

Culture Condition of *Pseudomonas aeruginosa* F722 for Biosurfactant Production

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Abstract *Pseudomonas aeruginosa* F722 produces a biosurfactant (BS) during its degradation of carbon and hydrocarbon compounds. The culture conditions for upgrading the biosurfactant productivity were investigated. The concentration of the biosurfactant produced by *P. aeruginosa* F722 was 0.78 g/L in C-medium; however, this increased to 1.66 g/L in BS medium, which was experimentally adjusted to optimal conditions. NaNO₂ was found to be most effective for microbial growth, with an O.D._{600nm} of 1.18 for 0.1% NaNO₂. Microbial growths, according to the O.D._{600nm} were 2.53, 2.68, 2.89, and 2.87 for glucose, glycerol, *n*-C₁₀, and *n*-C₂₂, respectively. Clear zone diameters (cm), indicating biosurfactant activity, were 9.0, 8.8, 5.7, and 8.5 for glucose, glycerol, *n*-C₁₀, and *n*-C₂₂, respectively. Microbial growth was not consistent with the biosurfactant activity. The best biosurfactant activity was found with a C/N ratio of 20. Under optimal culture condition, the average surface tension decreased from 70 to 30 mN/m after 5 days. With aeration of 1.0 vvm, the biosurfactant produced increased to 1.94 g/L (up to 20%) compared to that of 1.66 g/L with no aeration. With aeration, the velocities of glucose degradation during both the log and stationary growth phases increased from 0.25 and 0.18 h⁻¹ to 0.33 and 0.29 h⁻¹, respectively, and the time for the culture to arrive at the maximum clear zone diameter became shorter, from 80 down to 60 h with no aeration.

Keywords: *Pseudomonas aeruginosa* F722, biosurfactant, surface tension, aeration, degradation velocity

INTRODUCTION

Microbial surfactants are a structurally diverse group of surface-active molecules synthesized by microorganisms. They have been characterized by their ability to dissolve hydrophobic materials into aqueous solution more easily by reducing the interfacial and surface tensions [1-3]. The kinds of biosurfactants are glycolipids, lipopeptide, fatty acids, neutral lipids, phospholipids, polymeric surfactants, and particulate biosurfactants. Because of these characteristics, biosurfactants are used in many fields, such as petro-chemistry, bioremediation, cosmetics, agriculture, medicine, detergent, and the painting industry, etc. [1].

With the growth of environmental insight, chemical surfactants have been substituted by biosurfactants [2,4,5]. For example, Emulsan, a biosurfactant produced by Petroferm Co., USA, is largely used for cleaning oil-polluted tanks and electrical boards, etc.; however, only a few studies have become public because of the mainte-

nance of secrets and inter-company competition. Especially, concern over biosurfactants is fast growing in the fields of MEOR (Microbial Enhanced Oil Recovery) [6], macro molecular hydrocarbon and non-biodegradable phenanthrene degradations and heavy metal removal [7-14]. The industrial production of biosurfactants usually depends on microorganisms as they are easy to deal with and produce various surfactants in a short time.

There are various general degrading hydrophobic compounds, for example *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Nocardia*, *Arthrobacter*, *Vibrio*, *Bacillus*, *Micrococcus*, *Klebsiella*, and *Acinetobacter* [15-18]. Of these, biosurfactants are produced by *Pseudomonas*, *Nocardia*, *Bacillus*, and *Acinetobacter*, such as glycolipids, fatty acids, neutral lipids, phospholipids, and polymeric surfactants. *Pseudomonas aeruginosa* F722 is especially known to have many crude oil degradation [19,20], rhamnolipid production [21-25], and hexadecane degradation qualities [18]. The aim of this study was to examine the favorable culture conditions for the production of a biosurfactant using *P. aeruginosa* F722.

