Rhamnolipid Production in Batch and Fed-batch Fermentation Using *Pseudomonas aeruginosa* BYK-2 KCTC 18012P

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Abstract The optimization of culture conditions for the bacterium *Pseudomonas aeruginosa* BYK-2 KCTC 18012P, was performed to increase its rhamnolipid production. The optimum level for carbon, nitrogen sources, temperature and pH, for rhamnolipid production in a flask, were identified as 25 g/L fish oil, 0.01% (w/v) urea, 25 and pH 7.0, respectively. Optimum conditions for batch culture, using a 7-L jar fermentor, were 200 rpm of agitation speed and a 2.0 L/min aeration rate. Under the optimum conditions, on fish oil for 216 h, the final cell and rhamnolipid concentrations were 5.3 g/L and 17.0 g/L respectively. Fed-batch fermentation, with different feeding conditions, was carried out in order to increase, cell growth and rhamnolipid production by the *Pseudomonas aeruginosa,* BYK-2 KCTC 18012P. When 2.5 g of fish oil and 100 mL basal salts medium, containing 0.01% (w/v) urea, were fed intermittently during the fermentation, the final cell and rhamnolipid concentrations at 264 h, were 6.1 and 22.7 g/L respectively. The fed-batch culture resulted in a 1.2-fold increase in the dry cell mass and a 1.3-fold increase in rhamnolipid production, compared to the production of the batch culture. The rhamnolipid production-substrate conversion factor (0.75 g/g) was higher than that of the batch culture (0.68 g/g).

Keywords: production, rhamnolipids, *Pseudomonas aeruginosa,* batch fermentation, fed-batch fermentation

INTRODUCTION

Biosurfactants, with both hydrophilic and hydrophobic structural moieties, seem to facilitate the uptake of hydrocarbons into cells. A wide spectra of microbial compounds, including lipopeptides, glycolipids, fatty acids and polymeric biosurfactants, have been found to have surface activity. Such compounds are able to reduce the surface tension and interfacial tension between the water and hydrocarbon phases [1,2].

Biosurfactants have important advantages, such as biodegradability, low toxicity and various possible structures, relative to chemically synthesized surfactants [3]. With environmental compatibility becoming an increasingly important factor in the selection of industrial chemicals, the use of biosurfactants in environmental applications, such as, bioremediation and the dispersion of oil spills, is increasing [2,4]. In addition, biosurfactants have other uses in the petroleum industry, such as, enhanced oil recovery [5] and in the transportation of crude oil. Other possible application fields are in the food, cosmetic and pharmaceutical industries. In these industries, most biosurfactants are used as emulsifiers [1,3].

The composition, structure and properties of a variety of surfactants produced by bacteria, yeasts and fungi, have been described [6,7]. Among the best-studied biosurfactants are the rhamnolipids of *Pseudomonas aeruginosa* [8]. The rhamnolipids from *Pseudomonas aeruginosa* were first described in 1949 [9], and studies on the biosynthesis of these compounds were carried out *in vivo* by Hauser and Karnofsky [10-12], who showed that these glycolipids were secreted into the growth medium during the stationary phase of growth. Rhamnolipid production is dependent on a central metabolic pathway, like fatty acid synthesis and dTDP-activated sugars, as well as on enzymes participating in the production of the exopolysaccharide alginate. Synthesis of these surfactants is regulated by a very complex genetic regulatory system that also controls different *P. aeruginosa* virulence-associated traits. Rhamnolipids have several potential

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industrial and environmental applications, including the production of fine chemicals, the characterization of surfaces and surface coatings, as additives for environmental remediation and as a biological control agent. The realization of this wide variety of applications requires economical commercial-scale production of rhamnolipids [13].

The *Pseudomonas aeruginosa* BYK-2 KCTC 18012P used in this study, was isolated from the southern sea of Korea [14]. Biosurfactants produced by this strain were identified as rhamnolipid (M.W. 650) and rhamnolipid methyl ester (M.W. 664) [15]. This study reports on the optimal production of rhamnolipids by fermentation of *Pseudomonas aeruginosa* BYK-2 KCTC 18012P.

MATERIALS AND METHODS

Microorganism and Medium Compositions

The microorganism used in this study was *Pseudomonas aeruginosa* BYK-2 KCTC 18012P, which was isolated from the southern sea of Korea, as reported by Kim *et al.* [14]. The basal salts medium used for cell growth and rhamnolipid production was of the following composition (g/L): $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; KH_2PO_4, 1.0; K_2HPO_4, 0.5; $NaNO_3$, 2.0; $CaCl_2$, 0.01 and KCl, 0.1. The medium was adjusted to pH 7.0 with 5 N HCl.

