

## Enhanced Production of Surfactin by *Bacillus subtilis* E8 Mutant Obtained by Ion Beam Implantation

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### Summary

The application of surfactin is hampered by its high production cost. In this study, a high-producing surfactin mutant obtained by ion beam implantation, *Bacillus subtilis* E8, has been used to produce surfactin. The production in a modified bioreactor and the properties of the surfactin produced by the mutant strain have been investigated. The results indicate that the modified bioreactor with a cell/foam recycler showed an advantage as compared to a simple reactor. The concentration of crude surfactin (including recovery from two parts of foam and broth fractionation) increased significantly from 0.75 to 10.26 g/L. Time course profiles showed that surfactin produced by *B. subtilis* E8 was growth-associated during the exponential phase of biomass, and the growth-associated product formation constant ( $\alpha$ ) was 894 mg per g of dry cell ( $R^2=0.918$ ). The concentration of crude surfactin and biomass reached a maximum (12.20 and 6.50 g/L) after cultivation for about 32 h. The crude and extracted surfactin (critical micelle concentrations were 15 and 13  $\mu\text{M}$ , respectively) exhibited excellent emulsification property, pH stability, thermal stability and resistance to salts, which widens its potential application.

*Key words:* *Bacillus subtilis*, surfactin, ion beam implantation, biosurfactant, modified bioreactor

### Introduction

Biosurfactants have gained considerable interest in recent years due to their low toxicity, biodegradable nature and diversity, which makes them superior to chemical surfactants. Surfactin is one of the most powerful lipopeptide biosurfactants produced by various strains of *Bacillus subtilis*. Apart from the characteristics of high surface activity, low toxicity, high biodegradability and biocompatibility, surfactin has also got antimicrobial properties and inhibits fibrin clot formation (1,2). These favourable properties have great prospects in oil industry, environmental control, special cosmetics, agriculture, food and pharmaceutical applications. Although surfactin exhibits those important characteristics that are extensively employed in industry, its application has been

hampered by the high production costs. Applications will be practical once the process of biosurfactant production has become economical and the production rates have met the commercial demands. Therefore, it is important to improve the yield and reduce the production cost of surfactin and other lipopeptide biosurfactants.

Encouraging results have been obtained during the last 4 decades in improving the yield of surfactin. When Arima *et al.* (1) first found the novel biosurfactant named 'surfactin', its output was only 50–100 mg/L in a 24-hour culture. Then Cooper *et al.* (3) used a mineral-salts medium, which resulted in improved surfactin yield of 780 mg/L with continuous product removal and metal cation addition. Mulligan *et al.* (4) found an ultraviolet mutant of *Bacillus subtilis* ATCC 21332 which produced over three times more surfactin (1124 mg/L). Sen and

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Swaminathan (5) optimized the fermentation medium and obtained a maximum surfactin production of 760 mg/L. As a breakthrough, Wei and Chu (6) used inorganic salt-enriched medium accompanied by an appropriate pH control to achieve a production yield of nearly 3500 mg/L, which seemed to be ready for commercialized applications. Yeh *et al.* (7) demonstrated an enhanced production of surfactin (3600 mg/L) with the addition of solid carriers. An innovative bioreactor was also used to produce surfactin and gained an excellent maximum concentration of 6.45 g/L (8). These latter efforts made it possible to reduce the costs of surfactin production.

Kosaric *et al.* (9) suggested that several factors (microbes, process, the microbial growth substrate or feedstock processing and by-products recovery) influence biosurfactant production costs. Apart from microbes, growth substrate and the process for by-product management and recovery, the process of surfactin production has become rather difficult due to foaming, which is generally a serious problem in biosurfactant production. Some methods have been applied for manipulating this drawback (8,10). Davis *et al.* (10) reported the utility of foaming as a method for the recovery of surface-active fermentation products, particularly when used in an integrated production/recovery system. Furthermore, Yeh *et al.* (8) used an innovative bioreactor integrated with a foam collector, a cell recycler, and a surfactin precipitation unit to cope with the rapid foam generation. In our previous study (11), a mutant *Bacillus subtilis* E8 with high-producing surfactin property was obtained by ion beam implantation. After medium optimization, the outcome of crude surfactin (purity of approx. 55 %) was reached at 11–12 g/L after incubation for 48 h (unpublished data) in Erlenmeyer flasks. For possible industrial application, the production using a modified bioreactor and the properties of the surfactin from the ion beam implantation mutant *Bacillus subtilis* E8 have been investigated in this study.

