equation, average shear rate around the impeller region is proportional to the impeller speed that is $\gamma \sim KN$ where $K = 12$ for Rushton type impeller (Hemrajani and Tatterson, 2004). Thus, the shear rate at 400 rpm ($6.67 \text{ s}^{-1}$) was $80 \text{ s}^{-1}$ and at 500 rpm ($8.33 \text{ s}^{-1}$) was $100 \text{ s}^{-1}$.

Furthermore, *P. aeruginosa* is a Gram negative bacteria where the cell wall is thinner compared to Gram positive bacteria (Madigan et al., 2009). This condition might cause cell damaged at higher shear rate and thus lower rhamnolipids were produced at 500 rpm as compared to at 400 rpm.

From the DO and stirring rate profile (Figure 4.21b and c), it was noticed that the agitation speed at controlled DO and pH was higher than agitation speed at controlled DO only. The increase agitation indirectly indicated that low DO level was detected in the fermentation medium which meant that oxygen had been consumed to metabolise the substrate. Comparing the leftover oil in Figure 4.23 and 4.24, it did show that more oil was consumed at controlled DO and pH (Figure 4.24) than at controlled DO only (Figure 4.23). During the intense agitation, about 49.7% of oil was consumed at controlled DO and pH whereas at controlled DO only 27.5% of oil was consumed. Thus, it signifies the vigorous agitation showed at controlled DO and pH as compared to controlled DO only.
From the time course profile, it was observed that the batch production of rhamnolipid with DO control and when DO and pH were not controlled, the rhamnolipid production did not necessarily start at the onset of stationary phase. Instead, the production happened at the deceleration phase (Figures 4.23, 4.25). In contrast, when pH or both DO and pH were controlled, the production was initiated at the onset of stationary phase and this fact is well known as stated in literature elsewhere (Figures 4.22, 4.24) (Leitermann et al., 2010; Walter et al., 2010). Furthermore, results in this study have shown that, rhamnolipid production was initiated by the limitation in nitrogen source (Figure 4.30) and not necessarily coincided with the onset of stationary phase. When the nitrogen was limited, the cell metabolism was switched from nitrogen to carbon source resulting in rhamnolipid production (Mulligan and Gibbs, 1989). In contrast with the result obtained in this study, Müller et al., (2011) reported that the rhamnolipid synthesis was already induced before nitrogen was depleted and still in excess. Previous research also showed that the nitrogen source depletion did not enhance the rhamnolipid (Benincasa et al., 2002). In fact, Soberon-Chavez et al., (2005) stated that nitrogen-limiting conditions did not essentially favour rhamnolipid production, but production started with the exhaustion of nitrogen. Indeed, in this study the rhamnolipid production increased at the late stationary phase. This is in the agreement with the fact that rhamnolipid synthesis is regulated by the quorum sensing system through one of its autoinducers that is Pseudomonas quinolone signal (PQS) (Lovaglio et al., 2015). It was reported that PQS was produced at maximum level when the culture reached the late stationary growth phase (McKnight et al., 2000) with a similar profile to the rhamnolipid biosynthesis (Reis et al., 2011). Soares Dos Santos et al., (2016) demonstrated that the increase in rhamnolipid production was related to the autoinducers that were naturally secreted at a later
stationary phase. The rhamnolipid production was improved by 64% when 5% (v/v) of spent broth containing the endogenous autoinducers was added at the start of new *P. aeruginosa* cultures.

In the culture where pH was controlled, it was observed that foaming was less than that in the culture without pH control. Unfortunately, no quantification was done to show that foam was less in the pH-controlled culture. This was noted through observation records of the culture from time to time throughout the incubation period. Foaming has been recognised as one of the major problems in rhamnolipid production (Heyd *et al.*, 2011). Heavy foaming would cause the medium nutrition and cells to be carried away with the foam and eventually overflowed, thus led to less production of rhamnolipid (Salwa *et al.*, 2009). Even though in this study the foam was recycled into the bioreactor through a recycler attached to it, the foam did not collapse completely. So, the carried over nutrient and cells were suspended in the foam and the cells were inactive for the rhamnolipid production compared to when they were in the broth. Thus, it led to lower rhamnolipid production in DO controlled culture than in pH controlled culture.

