JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2014. Vol. **8**(Spl. Edn. 1), p. 783-789

Benchtop Scale Study of Rhamnolipid Production by *Pseudomonas aeruginosa* USM-AR2

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(Received: 08 January 2014; accepted: 24 March 2014)

The potential of a greener and cheaper substrate (waste cooking oil) in place of diesel for rhamnolipid production in laboratory scale was addressed in this investigation. In shake flasks study, rhamnolipid production was comparable when either diesel or waste cooking oil was used as the main carbon source. The yield was 2.15 g/L and 2.49 g/L for waste cooking oil and diesel, respectively. Rhamnolipid production with waste cooking oil as a carbon source was further studied in a 3.6 L bioreactor at two different agitation speed, 400 and 500 rpm. The agitation speeds were selected based on the similar volumetric mass transfer coefficient (k_La) of 10.8/h, as determined earlier in a non respiring system. 2.23 g/L of rhamnolipid was produced at 400 rpm compared to 1.09 g/L in broth and 1.80 g/L in foam obtained in the same bioreactor agitated at 500 rpm. Thus, waste cooking oil could be utilized as an alternative carbon source for a low cost rhamnolipid production.

Key words: Rhamnolipid,Waste cooking oil, Diesel, Volumetric mass transfer coefficient.

Rhamnolipid is a glycolipid-type of biosurfactantand has been utilized in various industries such as food, petroleum, agricultural, cosmetics, environmental, pharmaceuticals and biomedical sciences[1]due to their environmental compatibility. Nonetheless, mass production of biosurfactants is currently less economical compared to synthetic surfactants due to high production cost and low production yield.Theuse of cheaper raw materials and indigenous microbial strains were among the common strategies utilized to overcome the problems.

Pseudomonas aeruginosa is a widely studied microorganism for rhamnolipid production. It is capable of utilizing different types of hydrophobic and hydrophilic type of carbon sources for high rhamnolipid production (Table 1). The highest production was from using plant base or vegetable oil as a carbon source.

Previously, an indigenous isolate, *Pseudomonas aeruginosa* USM-AR2, has been reported to produce rhamnolipid at a relatively high yield using diesel as a carbon source in a fed batch culture[8]. However, diesel's primary usage as transportation fuels will incur a prohibitively high cost to the production process. Hencea cheaper plant based substrate,waste cooking oil (WCO), was investigated for its potential as an alternative carbon source in producing high yields of rhamnolipid. Around the world, about 15 million tonnes of WCO were generated[9] and it could cause major water pollution if proper disposal and management were not implemented. So, WCO is suitable as a cost effective carbon source in low

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cost production ofbiosurfactant, while simultaneously contribute in minimizing environmental contamination.

The growth of *Pseudomonas aeruginosa* USM-AR2 was studied in abenchtop scale bioreactor with different stirring speedswhile maintaining aeration rate at the lowest value possible. The speed was selected based on the optimum volumetric mass transfer coefficient,$k_L a$, predetermined in a non fermentative system using static gassing out technique. Results of this work provided information for optimum production ofrhamnolipid in batch culture at benchtop scale.

MATERIALS AND METHODS

Activation of the microorganism

A glycerol stock was cultured in 100 ml shake flasks containing 20 ml nutrient broth at 25°C with 200 rpm agitation on a shaker (Certomatâ R, B. Braun). A loopful of broth was streaked on nutrient agar (HiMedia) andincubated at 25 °C. A single colony was transferred into 50 ml of nutrient broth and incubated at the same condition previously.