## Materials and Methods

### Microorganisms and culture conditions

*Bacillus subtilis* E8 (which can produce surfactin) is a high-yielding mutant when subjected to ion beam im-

plantation (11). The slant cultures of the strain were maintained at 4 °C in our laboratory.

The cells were grown in Landy medium (12), a semi-synthetic medium, which served as the seeding medium. The fermentation medium consisted of (in g/L): soluble starch 80, NaNO<sub>3</sub> 15, MgSO<sub>4</sub> 0.5, KCl 0.5, KH<sub>2</sub>PO<sub>4</sub> 1, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.15, MnSO<sub>4</sub> 0.005, yeast extract 1, and CuSO<sub>4</sub>·5H<sub>2</sub>O 0.16 mg/L (pH=7.0). The seeding medium (100 mL) was cultured at 33 °C on a rotary shaker at 200 rpm for 24 h, and then inoculated into the fermentor (3.7 L, Bioengineering, Switzerland) containing 2 L of fermentation medium. The bioreactor modified with two foam collectors is shown in Fig. 1. The size of the first and second foam collectors was 2.0 and 1.0 L, respectively. Foam and liquid culture were recycled into the bioreactor with gas pressure. Batch cultures were incubated at 37 °C with an agitation rate of 300 rpm and aeration rate of 1.0 vvm (2.0 L/min). The pH in the bioreactor was controlled not to be lower than 7 by automatic addition of 1 M NaOH.

### Analytical methods

The fermentation culture was centrifuged at 10 000×g for 30 min. The cell precipitate was dried at 80 °C, and measured as biomass. Surfactin was extracted from the culture broth by classical methods including acidic precipitation and extraction by organic solvents. Crude surfactin was precipitated from the cell-free supernatant by adjusting the broth pH to 2.0 using 6 M HCl and keeping it at 4 °C overnight, then collected by centrifugation. The crude surfactin was extracted with dichloromethane. Surface tension of the cell-free culture was measured by Wilhelmy plate (Sigma 703 surface/interfacial tensiometer, USA). The critical micelle concentration (CMC) was determined by measurements of surface tension of serial diluted samples. The purity of surfactin was determined by HPLC (6). A Waters HPLC system equipped with a 5- $\mu$ m Kromasil C18 column was used. The mobile phase of 3.8 mM trifluoroacetic acid (20 %) and acetonitrile (80 %) was used, with a flow rate of 1.0 mL/min. A 20- $\mu$ L prefiltered sample was injected, and the absorbance of the eluent was monitored at 205 nm. The surfactin from Sigma (purity above 98 %) was used as a standard.

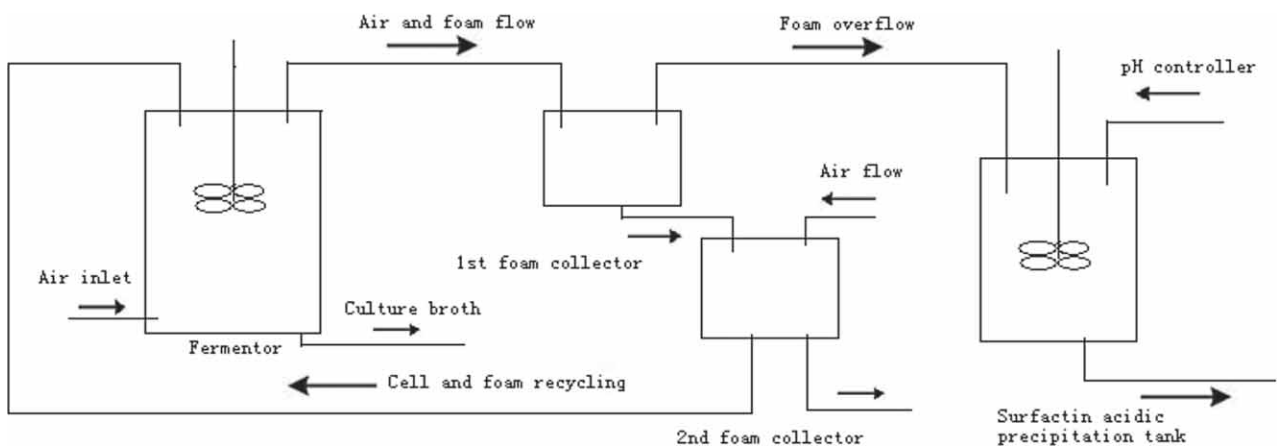


Fig. 1. Schematic description of bioreactor module for surfactin production

Emulsification activity was evaluated according to Healy *et al.* (13), whereby 6 mL of *n*-hexadecane were added to 4 mL of aqueous solution of biosurfactant (1 mg/mL) in a graduated tube and vortexed at a high speed for 2 min. The emulsion stability was determined after 24 h, and the emulsification index ( $E_{24}$ ) was calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying it by 100.

