Comparison between batch and fed-batch production of rhamnolipid by *Pseudomonas aeruginosa*

Mohammad Ghomi Avili, Mohammad Hassan Fazaelipoor*, Seyed Ali Jafari, Seyed Ahmad Ataei

Department of Chemical Engineering, Shahid Bahonar University of Kerman, P.O. Box 76169133, Kerman, I.R. Iran

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Abstract
This paper presents a comparison between batch and three different sets of fed batch fermentations for rhamnolipid production by *Pseudomonas aeruginosa*. The batch run was performed with 500 ml of culture medium having the initial glycerol and sodium nitrate concentrations of 30 and 8.3 g/l, respectively. For a fed batch run with nitrogen source in feed, 250 ml of the nitrogen excluded culture medium was in the bioreactor initially, and 250 ml culture medium containing 16.6 g/l sodium nitrate was fed to the bioreactor continuously. A similar procedure was repeated for fed batch runs with carbon, and phosphorus source in feed. Statistical analysis showed that fed batch runs were better than batch in term of rhamnolipid production, and among the fed batch runs the maximum amount of rhamnolipid. (4.12 g Rhamnose Equivalent/l) was for the fed batch run with the carbon source in feed.
Keywords**:** *Pseudomonas aeruginosa*; Rhamnolipid; Batch process; Fed batch process; Statistical analysis

INTRODUCTION

Biosurfactants or microbial surfactants are surface active biomolecules which are produced by a variety of microorganisms (Muthusamy *et al*., 2008). *Pseudomonas aeruginosa* produces mono and dirhamnolipid which are glycolipid type biosurfactants (Homayoon *et al*., 2010). Having such properties as higher biodegradability and lower toxicity compared to synthetic surfactants, biosurfactants have gained potential industrial applications. The major problems for industrial scale application of biosurfactants are low production yields and high recovery and purification costs (Muthusamy *et al*., 2008). Rhamnolipids are among the best characterized and the most effective biosurfactants known today (Salwa *et al*., 2009). Various factors such as the type of carbon and nitrogen sources, pH, temperature, aeration rate and agitation affect rhamnolipid production by *Pseudomonas aeruginosa* (Desai and Banat, 1997). A survey on the literature shows that most of the existing papers on rhamnolipid discuss the optimization of culture medium ingredients (Rodrigues *et al*., 2006 a,b), isolation of new rhamnolipid producer strains (Homayoon *et al*., 2010; Youssef *et al*., 2004), application of rhamnolipid in soil bioremediation (Urum *et al*., 2005; Rahman *et al*., 2002), and the use of agro-industrial wastes for rhamnlipid production (Wei *et al*., 2005; Dubey and Juwarkar, 2001).

Rhamnolipid is produced during the stationary phase and therefore batch fermentation is logically used to produce this material. Limitation of a nutrient in the microbial culture has been confirmed to promote rhamnolipid production (Sobern-chavez, 2011), and fed batch fermentation might be used to impose nutrient limitation for effective production of rhamnolipid.

*Correspondence to: **Mohammad Hassan Fazaelipoor, Ph.D.**
Tel: +98 913 3551807; Fax: +98 341 2118298
E-mail: fazaelipoor@uk.ac.ir; fazaelipoor@yahoo.com

