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# Fed-batch Cultivation of *Pseudomonas Aeruginosa* USM-AR2 Producing Rhamnolipid in Bioreactor through Pulse Feeding Strategy

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**Abstract.** *Pseudomonas aeruginosa* USM-AR2 culture was proven to produce a glycolipid type biosurfactant, rhamnolipid. Fed-batch cultivation of *P. aeruginosa* USM-AR2 with different feeding strategies was carried out in bioreactor to increase the rhamnolipid production and productivity. The quantification of cell biomass, rhamnolipid produced and rhamnolipid activity were determined using absorbance measurement, orcinol assay and surface tension measurement. Batch cultivation in bioreactor gave a biomass of 9.4 g $L^{-1}$ and rhamnolipid of 2.35 g $L^{-1}$ in 72 hours. Fed-batch cultivation with pulse feeding strategy using diesel as carbon substrate was carried out at different feeding time interval. The best feeding mode was recorded to be 12 h-feeding interval which resulted in 12.6 g $L^{-1}$ of biomass and 3.13 g $L^{-1}$ of rhamnolipid achieved in shorter time, 48 hours cultivation. Higher productivity was achieved by gradually feeding the carbon source while maintaining the optimal culture conditions, which was 0.065 g $L^{-1}$ $h^{-1}$ compared to batch culture, 0.033 g $L^{-1}$ $h^{-1}$.The highest biomass and rhamnolipid production, 24.1 g $L^{-1}$ and 13.4 g $L^{-1}$, respectively was obtained when multiple substrates were fed (diesel and yeast extract). However the productivity was lower, 0.054 g $L^{-1}$ $h^{-1}$ due to the extension of working time.

**Keywords:** *Pseudomonas aeruginosa,* Rhamnolipid, Biosurfactant, Diesel, Feeding strategies

## 1. Introduction

Biosurfactants were first discovered as extracellular amphiphilic compounds in research of hydrocarbon fermentation, which started in the late 1960s. They are produced by bacteria, yeasts and fungi growing on a water-immiscible substrate, to facilitate uptake of those substrates by the cell [1,2]. The increase of interest in the production of biosurfactants is motivated by the biological properties include their higher biodegradability, lower toxicity, better environmental compatibility, lower critical micelle concentration, higher surface activity, and the ability to be synthesized from renewable sources [1,3].

Most microbial surfactants are complex molecules which have both clearly defined hydrophobic and hydrophilic groups. They reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids or of a fluid and solid, consequently increase the surface areas of insoluble compounds leading to increased mobility, bioavailability and subsequent biodegradation [4]. Among bacteria, *Pseudomonas* species, especially *Pseudomonas aeruginosa* is well known for its capability to produce rhamnolipid biosurfactant when grown on different carbon substrates, especially in hydrocarbon rich medium. In particular, it offers special advantages because of their potent emulsifying activity and low critical micelle concentration [5,6]. The rhamnolipids from *P. aeruginosa* were first described in 1949 by Jarvis and Johnson, and studies on the biosynthesis of these compounds were carried out in vivo by Hauser and Karnovsky, who showed that these glycolipids were secreted into the medium during the stationary phase of growth when nitrogen is depleted in the medium [7].

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Numerous reports are available on rhamnolipid fermentation in aerobic processes that is suffered from heavy foaming even at low product concentration. Foaming is usually alleviated by mechanical foam breakers and/or chemical antifoaming agents. However, the foams in rhamnolipid fermentation appeared to be extremely fast and were too stable for these methods. This is generally caused by bubble aeration, stirring, and the presence of rhamnolipid itself [5]. Therefore, in this study, a special design of bioreactor was proposed by integrating a foam collector in its gaseous outlet. Foam containing biosurfactants were allowed to flow out of the bioreactor from the air outlet, collected in a separate vessel, and recycled back into the fermentation vessel [8,9]. An innovative bioreactor developed in this work was able to handle the foaming problem associated with production of rhamnolipid without the addition of any antifoam agent. The bacterial strain used in this research was *Pseudomonas aeruginosa* USM-AR2, isolated and identified as hydrocarbon utilizing and rhamnolipid producing bacterium [10]. Different fed-batch strategies were applied in bioreactor cultivation to enhance rhamnolipid production and its productivity.

# 2. Materials and Method

## 2.1. Production of Biomass and Rhamnolipid in Bioreactor

A suitable medium has been formulated from previous study containing 7% (v/v) diesel, 0.6% (w/v) yeast extract, 0.05% (w/v) $MgSO_4$ and 0.05% (v/v) Tween 8 [11]. Fermentations were carried out in 2.5 L bioreactor with 1 L working volume (Model Biostat B, B. Braun) equipped with Multi Function Control System (MFCS) software. During the course of fermentation, culture broth was periodically collected and analyzed for growth and rhamnolipid production. The control objective is met by a cascade control strategy which automatically changed the agitation speed with the goal of maintaining dissolved oxygen at 30% saturation. Pulse feeding strategy was carried out following batch cultivation in bioreactor at different feeding intervals, 6-h and 12-h. Further strategy was continued with multiple substrate feed, diesel and yeast extract. Initial diesel of 30 mL was supplemented and starting feeding time was set at 12 h, when the initial 30 mL diesel was depleted. Five mL diesel was fed per pulse.

