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Original Article

# Biosurfactant production in a stirred-tank fermenter under mechanical foam control

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## ABSTRACT

Production of surfactin, a lipopeptide-type biosurfactant, requires mechanical foam control using a foam-breaking apparatus to cultivate *Bacillus subtilis* ATCC 21332 without addition of an anti-foaming agent. The amount of surfactin produced through such mechanical foam control was greater than that by chemical foam control with addition of the anti-foam agent under identical culture conditions. The surfactin production rate was increased by raising the iron ion concentration during the cell growth decline phase. In a stirred-tank fermenter with mechanical foam control, the overall surfactin production rate reached 40.3 mg/(hr·L) under conditions where the iron ion concentration was increased to 8 mM after 18 hr of culture. This production rate was 10.9 times that obtained without adding iron ion during the culture period.

**KEYWORDS:** biosurfactant production, mechanical foam control, stirred-tank fermenter, surfactin.

## INTRODUCTION

Biosurfactants are biologically surface-active agents produced by microorganisms. Their superior properties of low toxicity and high biodegradability are environmentally acceptable [1-8]. For these reasons, they are applied in environmental techniques used for bioremediation, enhanced oil recovery, oil spill dispersion, etc. [9-11]. Moreover, biosurfactants have antibacterial activity, interfacial features, and several biological activities [8]. Therefore, their application in pharmaceuticals, biomedical fields, and cosmetic products is eagerly anticipated.

Many microorganisms that produce biosurfactants are cultivated in aerobic conditions. Sufficient quantities of oxygen for aerobic fermentation is supplied during the aeration and agitation processes, resulting in foams. The presence of surface-active solutes in the culture broth stabilizes the foams. We earlier recommended a mechanical foam control method in a reaction system in which foaming occurs violently [12-15]. A chemical foam control method incorporating anti-foaming agents markedly lowers the mass transfer rate, reaction inhibition, and toxicity or adverse effects on separation and purification of products [16-19]. For an earlier study [20], we applied a stirred-tank fermenter under mechanical foam control to produce a biosurfactant by *Bacillus subtilis* NT1, isolated from fermented soybean 'natto'. The biosurfactant was concentrated in the foam layer formed in the fermenter. The biosurfactant was recovered by drawing the foam from the fermenter during the culture period. However, the amount of biosurfactant produced was low. Foaming becomes stronger as more biosurfactant is produced. Under such strong foaming, investigation of the effectiveness of the mechanical foam control method is necessary. *Bacillus subtilis* ATCC 21332 is well studied as a microorganism

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producing a lipopeptide-type biosurfactant, 'surfactin' [21-24]. This study examined the conditions at which increased amount of surfactin is produced by this microorganism. The application of a stirred-tank fermenter equipped with a foam-breaking apparatus was attempted for surfactin production with strong foaming.

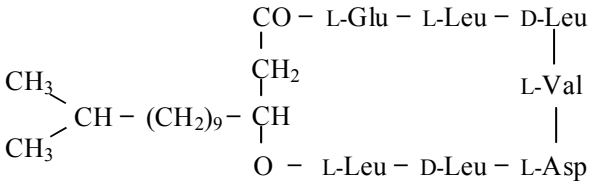


**Figure 1.** Structure of surfactin.

## MATERIALS AND METHODS

### Microorganism and culture conditions

*Bacillus subtilis* ATCC 21332 was used to produce surfactin (Figure 1). The strain was grown on a modified Cooper's medium [2] consisting of glucose (40 g/L), $NH_4NO_3$ (50 mM), $KH_2PO_4$ (30 mM), $Na_2HPO_4$ (40 mM), $MgSO_4 \cdot 7H_2O$ (0.8 mM), $CaCl_2$ (7 μM), $FeSO_4 \cdot 7H_2O$ (4 μM), sodium EDTA (4 μM), and yeast extract (5 g/L). Two loopfuls of cells grown on a nutrient agar slant were added to this medium, to make 200 mL of solution in a 500-mL Erlenmeyer flask. In shake-flask culture experiments, this was incubated at 303 K with shaking at 130 rpm. In addition, shake-flask culture was performed for 24 hr to produce seed cultures for experiments using a jar fermenter.