Inoculum preparation

Inoculum medium for shake flasks experiment comprised 0.05% (w/v) $MgSO_4.7H_2O$ (Bendosen), 0.6% (w/v) yeast extract (Oxoid), 0.4% (w/v) of glucose and 7% (v/v) waste cooking oil from a local campus cafe or diesel obtained from a local gas station. Similar composition of inoculum medium was applied for bioreactor experiments with the exception of glucose not being added and 4% (v/v) of WCO was used[10]. The inoculum medium was inoculated with 2% (v/v) of nutrient broth culture and incubated as previous.*Fermentation condition*

Pseudomonas aeruginosa USM-AR2 was grown in 500 ml shake flasks containing 100 ml of production medium with identical composition of the inoculum medium but without the addition of glucose, agitated at 200 rpm on an orbital shaker (Certomatâ R, B. Braun)for 6 days at 27 °C.Meanwhile, in 3.6 L bioreactor (Labfors 4, Infors HT, Switzerland) it was grown with a similar medium composition as the inoculum medium with 1.5 L working volume.The cultivation was set to run at 28°C, 400 rpm or 500 rpm agitation and 0.3 vvm (0.5 L/min) aeration without pH control. Samples were taken at predetermined intervals for rhamnolipid and cell dry weight measurements.

Critical oxygen level (C_{crit}) determination

At the end of fermentation, aeration and agitation was stopped and the decline in dissolved oxygen level was recorded continuously with DO probe (InPro6820Mettler Toledo) for critical oxygen level, C_{crit} determination. It is the point at which the decline in oxygen concentration deviates from linear relationship with time.

Determination of $k_L a$

$k_L a$ was determined in a non- fermentative system by static gassing out method[11].Increase in DO (%) was measured using a polarographic O_2 sensor (InPro6820 Mettler Toledo) and the data was captured by IRIS Software, Version 5.2. The effect of agitation speed on $k_L a$ was studied between 200 to 1000 rpm agitation. Cell-free broth containing rhamnolipidwas serially diluted to give concentrationsbetween 0 to 2.0 g/L and was used to study the effect of rhamnolipid concentrations on $k_L a$. During the experiment, the aeration was maintained at 1 vvm, the temperature at 28°C with 1.5 L working volume in distilled water system.

Analytical method

Cell growth was measured using a spectrophotometer (Genesys 20, Model 4001-04, USA) at 540 nm and expressed as cell dry weight (g/L) calculated from cell dry weight standard curve.Rhamnolipid production is quantified based on rhamnose (Acros) concentration using orcinol-sulphuric method[12]. First, 0.19% (w/v) of orcinol (Acros) was dissolved in 53% (v/v) of sulphuric acid (QReC). Then, 0.3 ml sample was mixed with 2.7 ml of orcinol-sulphuric acid solution and heated at 80°C for 30 minutes. The absorbance of the mixture was determined at 421 nm using a spectrophotometer (Genesys 20, Model 4001-04, USA) after the solution was allowed to cool at room temperature. A calibration curve was plotted using a standard solution of rhamnose with concentrations ranging from0 to 0.1g/L.

RESULTS AND DISCUSSION

Rhamnolipid production from two different carbon sources, waste cooking oil and diesel

In shake flasks study, rhamnolipid production grown in diesel oil was slightly higher with 2.49 g/L rhamnolipid compared to 2.15 g/L of rhamnolipid grown in waste cooking oil (Figure

1).Rhamnolipid concentration rapidly increased after 72 hours of incubation using both carbon sources with maximum productivity of 0.034 g/L/h for WCO and 0.030 g/L/h for diesel. During this maximum productivity, the yield of product over biomass ($Y_{p/x}$) was 0.9 and 1.1 for WCO and diesel, respectively. The maximum biomass was 12.5 g/L at 102 hour of incubation and 10.6 g/L at 120 hour of incubation for WCO and diesel, respectively.

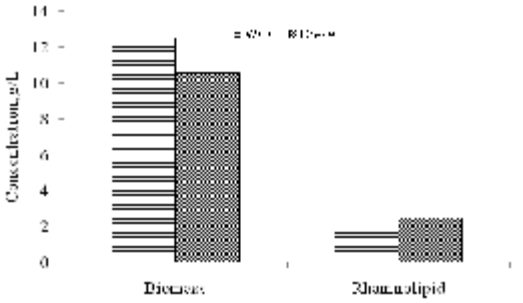


Fig. 1. Maximum biomass and rhamnolipid production with different carbon sources

Commercial diesel for car fuels contained (by weight percent) 84% to 86% of carbon[13] while base on analysis by CHN Analyzer, WCO contain around 76% to 77% of carbon. Carbon molecules were slightly lower in WCO compared to diesel, which translate into lower carbon to nitrogen ratio for medium with WCO. Therefore more biomass was formed through nitrogen metabolism at lower C/N ratio. Furthermore, WCO contains about 30% (v/v) polar compounds such as free fatty acid, after long and repetitive frying at a rather high temperature[14], which made it more soluble with aqueous phase. Hence, improved miscibility of WCO with fermentation medium might have accounted for the increased bioavailability of the carbon source for cell consumption. However, higher biomass with WCO did not support high rhamnolipid production.