Batch production of rhamnolipid in this study showed a different growth and production behaviours respective to types of control being applied that were pH and/or DO control. Thus, the kinetics of batch culture was estimated to elucidate the rhamnolipid production characteristics further. Understanding of the production behaviour is crucial to determine suitable strategy for enhancement of rhamnolipid production.
5.2.3 Kinetics study

The kinetics of rhamnolipid production in batch culture were carried out to determine whether rhamnolipid are growth-associated, non-growth associated or mixed-growth associated types of product. In this study, it was indicated that rhamnolipid produced by *P. aeruginosa* USM-AR2 was a non-growth associated type of product when grew on waste cooking oil as the sole carbon source. The formation of this product took place during the stationary phase when the growth rate is zero. Sana *et al.*, (2017) had determined that rhamnolipid was a growth-associated products when *P. aeruginosa* C2 grew on a combination of unprocessed waste of *Catla catla* fish fat and glucose. However, it had been shown earlier that the rhamnolipid was a non-growth-associated product when *P. aeruginosa* BS2 was grown on whey waste but the rhamnolipid was growth-associated when the same bacteria was grown on distillery waste and synthetic media (Sudhakar Babu *et al.*, 1996). Chen *et al.*, 2007 also claimed that the rhamnolipid produced by *P. aeruginosa* S2 was a non-growth-associated. Nevertheless, the claims from both researchers were not supported by kinetic analyses.

Analyses of the data using the Luedeking-Piret equation \( q_p = \alpha \mu + \beta \) which shows a relationship between specific product formation rate, \( q_p \), and specific growth rate, \( \mu \), can provide a better understanding whether the product formation was growth- or non-growth associated. The product is considered as growth associated if \( \beta = 0 \) so that \( q_p = \alpha \mu \) which mean the specific product formation rate has a positive relationship with growth (specific growth rate). If the product is non-growth-associated, the value of \( \alpha = 0 \) so that \( q_p = \beta \) which mean specific product formation rate is not related to growth. Meanwhile, the product is mixed-growth-associated if both \( \alpha \) and \( \beta \) are not zero. In this study, it was clearly shown in Figure 4.33(a) that the plot of \( q_p \) versus \( \mu \)
was at 0 value and $q_p$ had a negative relationship with $\mu$ in Figure 4.33(b). Thus, it was considered that the rhamnolipid produced in this study was non-growth associated since $q_p$ was not related to specific growth rate ($\alpha = 0$).

This correlated with findings that rhamnolipid are secondary metabolites where they were not essential for the growth of the producing cultures but served diverse survival functions in nature (Ruiz et al., 2010). In this case, rhamnolipids might involve in cell motility and biofilm formation (Chrzanowski et al., 2012). It is suggested that rhamnolipid produced particularly by the *P. aeruginosa* USM-AR2 might not involve in the uptake of the immiscible substrate at the early stage of bacterial growth. However, the substrate availability for uptake by *P. aeruginosa* could be through directly adhere to the oil droplets by changing the microbial cell surface hydrophobicity (as discussed in section 5.1). Furthermore, it is well known that *P. aeruginosa* is one of the microorganisms that are able to grow and transform vegetable oils due to the excretion of lipase, provided that the lipid substrates are available to cells (Haba et al., 2000; Hasanuzzaman et al., 2004). Lipase is an extracellular enzyme that catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids (Sharma et al., 2001).

The findings in this study suggested that rhamnolipid produced in this research, particularly by *P. aeruginosa* USM-AR2 with waste cooking oil as a sole carbon source, was a non-growth-associated product.

