The influence of agitation on oil substrate dispersion and oxygen transfer in *Pseudomonas aeruginosa* USM-AR2 fermentation producing rhamnolipid in a stirred tank bioreactor

M. N. Nur Asshifa1 · Nor Syafirah Zambry1 · M. S. Salwa1 · Ahmad R.M. Yahya1

Received: 14 February 2017/Accepted: 18 April 2017
© Springer-Verlag GmbH Germany 2017

Abstract Water-immiscible substrate, diesel, was supplied as the main substrate in the fermentation of *Pseudomonas aeruginosa* USM-AR2 producing rhamnolipid biosurfactant, in a stirred tank bioreactor. In addition to the typical gas–aqueous system, this system includes gas–hydrocarbon–aqueous phases and the presence of surfactant (rhamnolipid) in the fermentation broth. The effect of diesel dispersion on volumetric oxygen transfer coefficient, \( k_{L}a \), and thus oxygen transfer, was evaluated at different agitations of 400, 500 and 600 rpm. The oxygen transfer in this oil–water–surfactant system was shown to be affected by different oil dispersion at those agitation rates. The highest diesel dispersion was obtained at 500 rpm or impeller tip speed of 1.31 m/s, compared to 400 and 600 rpm, which led to the highest \( k_{L}a \), growth and rhamnolipid production by *P. aeruginosa* USM-AR2. This showed the highest substrate mixing and homogenization at this agitation speed that led to the efficient substrate utilization by the cells. The oxygen uptake rate of *P. aeruginosa* USM-AR2 was 5.55 mmol/L/h, which showed that even the lowest \( k_{L}a \) (48.21 h\(^{-1}\)) and hence OTR (57.71 mmol/L/h) obtained at 400 rpm was sufficient to fulfill the oxygen demand of the cells. The effect of rhamnolipid concentration on \( k_{L}a \) showed that \( k_{L}a \) increased as rhamnolipid concentration increased to 0.6 g/L before reaching a plateau. This trend was similar for all agitation rates of 400, 500 and 600 rpm, which might be due to the increase in the resistance to oxygen transfer (\( k_{L} \) decrease) and the increase in the specific interfacial area (\( a \)).

Keywords Bioreactor · Oil dispersion · Oxygen transfer rate · Oxygen uptake rate · *Pseudomonas aeruginosa* · Rhamnolipid

Introduction

Biosurfactants are amphiphilic compounds that tend to accumulate at interfaces by reducing the surface (gas–liquid) and interfacial (liquid–liquid or solid–liquid) tensions, reduce the repulsive forces between two dissimilar phases and allow the different phases to mix and interact more readily (Chandran and Das 2010; Makkar et al. 2011; Rahman et al. 2003). They are produced by bacteria, yeasts and fungi, especially when grown on water-immiscible carbon substrates. Biosurfactants are non-polluting and sustainable and show excellent physicochemical properties which make them interesting for various applications (Singh et al. 2006; Muthusamy et al. 2008; Chrzanowski et al. 2012).

Unlike chemical surfactants, which are mostly derived from petroleum feedstock, biosurfactants can be produced naturally by microbial fermentation processes using agro-based substrates and waste materials (Espuny et al. 1996; Mukherjee et al. 2006). Thus, biosurfactants offer higher biodegradability, lower toxicity, better environmental compatibility, lower critical micelle concentration, higher surface activity, higher selectivity and can be synthesized from renewable sources (Bhardwaj et al. 2013; Muthusamy et al. 2008; Noudeh et al. 2010). Their unique chemical characteristics make them desirable in various industrial processes such as food processing, pharmaceutical
formulations, enhanced oil recovery, cleaning of oil tankers, transportation of heavy crude oils and in environmental bioremediation (Marchant and Banat 2012; Soberón-Chávez and Maier 2011; Saharan et al. 2012).

Among the groups of biosurfactants, rhamnolipids are a group of high-value and effective glycolipid that influences various processes related to hydrocarbon degradation. In particular, rhamnolipid offers special advantages compared to other biosurfactants because of its potent emulsifying activity, high surface activities and low critical micelle concentration (Maier and Soberón-Chávez 2000; Rashedi et al. 2005; Raza et al. 2007). Consequently, rhamnolipid is a possible ‘green’ candidate to replace chemical or synthetic surfactants.

Water-immiscible substrates have been the substrate of choice to produce biosurfactants, as they are produced to aid in oil metabolism (Gomathy and Senthilkumar 2013; Muthusamy et al. 2008). In this study, diesel was used as the main carbon substrate. Diesel is a fuel product of petroleum fractional distillation which consists of mixtures of hydrocarbon compounds. It has been proven to be effective in rhamnolipid production by P. aeruginosa USM-AR2 compared to other substrates, such as glucose, glycerol, kerosene, palm oil, olive oil and corn oil, tested in a preliminary study.