Flask Cultures

Pseudomonas aeruginosa BYK-2 KCTC 18012P was initially grown on an agar plate containing tryptone and yeast extract: it was then transferred to the seed culture medium. The seed culture was grown in a 250-mL flask containing 50 mL of the basal salts medium at 25°C, on a rotary shaker at 180 rpm for 14 h. To discover the optimal carbon, nitrogen source, temperature and pH for the medium, the flask culture experiments were performed in a 250 mL Erlenmeyer flask containing 50 mL of the basal salts medium inoculated with 1.0% (v/v) of the seed culture.

Fermentation Conditions

The seed culture to be inoculated into the fermentor was prepared by cultivating cells in a 250-mL Erlenmeyer flask containing 50-mL of basal salts medium and 10 g/L of fish oil, at 25°C for 40 h on a rotary shaker (180 rpm). Fermentor experiments were performed with a 7-L jar fermentor (Best Korea Co. Ltd., Korea) containing 3 L of basal salts medium at 25°C and pH 7.0. The fermentation culture medium was supplemented with 25 g/L fish oil (E-Hwa Oil and Fat Ind. Co., Ltd., Korea) and 0.01% (w/v) urea, as the carbon source and nitrogen source, respectively. The agitation speed and aeration rate in the batch culture were in the range of 100~350 rpm and 1.0~3.0 L/min, respectively.

High rhamnolipid concentration were obtained from fed-batch cultures by varying the concentration of fish oil and the feeding medium volume in the same fermentor. The feeding medium was the basal salts medium containing 0.01% (w/v) urea. The feeding medium and fish oil were pumped into the reactor using a peristaltic pump.

Analytical Methods

The culture broth was centrifuged (10,000 × g at 4°C for 10 min) and the cells were washed with distilled water and dried at 110°C until the weight was constant. The emulsifying activity of the culture supernatants was measured by the method of modifying Rosenberg *et al.* [16]. In brief, 1.25 mL of the culture supernatant and 0.05 mL of a mixture of hexadecane/2-methylnaphthalene (1:1, v/v) were introduced into a test tube containing 3.7 mL of 20 mM Tris-HCl buffer (pH 7.0). After shaking for 1 min and standing for 10 min, the absorbance of the mixture was measured at 620 nm using a UV/VIS spectrophotometer (Ultrospec 3000, Pharmacia Biotech Ltd., UK). One unit of emulsifying activity was defined as 0.1 of absorbance.

The orcinol assay [17] was used to directly assess the concentration of the biosurfactant in the sample: 0.1 mL of the culture supernatant was mixed with 0.9 mL of a solution containing 0.19% (v/v) orcinol in 53% (v/v) H_2SO_4 and boiled for 20 min. After cooling at room temperature for 15 min, the sample was measured at 421 nm. A standard curve was prepared using the purified rhamnolipids [15].

RESULTS AND DISCUSSION

Effect of Carbon Sources on Rhamnolipid Production

The effect of the carbon source on rhamnolipid production by *Pseudomonas aeruginosa* BYK-2 KCTC 18012P, was investigated in the basal salts medium containing 1% (w/v) of a variety of carbon sources, such as, hydrocarbons (Arabian light crude oil, Kuwait crude oil, bunker C oil, liquid paraffin *n*-hexadecane and *n*-tetradecane), fatty acids (oleic acid, olive oil, lecithin, and fish oil) and sugars (arabinose, trehalose, dextrose, galactose, lactose, fructose, maltose, D-sorbitol and saccharose), respectively. The cell growth and emulsifying activity were measured after 48 h of cultivation. As shown in Table 1, the fish oil was the most effective carbon source for emulsifying activity among the 19 different kinds of carbon sources tested.

In order to determine the effect of the initial fish oil concentration, the concentration of fish oil was increased sequentially from 5 to 30 g/L. The rhamnolipid production decreased significantly with a concentration higher or lower than 25 g/L (Fig. 1). When the concentration of fish oil was 25 g/L, the final rhamnolipid production was 13.6 g/L. Therefore, the optimum fish oil concentration was 25 g/L.

