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

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

**by**

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## LIST OF SYMBOLS

| | |
|---|---|
| $a$ | specific surface area |
| $\alpha$, $\beta$ | Luedeking-Piret constant |
| $C_{crit}$ | Critical oxygen level |
| $C_L$ | Oxygen concentration in liquid phase |
| $C^*$ | Saturated dissolve oxygen |
| $\mu$ | Specific growth rate ($h^{-1}$) |
| $\mu_{max}$ | Maximum specific growth rate ($h^{-1}$) |
| $q_p$ | Specific product synthesis rate (g/g.h) |
| $q_{pmax}$ | Maximum specific product synthesis rate (g/g.h) |
| $q_s$ | Specific rate of substrate utilization ($h^{-1}$) |
| $q_{O_2}$ | Specific oxygen uptake rate (mg/g.h) |
| $Y_{P/x}$ | Yield of product over biomass (g/g) |
| $Y_{P/S}$ | Yield of product over substrate (g/g) |
| $Y_{P/N}$ | Yield of product over nitrogen (g/g) |
| $Y_{x/S}$ | Yield of biomass over substrate (g/g) |
| $k_e$ | maintenance coefficient ($h^{-1}$) |
| $k_La$ | volumetric mass transfer rate ($h^{-1}$) |
| $k_L$ | liquid phase mass transfer coefficient |
| $F_g$ | Calculated air flow rate |
| $x$ | cell concentration |
| $x_t$ | cell concentration at time t |
| $x_0$ | initial cell concentration |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| CDW | Cell dry weight |
| $CDW_{max}$ | Maximum cell dry weight |
| CER | Carbon dioxide evolution rate |
| CMC | Critical micelle concentration |
| C/N | Carbon to nitrogen ratio |
| CS | Carbon source |
| DO | Dissolved oxygen |
| $EC_{50}$ | Half maximal effective concentartion |
| MSUR | Maximum substrate uptake rate |
| OUR | Oxygen uptake rate |
| OTR | Oxygen transfer rate |
| RL | Rhamnolipid |
| $RL_{max}$ | Maximum rhamnolipid |
| *rhl* | Rhamnolipid-related gene |
| rpm | rotation per min |
| vvm | volume per volume per min |
| WCO | Waste cooking oil |

**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: $NO_3^-$, $Mg^+$, $K^+$, $PO_4^{3-}$, unsur surih dan minyak masak terpakai dengan nisbah C/N bersamaan dengan 18. Penambahan 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: $NO_3^-$, $Mg^+$, $K^+$, $PO_4^{3-}$, 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 $k_La$. The $k_La$ 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 Science$^{SM}$ 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 Asshifa *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.