In aerobic fermentation, oxygen molecules must first be transferred from gas bulk and overcome a series of transport resistances before it can be utilized by the cells. As oxygen is the key substrate in aerobic bioprocesses, a continuous supply is needed due to its low solubility in aqueous media (Bailey and Ollis 1986). Generally, gas–liquid oxygen transfer in bioreactors is characterized into parameters related to transport (volumetric oxygen transfer coefficient, \( k_{L}\alpha \)), and hence oxygen transfer rate (OTR) and the oxygen uptake rate (OUR) by the microorganism. The \( k_{L}\alpha \) is most commonly used to evaluate the aeration efficiency in aerobic fermentation and plays a decisive role in the performance of bioreactors. The measurement of OTR and OUR in a fermentation system is important, as the balance between oxygen demand and supply is to ensure that OUR of the culture does not exceed the OTR in the fermenter, creating anoxic conditions in the culture. Both \( k_{L}\alpha \) and OUR are affected by many factors, including the configuration of the bioreactor, the liquid medium properties (viscosity, surface tension and other physicochemical properties of the broth), temperature, pressure and the energy dissipation in the fluid, which generally depends on the aeration and agitation (Arjunwadkar et al. 1998; Garcia-Ochoa and Gomez 2009; Karimi et al. 2013; Sivapra­kasam et al. 2008).

The use of hydrocarbon substrate in biological processes introduces several challenges related to the nature of the substrate. These challenges are common to all hydrocarbon fermentations regardless of the product formed, which include flammability, volatility, insolubility and mass transfer limitations with respect to both oxygen and substrate. Early review by Rols and Goma (1989) presented the use of oxygen vectors having higher oxygen solubility to improve oxygen supply in aerobic fermentations. Typically, the oxygen vectors used are insoluble liquid in the fermentation media. Another study reported the use of hydrocarbon as oxygen vector to increase \( k_{L}\alpha \) (Jia et al. 1996).

In particular, the provision of adequate oxygen transfer to the microorganism in hydrocarbon-based bioprocesses is exacerbated by the deficiency of oxygen in the hydrocarbon molecular structure. This is in contrast to carbohydrate-based bioprocesses, where the carbohydrate itself supplies about half of the oxygen required. Therefore, the metabolic oxygen requirement in hydrocarbon-based bioprocesses has to be met entirely by the transfer of oxygen to the broth (Clarke et al. 2006; Clarke and Correia 2008). In view of the importance of adequate oxygen transfer in a stirred tank bioreactor, an attempt has been made to evaluate the effect of oil (diesel) substrate dispersion at different agitation rates toward oxygen transfer in the system, and thus its effect on cell growth and rhamnolipid production. This is because mass transfer between phases may often become the limiting step of the overall process rate (Karimi et al. 2013), especially in a water-immiscible system as in this work.

On the other hand, the influence of surfactant in the oil–water system may also affect the oxygen transfer through its effects on solubility and interfaces. This amphiphatic compound contains hydrophobic and hydrophilic moieties, which tend to concentrate at the gas–liquid and liquid–liquid interfaces. Therefore, another aim is to evaluate the effect of rhamnolipid on \( k_{L}\alpha \), as this biosurfactant will be produced throughout the fermentation process.

Materials and methods

Microorganism

The bacterial strain used in this work was a Gram-negative bacterium isolated from crude oil, identified as Pseudomonas aeruginosa and designated USM-AR2 from a previous study (Nur Asshifa 2009). This isolate was found to utilize hydrocarbon substrates and secrete copious amounts of rhamnolipid.

Culture maintenance

The bacterial culture was stored in 40% (v/v) glycerol at −20°C. Single bacterial colonies from a plate were picked
and grown for 24 h in nutrient broth containing the ingredients as follows per liter: 0.5% (w/v) peptone, 0.2% (w/v) yeast extract, 0.1% (w/v) beef extract, 0.5% (w/v) MgSO4, 0.05% (v/v) NaCl. To store the bacteria, a volume of 0.5 mL of a 24 h culture was added into 0.5 mL of 40% (v/v) sterile glycerol in a sterile microcentrifuge tube.

Production of biomass and rhamnolipid in the bioreactor

Experiments were performed in a 3.0 L bioreactor (BioFlo 115, New Brunswick, Hamburg, Germany) with 1.5 L working volume at 27 °C, 500 rpm agitation, aerated at an air flow rate of 0.3 vvm and a medium initial pH of 5. The agitation system of the bioreactor was equipped with six-blade Rushton turbine impeller with 5 cm diameter and positioned accordingly. The pH was left uncontrolled based on previous finding that showed uncontrolled pH resulted in higher growth compared to pH controlled at 6, 7 and 8. A production medium was used to cultivate P. aeruginosa USM-AR2 containing the ingredients as follows per liter: 0.6% (w/v) yeast extract, 0.05% (w/v) MgSO4, 0.05% (v/v) Tween 80, 20 mL initial diesel. Seed culture was prepared in nutrient broth before inoculation into the production medium. A 2% (v/v) cell suspension of a 24 h culture (OD540 = 2) was used as the inoculum.

Biomass quantification

Cells were harvested by centrifugation at 10 000×g for 5 min (Eppendorf Centrifuge 5424), rinsed with acetone to remove the adhering hydrocarbon (diesel) and washed twice with distilled water to remove traces of nutrients. The washed cell pellet was suspended in 3 mL distilled water and the bacterial cell growth was monitored by optical density (OD540) with a spectrophotometer (Genesys 20, Model 4001-04, USA). Distilled water was used as blank and the mixtures were subjected to the same procedure as the sample. The rhamnolipid concentrations were calculated from a standard curve prepared with l-rhamnose and expressed as rhamnolipid equivalent (RE).