### Culture using a jar fermenter

A schematic diagram of a 10-l jar fermenter (TEJC-L; Chiyoda Seisakusho Co., Ltd., Japan) is presented in Figure 2. The jar fermenter, with 0.186 m vessel diameter and 0.375 m height, was equipped with four baffles. The working liquid volume was 4 L in the 0.4 L of the culture broth of the seed culture and 3.6 L of the medium. The temperature was maintained at 303 K; pH was not controlled. A rod impeller was used as the agitation impeller (Figure 3(a)). The rod impeller can double its rotational speed at the same level of agitation power as the conventional six-blade turbine impeller, as described in a report of an earlier study [15]. A six-bladed vaned disk (Figure 3(b)), used as a foam-breaking impeller, was set at 0.31 m from the vessel bottom on the same shaft as the agitating impeller. The liquid in the jar fermenter was supplied at 4.67 mL/s onto the rotating foam-breaking impeller. The resulting liquid is dispersed from the circumference of the foam-breaking impeller by the centrifugal force. It has effect of defoaming. In the chemical foam control method, an anti-foaming agent (silicon oil

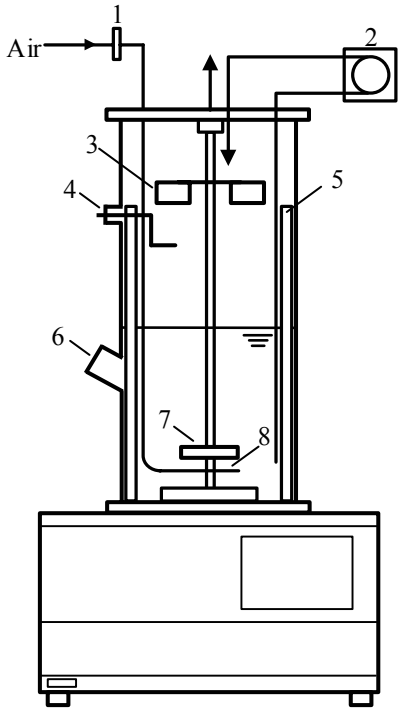


**Figure 2.** Schematic diagram of experimental apparatus: (1) filter, (2) pump, (3) foam-breaking impeller, (4) foam-sampling tap, (5) baffle plate, (6) oxygen electrode socket, (7) agitation impeller, and (8) ring sparger.

KM-70; Shin-Etsu Chemical Co., Ltd., Japan) was added at 1000 ppm. The air sparge rate and impeller rotational speed were maintained at 2.0 L/min and 800 rpm, respectively.

Growth was monitored by measuring the optical density of the culture broth at 600 nm ($OD_{600}$) on a UV-visible spectrophotometer (U-3210; Hitachi Ltd., Japan). The cell concentration was expressed as dry weight at the time of collection and was calculated using the following equation, which is derived from the relation between cell concentration ($C$, g/L) and $OD_{600}$.

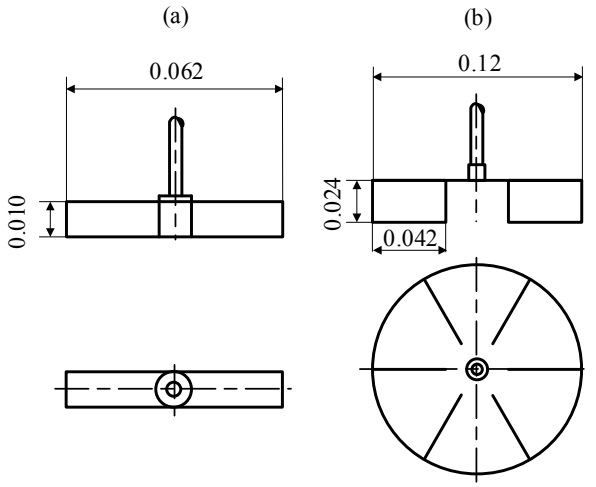


**Figure 3.** Designs and dimensions (m) of agitation and foam-breaking impellers: (a) agitation impeller, rod impeller; and (b) foam-breaking impeller, six-blade vaned disk.

$$C = 0.323 \times OD_{600} \quad (1)$$

The dissolved oxygen concentration was measured using an oxygen electrode (DO-1; Tokyo Rikakikai Co., Ltd., Japan). The height of the foam section and the liquid holdup in the foam were measured to evaluate the liquid volume in the foam layer. The liquid holdup in the foam was measured using the foam sampling technique [12].