## Results and Discussion

### The production of surfactin in the modified bioreactor

The production of surfactin was investigated under defined aeration (1.0 vvm) and agitation rate (300 rpm). The fermentations of batch 1 and batch 2 were manipulated in a bioreactor without cell/foam recycler, but the overflowed foam was collected. The method for surfactin recovery was similar to the one demonstrated by Davis *et al.* (10). The last 5 batches were carried out for antifoam-free fermentation in the modified bioreactor. The method of production was similar to the one applied by Yeh *et al.* (8). The results in Fig. 2 indicate that the modified bioreactor with cell/foam recycler gave better results as compared to the bioreactor without the recycler. The concentration of crude surfactin (including recovery from both foam and broth fractionation) increased significantly from (0.75±0.37) g/L (batches 1 and 2) to (10.26±0.78) g/L (batches 3–7). The crude surfactin was mainly concentrated in the foam fractionation in batches 1 and 2, but some was recovered from broth fractionation. In contrast, the quantity of surfactin recovered from the broth (batches 3–7) was superior to the one from the foam fractionation.

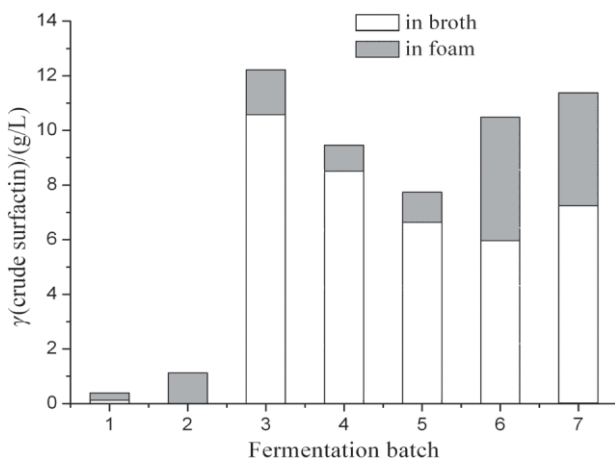


Fig. 2. Surfactin production in batch fermentation of *Bacillus subtilis* E8

Under the conditions of high agitation (350 rpm) and aeration (1.5 vvm) rates, the runs with only one foam collector had difficulties in avoiding the loss of broth due to rapid foam formation, and the effect of increasing the volume of foam collector or speed of peristaltic pump was limited (8). The design of two collectors and recycling by gas pressure can resolve the problem and is suitable for large-scale fermentation. By manipu-

lating the gas pressure of the two collectors, the process of liquefying the foam was enhanced. Foam and liquid broth in the second collector were transmitted into the bioreactor rapidly by air pressure. If only a single collector had been used, the air flow would have caused the failure of the total system.

The time course profiles of surfactin production in the modified bioreactor are shown in Fig. 3. It was revealed that in the exponential phase of biomass, the surfactin production was growth-associated. A parallel relationship was observed between growth and surfactin production in this period. According to the growth associated kinetics,  $q_p = (1/X)(dP/dt) = \alpha\mu$ , where  $q_p$  denotes specific surfactin production rate,  $P$  is surfactin concentration,  $X$  is cell concentration,  $\alpha$  is growth-associated product formation constant and  $\mu$  is specific growth rate  $= (1/X)(dX/dt)$ ; the  $\alpha$  value was calculated as 894 mg per g of dry cell ( $R^2=0.918$ ). This growth-associated relationship in biosurfactant production had been reported in some other *B. subtilis* strains (7,14). The concentrations of crude surfactin and biomass reached maximum (12.20 and 6.50 g/L, respectively) after cultivation for about 32 h, and the concentration of surfactin measured by HPLC method reached maximum (5.89 g/L) in 40 h. This result indicates that the surfactin production by the mutant strain could be terminated at 32–40 h under the culture conditions, thus the culture time was shorter as compared to the Erlenmeyer flask cultivation.