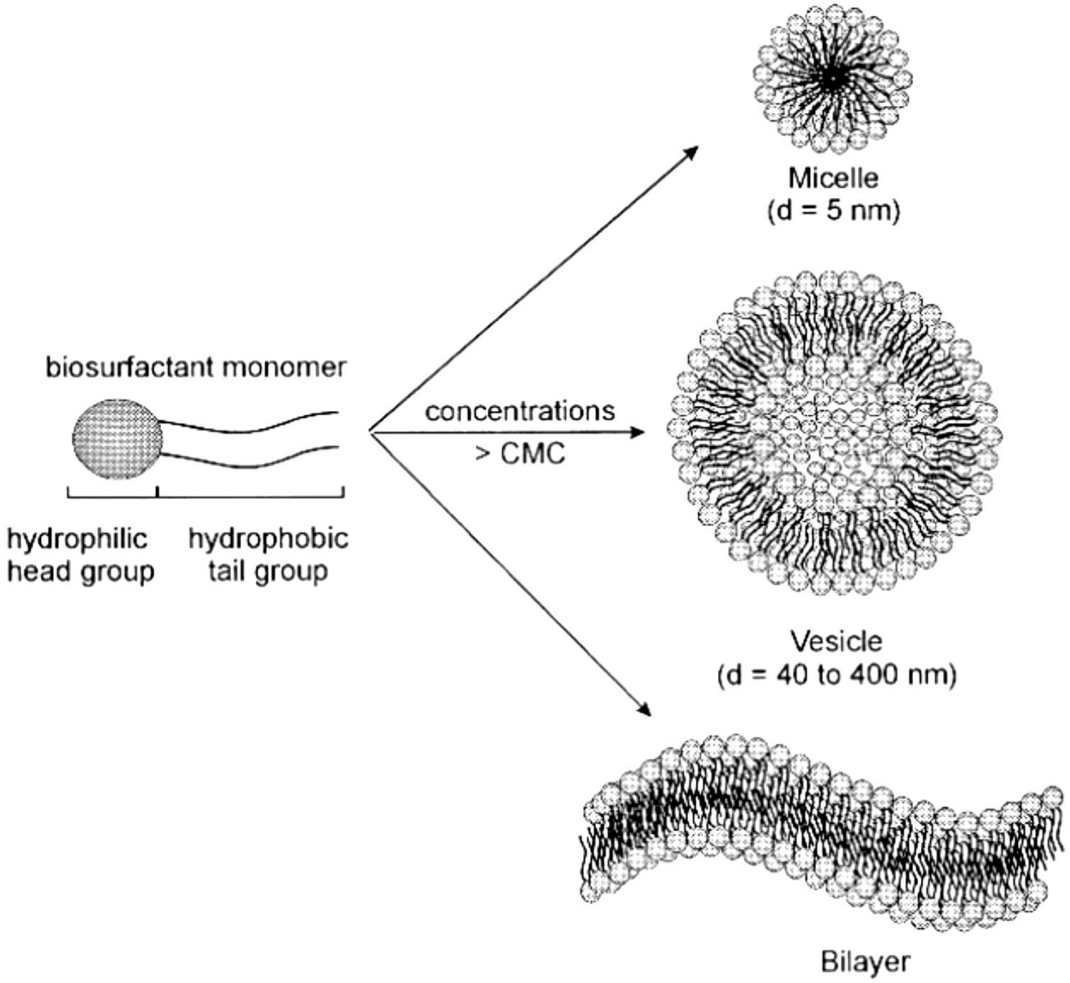


**Figure 2.1** Formation of biosurfactant structures at a concentration above the critical micelle concentration (Herman and Maier, 2002)