### Biosurfactant purification

The bacterial cells were removed from the culture broth by centrifugation at 10,000 × *g* for 10 min at 277 K. The cell-free culture broth was adjusted to pH 2.0 with 6 M HCl and was kept overnight. The precipitate formed was collected by centrifugation at 14,000 × *g* for 90 min at 277 K. After the biosurfactant was extracted from the precipitate using chloroform–methanol (2 : 1, by volume), the solvent was removed using a centrifugal vacuum concentrator (VC-36N; Taitec Corp., Japan). Reversed-phase HPLC was performed using a Hitachi model (L-6320, L-5030; Hitachi High-Technologies Corp., Japan) on a column (5 μm, 250 × 4.6 mm, Asahipak ODP-50; Showa Denko K. K., Japan) and a mobile phase of acetonitrile − 50 mM ammonium acetate buffer (51 : 49, by volume) at a flow rate of 0.8 mL/min. Separation was monitored using UV detection (L-4000H; Hitachi High-Technologies Corp., Japan) at 210 nm. A commercially available surfactin (Sigma-Aldrich Co. LLC, USA) was used as the authentic standard.

## RESULTS AND DISCUSSION

### Production of biosurfactant in a stirred-tank fermenter under mechanical and chemical foam control

Both mechanical and chemical foam control methods are useful for foam control during aerobic culture. *Bacillus subtilis* ATCC 21332 was cultivated to produce surfactin in a stirred-tank fermenter using both foam control methods. According to the mechanical foam control method, foam breaking was accomplished in 48 hr. Because of this process, a foam layer was formed in the fermenter. As demonstrated in an earlier study [20], the produced biosurfactant concentrated in the foam layer. The fermenter under mechanical foam control can be expected to achieve production and enrichment of surfactin simultaneously. In the chemical foam control method, an anti-foaming agent was added at 1000 ppm concentration. No foam formation occurred during the culture period. Table 1 presents results of surfactin production using both foam control methods after 48 hr. Surfactin production using the mechanical foam control method was much higher than that using the chemical foam control method. Our earlier studies [15, 18] showed that addition of anti-foaming agents markedly lowered the volumetric mass transfer coefficient, which is related to oxygen supply rate. The decrease in oxygen supply rate affects cell growth and biosurfactant production. The difference in the amounts of surfactin produced might be attributable to the difference in oxygen supply rate. Application of the fermenter under mechanical foam control to surfactin production by *Bacillus subtilis* ATCC 21332 was effective. However, the overall surfactin production rate in the mechanical foam control method was 3.70 mg/(hr·L), which was lower than the results reported by earlier studies [22, 24].

### Effects of iron ion concentration on surfactin production in shake-flask culture

The iron ion concentration in the culture broth reportedly affects surfactin production [2, 24].

**Table 1.** Surfactin production by *Bacillus subtilis* ATCC 21332 in a stirred-tank fermenter using different foam control methods.

| Foam control method | Surfactin production* (mg) | Overall surfactin production rate (mg/(hr·L)) |
|---|---|---|
| Mechanical foam control | 710 | 3.70 |
| Chemical foam control | 198 | 1.03 |

*Value at 48 hr.

Before culture using a jar fermenter, the effects of iron ion concentration on cell and surfactin concentrations were examined in a shake-flask culture. The iron ion concentration was adjusted with $FeSO_4 \cdot 7H_2O$ of the medium component. The iron ion concentration of the modified Cooper's medium used was 4 μM. Its concentration was increased from 4 μM to 2 mM. The cells were cultured at different timings to increase the iron ion concentration. Figure 4 presents the change of cell concentration, together with the result at 4 μM. An increase in the iron ion concentration at the start of culture (0 hr) accelerated cell growth but stopped it after 8 hr. The final cell concentration was approximately the same as that found at 4 μM. An increase in the iron ion concentration at 9 hr, corresponding to the logarithmic growth phase, doubled the final cell concentration, but cell growth ceased after 18 hr. An increase in the iron ion concentration at 18 hr, corresponding to the growth decline phase, allowed the cells to grow until 46 hr. This result suggests that the cells cultured at different iron ion concentrations increased after 18 hr of culture. Its concentration was varied: 2-8 mM. Figures 5(a) and 5(b) show the cell concentration and surfactin concentration, respectively. The cell concentration was highest at the iron ion concentration of 8 mM. As for surfactin production, the increase in iron ion concentration after 18 hr caused the surfactin concentration to increase sharply at 24 hr, as shown in Figure 5(b). The surfactin concentration after 48 hr was the highest at the iron ion concentration of 8 mM, as was the cell concentration. The increase in the iron ion concentration of 8 mM after 18 hr increased the surfactin concentration from 7.37 mg/L, obtained without adding iron ion, to 2127 mg/L.