Rhamnolipid quantification: orcinol assay

Rhamnolipid was quantified through an indirect way, using rhamnose as a reference, as rhamnose is a by-product of acid hydrolysis of rhamnolipid (Jeong et al. 2004). The orcinol reagent (Sigma-Aldrich) was prepared by dissolving orcinol in a concentration of 0.19% (w/v) in a 53% (v/v) sulfuric acid solution. To 0.3 mL of each sample, 2.7 mL of a solution containing 0.19% orcinol (in 53% (v/v) sulfuric acid) was added. After heating for 45 min at 70 °C, the mixtures were cooled at room temperature and the optical density (OD421) was measured using a spectrophotometer (Genesys 20, Model 4001-04, USA). Distilled water was used as blank and the mixtures were subjected to the same procedure as the sample. The rhamnolipid concentrations were calculated from a standard curve prepared with l-rhamnose and expressed as rhamnolipid equivalent (RE).

Determination of volumetric oxygen transfer coefficient (kLa) and oxygen transfer rate (OTR)

The volumetric oxygen transfer coefficient (kLa) in the bioreactor was determined by implementing the ‘static gassing out’ (non-fermentative) technique in a 3 L stirred tank bioreactor (BioFlo 115, New Brunswick) in the absence of respiring organism (Sabra et al. 2002), minimizing the interference of biomass which changed throughout fermentation. First, the bioreactor was filled with distilled water added with oil (diesel), as the medium for P. aeruginosa USM-AR2 fermentation contains diesel as the carbon substrate. The C* was measured. Distilled water with diesel was sparged with nitrogen to remove oxygen from the broth. Then, air was supplied and the deoxygenated liquid was aerated and agitated accordingly. The increase in dissolved oxygen concentration (C_L) until saturation was measured by a polarographic oxygen probe (InPro6830/12/220 Mettler-Toledo). The response time of the oxygen probe was within 30 s.

The increase in the dissolved oxygen concentration (C_L) can be described by Eq. (1):

\[ \ln(C^* - C_L) = -k_{La}t, \]

where C* is the dissolved oxygen concentration in equilibrium at the gas–liquid interface, C_L the dissolved oxygen concentration in the fermentation broth, k_L the liquid phase oxygen transfer coefficient, a the gas–liquid interfacial area per liquid volume and t the time.

The oxygen transfer coefficient, k_{La}, was then calculated by plotting a graph of \( \ln(C^* - C_L) \) against time, which gave a straight line with the slope of k_{La} (Arjunwadkar et al. 1998). The k_{La} values were calculated for different impeller speeds: 400, 500 and 600 rpm at an air flow rate of 0.3 vvm. The impeller tip speed for each agitation rate was also calculated using Eq. (2):

\[ \text{Impeller tip speed} = \pi ND, \]

where \( \pi = 3.142, N \) the agitation rate (rps) and D the diameter of the impeller = 0.05 m.
The effect of rhamnolipid in the broth toward $k_{1a}$ value was also performed by repeating the same static gassing out technique. The profile of $k_{1a}$ values over certain concentrations of rhamnolipid was determined for different impeller speeds: 400, 500 and 600 rpm at an air flow rate of 0.3vvm.

The rate of oxygen transfer from the gas phase (air bubbles) to the liquid phase (cultivation broth) is represented by Eq. (3) (Garcia-Ochoa and Gomez 2009):

\[
\text{Oxygen transfer rate (OTR)} = \frac{dC_L}{dt} = k_{1a}(C^* - C_L),
\]

where $\frac{dC_L}{dt}$ is the rate of change in oxygen concentration in the liquid phase, $k_{1a}$ the volumetric mass transfer coefficient, $(C^* - C_L)$ the difference between the oxygen saturation concentration at equilibrium and the actual oxygen concentration in the liquid, and oxygen solubility at 25°C, 1 atm = 1.26 mmol/L.

**Determination of oxygen uptake rate (OUR)**

The measurement of OUR was made using the ‘dynamic technique’ (fermentative) during batch cultivation of *P. aeruginosa* USM-AR2 in 3.0 L bioreactor (Bioflo 115, New Brunswick) with 1.5 L working volume at 27°C, 500 rpm agitation, aerated at an air flow rate of 0.3 vvm and a medium initial pH of 5. The method relies on measuring the DO consumption over time during the cultivation of *P. aeruginosa* USM-AR2 in the bioreactor. The specific OUR, $q_o$, was calculated based on the relationship between the OUR and cell concentration, given by Eq. (4):

\[
\text{Oxygen uptake rate (OUR)} = \frac{dC_L}{dt} = -q_o \times X,
\]

where $q_o$ is the specific oxygen consumption or respiration rate (mmol/g/h) and $X$ the cell biomass concentration (g/L).

**Quantification of diesel**

The quantification of diesel in the aqueous phase was determined at different agitation rates of 400, 500 and 600 rpm. To represent the production medium, distilled water was mixed with diesel and 0.05% (v/v) of Tween® 80 (originally existing in the medium) in the bioreactor. The agitation was set at 400, 500 or 600 rpm for each experiment to compare the diesel mixing at these different agitation rates. After 1 h of agitation, samples were taken out. The bottom layer (water with dispersed oil) of the samples was pipetted out and mixed with hexane at a ratio of 1:1, where 25 mL of the mixture was mixed with 25 mL hexane in a Falcon tube and vortexed for 5 min. 15 mL of the upper phase was transferred in a pre-weighed aluminum cup and hexane was evaporated at room temperature for 24 h. The residual oil was determined gravimetrically (Costa et al. 2009). The procedure was repeated for distilled water added with 0.05 g/L of rhamnolipid at different agitation rates. For each experiment, samples were measured in five replicates.