### 5.3 Fed-batch production of rhamnolipid

Based on kinetics study, it was suggested that rhamnolipid is a non-growth-associated product, thus the best strategy to enhance the production was through fed-batch culture. The idea of fed-batch is to maintain a growth rate at almost zero so
product formation is favoured instead of bacterial growth. The carbon source which is the waste cooking oil is immiscible with aqueous phase thus it has to be supplied in limited amounts. Subsequently the oil was dispersed by agitation hence increasing substrate availability to cells (Nurashifa et al., 2017). However, over feeding of the oil will cause it to float above the aqueous phase and make it unavailable for substrate uptake. It is therefore important to determine the best feeding strategy that could supply enough oil for consumption and avoid excess feeding. Four feeding strategies had been tested for rhamnolipid production: manual feeding; DO-stat feeding strategy; MSUR feeding strategy and constant rate feeding strategy. Fed-batch was started as batch-mode and the feeding of carbon source began when the DO began to rise. Oxygen was consumed during carbon metabolism that causing a decrease in DO level. The DO would rise when the carbon source was depleted which meant oxygen was not used for aerobic carbon metabolism (Noh et al., 2014).

In the first feeding strategy, the oil was fed manually and the amount fed was based on the maximum substrate uptake rate for the duration of 24 h before the second feeding. However, the amount fed finished within 14 h after the first feeding showing that the consumption rate was high and led to starvation phase for almost 10 h. To overcome the problem, an automatic feeding strategy based on DO level was deployed. The automatic feeding strategies were MSUR feeding strategy and DO-stat feeding strategy. Even though both strategies were automatic, but the difference was in terms of the amount of oil fed every time the pump was triggered for feeding. In MSUR feeding strategy, the oil fed was estimated from the consumption rate of the bacteria and in DO-stat, the oil was fed at a constant rate. However both strategies suffered the same problem where after two (for DO-stat strategy) or three days (MSUR strategy) of incubation, no drop in the DO level was observed which indicated that the oxygen
might have not been used to metabolise the substrate. The viability of the cells might be affected due to nitrogen source limitation since in both strategies oil was the only substrate fed into the medium. An experiment was attempted by feeding both the oil and the nitrogen source which is NaNO₃, but the strategy did not improve either the rhamnolipid or the cells production. However, the result of this attempt was not reported in detail in this dissertation.

The oil was continuously pumped into the fermentation medium since the pump will only stop when the DO level dropped below 30%. Excessive oil in the fermentation medium was detrimental to the production of rhamnolipid because it would affect the enzymes involved in rhamnolipid synthesis, particularly rhamnosyltransferase A and rhamnosyltransferase B. These enzymes catalyse the formation of the rhamnolipid precursor, 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA) and the assembly of a mono-rhamnolipid molecule (Henkel, 2014).

For a successful implementation of MSUR feeding strategy, it requires that the added substrate must be completely consumed (Oh et al., 1998) and the substrate exhaustion is detected through the rise of DO level. The complete consumption is possible if the substrate used is water-soluble such as glucose, but in the case of immiscible substrate, there is still oil remaining in the medium even though the DO level rose. Thus, to improve the production, it is suggested that the strategy can be combined with other conventional strategy such as repeated fed-batch which mean when the DO stop to respond, in this study after 3 days of incubation, the fermentation medium could be withdrawn and replaced with a fresh medium. The hypothesis is that, the new and fresh medium may restore the viability of the cells and at the same time it could reduce the inhibition effect by excessive oil or other toxic byproducts (He et al.,
2017). Consequently the cells may respire which will be indicated by a drop in DO level. The MSUR works effectively when the DO level responded well.

The highest rhamnolipid obtained through MSUR feeding strategy was 8.54 g/L and the method used to measure rhamnolipid in this study was the orcinol method. This method measures the sugar moiety of the biosurfactant which is rhamnose and the value is usually multiplied by a factor of 3.4 (Gong et al., 2015), or 3 (Cheng et al., 2017; Marchant and Banat, 2014). Various techniques have been implemented in measuring the rhamnolipid yield, thus it is suggested that the yields of biosurfactant from different fermentation systems cannot be directly compared and that great care should be exercised in evaluating claims for high production yields (Marchant and Banat, 2014; Irorere et al., 2017). On top of that, the yield of rhamnolipid achieved in this research was comparable with the yield reported by Luo et al. (2013). The final rhamnolipid production was 8.5 g/L (measured as rhamnose equivalent) with pulse feeding strategy using waste cooking oil as the carbon source (Luo et al., 2013).