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MATERIAL AND METHODS

Strain and Culture Medium

P. aeruginosa F722, from the Korean Agricultural Culture Collection (KACC) 91006, was used for biosurfactant production, which was isolated from soil. Before inoculation of the main culture, it was inoculated into nutritional medium LB (Luria Bertani: trypton, 10 g; yeast extract, 5 g; NaCl, 10 g; bacto agar, 15 g, and distilled water, 1,000 mL) using a platinum loop, and then cultured at 35°C, 150 rpm, pH 7.0 for 12 h in a shaking incubator. For the main culture, a 1.0% (v/v) strain was inoculated into C-medium (KH₂PO₄, 1.0 g; K₂HPO₄, 2.0 g; MgSO₄·7H₂O, 0.2 g; NaCl, 2.0 g; CaCl₂, 0.01 g; FeSO₄·7H₂O, 0.01 g; trace elements solution, 2 mL; pH, 6.8; distilled water, 1,000 mL, and the composition of trace elements solution: MoO₃, 1.0 mg; ZnSO₄·5H₂O, 7.0 mg; CuSO₄·5H₂O, 0.5 mg; H₃BO₃, 1.0 mg; MnSO₄·5H₂O, 1.0 mg; CoCl₂·6H₂O, 1.0 mg, and NiSO₄·7H₂O, 1.0 mg; distilled water, 1,000 mL) [20]. The carbon sources used were glucose, glycerol, *n*-C₁₀ to *n*-C₂₂, and diesel oil, added as 2.0% (v/v), and then run at 35°C, pH 7.0, and 150 rpm for 5 days.

Effect of Nitrogen and Carbon Sources

As well as the carbon source, nitrogen sources also affect the production of biosurfactants [1]. The effect of different nitrogen sources on the biosurfactant productivities and activities were also investigated. NaNO₃, NaNO₂, NH₄Cl, and (NH₄)₂SO₄ were used as inorganic nitrogen sources, with yeast extract, beef extract, malt extract, and trypton used as organic nitrogen sources which were added to the C-medium at concentrations of 0.01, 0.05, 0.1, and 0.5% (w/v), respectively. We checked their effects by the germ growth and biosurfactant activity [19,20]. Glycerol and glucose were used as water-soluble carbon sources, with diesel and *n*-alkanes as water-immiscible carbon sources. Three or five parallel experiments were performed to reduce the experimental errors. The data used were the mean values of three determinations from multiple measurements. The C/N ratio was controlled by changing the carbon concentration.

Biosurfactant Extraction

P. aeruginosa F722 culture broth was cultivated in BS medium, with the concentrations of NH₄Cl, yeast extract, and glucose in the C-medium adjusted to 0.05, 0.1, and 3% (w/v), respectively, at 35°C, 150 rpm, and pH 7.0 for 5 days. BS medium, from other experimental work, has been reported to be suitable for checking the production of biosurfactants and the measurement of their activities [26,27].

Extraction of the biosurfactant was achieved as follows. The culture broth was centrifuged at 12,000 × g and 4°C for 15 min. The pellet was discarded, and the supernatant adjusted pH to 2.0 with 2 M HCl, and kept at a low temperature for 12 h. The biosurfactant was recov-

ered using chloroform-methanol (1:1, v/v) liquid, which was previously identified to be effective for this use by Kuyukina *et al.* [28]. After evaporating the organic solvent from the recovered organic solvent layer at 50°C, the residual biosurfactant was collected [29], and then the clear zone and surface area was checked to evaluate the biosurfactant activity.

Analytical Methods

The microorganism growth was measured using both a Spectrophotometer (UV-1601, Shimadzu, Japan) at O.D_{600nm} (Optical Density) and the colony-forming units of indirect and direct enumeration methods, respectively. The residual glucose was analyzed using a Glucose-E Kit (BC 103-E). With increasing biosurfactant activity, the surface tension and clear zone size decreased and increased, respectively. The biosurfactant produced and its activity was efficiently monitored by measuring the clear zone size and surface tension, respectively. To measure the clear zone size, 30 mL of tap water was dispensed onto a petri-dish (90 × 15 mm), with 50 μL of *Eleuthera* dropped to form an oil film, and then the size measured with a rule. The surface tension of culture supernatant was measured at room temperature using a Du Nouy tensiometer (Du Nouy, model No. 3010, Japan) [1].