**Gas chromatography mass spectrometry (GC–MS) analysis of diesel**

Diesel used as the carbon source in the fermentation was analyzed by gas chromatography mass spectrometry (GC–MS) for component identification. The peaks in the chromatogram represent the qualitative concentration of each component. GC–MS analysis was performed using Shimadzu QP 2010 with a BPX-5 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and SGE 10 μL syringe. Helium was used as the carrier gas at a flow rate of 1 mL/min. The injection port was maintained at 250°C. The method relies on measuring the DO consumption over time during the cultivation of *P. aeruginosa* USM-AR2 in the bioreactor. The specific OUR, $q_o$, was calculated based on the relationship between the OUR and cell concentration, given by Eq. (4):

\[
\text{Oxygen uptake rate (OUR)} = \frac{dC_L}{dt} = -q_o \times X,
\]

where $q_o$ is the specific oxygen consumption or respiration rate (mmol/g/h) and $X$ the cell biomass concentration (g/L).

**Results and discussion**

In typical mechanically agitated bioreactors, the impeller is the main gas-dispersing tool and plays a major role in breaking up bubbles to maintain high interfacial area which influenced the $k_{1a}$ value and oxygen transfer (Casas-López et al. 2006; Garcia-Ochoa and Gomez 2009). Therefore, the oxygen transfer study was initiated with the determination of $k_{1a}$ in the 3.0 L bioreactor using combinations of agitation rates of 400, 500 and 600 rpm and air flow rate of 0.3 vvm. These three speeds were chosen because preliminary study showed that the rhamnolipid production was most influenced at these speeds (Salwa et al. 2010). The growth and rhamnolipid production by *P. aeruginosa* USM-AR2 at different impeller speeds were the highest at 500 rpm (9.4 g/L biomass, 3.23 g/L rhamnolipid), compared to 400 rpm (4.3 g/L biomass, 0.86 g/L rhamnolipid) and 600 rpm (8.2 g/L biomass, 1.55 g/L rhamnolipid). The impeller tip speed values for each agitation are also presented in Table 1. Referring to Table 1, as the agitation rate increases from 400 to 500 rpm, the $k_{1a}$ value also increases from 48.21 to 70.38 h⁻¹ when the agitation increases to 600 rpm, the $k_{1a}$ value is slightly lower, which is 65.31 h⁻¹. It is speculated that the $k_{1a}$ values approach a plateau and subsequently decrease at higher agitation (600 rpm) as it is already limited by the surface area, $a$, for oxygen transfer. This means that the surface area of the dispersed air bubbles no longer increases at
any higher agitation (small bubble size). Karimi and co-
researchers (2013) reported the similar trend where
\( k_La \) increment was observed using Rushton turbine impeller
begins from 200 to 700 rpm, and this trend approaches a
plateau at higher agitations of 800–1000 rpm.

The highest \( k_La \) at 500 rpm or impeller tip speed of
1.31 m/s was paralleled with the highest oil dispersion
among all agitation rates tested. Diesel dispersion at dif-
ferent agitations of 400, 500 and 600 rpm in the medium is
depicted in Fig. 1 for conditions without and with rham-
nolipid addition. Rhamnolipid was added at 0.05 g/L to
represent a low concentration to evaluate whether rham-
nolipid affects oil dispersion, even at very low concentra-
tion. Referring to Fig. 1, diesel is most dispersed at
500 rpm, which could lead to easier uptake by the cells and
rhamnolipid secreton. Diesel dispersion with and without
rhamnolipid presence showed that rhamnolipid increases
the oxygen transfer at a concentration of 0.05 g/L for all
agitations. This can be further related to the \( k_La \) profile at
different rhamnolipid concentrations which is discussed
later.

Figure 2 shows an example of diesel oil extracted from
the broth using hexane. Due to the insolubility of diesel in
water, optimum dispersion was very important to ensure
homogenization of the culture broth in the fermentation
system. Upon good mixing, both the gas phase and the
hydrocarbon phase are dispersed to fine bubbles and oil
droplets of various sizes that play an important role in the
oxygen transportation from bulk to microorganisms
(Clarke et al. 2006; Clarke and Correia 2008; Gomes et al.
2007). Therefore, the highest dispersion of this oil at
500 rpm compared to 400 and 600 rpm, both with and
without rhamnolipid presence in the broth, is related to the
highest \( k_La \) at 500 rpm, and thus the highest growth of \( P. \)
aeruginosa USM-AR2 and rhamnolipid production.