Therefore, it appears that MSUR feeding strategy is possible to be implemented with immiscible substrate and could be promising strategy for improvement of rhamnolipid production.
CHAPTER 6 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Microbial production of rhamnolipid has been widely investigated and its potential as the next generation of surfactants has been acknowledged. However, their production at commercial scale is still limited. The focus of this research is to enhance rhamnolipid production by *P. aeruginosa* USM-AR2 using waste cooking oil as a sole carbon source. Due to that, several objectives were to be met.

The first objective was to evaluate and select different media formulation from literature. It was shown that rhamnolipid production was significantly high in medium A containing NaNO₃ as the nitrogen source compared to the production in medium C which contained yeast extract as the nitrogen source and waste cooking oil as the carbon source. Other than that, the C/N ratio of 18 significantly improved the rhamnolipid production compared to C/N ratios of 9 and 27. However, addition of commercial emulsifier such as Tween 80 had no effect in rhamnolipid production. Thus, medium A with the following compositions: 5.5 g/L of NaNO₃, 0.5 g/L MgSO₄·7H₂O, 1.0 g/L KCl, 0.3 g/L K₂HPO₄, 1.0 mL Trace elements and 18.4 g/L WCO with C/N equivalent to 18 was utilised throughout the study.

The second objective is to determine the effect of operational conditions on rhamnolipid production and the behaviour of oxygen transfer in batch culture. The results showed that rhamnolipid production at the lower tip speed, which corresponded to 400 rpm agitation, was 1.5 times higher than rhamnolipid production at higher tip speed (500 rpm). The maximum rhamnolipid concentration produced was 4.86 g/L when pH of the culture was controlled. The agitation speed of 400 rpm and aeration rate of 0.3 vvm were chosen as the operational conditions of the bioreactor since adequate oxygen transfer could be provided to the fermentation system. In addition, it
was shown that the waste cooking oil had no impact on $k_{La}$ within the range of concentrations tested. The $k_{La}$ increased with the increase of rhamnolipid concentration up to 1.0 g/L but further increase in rhamnolipid concentration caused the $k_{La}$ to decrease. However, the $k_{La}$ behaviour demonstrated in this study was examined within 0.0 to 2.0 g/L of rhamnolipid only.

The third objective was to analyse the kinetics of rhamnolipid production in batch culture. It was found that rhamnolipid was a non-growth-associated type of product. The kinetic properties were consistent for all batch cultures under different control schemes.

The final objective was to identify the best feeding strategy to improve rhamnolipid production in fed-batch culture. Two feeding strategies namely, the automatic MSUR feeding and DO-stat feeding strategies, were comparable in their performances to increase rhamnolipid production. Despite that, the automatic MSUR feeding strategy showed its potential as an effective technique for enhancing the rhamnolipid production.

Finally, it is important to highlight that the rhamnolipid production was enhanced by 76.4% in fed-batch culture compared to the production in batch culture. Thus, these results suggested that rhamnolipid production was enhanced through improvement of medium formulation coupled with the use of cheaper substrate and effective bioprocessing strategy employed in this study.

6.2 Recommendations

Results of this study suggested that the strategies used improved rhamnolipid production. But still some limitations are important to be acknowledged and
recommendations for improvement are highlighted. The limitations to be taken into account are:

i. Increase variation of dissolved oxygen level that failed to be detected by the control sequence which led to excessive addition of oil.

ii. No reduction in DO level after 60 h of incubation caused excessive addition of oil. This was because the control sequence worked based on the feedback by DO level.

The $k_{la}$ was examined in batch culture but not in fed-batch culture. In fed-batch culture, the rhamnolipid produced and total concentration of waste cooking oil utilised was higher than the concentration range used to study the effect of these two surface active materials in batch culture. So, it suggested to study the oxygen transfer in such a system to confirm that no limitation caused by the oxygen transfer might affect the rhamnolipid production.