### 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.

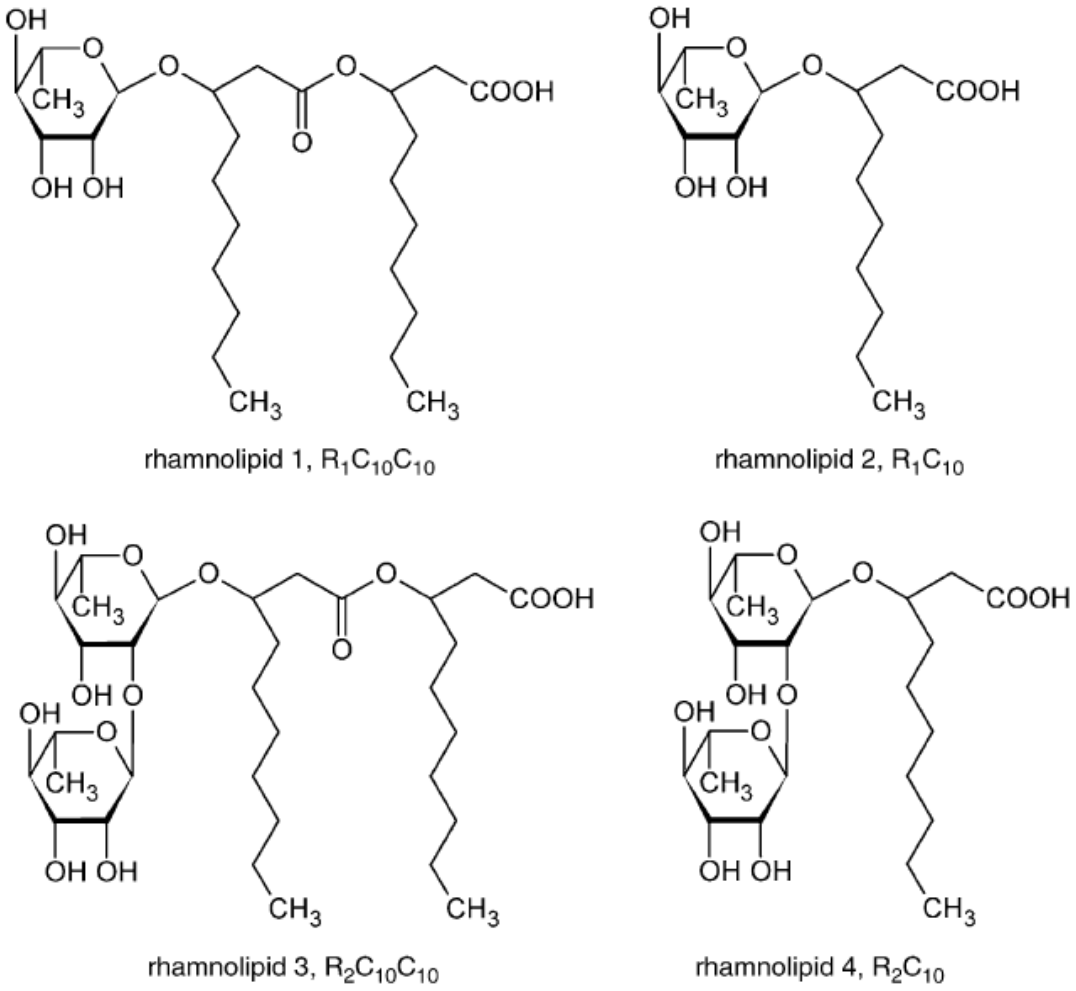


**Figure 2.2** Molecular structure of four different types of rhamnolipids (Leitermann *et al.*, 2010)

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 $3.96\times10^{5}$ mol/L for RL1 and $3.07\times10^{5}$ mol/L for RL3. The CMC of sodium dodecyl sulphate is much higher, i.e. $8.39\times10^{3}$ 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., $NaOCH_3$–) 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)

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

## 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)

| Strain | Surface tension (mN/m) | CMC (mg/L) | Rhamnolipids (g/L) |
|---|---|---|---|
| *P. putida* | 31.2 | 91 | 4.1 |
| *P.alcaligenes* | 28 | 30 | 2.3 |
| *P.fluorescens* | 35 | 20 | 2.0 |
| *P.chlororaphis* | 25-30 | n.d. | 1.0 |
| *P.stutzeri* | n.d. | n.d. | 0.5 |
| *P.luteola* | n.d. | n.d. | 0.38 |
| *P.aeruginosa*[a] | 27.9 | 9 | 12.5 |
| *P.aeruginosa* | 27.3 | 13.9 | 3.9 |
| *P.aeruginosa*[b] | 28.3 | 46.8 | 46 |

n.d. not determined, CMC Critical micelle concentration
[a] Mutant strain, [b] Solid state fermentation

A non-*Pseudomonas* species such as *Burkholderia plantarii* DSM 9509$^T$ 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$^T$ was also reported as a natural rhamnolipid producer. It was identified that *B. kururiensis* KP23$^T$ 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.