Figure 3 shows a chromatogram of diesel used in the
fermentation of \( P. \) aeruginosa USM-AR2 producing
rhamnolipid, as analyzed by GC–MS. The major peaks of
the chromatogram represent aliphatic hydrocarbon com-
ounds: undecane (1), dodecane (2), tetradecane (3),
dodecane (4), hexadecane (5, 6), heptadecane (7, 8),
octadecane (9, 10), heneicosane (11, 12), eicosane (13),

### Table 1

| Agitation (rpm) | Impeller tip speed (m/s) | \( k_La \) (h\(^{-1}\)) | Growth (g/L) | Rhamnolipid (g/L) |
|----------------|-------------------------|----------------------|-------------|------------------|
| 400            | 1.05                    | 48.21 ± 4.49         | 4.3         | 0.86             |
| 500            | 1.31                    | 70.38 ± 2.14         | 9.4         | 3.23             |
| 600            | 1.57                    | 65.31 ± 2.70         | 8.2         | 1.55             |

![Figure 1](image1.png)

**Fig. 1** Amount of diesel oil extracted from aqueous phase at different agitation rates with and without rhamnolipid (RL) of 0.05 g/L in the absence of bacterial cells.
tetracosane (14), tetratriacontane (15), tetracontane (16, 17), and hexatriacontane (18, 19). Several studies reported the capability of \textit{P. aeruginosa} strain in utilizing various hydrocarbons as the source of carbon and secreting biosurfactant (rhamnolipid) while growing on a hydrocarbon-rich medium (Das et al. 2008; Chaerun et al. 2004). The volumetric oxygen uptake rate (OUR) of \textit{P. aeruginosa} USM-AR2 was determined during the cultivation at 500 rpm and 0.3 vvm air flow rate. The rate at which oxygen is consumed by cells determines the minimum rate at which oxygen must be transferred from gas to broth (Kirk and Szita 2013). Figure 4 shows that the OUR during the exponential phase at 24 h is 3.53 mmol/L/h, while the specific oxygen uptake rate \((q_{o2})\) is 0.59 mmol/g/h. The \(q_{o2}\) value is rather low when compared with those of most bacteria where the \(q_{o2}\) value typically lies in the range of 2–12 mmol/g/h (Shuler and Kargi 2002). It is important to note that \textit{P. aeruginosa} USM-AR2 is a facultative anaerobe organism which does not require high oxygen to grow. Another strain of \textit{P. aeruginosa} was also reported to prefer microaerobic condition for growth and metabolite formation, especially when grown in hydrocarbons (Chayabutra and Ju 2000; Sabra et al. 2002). The maximum OUR for \textit{P. aeruginosa} USM-AR2 when \(q_{o2}\) was multiplied with the highest biomass, 9.4 g/L, was 5.55 mmol/L/h.

Throughout the cultivation, the OUR and \(q_{o2}\) declined as the growth rate slowed, indicating lower oxygen consumption rates by the cells. The bacterial cells were no longer utilizing oxygen as quickly as the consumption during the early cultivation period. Typically, oxygen demand is at maximum in the early exponential phase, as illustrated in Fig. 4, even though the cell concentration is relatively low at that time. The higher OUR during early cultivation is interpreted as a higher respiratory activity of bacterial cells. This is in agreement with Bailey and Ollis (1986) who claimed that the maximum oxygen demand occurs in the early exponential phase, although the cell concentration is larger at a later time.

The \(k_{L}a\) and OTR at different agitation rates in Table 2 documents that the lowest \(k_{L}a\) (48.21 h\(^{-1}\)) and hence OTR Fig. 2 Diesel oil extracted from the aqueous phase in an aluminum dish (indicated by arrow)

Fig. 3 Time course of a growth, rhamnolipid production, b diesel utilization and c dissolved oxygen in batch cultivation of \textit{P. aeruginosa} USM-AR2
(57.71 mmol/L/h) were obtained at 400 rpm, showing that the oxygen supplied in the bioreactor was sufficient to meet the requirement of the oxygen demand by the cells, as the oxygen requirement by \textit{P. aeruginosa} USM-AR2 at agitation that gave the highest growth (500 rpm) was 5.55 mmol/L/h, which is far below the oxygen transfer at all agitation rates tested.

Batch cultivation of \textit{P. aeruginosa} USM-AR2 producing rhamnolipid was carried out in the stirred tank bioreactor. The batch fermentation in Fig. 5 had been discussed in terms of cell growth and rhamnolipid production by Md Noh et al. (2014). However, this study presented that the oil substrate utilization and dissolved oxygen (DO) profile were related to oil substrate dispersion and oxygen transfer. During early cultivation, diesel was the least dispersed in the medium (aqueous phase), as represented by lower diesel extracted from the culture. This is because, initially, a macroemulsion was created by agitation or aeration forces in the bioreactor (Kosaric et al. 1987). After 4 h, oil dispersion in the broth started to increase and resulted in 22.43 g/L of extracted diesel due to the mixing and increasing rhamnolipid production (0.06 g/L). This result was similar to the finding in Fig. 4, where the diesel concentration in the aqueous phase was 24.75 g/L at a rhamnolipid concentration of 0.05 g/L. At 12 h, as cell concentration and rhamnolipid production increased to 3.7 and 0.38 g/L, respectively, the diesel concentration in the culture medium was 39.8 g/L, suggesting the higher dispersion into aqueous phase after a longer mixing time (12 h) compared to the previous points. However, this is also subjected to utilization by cells as cells are growing.