Air Supply

To investigate the effect of aeration on the production of the biosurfactant, air was supplied at 0.5 to 1.0, 1.5, 2.0, and 2.5 vvm (air/liquid/min) [30]. A 2,000 mL laboratory bottle, containing 200 mL BS medium, was inoculated with 1% (v/v) cell broth (about 10⁹ cells mL⁻¹), which had been previously cultured for 12 h in LB medium, and incubated on a rotary shaker at 35°C and 150 rpm, both with and without aeration.

RESULT AND DISCUSSION

Effect of Nitrogen and Carbon Sources

Table 1 shows the experimental results for the different nitrogen sources. The growth of *P. aeruginosa* F722 was most favorable with 0.1% NaNO₂ and 0.05% NH₄Cl as the inorganic nitrogen sources, and trypton and yeast extract as the organic nitrogen sources. The microbial growths (O.D_{600nm}) were 1.88, 1.58, 0.70, and 0.61 for NaNO₂, NH₄Cl, trypton, and yeast extract, respectively.

In an experiment to find the optimum ratio of inorganic/organic nitrogen source, a mixing ratio of 0.05% (w/v) NH₄Cl and 0.1% (w/v) yeast extract (Table 1) was found to be best. Luis *et al.* [26] reported that yeast extract, now ordinarily using for biosurfactant production, was not a favorable nitrogen sources due to its complexity. However, it is still used as no substitute for yeast extract has been found. Conversely, Benincasa *et al.* [5] reported on the importance of carbon sources in affecting productivity increases.

Table 1. Effect of nitrogen sources on microbial growth

		Growth ^a (O.D. 600 nm)
Inorganic	0.1% NaNO ₂	1.88 (± 0.11)
	0.5% NaNO ₃	0.62 (± 0.03)
	0.05% NH ₄ Cl	1.58 (± 0.09)
	0.5% (NH ₄) ₂ SO ₄	0.69 (± 0.04)
Organic (0.5%)	Beef extract	0.44 (± 0.02)
	Malt extract	0.22 (± 0.01)
	Trypton	0.70 (± 0.04)
	Yeast extract	0.61 (± 0.02)

Crude oil was used as the sole carbon source.

^aValues were means of three determinations in five parallel experiments (± standard deviations).

Table 2. Effect of carbon sources on microbial growth and biosurfactant activity

Carbon sources (2.0%)		Growth ^a (O.D. 600 nm)	Clear zone ^b (cm)
Soluble	Glucose	2.53 (± 0.08)	9.0 (± 0.1)
	Glycerol	2.68 (± 0.12)	8.8 (± 0.2)
Insoluble	<i>n</i> -C ₁₀	2.89 (± 0.13)	5.7 (± 0.2)
	<i>n</i> -C ₁₂	1.50 (± 0.15)	1.0 (± 0.2)
	<i>n</i> -C ₁₄	1.17 (± 0.19)	1.2 (± 0.3)
	<i>n</i> -C ₁₆	1.22 (± 0.17)	3.7 (± 0.1)
	<i>n</i> -C ₁₈	2.87 (± 0.21)	8.5 (± 0.4)
	<i>n</i> -C ₂₂	1.98 (± 0.25)	0.2 (± 0.5)
	Diesel	1.99 (± 0.07)	2.1 (± 0.2)

Nitrogen sources were 0.05% (w/v) NH₄Cl and 0.1% (w/v) yeast extract.

^{a,b}Values were means of three determinations in three parallel experiments (± standard deviations).

According to Table 2, the clear zone size was highest in the soluble carbon sources. The microbial growths (O.D._{600nm}) and clear zone sizes for glucose and glycerol were 2.53 and 9.0 cm, and 2.68 and 8.8 cm, respectively. The clear zone sizes with the insoluble carbon sources were smaller than those for the soluble sources, despite the higher microbial growth in *n*-C₁₀ and *n*-C₁₈. However, in diesel, the microbial growth was very low, so reuse of the carbon source was difficult. Between glucose, glycerol, and *n*-C₁₈, glucose was selected for its ease of analysis and effectiveness in biosurfactant production.