## 2.2. Biomass Quantification

Cells were harvested by centrifugation at 10 000 x *g* for 5 min, treated with acetone to remove the adhering hydrocarbon (diesel) and washed twice with distilled water to remove traces of nutrient. The cell pellet was suspended in 3 mL of distilled water and the biomass, expressed in dry weight (g $L^{-1}$), was obtained from a calibration curve. Bacterial growth was monitored by $OD_{540}$ nm with a spectrophotometer (Genesys 20, Model 4001-04, USA).

## 2.3. Rhamnolipid Quantification: Orcinol Assay

The quantification of the rhamnolipids was carried out through an indirect way, using rhamnose as reference as rhamnose is a byproduct of acid hydrolysis of rhamnolipids [12]. To 0.3 ml of each sample, 2.7 ml of a solution containing 0.19% orcinol (in 53% $H_2SO_4$) was added. After heating for 40 min at 70 ºC the samples were cooled at room temperature and the $OD_{421}$ nm was measured using spectrophotometer. The rhamnolipid concentrations were calculated from a standard curves prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (g $L^{-1}$).

## 2.4. Surface Tension Measurement

Biosurfactant activity was evaluated by surface tension measurement of distilled water mixed with culture with a Surface Tension Analyzer. Samples were withdrawn every 24 hours to analyze the surface activity. 10 mL of the culture was mixed with 70 mL of distilled water in the cylinder. The apparatus was set up vertically in a 30 °C water bath. Air was expelled through the tubulation until sample comes out of the top of the capillary. The meniscus inside the capillary was allowed to come to equilibrium. The distance between the meniscus inside the capillary and the meniscus inside the cylinder was recorded. The surface tension was calculated from the following equation, $\gamma = (1/2)\,(h)(r)(\rho)(g)$, where :

$\gamma$= surface tension (dynes/cm)
h= distance between meniscus (cm)
r= radius of capillary (cm) = 0.025 cm

ρ= density of sample (g/cm$^3$ at measuring temperature)
g= acceleration due to gravity (cm/s$^2$) = 981 g cm/s$^2$

## 3. Results and Discussion

Batch cultivation of *P. aeruginosa* USM-AR2 showed its growth (9.4 g L$^{-1}$) was accompanied by rhamnolipid production (2.35 g L$^{-1}$) (Figure 1). They produce rhamnolipid as they breakdown the diesel existed in the medium. This is because the contact between the cells and hydrocarbon droplets are a prerequisite for its growth. Enhanced rhamnolipid production was established at the stationary growth phase, suggesting the accumulation of rhamnolipid as secondary metabolites [13].

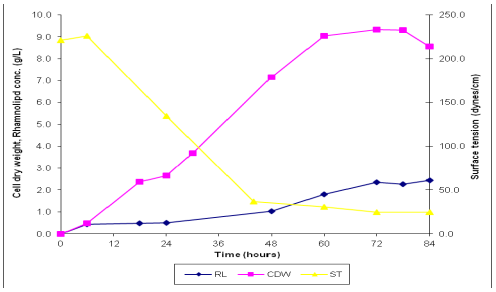


Fig. 1: Growth, rhamnolipid production and surface tension reduction in batch culture of *P. aeruginosa* USM-AR2

Fed-batch might be the only option for fermentation dealing with low solubility substrates such as diesel, as the high proportion of unproductive down time in batch operation may reduce the overall productivity of the process. In this study, diesel was used as the main feed as it was proven to be efficient in rhamnolipid production (data not shown) [11]. The performance of 6-h interval strategy was no different from batch cultivation. The feed seems excess as after 24 h, the diesel added had no effect in oxygen consumption. Cells were no longer utilizing oxygen as quick as the consumption during the early cultivation period.

In 12-h interval strategy, when diesel was fed at 12-h, stirrer increased to 500 rpm indicating that cells grow actively by consuming oxygen (Figure 2). The increase of agitation rate indicated an increment in the uptake of dissolved oxygen level (DO) by the culture. At this point, carbon source was the limiting factor for the bacterial growth, where the substrate (diesel) pumped in was directly used by the cells. The highest biomass was 12.6 g L$^{-1}$, while rhamnolipid was produced at 3.13 g L$^{-1}$ and achieved in a shorter time, after 48 h (Figure 3) compared to 72 h in batch and fed-batch at 6-h interval (not shown). Higher productivity compared to the batch culture was in general agreement with fed-batch principle. In addition, the excess diesel present in the culture broth (as in batch and 6-h interval feeding) due to thick diesel layer may inhibit oxygen transfer into the cells, thus resulted in slower growth as oil is insoluble in water. As the fermentation progressed, the oil was utilized by the cells. The reduction of oil after some time enhanced oxygen transfer into cells and promoted growth.