Wei and coworkers, 2005 reported less rhamnolipid production in diesel compared to other hydrophilic and hydrophobic carbon sources such as glycerol, glucose, olive oil, sunflower oil and grape seed oil. The maximum production was 1.3 g/L of rhamnolipid with 6% of diesel. Using fed-batch fermentation, a maximum of 13.4 g/L rhamnolipid with diesel was produced in 2.5 L bioreactor[8]. Recently, 6.6 g/L of rhamnolipid was produced with waste frying oil in a shake flasks experiment and further improved to 8.5 g/L of rhamnolipid through fed-batch fermentation[16]. Rhamnolipid production increased 63% from 2.8 g/L to 7.5 g/L when grown in waste frying oil treated with activated earth treatment to reduce toxic compound such as peroxide that may interfere with the oils metabolism[17]. With a mutant strain, *Pseudomonas aeruginosa* zju.u1M, rhamnolipid production was enhanced to 24.61 g/L when grown on waste frying oil[10].

In this study, rhamnolipid production was lower with WCO compared to the production reported in literature[16]. However, the experiment conducted was not at the optimum condition using wild type strain of indigenously isolated *Pseudomonas aeruginosa* strain. But with the promising rhamnolipid production reported with WCO, the potential of this cheap and greener substrate is worth to be explored further.

Effect of agitation speed on rhamnolipid production

Rhamnolipid production was studied in a 3.6 L bioreactor, Labfors 4. For an aerobic fermentation, the oxygen supply must be sufficient for growth and production. Oxygen transfer rate (OTR) must be at least equivalent to the oxygen uptake rate (OUR). k_La was measured at different agitation speeds with constant aeration of 1 vvmin a non fermentative system. It was found that, k_La increased with agitation speed and reached an optimum value (28.8/h) at 400 rpm and constant afterwards (Figure 2).Therefore,rhamnolipid production was compared at 400 rpm and 500 rpm at lower aeration (0.3 vvm) with k_La at both agitations was 10.8/h.

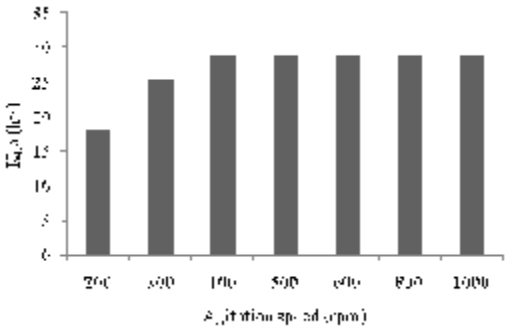


Fig. 2. Effect of agitation speed on k_La with aeration maintained at 0.3 vvm

The value of k_La determined in a non-fermentative system was only correct during the absence of biosurfactant. In a biosurfactant

Table 1. Rhamnolipid production from various strains of *Pseudomonas aeruginosa* with hydrophobic and hydrophilic type of carbon sources

Production Strain	Carbon source	Process	Production scale (L)	Rhamnolipid$_{max}$ (g/L)	Productivity (g/L/h)	Reference
PAO1	Sunflower oil	Batch	30	39.0	0.433	Müller *et al.*[2] (2010)
BYK-2 KCTC 18012P	Fish oil	Batch	3	17.0	0.079	Lee *et al.*[3] (2004)
BYK-2 KCTC 18012P	Fish oil	Fed Batch	3	22.7	0.086	Lee et al.[3] (2004)
LB1	Soap stock	Fed Batch	1.2	15.9	0.2	Benincasa *et al.*[4] (2002)
S2	Glucose	Fed Batch	2	9.4	0.026	Chen *et al.*[5] (2007)
LB1	Soap stock	Fed Batch	2	16.9	0.14	Lovaglio *et al.*[6] (2010)
O-2-2	Soybean oil	Fed Batch	2.5	70	0.73	Zhu *et al.*[7] (2012)