Only a few reports exist on the fed batch production of rhamnolipid. Lee *et al*. (2004) compared batch and fed batch fermentation for rhamnolipid production using fish oil as the carbon source. The fermentation started with 25 g/l fish oil, and intermittent addition of fish oil to the microbial culture was adopted as the feeding strategy. The final concentration of rhamnolipid was 22.7 g/l after 264 h which was 1.3 fold greater than the concentration obtained in batch mode. Chen *et al*. (2007) used a pH-stat fed batch fermenter for rhamnolipid production and demonstrated the superiority of fed batch fermentation for the production of rhamnolipid. To the best of our knowledge, however, no exact comparison has been performed between batch and fed batch fermentation for rhamnolipid production. In this research work batch and fed-batch processes were compared for rhamnolipid production by *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Growth medium: A basal mineral medium with the following composition was used for microbial growth (Homayoon *et al*., 2010): 0.2 g/l $MgSO_4.7H_2O$, 0.04 g/l $CaCl_2.2H_2O$, 0.04 g/l $FeSO_4$. The mineral medium was supplemented with a trace element solution comparised of: 0.068 g/l $ZnSO_4$, 0.006 g/l $CuSO_4$, 0.07 g/l $NaMoO_4$, 0.015 g/l H_3BO_3, 0.4 g/l $ZnCl_2$. Each liter of the final mineral medium contained 975 ml of the basal medium and 25 ml of the trace element solution. The concentration of glycerol, $NaNO_3$, KH_2PO_4, and K_2HPO_4 for each run of the experiments are given in the section of experimental design (below).

Microorganism: A strain of *Pseudomonas aeruginosa* (ATCC 53752) was used in the experiments. The cultures were maintained on *Pseudomonas aeruginosa* isolation agar (HIMEDIA) slants at 4ºC. To prepare a seed culture a loop of the microorganism was inoculated to the mineral medium containing 15 g/l glycerol, 4 g/l sodium nitrate, 3.4 g/l KH_2PO_4, and 4.3 g/l K_2HPO_4. The culture was incubated for 24 h at 37ºC. The seed culture was prepared in 1 liter sterilizable glass vessel. The culture medium was aerated using a small air pump with constant flow rate of 1 l/min. The inoculum size was 10% by volume of the growth medium.

Biosurfactant production: Biosurfactant production was performed in a sterilizable glass vessel. The culture medium was aerated using a small air pump with constant flow rate of 1 l/min. The air was sterilized by air filters. The aseptic condition of fermentation broth was verified by streaking a sample of the fermentation medium on the *Pseudomonas aeruginosa* isolation agar. The vessel was placed in a water bath at 37ºC. A schematic of the system has been shown in Figure 1.

Biomass measurement: A sample of fermentation broth (20 ml) was centrifuged at 6000 rpm (4000 g) for 25 minutes. The pellet was then suspended in a small amount of distilled water and dried in an oven at 105ºC for 24 h (Silva *et al*., 2010).

Rhamnolipid quantification: Rhamnolipid (expressed as Rhamnose Equivalent, RE) was measured in the cell free culture using phenol-sulfuric acid method (Silva *et al*., 2010; Tatsuya *et al*., 2005). A calibration curve was prepared with an analytical grade rhamnose from Merck.

Experimental design: A full factorial experimental design was employed. The method of fermentation and fermentation time were the factors, and the biomass and rhamnolipid concentrations were the responses. The method of fermentation had four levels: batch fermentation, fed batch with nitrogen source in feed, fed batch with phosphorus source in feed, and fed batch with carbon source in feed. The factor levels for time

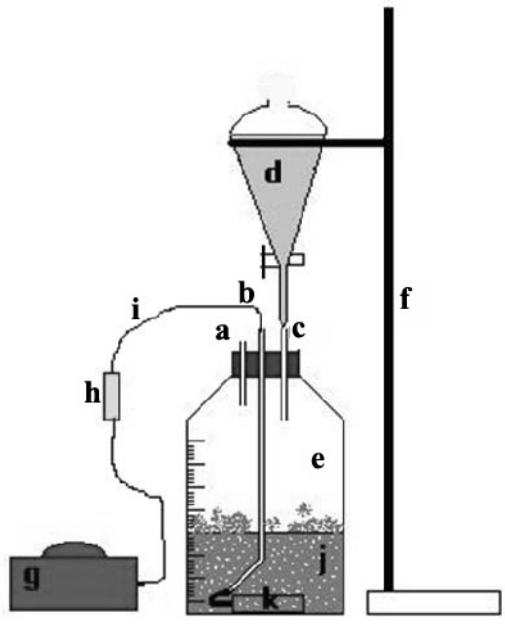

Figure 1. The experimental setup: a: air outlet, b: air inlet, c: feed inlet, d: feed, e: glass vessel, f: feed reservoir holder, g: air pump, h: air filter, i: silicone rubber pipe, j: fermentation medium, k: air sparger. (for batch experiments the feed reservoir was removed).

were 48 and 72 h. All runs were replicated twice.