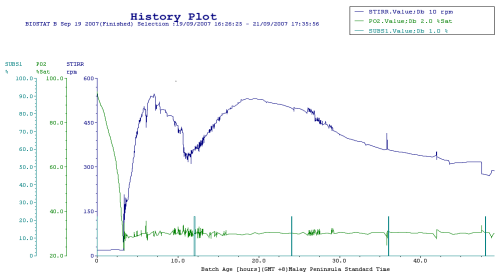


Fig. 2: Profile of dissolved oxygen, agitation and 12-h interval feeding in *P. aeruginosa* USM-AR2 culture.

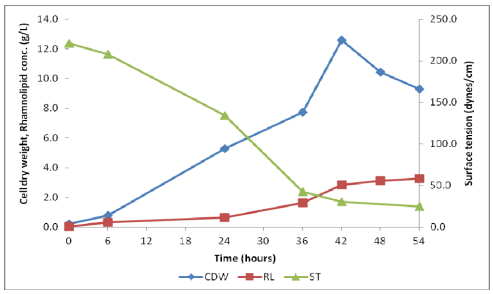


Fig. 3: Growth, rhamnolipid production and surface tension reduction in 12-h interval fed-batch culture of *P. aeruginosa* USM-AR2

Referring to MFCS graph (Figure 2), feeding at 36 and 48 h did not affect oxygen utilization by the cells, indicated by the decrease in stirring. This indicated another limiting factor that may inhibit the growth, which

is believed to be nitrogen. Usually, the limitation of hydrocarbon utilization by the cells is due to a nutritional imbalance between the carbon supplied and the nitrogen required for microbial growth [14]. Therefore, other nutrient that contains nitrogen should be fed whenever needed. From the dissolved oxygen and agitation profile, the feeding of diesel should be held after the second feeding (at 24 h), where the second substrate should be initiated. Therefore, in further cultivation, fermentation was carried out with multiple substrates feeding which were diesel and yeast extract. Organic nitrogen source such as yeast extract are reported to stimulate the growth of *P. aeruginosa* strain [15]. In addition, the step taken by feeding yeast extract together with diesel was to improve substrate affinity, as the presence of yeast extract may engender high hydrocarbon degradation by supporting adequate biomass to carry out the substrate utilization. Result obtained showed that the growth of *P. aeruginosa* USM-AR2 and rhamnolipid production was enhanced by a mixture of substrates, supporting that growth and production may not be controlled by only a single nutrient but by two or more nutrients simultaneously. Figure 4 illustrated the performance of fed-batch cultivation with multiple substrate feeding in *P. aeruginosa* USM-AR2 culture producing rhamnolipid.

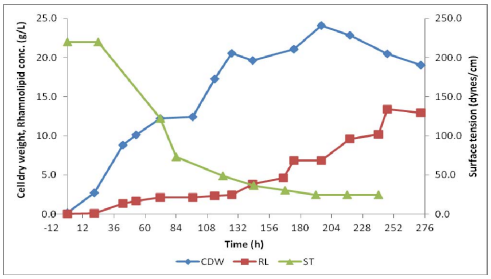


Fig. 4: Growth, rhamnolipid production and surface tension reduction in fed-batch culture of *P. aeruginosa* USM-AR2 with multiple substrate feeding

Although the rhamnolipid concentration was the highest compared to all runs, the productivity of rhamnolipid was low, 0.054 g $L^{-1}$ $h^{-1}$ because of a long period needed to produce such amount. However, the advantage of this strategy is the production of high cell densities and product due to the extension of working time. The details of biomass and rhamnolipid production of *P. aeruginosa* USM-AR2 at different strategies were summarized in Table 1. In comparison, cultivation at 12-h interval strategy gave the highest productivity, 0.065 g $L^{-1}$ $h^{-1}$.