Luis *et al.* [26] reported the optimum C/N ratio for biosurfactant production was 18, and could be restricted below a ratio of 11. In our experiments, the ratio was controlled at 7, 13, 20, and 27 by varying the glucose concentration to 10, 20, 30, and 40 g/L, respectively. With increasing C/N ratio, the cell growth gradually increased, but the clear zone size remained reasonably unchanged (Table 3). Therefore, the microbial growth

Table 3. Effect of glucose concentration on biosurfactant activity, microbial growth, and C/N ratio

Glucose (g/L)	Growth ^a (g/L)	Clear zone ^b (cm)	C/N ratio
10	1.91 (± 0.14)	8.8 (± 0.3)	7
20	2.30 (± 0.17)	9.0 (± 0.1)	13
30	2.98 (± 0.21)	9.0 (± 0.1)	20
40	3.38 (± 0.28)	8.9 (± 0.2)	27

Nitrogen sources were 0.05% (w/v) NH₄Cl and 0.1% (w/v) yeast extract.

^{a,b}Values were means of three determinations in five parallel experiments (± standard deviations).

were inconsistent with clear zone size. From our results, the optimum C/N ratio for microbial growth and biosurfactant production was found to be 20.

Effect of Aeration

The effect of aeration on the production of biosurfactant was investigated. Table 4 shows the velocities of the glucose degradation during each microbial growth phase, as well as the biosurfactant productivity and surface tension due to aeration. With aeration, the cultivation time to produce the biosurfactant was shorter than with no aeration. The rate of glucose removal due to the microorganism increased, and was highest at the log phase. With aeration of 1.0 vvm, the cultivation time was short up to 108 h, compared to 144 h with no aeration, with the BS productivity increasing to 1.94 g/L compared to 1.67 g/L with no aeration.

The surface tension, which was dependent on the biosurfactant activity, was lowest at 1.5 vvm, and decreased to 30.27 mN/m with aeration, compared to 31.27 mN/m with no aeration. Aeration was effective at increasing the production of biosurfactant, reducing the surface tension and increasing the microbial growth. Our results were similar to those of Desal and Banat [1], in that the surface tension of the biosurfactant produced by *Pseudomonas* spp. was 25~30 mN/m. It was also consistent with the report by Kim [30], in which the air supply was effective at increasing the biosurfactant activity.

Changes of Each Factor with Cultivation Time

The microbial growth, glucose degradation, pH, surface tension, and clear zone were investigated in relation to the culture time and aeration. Fig. 1 shows the change in each factor at a 30 g/L (= 20 C/N) initial glucose concentration with no aeration. The residual glucose concentration gradually decreased with cultivation time, and the pH was also found to drop to 5 after 30 h, which was suspected to the accumulation of organic acids due to glucose degradation. The microbial growth rapidly increased with glucose degradation, but slightly decreased after 40 h, despite the 20 g/L of high residual glucose concentration, but finally stabilized at 3.5 O.D._{600nm}. The clear zone increased with increasing mi-

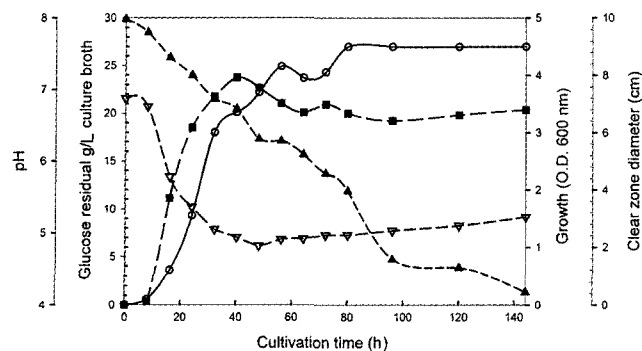


Fig. 1. The changes in the pH, glucose degradation, microbial growth, and clear zone diameter with culture time in modified C-medium (usually called BS-medium) with no aeration. Symbols: —■—, growth (O.D. 600 nm); —▲—, glucose residual g/L culture broth; —▽—, pH; —○—, clear zone diameter (cm).