Batch fermentation was carried out with 500 ml of the growth medium containing the initial glycerol, $NaNO_3$, KH_2PO_4, and K_2HPO_4 concentrations of 30, 8.3, 3.4, and 4.3 g/l, respectively. The fed batch runs were conducted in three sets. In each set 250 ml of the growth medium was in the glass vessel initially. The medium contained all of the above mentioned components except one. The remaining component (dissolved in the basal mineral medium) was fed to the vessel during fermentation. The volume of feed for all fed batch runs was 250 ml. So the total volume of the growth medium at the end of each fed batch run was the same as the volume in batch experiments (500 ml). In the first set, $NaNO_3$ at concentration of 16.6 g/l was fed to the bioreactor. The initial concentrations for glycerol, K_2HPO_4, and KH_2PO_4 in the vessel were 60, 8.6, and 6.8 g/l, respectively. Two separate runs were done in this set with the feed flow rates of 5.2 ml/h (48 h of feeding) and 3.5 ml/h (72 h of feeding), respectively. In the second set of fed batch experiments the feed was 250 ml of the basal mineral medium with the glycerol concentration of 60 g/l. The initial concentrations for $NaNO_3$, K_2HPO_4, and KH_2PO_4 in the vessel were 16.6, 8.6, and 6.8 g/l, respectively. Two separate runs were conducted with two different feed flow rates. In the third set of fed batch experiments the feed was 250 ml of mineral medium with K_2HPO_4 and KH_2PO_4 concentration of 8.6 g/l and 6.8 g/l, respectively. The medium in the bioreactor contained 60 g/l glycerol, and 16.6 g/l $NaNO_3$. Two separate runs were also conducted in this set with two different feed flow rates similar to the previous sets.

Statistical analysis: The method of analysis of variance was used to analyze the results. The analysis was performed using the commercial software MINITAB 16. For each response (cell dry weight and rhamnolipid concentration), the 95% confidence interval for the average values were presented using pooled variance of all data points (Montgomery, 2001).

RESULTS

Tables 1 and 2 show the results of analysis of variance for biomass cell dry weight, and rhamnolipid concentration as a function of the method of fermentation and time, respectively. The results indicate that the method of fermentation affects biomass and rhamnolipid production significantly (very low P-values). Over the examined time interval, however, the variations in biomass cell dry weight and rhamnolipid concentration were not significant for confidence intervals greater than 90% (P-values for both are greater than o.1). Figures 2 and 3 show the normal probabilities plot for the performed analysis of variances. The Figures indicate normal distribution of residuals and the validity of the analyses (Montgomery, 2001). Figures 4 and 5 show the biomass cell dry weight and rhamnolipid

Table 1. Analysis of variance for cell dry weight data.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Method of fermentation	3	11.0439	11.0439	3.6813	58.59	0.000
Time	1	0.1010	0.1010	0.1010	1.61	0.240
Interaction	3	0.0190	0.0190	0.0063	0.10	0.957
Error	8	0.5026	0.5026	0.0628		
Total	15	11.6665				

Table 2. Analysis of variance for rhamnolipid data.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Method of fermentation	3	22.8386	22.8386	7.6129	51.15	0.000
Time	1	0.3189	0.3189	0.3189	2.14	0.181
Interaction	3	0.1150	0.1150	0.0383	0.26	0.854
Error	8	1.1906	1.1906	0.1488		
Total	15	24.4631				