As the fermentation progressed, diesel was utilized by the cells as indicated by the decreasing diesel leftover. The diesel consumption throughout the cultivation increased oxygen consumption by the cells, thus promoting growth up to 9.5 g/L after 50 h. After this period, eventually, the bacteria entered the stationary phase after the limiting nutrient (diesel) was depleted. This observation was also supported by the DO profile which showed a spike after ~50 h cultivation, due to the exhaustion of carbon supplied by the diesel. The highest growth was recorded at 9.5 g/L with the highest rhamnolipid concentration at 2.61 g/L. The rate of rhamnolipid production was generally constant during the exponential (0 ~ 30 h) and stationary (~30 ~ 50 h) growth phases. The production of rhamnolipid stabilizes during the decline of growth (Fig. 5).

It was shown that $k_{L}a$ values may increase, decrease, or remain constant, depending on the nature of the compound added and the operating conditions (Dumont and Delmas 2003; Gomes et al. 2007). Figure 6 shows that $k_{L}a$ is higher at 500 rpm compared to 400 and 600 rpm without rhamnolipid addition, as supported earlier by Table 1. Gomes et al. (2007) reported that the presence of surfactant in a

| Agitation (rpm) | OTR $k_{L}a \times \Delta C$ (mmol/L/h) |
|-----------------|----------------------------------------|
| 400             | 57.71                                  |
| 500             | 84.24                                  |
| 600             | 78.18                                  |

(57.71 mmol/L/h) were obtained at 400 rpm, showing that the oxygen supplied in the bioreactor was sufficient to meet the requirement of the oxygen demand by the cells, as the oxygen requirement by \textit{P. aeruginosa} USM-AR2 at agitation that gave the highest growth (500 rpm) was 5.55 mmol/L/h, which is far below the oxygen transfer at all agitation rates tested.
medium increases the $k_L a$ value of a system. To relate this with the present findings, this is only true for rhamnolipid concentrations below 0.6 g/L at all agitations. This effect might be attributed to the increase of the interfacial area, $a$, as surfactants' concentration increases. The increase in $a$ is related to the size of gas bubbles and their dispersion throughout the fermentation vessel. This is because the decrease in the surface tension of the liquid phase when surfactant concentration increases prevents the coalescence of bubbles. This will result in the formation of smaller

**Fig. 5** Time course of growth, rhamnolipid production, diesel utilization and dissolved oxygen in batch cultivation of *P. aeruginosa* USM-AR2
bubble size, which increase the $a$, and therefore a better oxygen transfer rate (Gomez-Diaz et al. 2010; Najafpour 2007). Moreover, rhamnolipid is an anionic biosurfactant, which means it is negatively charged. The $k_La$ typically increases when ion concentration in the solution is raised, due to its influence on decreasing bubble size and the non-coalescence effect (García-Ochoa and Gomez 2009).

An increase of $k_La$ is observed when rhamnolipid is added up to a certain concentration until a plateau is observed in Fig. 6 (above 0.6 g/L) for all agitation rates. Two opposing mechanisms may affect the oxygen transfer in the fermentation vessel, which can be due to the effect on the liquid film coefficient, $k_L$, and the surface area of bubbles, $a$. Their combined effect cannot be easily predicted (Alves et al. 2004). This is as a result of the balance in the two competitive effects of surfactants, which is the increase in the resistance to oxygen transfer ($k_L$ decrease) and the increase in the specific interfacial area ($a$) (Gomes et al. 2007; Wagner and Popel 1996).

The increase in $a$ may promote mass transfer, whereas the decrease in $k_L$ may inhibit mass transfer. The decrease in $k_L$ on the addition of surfactant is due to changes in the hydrodynamic characteristics of the system. Adsorption of surfactants at the interface is a spontaneous process, where the surface tension is reduced, leading to higher values of the interfacial area per volume, $a$. The tendency of increased $a$ is countered by the effect of surfactant films on the $k_L$, as its adsorption on the film results in a stagnant and rigid interface. This is because the adsorption of surfactants onto the gas–liquid interface forms a film which gives additional resistance to mass transfer across the interface. This retards surface flow by the surface tension gradient at the interface, and hence decreases the $k_L$ value (Bailey and Ollis 1986; Koide et al. 1985). In addition, other researchers have pointed out the barrier effect of these substances, due to a monolayer of the surfactants forming at the interface, resulting in a higher surface viscosity and an increased thickness of the liquid film around the bubbles, thus increasing the resistance to oxygen transfer ($k_L$ decrease) (Rosso et al. 2006).

It was stated that oil that spreads over a gas–liquid interface increases the interfacial area, $a$, by reducing the average bubble diameter, but decreases the mass transfer coefficient, $k_L$, by increasing the liquid phase mass transfer resistance (Cents et al. 2001; Dumont and Delmas 2003). The initial decrease in $k_La$ in Fig. 6 might be due to a decrease in $a$ due to the coalescence of oil droplets, while $k_L$ remains constant. At this initial point, the oil was least distributed and rhamnolipid concentration was very low, which was 0.01 g/L. It was reported that low surfactant concentration promotes coalescence, where surfactants act as a ‘bridge’ between oil droplets. As surfactant increases, coalescence decreases (Narsimhan and Wang 2008).