The kinetic parameters such as product yield reported in this study was based on apparent yield which was not a real constant value. So, it is proposed to study these kinetic parameters through a continuous culture where a true yield could be obtained. It is also suggested that the effect of substrate on specific growth rate of the microorganism based on the Monod equation to be determined as well as the true biomass yield to be estimated to ensure that the maximum cell growth could be obtained. In this study, rhamnolipid production was found to be a nongrowth-associated type of product which means they depend on high cell concentration for maximum production. Therefore, if high biomass could be maintained in this strategy, the yield of rhamnolipid could be enhanced as well.
The limitation in fed-batch culture was highlighted in section 6.1. It is
recommended that other means of substrate utilisation estimation to be employed to
improve the automatic MSUR feeding technique. For instance, exit gas composition
in real time and estimate the oxygen uptake rate are to be measured. The amount of
substrate consumed could be estimated online using cumulative oxygen consumption
and the stoichiometric relation between substrate and oxygen consumption.


He, Ni, Tingyu Wu, Jingjing Jiang, Xuwei Long, Bing Shao, Qin Meng. Toward high-efficiency production of biosurfactant rhamnolipid using sequential fed-batch
fermentation based on a fill-and-draw strategy, Colloids and Surfaces B: Biointerfaces http://dx.doi.org/10.1016/j.colsurfb.2017.06.007


Irorere, V. U., Tripathi, L., Marchant, R., McClean, S., Banat, I. M. 2017. Microbial rhamnolipid production: a critical re-evaluation of published data and


APPENDICES

A. STANDARD CURVES

Figure A.1 Standard curve for cell dry weight

Figure A.2 Standard curve for rhamnolipid concentration
B. CONTROL SEQUENCE FOR FED-BATCH CULTURE

#0, Waiting
IF(Elapsed > time(20:00)) {Seq = 1}
#1, ActiveFeed, 10
IF(pO2.v > 50) {Feed.sp = 2.3; Seq = 2}
IF(Elapsed < time(25:00) AND pO2.v < 30) {Seq = 1}
#2, TotalFeed
VAR1=seq_time
Feed_Total.v=((VAR1/60)*1.9614*(Feed.sp/100))
Feed_Total.vv=Feed_Total.v
IF(Seq_time > Time(01:00)) {Seq = 3}
IF(pO2.v < 40 OR pO2.v < 30) {Seq = 4}
#3, TestPhase, 10
Feed.sp=0
IF(pO2.v > 50) {Seq=3}
IF(pO2.v < 40 OR pO2.v < 30) {Seq = 4}
#4, SubstrateConsumptionRate, 60
Feed.sp=0
IF(pO2.v < 40 OR pO2.v < 30) {Seq=4}
Counter.v = Counter.v + 1
FlowRate.v = (Feed_Total.v / Counter.v)
IF(pO2.v > 50) {Seq=5}
#5, CalculatedFlow, 10
DEF maxFlow=198
Feed.sp = ((FlowRate.v^100)/(maxFlow/60))
//if(Feed.sp<1) {Feed.sp = 2.3}
if(Feed.sp>100) {Feed.sp=2.3}
Seq = 6
#6, WaitForReset, 20
Counter.v = 0
Feed_Total.v = 0
Seq = 2

Figure B.1 Sequence for automatic MSUR feeding strategy

#0, Control pO2
if(pO2.v > 50.0) {feed_pump.sp=2.34; Seq=1}
if(pO2.v < 45.0) {feed_pump.sp=0; Seq=1}
#1, waiting
if(seq_time > 120) {seq=0}

Figure B.2 Sequence for DO-stat feeding strategy

#0, Control pO2
if((seq_time > 3600 AND pO2.v > 10.0) {feed_pump.sp=1.67}
if((seq_time > 28800) {feed_pump.sp = 1.67}

Figure B.3 Sequence for constant feed rate feeding strategy
LIST OF PUBLICATIONS