production process, $k_L a$ value was greatly affected by the concentration of rhamnolipid produced (Figure 3a). It was found that $k_L a$ fluctuated with the increase of rhamnolipid concentration in the fermentation medium. It increased from 10.8/h at 0 g/L concentration to 31.68/h at 1.0 g/L and dropped to 23.28/h at 2.0 g/L of rhamnolipid. However, in this study the addition of oil in the fermentation system exhibited minimal effect on $k_L a$ (Figure 3b). Nonetheless, mass transfer was not limiting since oxygen transfer rate (OTR) calculated at the lowest $k_L a$ value, 12.1 mmol/Lh was higher than maximum oxygen uptake rate (OUR), 3.53 mmol/Lh of the microorganism used in this study.

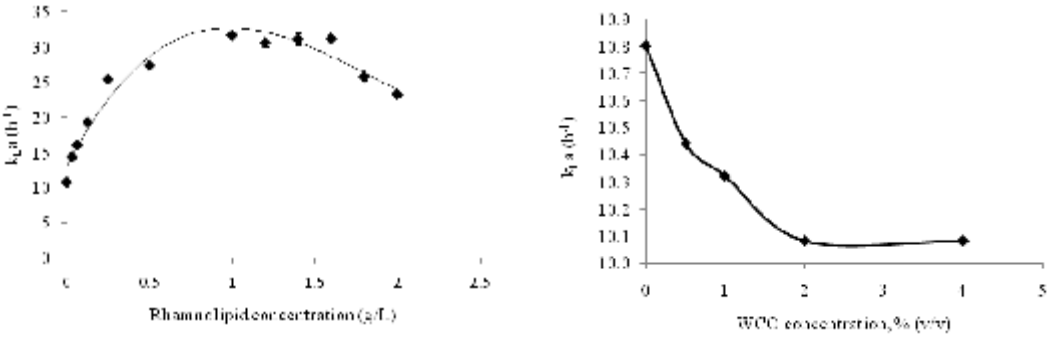
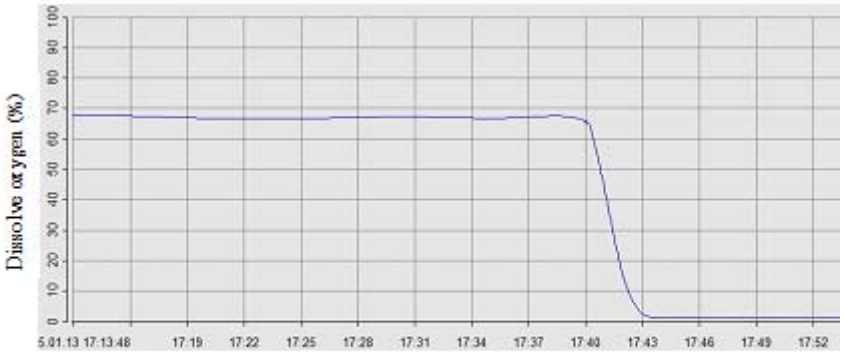


Fig. 3. Effect of rhamnolipid and WCO concentration on $k_L a$

Lower aeration which would create a microaerobic condition was selected due to minimum requirement of dissolved oxygen level by *Pseudomonasaeruginosa* for improved rhamnolipid production[18, 19, 20].The lower oxygen requirement was supported with low critical oxygen level (C_{crit}) of the microorganism determined in this fermentation system, which was around 3.7% to 4.2% (Figure 4). However, anerobic fermentation of *Pseudomonas aeruginosa* for rhamnolipid production was not preferred since the production was less compared to aerobic fermentation[21]. It was proven that, oxygen was required to stimulate Pseudomonas Quinolone Signal (PQS), a component of quorum sensing (QS) system which regulates the production of *Pseudomonas aeruginosa* virulence factors which include rhamnolipid[22]. In addition, the problem with intense foaming due to higher aeration could be minimized with lower aeration[23]. So, rhamnolipid production was conducted at very low aeration in this investigation.