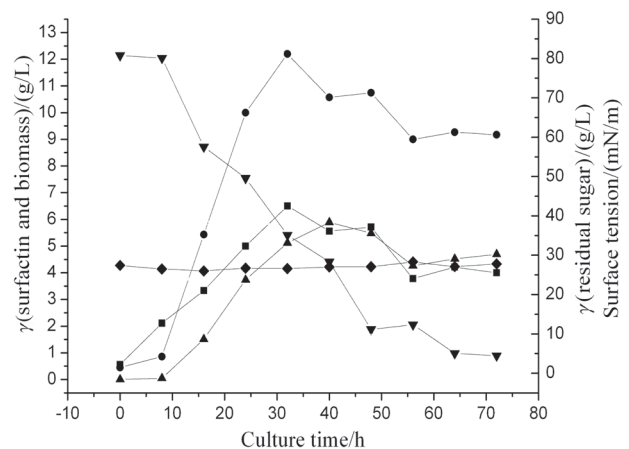


Fig. 3. Time course profiles of crude surfactin concentration (●), biomass concentration (■), sugar concentration (▼), surfactin concentration measured by HPLC method (▲), and broth surface tension (◆) in the modified bioreactor with *Bacillus subtilis* E8

### Properties of surfactin produced with *Bacillus subtilis* E8

#### Surface activity

Critical micelle concentrations of surfactants were investigated in 50 mM Tris-HCl buffer solution (pH=7.50). The results presented in Table 1 indicate that surfactin sodium salts exhibited excellent surface activity. CMC of surfactin was much lower than that of many chemically synthesized surfactants. As low as 13  $\mu$ M of extracted surfactin sodium salt reduced the surface ten-

Table 1. Surface activity of several biosurfactants

Biosurfactants	CMC	Surface tension
	$\mu\text{M}$	of CMC mN/m
Crude surfactin sodium salt	15	45.0
Extracted surfactin sodium salt	13	42.5
SDS	700	42.5
Triton X-100	28*	40.0
Tween 80	50*	49.7
Organic silicon surfactant	13*	32.5

\*concentration in mL/L

sion of water from 72.3 to 42.5 mN/m, whereas that of sodium dodecyl sulphate (SDS) was significantly higher (700  $\mu\text{M}$ ).

#### Emulsification activity

The emulsification activities of surfactants are shown in Table 2. The values of  $E_{24}$  revealed that the emulsification activity of surfactin obtained by fermentation with *Bacillus subtilis* E8 was close to the chemically synthesized surfactants (sometimes also called emulsifiers). The ability to form emulsions with oil and hydrocarbon suggests that *B. subtilis* E8 surfactin is a good candidate as a cleaning and emulsifying agent in food industry, and it has potential applications in microbial enhanced oil recovery (MEOR), environment preservation, medicine and cosmetics industry.

Table 2. Emulsification activity ( $E_{24}$ ) of several biosurfactants at the mass fraction of 0.1 % in 50 mM Tris-HCl buffer solution (pH=7.5)

Biosurfactants	$E_{24}/\%$
Crude surfactin sodium salt	68.0
Extracted surfactin sodium salt	69.1
SDS	71.7
Triton X-100	72.5
Tween 80	71.2
Organic silicon surfactant	69.1

#### pH stability

The effect of pH on *B. subtilis* E8 surfactin surface activity is shown in Fig. 4. The crude surfactin sodium salt and extracted surfactin sodium salt at the mass fraction of 0.1 % were investigated. The surface activities of the two surfactin sodium salt samples changed slightly when the pH value increased from 5 to 12. The surface tension increased when the pH value dropped below 4, which is due to precipitation of surfactin. The ability of holding surface activity stable against pH change (from 5 to 12) widens the areas of biosurfactant application.

#### Thermal stability

The two *B. subtilis* E8 surfactin samples (concentration of 0.01 %) were incubated at a temperature of 100 °C for 12 h, and surface tension was measured every hour. The effect of thermal treatment on surface activity

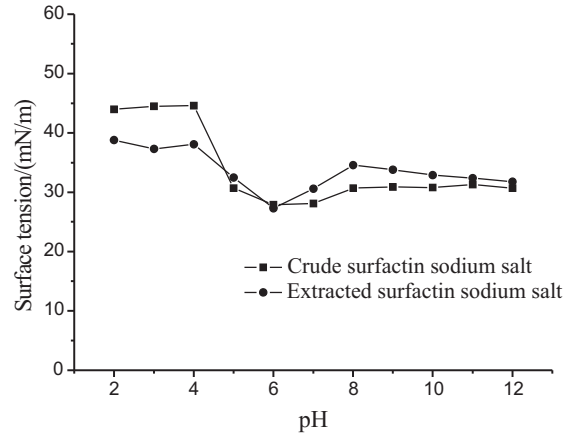


Fig. 4. The effect of pH on the surface activity of surfactin sodium salts

is shown in Fig. 5. The surface tension of the surfactin samples was stable for a long time after the treatment at high temperature. The ability of thermal stability makes the *B. subtilis* E8 surfactin available in some high temperature environments.