The rhamnolipid production by *Pseudomonas aeruginosa* BYK-2 KCTC 18012P has been found to be affected by carbon sources in the medium. Water-soluble carbon

Table 1. Effect of carbon sources on rhamnolipid production

Carbon sources	Cell growth ($O.D._{660\ nm}$)	Emulsifying Activity (Unit)
Arabian light crude oil	7.8	51.3
Kuwait crude oil	8.8	52.0
Bunker C oil	6.4	51.5
Liquid paraffin	4.7	26.0
n-Hexadecane	7.3	110.5
n-Tetradecane	5.5	79.5
Oleic acid	12.6	9.0
Olive oil	14.2	71.5
Lecithin	13.9	22.7
Fish oil	12.2	168.4
Arabinose	6.3	34.8
Trehalose	9.4	60.7
Dextrose	9.9	12.4
Galactose	6.1	42.0
Lactose	4.8	36.9
Fructose	9.5	85.4
Maltose	4.3	34.3
D-sorbitol	4.0	36.3
Saccharose	4.2	27.0

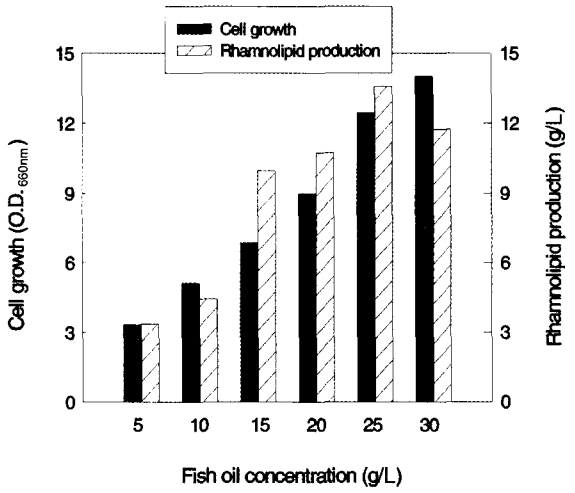

Fig. 1. Effect of fish oil concentration on the cell growth and rhamnolipid production by *Pseudomonas aeruginosa* BYK-2 at 25°C.

sources such as sugar (dextrose, fructose *etc.*) could be used for rhamnolipid production [19], but water-insoluble materials like alkane and oil are superior to them. Thus, it is thought that the microorganism produces rhamnolipids to emulsify these water-insoluble carbon sources for good assimilation.

Table 2. Effect of nitrogen sources on rhamnolipid production

Nitrogen sources	Cell growth ($O.D._{660\ nm}$)	Emulsifying Activity (Unit)
1.0% Tryptone + 0.5% yeast extract[1]	**7.6**	**167.1**
1.0% tryptone[1]	4.7	62.6
0.5% yeast extract[1]	2.4	58.1
0.5% peptone[1]	2.5	59.0
0.5% tryptone[1]	2.4	59.8
0.5% tryptone + 0.5% yeast extract[1]	6.6	122.5
0.5% peptone + 0.5% yeast extract[1]	6.6	88.7
0.2% urea[2]	6.9	147.9

[1] Basal salt medium (BSM): 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 1.0 g KH_2PO_4, 0.5 g, K_2HPO_4, 2.0 g $NaNO_3$, 0.01 g $CaCl_2$ and 0.1 g KCl in 1 L distilled water (pH 7.0)
[2] Basal salt medium (absence 2.0 g $NaNO_3$)

Effect of Nitrogen Sources on Rhamnolipid Production

Medium constituents, other than the carbon source, also affect rhamnolipid production. Nitrogen can be an important key to the regulation of rhamnolipid synthesis, and there is evidence that nitrogen plays a significant role in the production of rhamnolipids by microbes [20,21]. To examine the effect of various nitrogen sources on rhamnolipid production, complex nitrogen sources such as yeast extract, peptone and tryptone, as well as inorganic nitrogen sources such as urea were used. The cell growth and emulsifying activity were measured after 48 h of cultivation. Among the nitrogen sources tested, the maximum emulsifying activity was observed when the cells were grown on 1.0% tryptone and 0.5% of a yeast extract complex medium (Table 2). However, in terms of industrial application, urea is about 10 times cheaper than Luria Broth. Therefore, urea was chosen as the optimum nitrogen source.

To determine the effect of the initial urea concentration, urea concentrations in the range of 0.01~0.1% (w/v) were utilized. The cell mass was maximal at 0.08% (w/v) urea whereas the rhamnolipid production was maximum at 0.01% (w/v) urea (Fig. 2). Therefore, the optimum concentration of urea for rhamnolipid production was determined to be 0.01% (w/v) urea.