(a)

(b)

Fig. 4. Dissolved oxygen profile for critical oxygen level, C_{crit}, determination of *Pseudomonas aeruginosa* USM-AR2 from two different fermentation batches.

Figure 5

Production was slightly higher at lower agitation speed. At 400 rpm (0.230 m/s tip speed), the maximum biomass was 11.13 g/Lcompared to only 7.85 g/L (biomass in broth) and 9.75 g/L (biomass in foam) for 500 rpm (0.288 m/s). The maximum rhamnolipid production was attained at the end of fermentation time for both experiments. The highest rhamnolipid concentration for the lower tip speed was 2.23 g/L with 0.023 g/L/h productivity (Figure 5). Conversely, lowerrhamnolipid concentration was obtained at the higher tip speed, 1.09 g/L and 1.80 g/L in broth and foam, respectively.The productivity based on total concentration was 0.021 g/L.

It was known that rhamnolipid production was regulated by quorum sensing, a cell-to-cell communication system, which was cell-density dependent[24]. Eventhough direct relation between rhamnolipid production and cell density was not shown, Zhu and co workers, 2012, stated that cell biomass should be maintained within a certain range for high rhamnolipid production. In their case, the biomass was retained above 30 g/L. Higher agitation caused foam formation to occur and consequently caused partitioning of nutrient and biomass into the foam. Since mass transfer is less efficient in foam (non-agitated), the cells in the foam are less productive for both biomass and rhamnolipid. This caused less cells remained in broth; therefore low biomass andrhamnolipid were generated.

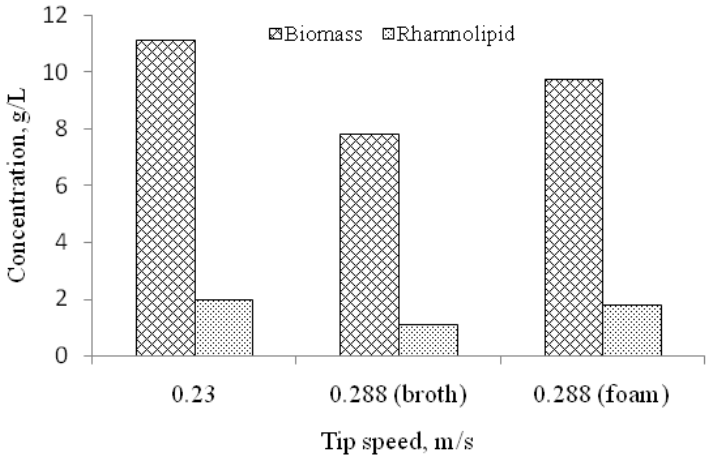


Fig. 5. Maximum rhamnolipid and biomass concentration at two different tip speeds. At lower tip speed, the concentration was determined from broth only

de Lima and co workers, 2009, stated a maximum of 3.3 g/L rhamnolipid was produced at 550 rpm agitation and 0.5vvm aeration with $k_L a$ of 10.2/h. Meanwhile, 19.54 g/L of rhamnolipid was obtained in 50 L bioreactor, with 25 L working volume and 300 rpm using mutant strain zju.1M[10]. Therefore, the condition used and investigated in this study was within the range reported by other researcher to obtain optimum production of rhamnolipid.

CONCLUSION

A comparablebiosurfactant production was attained, 2.49 g/L and 2.15 g/L of rhamnolipid when diesel and waste cooking oil was used as carbon source, respectively, suggesting that WCO could replace diesel as a good carbon source for rhamnolipid production. In the small scale bioreactor, rhamnolipid production was somewhat higher (2.23 g/L) at lower tip speed compared to higher tip speed (1.09 g/L in broth and 1.80 g/L in foam). The finding in this study shows that waste cooking oilmay be a promising carbon source for a low cost production of rhamnolipid.

ACKNOWLEDGEMENTS

The authors would like to acknowledge UniversitiSains Malaysia (USM) for supporting this research through grant noAPEX. 1002/PBIOLOGI/ 910308.

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