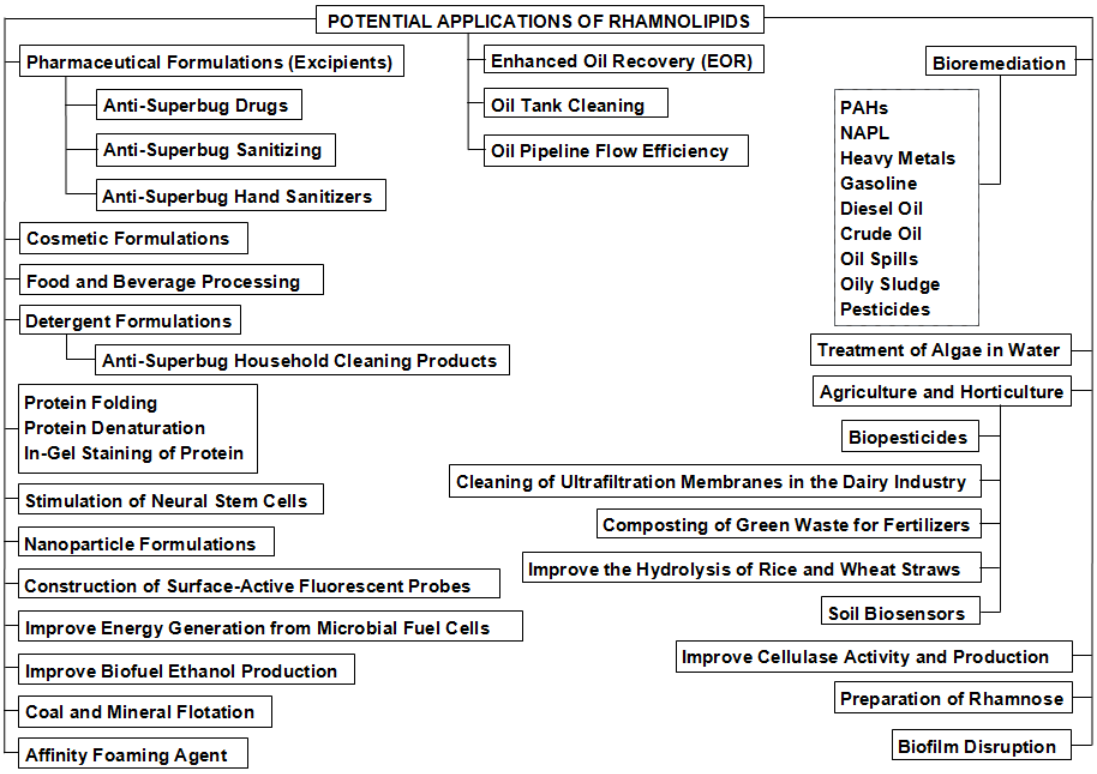


**Figure 2.3** Some of the potential application of rhamnolipid in various industries produced (www.agaetech.com)

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 2.3** Range of fatty acids in waste and fresh cooking oils

| | Range of fatty acids (%) | | | | | Reference |
|---|---|---|---|---|---|---|
| | Myristic | Palmitic | Stearic | Oleic | Linoleic | |
| Waste cooking oil | 0.8 – 3.21 | 21.47 – 39.0 | 4.5 – 13.0 | 28.64 – 44.6 | 10.1 – 13.58 | (Muhamad Ghazali *et al.*,2014; Taufiqurrahmi *et al.*, 2011; Chuah *et al.*, 2016; Lam *et al.*, 2016) |
| Fresh cooking oil | 0.7 – 1.0 | 36.7 – 39.4 | 3.6 – 4.4 | 43.6 – 45.3 | 10.8 – 12.1 | MPOB* |

* 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)

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

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

| Biosurfactant | Producer | Maximum Production (g/L) | References |
|---|---|---|---|
| Extracellular glycolipids | *Rhodococcus erythropolis* 16 LM.USTHB | n.a | Sadouk *et al.*, 2008 |
| Surfactin | *Bacillus subtilis MTCC 2423* | 0.45 | Vedaraman and Venkatesh, 2011 |
| Sophorolipid | *Candida bombicola* | 50 | Fleurackers, 2006 |
| | *Candida bombicola* | 42 | Shah *et al.*, 2007 |
| Rhamnolipid | *P. aeruginosa* ATCC 9027 | 8.5 | Luo *et al.*, 2013 |
| | *P. aeruginosa* zju1.m | 20 | Zhu *et al.*, 2007 |
| | *P. aeruginosa* ATCC 10145 | 7.5 | Wadekar *et al.*, 2012 |
| | *P. aeruginosa* mutant EBN-8 | 9.3 | Raza *et al.*, 2006 |
| | *P. aeruginosa* D | 2.26 | George and Jayachandran, 2013 |

*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 accesibility 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

| Agitation (rpm) | Aeration (vvm) | RL produced (g/L) | Reference |
|---|---|---|---|
| 800 | 1.0 | 7.60 | Lovaglio *et al*., 2010 |
| 550 | 0.5 | 3.30 | de Lima *et al*., 2009 |
| 600 | 1.2 | 5.37 | Borges *et al*., 2015 |

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 o rhamnolipid production

| Agitation (rpm) | Aeration (vvm) | RL yield (%) | Reference |
|---|---|---|---|
| 500 | 1.0 | 4.1 | Lovaglio *et al*., 2010 |
| 500 | 2.0 | 5.3 | |
| 800 | 1.0 | 7.6 | |
| 800 | 2.0 | 16.9 | |

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