**Conclusion**

The fermentation system under this study presented additional factors affecting $k_La$: the rhamnolipid concentration and the immiscible substrate, diesel, in the culture broth. Diesel was most dispersed at 500 rpm or impeller tip speed of 1.31 m/s, which is related to the highest $k_La$ and oxygen transfer, growth and rhamnolipid production by *P. aeruginosa* USM-AR2 achieved at this speed. In addition, $k_La$ increased when rhamnolipid concentration increased to 0.6 g/L before reaching a plateau for all agitation rates of 400, 500 and 600 rpm. This phenomenon might be due to the increase in the resistance to oxygen transfer and the
increase in the specific interfacial area, as the increase in $a$ may promote mass transfer, whereas the decrease in $k_t$ may inhibit mass transfer.

Acknowledgements This research was supported in part by USM Research University Individual (RUI) Grant Scheme (1001/PIBIOLOGI/811242) and Ministry of Education Malaysia, Fundamental Research Grant Scheme (FRGS) (203/P/PIBIOLOGI/6711494).

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

References

Alves SS, Maia CL, Vasconcelos JMT (2004) Gas-liquid mass transfer coefficient in stirred tanks interpreted through bubble contamination kinetics. Chem Eng Proc 43:823–830

Arjunwadkar SJ, Sarvanan K, Kulkarni PR, Pandit AB (1998) Gas liquid mass transfer in dual impeller bioreactor. Biochem Eng J 1:99–106

Bailey JE, Ollis DF (1986) Biochemical engineering fundamentals. McGraw-Hill, Singapore

Bhardwaj G, Cameotra SS, Chopra HK (2013) Mini review: utilization of oleo-chemical industry by-products for biosurfactant production. AMB Express 3(68):1–5

Casas-López JL, Porcel EMR, Alberola IO, Martín MMB, Pérez JAS, Sevilla JMF, Chisti Y (2006) Simultaneous determination of oxygen consumption rate and volumetric oxygen transfer coefficient in pneumatically agitated bioreactors. Ind Eng Chem Res 45(3):1167–1171

Cents AHG, Brilman DW, Versteeg GF (2001) Gas absorption in an agitated gas-liquid-liquid system. Chem Eng Sci 56:1075–1083

Chaerun SK, Tazaki K, Asada R, Kogure K (2004) Bioremediation of coastal areas 5 years after the Nakhodka oil spill in the Sea of Japan: Isolation and characterization of hydrocarbon degrading bacteria. Environ Int 30(7):911–922

Chandran P, Das N (2010) Biosurfactant production and diesel oil degradation by yeast species Trichosporon asahii isolated from petroleum hydrocarbon contaminated soil. Int J Eng Sci Technol 2(12):6942–6953

Chayabutra C, Ju LK (2000) Degradation of n-hexadecane and its metabolites by Pseudomonas aeruginosa under microaerobic and anaerobic denitrifying conditions. Appl Environ Microbiol 66(2):493–498

Chrzanoski L, Lawniczak L, Czaczyk K (2012) Why do microorganisms produce rhamnolipids? World J Microbiol Biotechnol 28:401–419

Clarke KG, Correia LDC (2008) Oxygen transfer in hydrocarbon-aqueous dispersions and its applicability to alkane bioprocesses: A review. Biochem Eng J 39:405–429

Clarke KG, Williams PC, Smit MS, Harrison STL (2006) Enhancement and repression of the volumetric oxygen transfer coefficient through hydrocarbon addition and its influence on oxygen transfer rate in stirred tank bioreactors. Biochem Eng J 28:237–242

Costa SGVAO, Lepine F, Milot S, Deziel E, Nitschke M, Contiero J (2009) Cassava wastewater as a substrate for the simultaneous production rhamnolipids and polyhydroxyalkanoates by Pseudomonas aeruginosa. J Ind Microbiol Biotechnol 36:1063–1072

Das P, Mukherjee S, Sen R (2008) Genetic regulations of the biosynthesis of microbial surfactants: an overview. Biotechnol and Genetic Eng Rev 25(1):165–186

Dumont E, Delmas H (2003) Mass transfer enhancement of gas absorption in oil-in-water systems: a review. Chem Eng Proc 42:419–438

Espuny MJ, Egidio S, Rodon I, Manresa A, Mercade ME (1996) Nutritional requirements of a biosurfactant producing strain Rhodococcus sp. 5177. Biotechnol Lett 18(5):521–526

Garcia-Ochoa F, Gomez E (2009) Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. Biotechnol Adv 27:153–176

Gomathy C, Senthilkumar R (2013) Production of rhamnolipid biosurfactant from a marine Pseudomonas aeruginosa. Int J Res Environ Sci Technol 3(3):86–91

Gomes N, Aguedo M, Teixeira J, Belo I (2007) Oxygen mass transfer in a biphasic medium: influence on the biotransformation of methyl ricinoleate into y-decalactone by the yeast Yarrowia lipolytica. Biochem Eng J 35:380–386

Gomez-Diaz D, Gomes N, Teixeira JA, Belo I (2010) Gas-liquid interfacial area in the oxygen absorption to oil-in-water emulsions in an airlift reactor. Can J Chem Eng 88:561–564

Jeong HS, Dong-Jung L, Sun-Hee H, Soon-Duck H, Jai-Yul K (2004) Rhamnolipid production by Pseudomonas aeruginosa immobilized in polyvinyl alcohol beads. Biotechnol Lett 26(1):35–39

Jia S, Li P, Park YS, Okabe M (1996) Enhanced oxygen transfer in tower bioreactor on addition of liquid hydrocarbons. J Ferment Bioeng 82(2):191–193