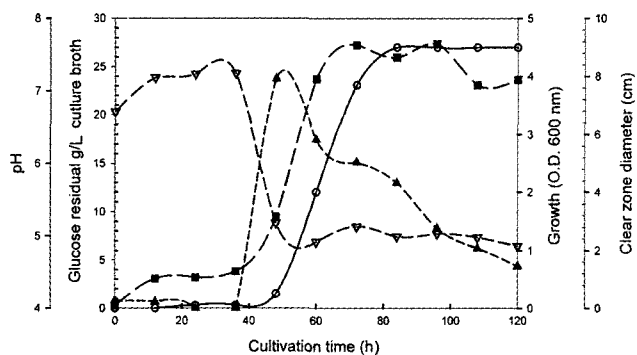


Fig. 2. The changes in the pH, glucose degradation, microbial growth, and clear zone diameter with culture time in modified C-medium (usually called BS-medium) with no aeration. The carbon source (glucose 30 g/L) was added after the 36 h. Symbols: —■—, growth (O.D. 600 nm); —▲—, glucose residual (g/L culture broth); —▽—, pH; —○—, clear zone diameter (cm).

crobial growth, and stabilized at 9.1 cm after 80 h. We suspected that the microbial growth and clear zone diameter could be restricted by low pH after 40 h.

The data in Fig. 2 were obtained under the same conditions as those of Fig. 1, with the exception of a glucose injection after 36 h. There have been many reports on additional injection of carbon sources for increasing the biosurfactant productivity at the point of nitrogen sources exhausted [1,5,26,27]. Based on these reports, we added 3.0% glucose to the culture broth after 36 h. However, when the carbon sources were added at this point, the O.D. 600 nm neared 4.5 after 72 h, with a clear zone diameter of 9.0 cm at 80 h, which was similar to the result of Benincasa *et al.* [5] (Fig. 2).

Initially (before 36 h), the glucose concentration was very low; therefore, the pH did not drop. Low microbial growth leads to low biosurfactant production and activity, and in the result would be suspected to lead to a lower clear zone size. After 40 h, the point of glucose addition, Fig. 2 has a similar curve to those of Fig. 1, with the exception of the higher microbial growth. Clear zone di-

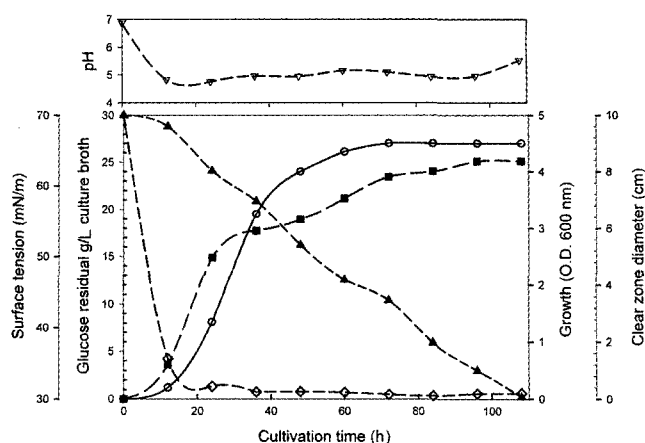


Fig. 3. The changes in the pH, glucose degradation, microbial growth, and clear zone diameter with culture time in modified C-medium (usually called BS-medium) with aeration at 1.0 vvm. Carbon source (glucose 30 g/L) was added after the 36 h. Symbols: —■—, growth (O.D. 600 nm); —▲—, glucose residual g/L culture broth; —▽—, pH; —○—, clear zone diameter (cm); —◇—, surface tension (mN/m).

ameter became stable after 80 h.