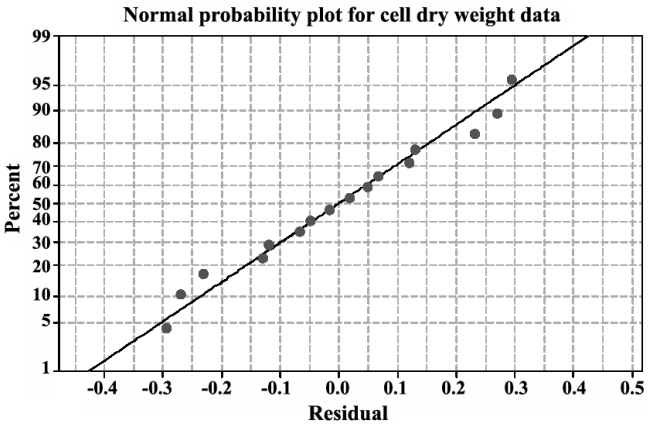

Figure 2. Normal probability plot of residuals for cell dry weight data.

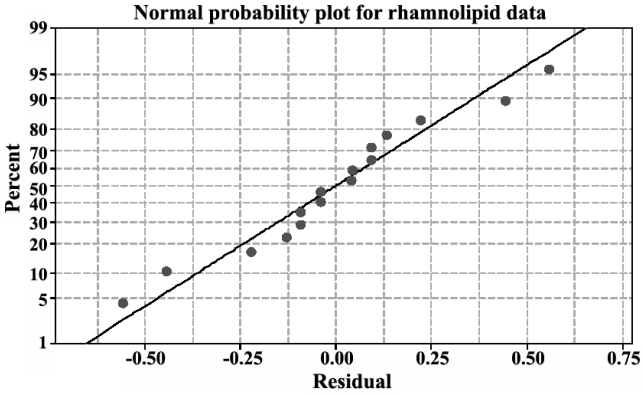

Figure 3. Normal probability plot of residuals for rhamnolipid data.

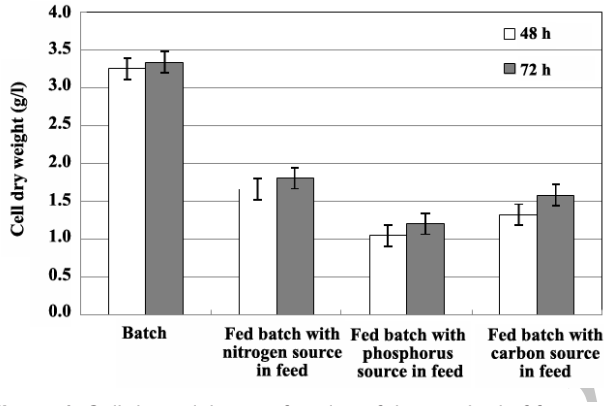

Figure 4. Cell dry weight as a function of the method of fermentation.

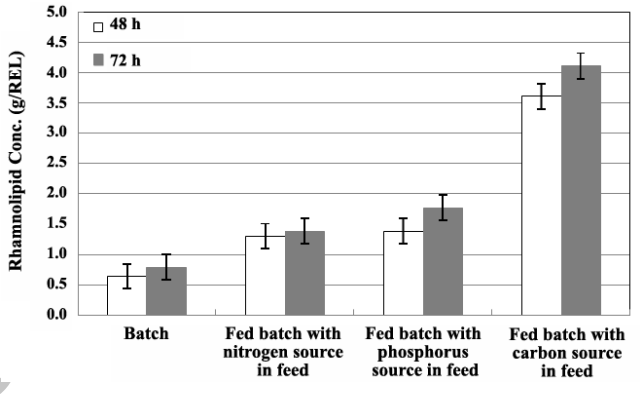

Figure 5. Rhamnolipid concentration as a function of the method of fermentation.

concentration as a function of the method of fermentation. The biomass concentration was maximum for the batch fermentation, and the fed batch fermentations gave close values of biomass. For rhamnolipid concentration the lowest value was for the batch fermentation and the largest value was for the fed batch fermentation with glycerol in feed.