Effects of Temperature and Initial pH on Rhamnolipid Production

The effects of the initial pH and temperature on rhamnolipid production by *Pseudomonas aeruginosa* BYK-2

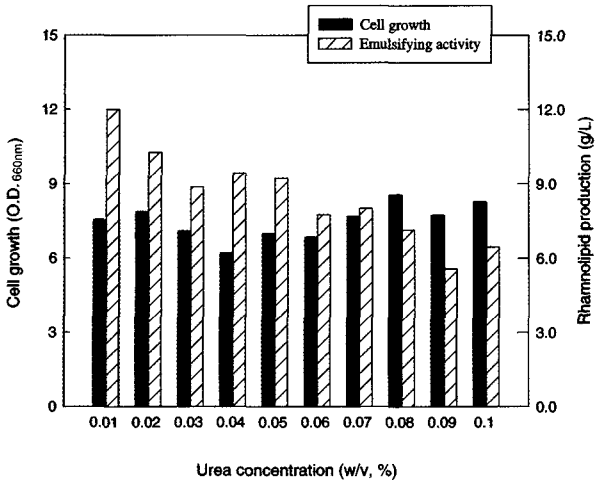

Fig. 2. Effect of urea concentration on the cell growth and emulsifying activity of *Pseudomonas aeruginosa* BYK-2 KCTC 18012P.

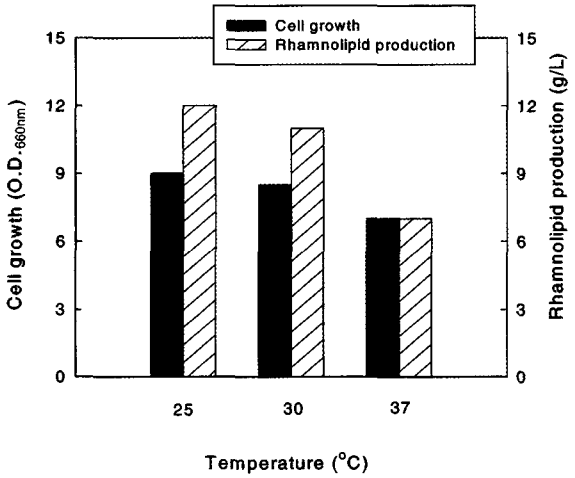

Fig. 3. Effect of temperature on the cell growth and rhamnolipid production by *Pseudomonas aeruginosa* BYK-2 KCTC 18012P.

KCTC 18012P, were examined after the strain was cultured for 144 h in an optimum medium containing 25 g/L fish oil and 0.01% (w/v) urea. The effect of temperature on rhamnolipid production is shown in Fig. 3. The maximum rhamnolipid production by *Pseudomonas aeruginosa* BYK-2 KCTC 18012P was observed at 25°C.

In order to investigate the effect of the initial pH on cell growth and rhamnolipid production, the pH level of the media was varied from 3.0 to 11.0. The pH was adjusted with 2N HCl or 2N NaOH. The optimum culture pH for both cell growth and rhamnolipid production was pH 7.0 (Fig. 4).

Effect of Agitation Speed on Rhamnolipid Production

To investigate the effect of agitation speed on rham-

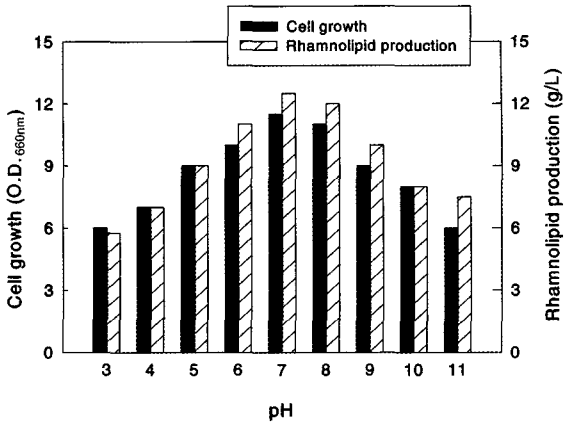

Fig. 4. Effect of pH on cell growth and rhamnolipid production by *Psedomonas aeroginosa* BYK-2 KCTC 18012P.

