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

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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_La , 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_La , 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_La (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_La showed that k_La 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

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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_La), and hence oxygen transfer rate (OTR) and the oxygen uptake rate (OUR) by the microorganism. The k_La 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_La 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; Sivaprasam 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_La (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_La , 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 $-20\text{ }^{\circ}\text{C}$. 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) 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) MgSO_4 , 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 ($\text{OD}_{540} = 2$) was used as the inoculum.

Biomass quantification

Cells were harvested by centrifugation at $10\,000 \times g$ 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 (OD_{540}) with a spectrophotometer (Genesys 20, Model 4001-04, USA). The biomass, expressed in dry weight (g/L), was obtained by comparison to a previously constructed calibration curve, where 1.0 OD was equivalent to 0.5 g/L of cell biomass. The calibration curve was constructed by plotting the optical density (*y axis*) of cell suspension at different concentration against the cell dry weight (*x axis*). Samples with absorbance readings exceeding 0.5 were subjected to dilution to ensure cell density and absorbance linearity.

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 (OD_{421}) 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 (k_La) and oxygen transfer rate (OTR)

The volumetric oxygen transfer coefficient (k_La) 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):

$$\ln(C^* - C_L) = -k_Lat, \quad (1)$$

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):

$$\text{Impeller tip speed} = \pi ND, \quad (2)$$

where π is 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_La value was also performed by repeating the same static gassing out technique. The profile of k_La values over certain concentrations of rhamnolipid was determined for different impeller speeds: 400, 500 and 600 rpm at an air flow rate of 0.3 vvm.

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):

$$\text{Oxygen transfer rate (OTR)} = \frac{dC_L}{dt} = k_La(C^* - C_L), \quad (3)$$

where $\frac{dC_L}{dt}$ is the rate of change in oxygen concentration in the liquid phase, k_La 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_2} was calculated based on the relationship between the OUR and cell concentration, given by Eq. (4):

$$\text{Oxygen uptake rate (OUR)} = \frac{dC_L}{dt} = -q_{O_2} \times X, \quad (4)$$

where q_{O_2} 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 and the split ratio was 1:100. One microliter of diesel was injected. The oven temperatures were programmed from 50 to 280 °C at the rate of 10 °C/min and then isothermally held for 10 min.

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_La 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_La 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_La value also increases from 48.21 to 70.38 h^{−1}. When the agitation increases to 600 rpm, the k_La value is slightly lower, which is 65.31 h^{−1}. It is speculated that the k_La 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

Table 1 Effect of agitation speed on k_La (determined separately without biomass and rhamnolipid) in a 3.0 L stirred tank bioreactor in oil–water–surfactant system at an air flow rate of 0.3 vvm

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

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

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 different agitations of 400, 500 and 600 rpm in the medium is depicted in Fig. 1 for conditions without and with rhamnolipid addition. Rhamnolipid was added at 0.05 g/L to represent a low concentration to evaluate whether rhamnolipid affects oil dispersion, even at very low concentration. Referring to Fig. 1, diesel is most dispersed at 500 rpm, which could lead to easier uptake by the cells and rhamnolipid secretion. 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 compounds: undecane (1), dodecane (2), tetradecane (3), dodecane (4), hexadecane (5, 6), heptadecane (7, 8), octadecane (9, 10), heneicosane (11, 12), eicosane (13),

Fig. 2 Diesel oil extracted from the aqueous phase in an aluminum dish (indicated by arrow)

tetracosane (14), tetratriacontane (15), tetracontane (16, 17), and hexatriacontane (18, 19). Several studies reported the capability of *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).

Fig. 3 Time course of **a** growth, rhamnolipid production, **b** diesel utilization and **c** dissolved oxygen in batch cultivation of *P. aeruginosa* USM-AR2

The volumetric oxygen uptake rate (OUR) of *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_{O_2}) is 0.59 mmol/g/h. The q_{O_2} value is rather low when compared with those of most bacteria where the q_{O_2} value typically lies in the range of 2–12 mmol/g/h (Shuler and Kargi 2002). It is important to note that *P. aeruginosa* USM-AR2 is a facultative anaerobe organism which does not require high oxygen to grow. Another strain of *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 *P. aeruginosa* USM-AR2 when q_{O_2} was multiplied with the highest biomass, 9.4 g/L, was 5.55 mmol/L/h.

Throughout the cultivation, the OUR and q_{O_2} 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_La and OTR at different agitation rates in Table 2 documents that the lowest k_La (48.21 h⁻¹) and hence OTR

Fig. 4 Oxygen uptake rate (OUR), specific oxygen uptake rate (q_{O_2}) and growth (CDW) of *P. aeruginosa* USM-AR2 during batch cultivation in a bioreactor

Table 2 Comparison of oxygen transfer rate at different agitation speeds

Agitation (rpm)	OTR $k_{La} \times \Delta C$ (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 *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 *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_{La} 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_{La} 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

Fig. 5 Time course of growth, rhamnolipid production, diesel utilization and dissolved oxygen in batch cultivation of *P. aeruginosa* USM-AR2

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. 6 Effect of rhamnolipid addition on $k_L a$ at different agitation speeds in a 3.0 L stirred tank bioreactor

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 $k_L a$ typically increases when ion concentration in the solution is raised, due to its influence on decreasing bubble size and the non-coalescence effect (Garcia-Ochoa and Gomez 2009).

An increase of $k_L a$ 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 $k_L a$ 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 $k_L a$: 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 $k_L a$ and oxygen transfer, growth and rhamnolipid production by *P. aeruginosa* USM-AR2 achieved at this speed. In addition, $k_L a$ 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_L may inhibit mass transfer.

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Compliance with ethical standards

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

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