DISCUSSION

Production of rhamnolipid in batch mode has been widely investigated. In our batch experiments the biomass concentration reached its maximum value after 20 h, while rhamnliopid accumulated in the medium up to 100 h with the maximum concentration of about 1 g/RE L (Fig. 6 Note that we collected data up to 150 h of batch fermentation, but only the data at 48 and 72 h were used to compare with fed batch fermentations, because for fed batch fermentations we had data only at these two times). The average dried biomass empirical formula for bacterial biomass is $C_{45}H_{85}O_{15}N_{10}P$ (Schaechter, 2004). It can be concluded from this formula that the approximate balanced molar ratio for C:N:P in bacterial growth media is 100:10:1 (considering some carbon conversion to carbon dioxide). For the growth medium which was used for batch fermentation this ratio was 100:10:0.5. So the medium was evidently limiting in term of phosphorus concentration. In batch fermentation considerable amount of biomass is accumulated before the exhaustion of the limiting nutrient resulting in the relative low conversion of the carbon source to rhamnolipid. Table 3 compares the results of batch fermentation in this study with some published data in the literature. The rather large variations in the rhamnolipid concentration can be attributed to the variations in the strains, substrates, and fermentation conditions. Overall in batch fermen-

Table 3. Reported values of rhamnolipid concentration in batch fermentation.

Substrate	Initial substrate conc.	Fermentation duration (h)	Maximum rhamnolipid conc.	Reference
Glucose	4%	120	1.6 g/l	Wei *et al*., 2005
Naphthalene	2%	180	2 g/RE l	Deziei *et al*., 1996
Mannitol	2%	120	0.3 g/RE l	Nayak *et al*., 2009
Palm oil	2%	48	0.36 g/RE l	Sarachat *et al*., 2010
Soy bean oil	2%	240	4 g/l	Raza *et al*., 2006
Casamino acid	5.3 g/l	65	3.6 g/RE l	Al-Araji *et al*., 2007
Glycerol	30 g/l	168	0.64 g/l	Matsufuji *et al*., 1997
Ethanol	30 g/l	168	3.6 g/l	Matsufuji *et al*., 1997
Glucose	30 g/l	168	0.284 g/l	Matsufuji *et al*., 1997
Rape seed oil	30 g/l	168	4.27 g/l	Matsufuji *et al*., 1997
Sunflower oil	250 g/l	120	37 g/l	Müller *et al*., 2011
Sunflower oil	15 g/l	40	0.3 g/l	Pantazaki *et al*., 2010
Oleic acid	9.4 g/l	60	0.25 g/l	Pantazaki *et al*., 2010
Glucose	3%	103	1.3 g/REl	Clarke *et al*., 2010
Fish oil	25 g/l	216	17 g/l	Lee *et al*., 2004
Glycerol	30 g/l	94	1.07 g/RE l	This study

tation, after 72 h the biomass and rhamnolipid concentrations were 3.33 g/l ± 0.14 and 0.79 g/REL ± 0.21, respectively.

Nutrient limiting conditions can be achieved in fed batch fermentation by exclusion of one of the nutrients from the growth medium. The limiting nutrient is fed to the bioreactor in a low rate continuously. This strategy ensures the limiting conditions from the beginning of the fermentation.

Nitrogen is an essential nutrient for microbial growth, and ammonium is a preferred nitrogen source for most bacteria (Prescott *et al*., 2002). Nitrogen in ammonium is in reduced form and it can be incorporated easily into proteins and nucleic acids. For rhamnolipid production, however, nitrate has been shown to be the best nitrogen source (Desai and Banat, 1997). Microbes need to convert nitrate to a reduced form before it can be incorporated into biomass. Since nitrate reduction is an energy consuming process, nitrate is not a favorable nitrogen source for microbial growth compared to ammonium. So it is not surprising that rhamnolipid production (as a secondary metabolite) is stimulated with nitrate as the nitrogen source. Fed batch fermentation with nitrate in feed makes conditions more severe for microbes in term of nitrogen limitation. Active microbes from seed culture enter to a medium with no nitrate. With gradual introduction of nitrate, microbes encounter a growth condition with limited nitrogen amounts which results in glycerol conversion to rhamnolipid. Under these conditions, with 72 h of fed batch fermentation the biomass and rhamnolipid concentrations were 1.80 g/l ± 0.14, and 1.38 g/REL ± 0.21, respectively. These values indicate that less biomass and more rhamnolipid were produced compared to batch fermentation.