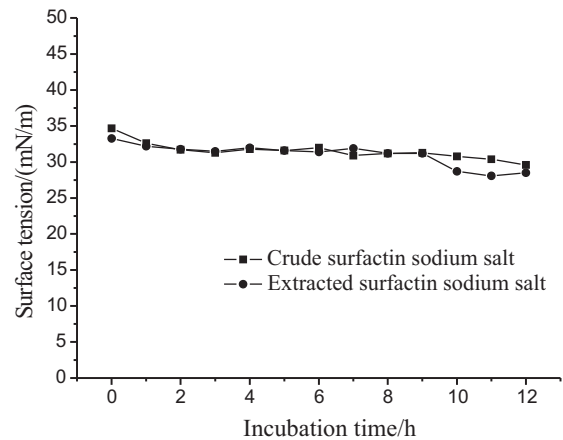


Fig. 5. The effect of thermal treatment (100 °C) on the surface activity of surfactin sodium salts

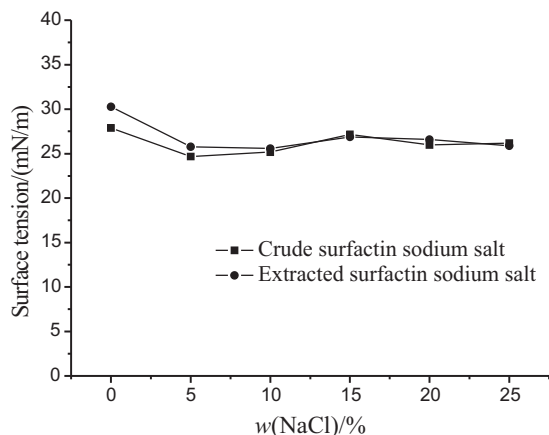
#### Resistance to high salt concentrations

In order to examine the potential ability for application of surfactin in high salt environments (marine environment or some industrial wastewater), the resistance to salts was investigated. The results shown in Table 3 and Fig. 6 indicate that the surfactin sodium samples

Table 3. The effect of  $\text{CaCl}_2$  concentration on the surface activity

$\gamma(\text{CaCl}_2)/(\text{mg/L})$	Surface tension/(mN/m)	
	Crude surfactin sodium salt	Extracted surfactin sodium salt
0	27.3	29.5
5	27.0	28.5
50	27.3	25.7
500	29.0	28.8
5000	28.0	27.3





**Fig. 6.** The effect of NaCl mass fraction on the surface activity of surfactin sodium salts

(mass fraction of 0.1 %) have a strong resistance to salts, even when the mass fraction of NaCl increased up to 25 % and  $\text{CaCl}_2$  concentration to 5 g/L. The excellent resistance of crude or extracted surfactin from *B. subtilis* E8 to salts ensured that they could work in high salt environments such as hard water areas, marine environment, some industrial wastewater, *etc.*

## Conclusions

In this study, the results of surfactin production by a high-producing surfactin mutant *Bacillus subtilis* E8, which was obtained by ion beam implantation, indicate that the modified bioreactor with a cell/foam recycler has an advantage over the bioreactor without the recycler. The concentration of crude surfactin (including recovery from both foam and broth fractionation) increased significantly from 0.75 to 10.26 g/L. Time course profiles showed that surfactin produced by *B. subtilis* E8 was growth-associated during a growth stage up to 32 h. The concentration of crude surfactin and biomass reached maximum (12.20 and 6.50 g/L, respectively) after cultivation for 32 h, and the concentration of surfactin measured by HPLC reached maximum (5.89 g/L) after 40 h. The process of surfactin production seemed to achieve the requirements for commercial applications.

The low CMC values of crude and extracted surfactin from *B. subtilis* E8 (15 and 13  $\mu\text{M}$ , respectively) were favourable compared to those of the chemically synthesized surfactants. The crude and extracted surfactin sodium salts exhibited excellent emulsification property, pH stability, thermal stability, and resistance to salts, which indicated that this biosurfactant could be a favourable candidate for oil industry (MEOR), environmental remediation (*e.g.* to eliminate oil pollution, remove toxic heavy metals, enhance pesticide biodegradation and soil bioremediation, *etc.*), health care and cosmetics industry, food industry (as bioemulsifier for

food additives), agricultural and pharmaceutical applications.

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