Table 1: Details on biomass and rhamnolipid production by *P. aeruginosa* USM-AR2 at different batch cultivations

| **Run/Feeding strategy** | **Biomass (g $L^{-1}$)** | **Highest rhamnolipid(g$L^{-1}$)** | **Productivity (g $L^{-1}$ $h^{-1}$)** | **Surface tension (dynes/cm)** |
|---|---|---|---|---|
| Batch | 9.4 | 2.35 | 0.033 | 24.4 |
| Diesel 6-h interval | 9.6 | 2.48 | 0.034 | 24.4 |
| Diesel 12-h interval | 12.6 | 3.13 | 0.065 | 24.4 |
| Diesel and yeast extract | 24.1 | 13.40 | 0.054 | 24.4 |

Rhamnolipid produced by *P. aeruginosa* USM-AR2 had the ability to lower the surface tension as can be observed in all cultivations (Table 1). The main physiological role of rhamnolipid is to permit the bacteria to grow on hydrocarbon substrate by reducing the surface tension at the phase boundary, therefore making the hydrophobic substrate more readily available for uptake and metabolism [13]. A special property of rhamnolipid is their ability to reduce the surface tension of water to below 35 dynes/cm [16]. From this study, the minimal surface tension values were recorded at 24.4 dynes/cm, which coincided with the initiation of rapid increase in rhamnolipid secretion.

In summary, growth, rhamnolipid production and productivity has been improved from batch to fed-batch cultivation of *P. aeruginosa* USM-AR2 in bioreactor. Moreover, the multiple-substrate pulse feeding of yeast extract and diesel was effective in improving the growth of *P. aeruginosa* USM-AR2, thus enhancing rhamnolipid production. However, the productivity is low compared to 12-h interval feeding that resulted in the highest productivity.

## 4. Acknowledgements

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## 5. References

[1] G.R. Dehghan Noudeh, B.S.F. Bazaz, and M.R. Housaindokht. Comparative study of the hemolytic and surface activities of the biosurfactant produced by *Bacillus subtilis* ATCC6633 with some synthetic surfactants. *Iranian J. Basic Med. Sci.* 2003, **6**(19): 6-13.

[2] A. Singh, J.D. Van Hamme, and O.P. Ward. Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotechnol. Adv.* 2007, **25**(1): 99-121.

[3] S.B. Batista, A.H. Mounteer, F.R. Amorim, and M.R. Totola. Isolation and characterization of biosurfactant/bioemulsifier-producing bacteria from petroleum contaminated sites. *Bioresour Technol.* 2006, **97**(6): 868-875.

[4] E.Z. Ron, and E. Rosenberg. Natural roles of biosurfactants. *Environ. Microbiol.* 2001, **3**(4): 229-236.

[5] H. Rashedi, M.M. Assadi, B. Bonakdarpour, and E. Jamshidi. Isolation and production of biosurfactant from *Pseudomonas aeruginosa* isolated from Iranian southern wells oil. *Int. J. Environ. Sci. Technol*. 2005, **2**(2): 121-127.

[6] Z. Ali Raza, A. Rehman, M.S.Khan, and Z.M. Khalid. Improved production of biosurfactant by a *Pseudomonas aeruginosa* mutant using vegetable oil refinery wastes. *Biodegradation.* 2007, **18**(1): 115-121.

[7] A.K. Koch, O. Kappeli, A. Fiechter, and J. Reiser. Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. *J. Bacteriol.* 1991, **173**(13): 4212-4219.

[8] M.S. Yeh, W. Yu-Hong, and C. Jo-Shu. Bioreactor design for enhanced carrier-assisted surfactin production with *Bacillus subtilis*. *Proc. Biochem*. 2006, **41**(8): 1799-1805.

[9] M.S. Salwa, M.N. Nur Asshifa, A.A. Amirul, and M.Y. Ahmad Ramli. Different feeding strategy for the production of biosurfactant from *Pseudomonas aeruginosa* USM-AR2 in modified bioreactor. *Biotechnol. Bioproc. Eng.* 2010, **14**: 763-768.

[10] M.N. Nur Asshifa, A.A. Amirul, M.I. Mohamad Nasir, and M.Y. Ahmad Ramli. Rhamnolipid produced by *Pseudomonas aeruginosa* USM-AR2 facilitates crude oil distillation. *J. Gen. Appl. Microbiol.* 2012, **58**(2): 153-161.

[11] M.N. Nur Asshifa. *The effects of Pseudomonas aeruginosa USM-AR2 on crude oil biomodification and its potential in facilitating distillation.* Master of Science thesis (Biotechnology), Universiti Sains Malaysia, 2009.

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

[13] J.D. Van Hamme, A. Singh, and O.P. Ward. Recent advances in petroleum microbiology. *Microbiol. Mol. Biol. Rev.* 2003, **67**(4): 503-549.

[14] J.T. Dibble, and R. Bartha. Effect of environmental parameters on the degradation of oil sludge. Appl. *Environ. Microbiol.*1979, **37**(4): 729-739.

[15] G.L. Zhang, W. Yue-ting, Q. Xin-ping, and M. Qin. Biodegradation of crude oil by *Pseudomonas aeruginosa* in the presence of rhamnolipids. *J. Zheijiang University Sci.* 2005, **6B**(8): 725-730.

[16] J.D. Desai, and I.M. Banat. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.* 1997, **61(**1): 47-64.

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