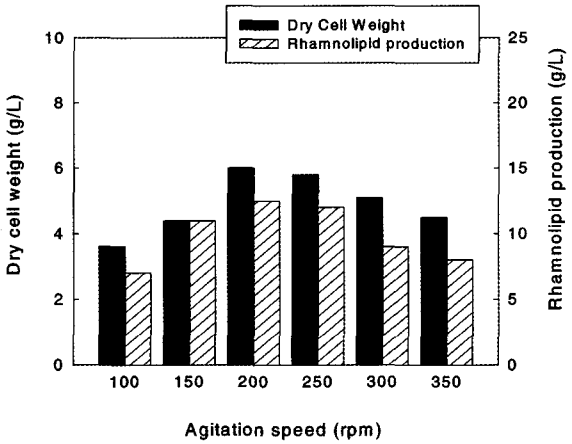

Fig. 5. Effect of the agitation speed on dry cell weight and rhamnolipid production during the 7 L batch fermentation.

nolipid production, *Pseudomonas aeruginosa* BYK-2 KCTC 18012P was cultivated with an agitation speed of 100, 150, 200, 250, 300, and 350 rpm. At an agitation speed of 200 rpm, the concentrations of the cells and biosurfactant were 6.0 and 12.5 g/L, respectively, and were significantly higher than at the other speeds (Fig. 5). A high agitation speed (250~350 rpm) was found to be detrimental to cell growth, due to shear damage, even though it provided sufficient dissolved oxygen. On the other hand, a low agitation speed (100, 150 rpm) caused stagnant regions in the fermentor due to improper mixing [22].

Therefore, it is suggested that the agitation speed used on *Pseudomonas aeruginosa* BYK-2 KCTC 18012P for rhamnolipid production, should be carefully controlled to satisfy the requirements of both minimizing cell damage and maximizing the effect of mixing.

Effect of Aeration Rate on Rhamnolipid Production

The importance of aeration in aerobic fermentation

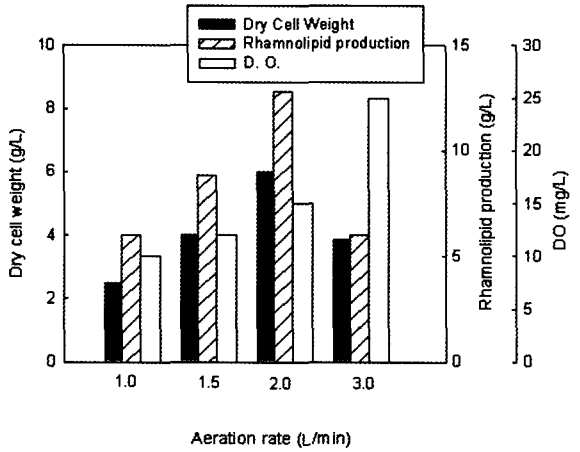

Fig. 6. Effect of the aeration rate on the dry cell weight and rhamnolipid production during 7 L batch fermentation.

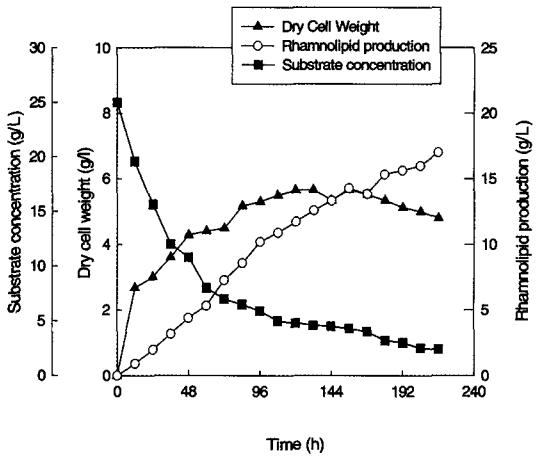

Fig. 7. Time course of the batch fermentation with 25 g/L fish oil and 0.01% (w/v) urea for rhamnolipid production by *Pseudomonas aeruginosa* BYK-2 KCTC 18012P during the 7 L batch fermentation.