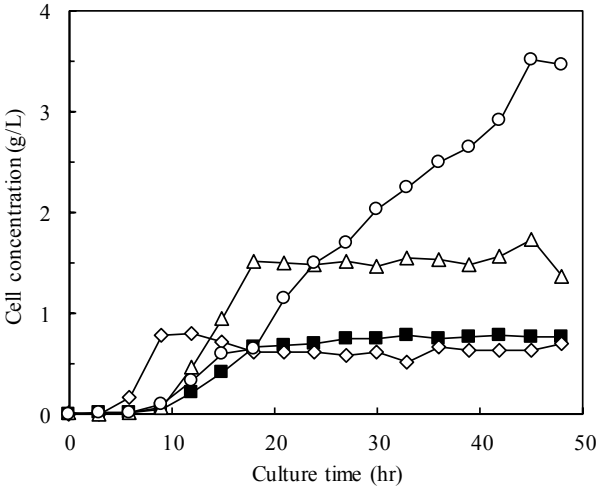


**Figure 4.** Effects of timing of increase of iron ion concentration to 2 mM on cell growth: (■) without adding iron ion, (◇) 0 hr, (Δ) 9 hr, and (○) 18 hr.

## Effects of iron ion addition on surfactin production in a stirred-tank fermenter equipped with a foam-breaking apparatus

Based on shake-flask culture results, a stirred-tank fermenter equipped with a foam-breaking apparatus was used for surfactin production with increased iron ion concentration during culture. The air sparge rate was set to 2.0 L/min for comparison with the results in Table 1. In the culture without adding iron ion, shown in Table 1, the cell mass in the fermenter began to become constant around 18 hr in the culture at 2.0 L/min. Therefore, the iron ion concentration was increased to 8 mM at 18 hr, corresponding to the growth decline phase. Figures 6(a) shows changes in the cell concentration in the foam section and culture broth section. Results of the effects of iron ion concentration of 4 μM without adding iron ion are

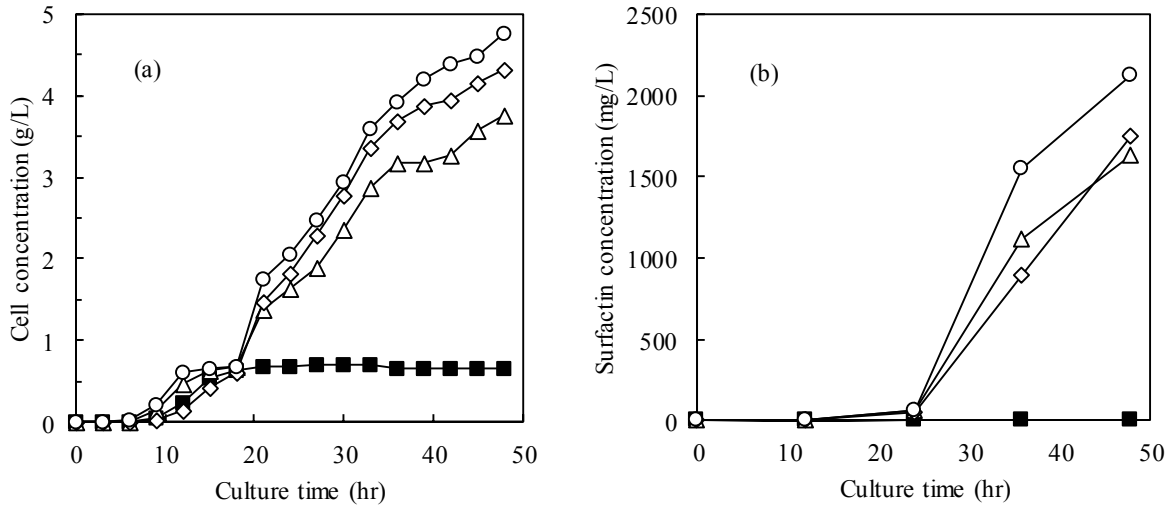


**Figure 5.** Effects of increasing iron ion concentration on cell growth and surfactin production: (a) cell concentration, (b) surfactin concentration; (■) without adding iron ion, (Δ) 2 mM, (◇) 6 mM, and (○) 8 mM.