Karimi A, Golbabaei F, Mehrnia MR, Neghab M, Mohammad K, Nikpey A, Pourmand MR (2013) Oxygen mass transfer in a stirred tank bioreactor using different impeller configurations for environmental purposes. Iranian J Environ Health Sci Eng 10(6):1–9

Kirk TV, Szita N (2013) Oxygen transfer characteristics of miniaturized bioreactor systems. Biotechnol Bioeng 110(4):1005–1019

Koide K, Yamazoe S, Harada S (1985) Effects of surface active substances on gas hold-up and gas-liquid mass transfer in bubble column. J Chem Eng Jpn 18(4):287–292

Kosaric NN, Gray CC, Cairns WL (1987) Exopolysaccharide bioemulsifiers. In: Biosurfactants and Biotechnology (ed) Surfactant science series, vol 25. Marcel Dekker, New York

Maier RM, Soberón-Chávez G (2000) Pseudomonas aeruginosa rhamnolipids: biosynthesis and potential applications. Appl Microbiol Biotechnol 54:625–633

Makkar RS, Cameotra SS, Banat IM (2011) Mini-Review: advances in utilization of renewable substrates for biosurfactant production. AMB Express 1(5):1–19

Marchant R, Banat IM (2012) Biosurfactants: a sustainable replacement for chemical surfactants? Biotechnol Lett 34:1597–1605

Md Noh NA, Salwa MS, Ahmad Ramli MY (2014) Enhanced rhamnolipid production by Pseudomonas aeruginosa USM-AR2 via fed-batch cultivation based on maximum substrate uptake rate. Lett Appl Microbiol 58(6):617–623

Mukherjee S, Das P, Sen R (2006) Towards commercial production of microbial surfactants. Trends Biotechnol 24(11):509–515

Muthusamy K, Gopalakrishnan S, Ravi TK, Sivachidambaram P (2008) Biosurfactants: properties, commercial production and application. Curr Sci 94(6):736–747

Najafpour GD (2007) Biochemical engineering and biotechnology. Elsevier, Amsterdam, The Netherlands

Narsimhan G, Wang Z (2008) Guidelines for processing emulsion-based food. In: Hasenhuettl GL, Iartel RW (eds) Food emulsifiers and their applications, Springer, Heidelberg

Noudeh GD, Noodeh AD, Moshafi MH, Behravan E, Afzadi MA, Sodagar M (2010) Investigation of cellular hydrophobicity and surface activity effects of biosynthesized biosurfactant from broth media of PTCC 1561. Afr J Microbiol Res 4(17):1814–1822
Nur Asshifa MN (2009) The effects of *Pseudomonas aeruginosa* USM-AR2 culture containing rhamnolipid in facilitating crude oil distillation. Masters in science (biotechnology). Universiti Sains Malaysia, Penang

Rahman KSM, Rahman TJ, Kourkoutas Y, Petsas I, Marchant R, Banat IM (2003) Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. Bioresour Technol 90:159–168

Rashedi H, Assadi MM, Jamshidi E, Bonakdarpour B (2005) Production of rhamnolipids by *Pseudomonas aeruginosa* growing on carbon sources. Int J Environ Sci Technol 3(3):297–303

Raza ZA, Rehman A, Khan MS, Khalid ZM (2007) Improved production of biosurfactant by a *Pseudomonas aeruginosa* mutant using vegetable oil refinery wastes. Biodegradation 18(1):115–121

Rols JL, Goma G (1989) Enhancement of oxygen transfer rates in fermentation using oxygen vectors. Biotechnol Adv 7(1):1–14

Rosso D, Huo DL, Stenstrom MK (2006) Effects of interfacial surfactant contamination on bubble gas transfer. Chem Eng Sci 61:5500–5514

Sabra W, Kim EJ, Zeng AP (2002) Physiological responses of *Pseudomonas aeruginosa* PAO1 to oxidative stress in controlled microaerobic and aerobic cultures. Microbiology 148:3195–3202

Saharan BS, Sahu RK, Sharma D (2012) A review on biosurfactants: fermentation, current developments and perspectives. Genet Eng Biotechnol J 2011:GEBJ-29

Salwa MS, Nur Asshifa MN, Amirul AA, Ahmad Ramli MY (2010) Different feeding strategy for the production of biosurfactant from *Pseudomonas aeruginosa* USM-AR2 in modified bioreactor. Biotechnol Bioproc Eng 14:763–768

Shuler ML, Kargi F (2002) Bioprocess engineering: basic concepts. Prentice Hall, New Jersey

Singh A, Van Hamme JD, Ward OP (2006) Surfactants in microbiology and biotechnology: part 2. Application aspects. Biotechnol Adv 25:99–121

Sivaprakasam S, Mahadevan S, Gopalaraman S (2008) Oxygen mass transfer studies on batch cultivation of *P. aeruginosa* in a biocalorimeter. Elect. J Biotechnol 11(1):1–13

Soberón-Chávez G, Maier RM (2011) Biosurfactants: a general overview. In: Soberón-Chávez G (ed) Biosurfactants, microbiology monographs 20. Springer, Heidelberg

Wagner M, Popel HJ (1996) Surface active agents and their influence on oxygen transfer. Water Sci Technol 34(3–4):249–256