Table 3. Comparison of rhamnolipid production by different strains of *Psedomonas aeruginosa* on different substrates

Microbial strain	Carbon Source	Product concentration (g/L)	Conversion yield ($Y_{p/s}$)	Reference
UI 29791	Corn oil	46.0	0.61	21
44T1	Olive oil	10.0	0.5	23
JAMM (NCIB 40044)	Waste material (Olive oil mill effluent medium)	1.4	0.058	24
DS 10-129	Soybean oil	4.31	0.716	25
DS 10-129	Safflower oil	2.98	0.496	25
DS 10-129	Glycerol	1.77	0.295	25
DSM 2659	Glucose	2.25	0.075	26
DSM 2874	*n*-Alkanes (resting cells)	13.2	0.24	29
DSM 2874	*n*-Alkanes (growing cells)	12.8	0.32	29, 30
BYK-2 KCTC 18012P	Fish oil	17.0	0.68	-

processes is well recognized by laboratory and industrial fermentation technologists. The primary objective of aeration and agitation is to supply the necessary oxygen to the microorganisms in order to achieve the proper metabolic activities. Oxygen is the most important gaseous substrate for microbial metabolism. A secondary function of aeration and agitation is to keep the microorganisms in suspension [23]. To examine the effect of dissolved oxygen on cell growth and rhamnolipid production, *Pseudomonas aeruginosa* BYK-2 KCTC 18012P, was cultivated with an aeration rate of 1.0, 1.5, 2.0, and 3.0 L/min. As shown in Fig. 6, both concentration of cells (dry cell weight of 6.0 g/L) and rhamnolipids (12.8 g/L) were the highest at 2.0 L/min. The cell growth and rhamnolipid production decreased significantly with an aeration rate higher or lower than 2.0 L/min.

Time Course of Rhamnolipid Production

Fig. 7 shows the time courses of the cell growth, substrate (fish oil) consumption and rhamnolipid production, during batch cultivation of *Pseudomonas aeruginosa* BYK-2 KCTC 18012P in optimum conditions. Maximum dry cell weight was 5.3 g/L, after the stationary phase of 132 h. The rhamnolipid concentration increased in proportion to increasing cell growth and reached the maximum level of 17.0 g/L after 216 h of cultivation. The residual fish oil concentration decreased during the entire period of the fermentation process.

This result indicates that the concentration of rhamnolipids from *Pseudomonas aeruginosa* BYK-2 KCTC 18012P is much higher than that of other rhamnolipid-producing microorganisms (Table 3) [18,24-30].

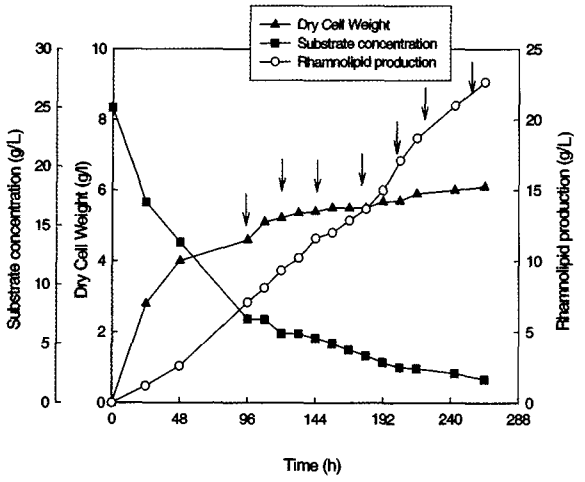

Fig. 8. Rhamnolipid production by *Pseudomonas aeruginosa* BYK-2 KCTC 18012P during the fed-batch fermentation at 200 rpm with 2.0 L/min oxygen. The arrows indicate the start of medium feeding each other (2.5 g fish oil/100 mL medium).

Fed-batch Culture

Fed-batch culture has been the most popular method used to achieve a high productivity and final concentration level of the desired product, by maintaining a high cell concentration and controlling the feeding medium during the production stage [31-33].

Among some intermittently fed-batch experiments tested (data not shown), the result shown in Fig. 8 was the best for rhamnolipid production. The investigators started their first feeding after 4 days and made additional feedings of the fish oil (2.5 g) and basal salts medium (100 mL) every 1 or 2 days. The cell growth and substrate consumption patterns were similar to those obtained with batch cultivation. The cell growth increased over the whole fermentation period, and reached a maximum concentration of 6.1 g/L in 264 h. During the period of fish oil and basal salts medium feeding, there was a steady increase in rhamnolipid concentration. In the fed-batch culture, a maximum of 22.7 g/L of rhamnolipid was produced from a total of 30.2 g/L of fish oil (0.75 g/g substrate conversion yield). The optimized fed-batch culture resulted in a 1.2-fold increase in cell mass and a 1.3-fold increase in rhamnolipid production compared with those of the batch culture.

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