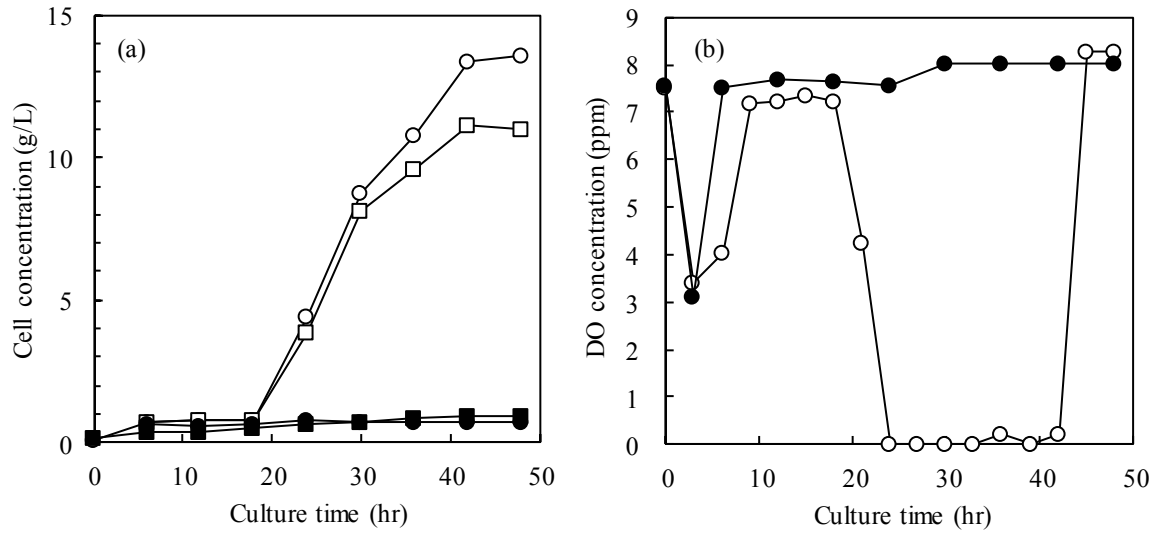


**Figure 6.** Comparison of cell growth and DO with and without adding iron ion: (a) cell concentration, (●) the foam section without adding iron ion, (■) the culture broth section without adding iron ion, (○) the foam section with addition of iron ion, (□) the culture broth section with addition of iron ion; (b) DO (●) without adding iron ion and (○) with addition of iron ion.

also shown for comparison. An increase in iron ion concentration sharply increased the cell concentrations of both sections, as in the shake-flask culture. Figures 6(b) show changes in the dissolved oxygen (DO) concentration in the culture solution. The DO concentration decreased rapidly when the iron ions were added. In fact, it was near zero until 42 hr, which suggests that the cells consume dissolved oxygen and that cell growth becomes active. The return of DO concentration after 42 hr indicated that the cell growth slowed down, as depicted in Figure 6(a).

Figure 7 presents changes in the amount of surfactin produced with and without increasing the iron ion concentration. The amount of surfactin produced is the sum of the amount of

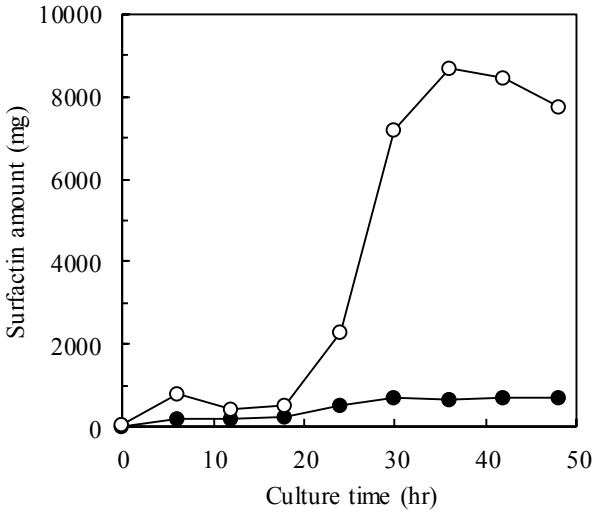


**Figure 7.** Comparison of surfactin production and added iron ion: (●) without adding iron ion and (○) with addition of iron ion.

foam and culture broth section. The amount of surfactin produced without increasing the iron ion concentration was 710 mg at 48 hr, as shown in Table 1. In contrast, the iron ion concentration rose markedly to 7740 mg at 48 hr. In addition, the overall surfactin production rate was 40.3 mg/(hr·L), which was 10.9 times that without increasing the iron ion concentration. Although considerably strong foaming was apparent by visual observation, mechanical foam control using this foam-breaking apparatus enhanced surfactin production by *Bacillus subtilis* ATCC 21332.

## CONCLUSIONS

Surfactin production by *Bacillus subtilis* ATCC 21332 was achieved in a stirred-tank fermenter equipped with a foam-breaking apparatus. The amount of surfactin produced using mechanical foam control with the foam-breaking apparatus was greater than that in the chemical foam control with addition of an anti-foam agent. An increase in iron ion concentration decreased dissolved oxygen and activated cell growth, causing an increase in surfactin concentration. Under the conditions of this cultivation experiment, the maximum amount of surfactin produced was 7740 mg for 48 hr. The overall production rate was 40.3 mg/(hr·L).

## CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest related to the content or composition of this article.

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