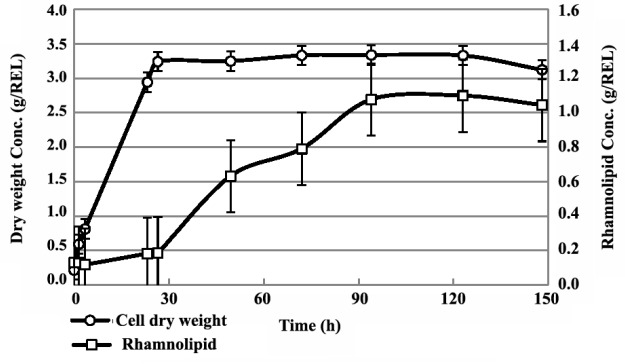

Figure 6. Cell dry weight and rhamnolipid concentration in batch fermentation for initial glycerol concentration of 30 g/l.

Phosphorus is another essential nutrient for microbial growth and phosphates are common sources of phosphorus for microbes. Limitation of phosphorus stimulates rhamnolipid accumulation in the growth medium. Fed batch fermentation with phosphates in feed keeps microorganisms under limited concentration of phosphorus. With these conditions, after 72 h of fed batch fermentation the biomass and rhamnolipid

concentrations were 1.2 g/l ± 0.14, and 1.77 g/REL ± 0.21, respectively. The results indicate that under phosphorus limitation more rhamnolipid is produced compared to nitrogen limitation. Chayabutra *et al*. (2001) obtained the same results. With hexadecane as the carbon source and under anaerobic denitrifying conditions, phosphorus limitations resulted in five fold higher productivity compared to nitrogen limited conditions. Clark *et al*. (2010) also showed that rhamnolipid production is considerably higher (up to 12 fold) under phosphorus limitation as compared to nitrogen limitation.

Gradual introduction of glycerol to the growth medium was another strategy for rhamnolipid production. This strategy seems to be illogical since it is well known that *Pseudomonas aeruginosa* produces rhamnolipid when carbon source is in excess in the growth medium. Nevertheless this strategy was tested as part of the experimental design. Surprisingly with this strategy more rhamnolipid was obtained compared to the batch and other fed batch experiments. After 72 h of fed batch fermentation the biomass and rhamnolipid concentrations were 1.59 g/l ± 0.14, and 4.12 g/REL ± 0.21, respectively. The results indicate that rhamnolipid is produced whenever the concentration of one of the nutrients is low. The limiting nutrient can be phosphorus, nitrogen, or carbon. Limitation in such elements as Fe and K has also been reported to promote rhamnolipid production (Soberon-Chavez, 2011). Similar results were obtained by Lee *et al*. (2004). In their research fish oil, as the carbon source, was added intermittently to the growth medium during fermentation. With this strategy of feeding the concentration of rhamnolipid was 1.3 fold higher compared to batch fermentation.

CONCLUSIONS

Method of fermentation affects rhamnolipid production by *Pseudomonas aeruginosa* significantly despite using the same amounts of materials for microbial growth. Batch fermentation results in the highest biomass and the lowest rhamnolipid concentrations compared to the fed batch alternatives. Exclusion of a nutrient from the culture medium and gradual introduction of the nutrient during fermentation stimulates rhamnolipid production. The excluded nutrient can be either nitrogen and phosphorus, or carbon source. This indicates non selective nature of rhamnolipid production as a function of nutrient limitation. Although limitation in a nutrient can stimulate rhamnolipid production, the amount of rhamnolipid is significantly different with the nature of limited nutrient. In this work the highest concentration of rhamnolipid in fed batch was obtained when the carbon source was fed to the vessel during fermentation.

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