Fig. 3 shows the curves with on aeration at 1.0 vvm. With aeration, the pH dropped more rapidly than with no aeration (Fig. 1), and rapidly dropped to 5 after 10 h with aeration compared to 30 h without aeration. However, the microbial growth remained almost unchanged compare to no aeration, despite the pH drop. The remaining glucose concentration also became lower with aeration compared to no aeration at the same culture time. As aeration reduced the influence of pH on microbial growth, we suspected the glucose degradation velocity by the microorganism had increased. With 1.5 vvm, as shown in Table 4, the glucose degradation velocity at the log and stationary phases were increased to 0.40 and 0.38 h^{-1} with aeration from 0.25 and 0.18 h^{-1} without aeration. The surface tension dropped to 30.27 mN/m after 20 h, and the clear zone diameter rapidly increased, but finally stabilized at 9 cm after 60 h. With aeration, the time needed to achieve a clear zone diameter of 9 cm became shorter to 60 h, compared to 80 h with no aeration. Therefore, aeration was effective in reducing the culture time for biosurfactant production.

CONCLUSION

P. aeruginosa F722 growth was more favorable at inorganic nitrogen sources, such as NaNO_2 and NH_4Cl , than organic nitrogen sources. *P. aeruginosa* F722 growth was higher in insoluble carbon sources, but the clear zone size was bigger in soluble carbon sources. The optimum C/N ratio for microbial growth was found to be 20. Aeration was very effective in reducing the culture time, microbial growth rate, biosurfactant production, and surface tension. Aeration reduced the influence of pH on the growth of *P. aeruginosa* F722. As the rate of glucose removal was

Table 4. Effect of aeration on glucose degradation rate, biosurfactant production, surface tension, and culture time

Volume of supplied air (vvm)	Lag phase ^a (h ⁻¹)	Log phase ^b (h ⁻¹)	Stationary phase ^c (h ⁻¹)	BS production ^d (g/L)	Surface tension ^e (mN/m)	Cultivation time ^f (h)
0	0.17	0.25	0.18	1.66	31.27	144
	(± 0.02)	(± 0.01)	(± 0.02)	(± 0.05)	(± 0.45)	(± 18)
0.5	0.52*	0.26*	0.17*	1.67*	30.82*	144*
	(± 0.03)	(± 0.01)	(± 0.01)	(± 0.04)	(± 0.53)	(± 12)
1.0	0.31	0.33	0.25	1.54	30.46	108
	(± 0.01)	(± 0.03)	(± 0.03)	(± 0.06)	(± 0.62)	(± 10)
1.5	0.10	0.33	0.29	1.94	30.46	108
	(± 0.01)	(± 0.03)	(± 0.02)	(± 0.12)	(± 0.57)	(± 16)
2.0	0.18	0.40	0.38	1.64	30.27	90
	(± 0.02)	(± 0.04)	(± 0.05)	(± 0.09)	(± 0.71)	(± 12)
2.5	0.16	0.38	0.38	1.45	30.58	84
	(± 0.03)	(± 0.04)	(± 0.04)	(± 0.08)	(± 0.68)	(± 8)
2.5	0.33	0.27	0.38	1.22	30.91	84
	(± 0.04)	(± 0.03)	(± 0.05)	(± 0.07)	(± 0.49)	(± 12)

*Glucose 30 g/L as carbon source was added after the 36 h without aeration.

^{a,b,c,d,e,f}Values were means of three determinations in three parallel experiments (± standard deviations).

highest during the log phase, a bio-reactor should be run at this phase for maximum biosurfactant production. With no aeration, the transition time from the log phase to the stationary phase was short. However, the time became longer with aeration, and was able to sustain the log phase for longer time. In the exponential and stationary phases, the rate of glucose removal with aeration was 0.08, 0.11 h⁻¹ higher than that with no aeration. With aeration, the biosurfactant productivity was also increased up to 18% compared to that with no aeration. However, excessive aeration reversely decreased the productivity; so it was necessary to check the optimum volume before commencing a new experiment. Conversely, the injection of a carbon source after 36 h did not effect on the microbial growth and biosurfactant activity.

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