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Effect of Oxygen Supply on Surfactin Production and Sporulation in Submerged Culture of *Bacillus subtilis* Y9

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Abstract: Fermentation parameters for surfactin production and sporulation in a submerged culture of *Bacillus subtilis* Y9 with various oxygen transfer rates in 5 L jar fermenters were investigated. The oxygen-uptake rate (OUR) was positively correlated with volumetric surfactin productivity. When OUR value increased from 0 to 250 s⁻¹, productivity increased up to 45 mg/L·h; however, no further increase was observed at OUR values above 255 s⁻¹. The volumetric mass transfer coefficient K_La increased with increasing agitation speed. However, a reduction in surfactin production was observed at the highest agitation speed of 500 rpm. Productivity sharply decreased after spore appearance, and remained low until the end of the culture. A mesh-type sparger was installed to generate micro-sized air bubbles. When the system was operated at 400 rpm with the mesh-type sparger, K_La was higher than that at 500 rpm with an original sparger. Under agitation at 400 rpm with the mesh-type sparger, productivity was maintained above 42.3 mg/L·h until 24 h, resulting in the highest surfactin concentration of 875 mg/L. Thus, a mesh-type sparger promotes K_La , leading to an increase in productivity.

Keywords: *Bacillus subtilis* Y9; mesh-type sparger; oxygen-uptake rate; surfactin; volumetric-mass transfer rate

1. Introduction

Biosurfactants are surface-active compounds that are produced by various micro-organisms, e.g., bacteria, yeast, and fungi [1,2]. Their amphipathic characteristics make it possible to enhance the solubility of hydrophobic organic materials, leading to various applications to detergents, emulsions, dispersion systems, fabric softeners, and paints over food texture [3,4]. Compared to chemical surfactants, biosurfactants show several advantages, i.e., high bioavailability, biodegradability, and low toxicity.

Surfactin is a lipopeptide biosurfactant that consists of four isomers, i.e., surfactin A–D, with different chain length and branches of its hydroxyl fatty acid components. It is mainly produced by a rod-shaped Gram-positive bacterium, *Bacillus subtilis*, and shows biological potential for antimicrobial, antiviral, and insecticidal activities [5,6]. However, it has not been industrialized because of the high cost of production and purification [7]. Although the cost competitiveness of surfactin is lower than that of chemical surfactant, it should be used in some high-value products such as cosmetics, medicine, and biocontrol agents, due to increasing demand for safety.

From an industrial point of view, high surfactin productivity in a submerged culture of *B. subtilis* is necessary to step forward toward commercial production. Various fermentation strategies, e.g., strain development, media optimization, trace-element optimization, fermentation types, reactor design, and cultivation conditions, were investigated to increase surfactin yield [8]. Among these parameters, the cultivation conditions of dissolved oxygen and oxygen-transfer efficiency have been actively investigated for surfactin production. Sufficiently dissolved oxygen had a positive effect on surfactin concentration in the submerged culture of *B. subtilis* [9–11]. Increases in agitation speed resulted in increasing oxygen-transfer efficiency and surfactin yield. However, the accumulation of foam at high agitation speed resulted in poor surfactin productivity, limiting the increase of agitation speed in the vessel [8].

We previously isolated the surfactin-producing *B. subtilis* Y9 [6], whose culture filtrate contained surfactin isomers (C₁₄[Leu₇], C₁₄ [Val₇], and C₁₅[Leu₇]). In this study, *B. subtilis* Y9 fermentation parameters, including oxygen-uptake rate, oxygen supply, volumetric productivity, and sporulation, were investigated under various agitation speeds. In addition, the effect of the use of equipment to enhance oxygen supply on the parameters was investigated to enhance surfactin concentration in the submerged culture of *B. subtilis* Y9.

2. Materials and Methods

2.1. Micro-Organisms and Media

The *B. subtilis* Y9 strain was used to produce surfactin [6]. The fermentation medium consisted of 3% (*w/v*) galactose (Daejung, Siheung, Korea), 4% (*w/v*) yeast extract (Angest, Hubei, China), 0.25% (*w/v*) KH₂PO₄ (Ducksan, Ansan, Korea), and 0.1% (*w/v*) NaCl (Ducksan, Ansan, Korea) in distilled water. For maintenance of the strain, spores from a 5 day culture on tryptic soy agar (Sigma-Aldrich, St. Louis, MO, USA) were harvested, suspended in 1.2 mL cryovial tubes (Simport, Beloeil, QC, Canada) containing 20% glycerol (Duksan, Ansan, Korea), and stored at −70 °C to avoid genetic mutation due to successive culturing.

2.2. Cultivation

For seed cultures, a cryovial tube of *B. subtilis* Y9 was thawed at 25 °C and the bacteria were cultured in 500 mL Erlenmeyer flasks containing 100 mL of tryptic soy broth (Sigma-Aldrich, St. Louis, MO, USA). The flasks were incubated in a rotary shaker at 200 rpm (IS-971RF; Jeitech, Daejeon, Korea) at 30 °C for 12 h. For 5 L scale fermentation, 2% (*v/v*) of seed culture was inoculated in a jar bioreactor (MARADO-05D-XS, BioCnS, Daejeon, Korea) containing 3 L of fermentation medium and equipped with a dissolved oxygen meter (InPro6820/12/220, Mettler Toledo, Greifensee, Switzerland) and a pH meter (InPro3030/12/220, Mettler Toledo, Greifensee, Switzerland). Cultivations with an original sparger were carried out at 30 °C for 48 h, with agitation speeds ranging from 300 to 500 rpm and an aeration rate of 1.0 vvm (volume of air added to liquid volume per minute). In addition, a mesh-type sparger (CNS-A302, BioCnS, Daejeon, Korea) was installed to generate 10 µm air bubbles. Cultivations with the mesh-type sparger were carried out at an agitation speed of 400 rpm and an aeration rate of 1.0 vvm.

2.3. Measurement of Surfactin

The culture broth of *B. subtilis* Y9 was centrifuged at 16,000 × *g* (5810R, fixed-angle type; Eppendorf, New York, NY, USA) for 5 min, serially diluted, and filtered through a 0.45 µm Polytetrafluoroethylene (PTFE) syringe filter (Whatman, Pittsburgh, PA, USA). The amount of surfactin was measured by High Pressure Liquid Chromatography (HPLC) (e2695 system; Waters Corp., Milford, MA, USA) using a C18 column (5 µm, 4.6 × 250 mm; Waters). Elution was carried out isocratically using 20% (*v/v*) trifluoroacetic acid and 80% (*v/v*) acetonitrile. The flow rate and detection wavelength were

1.0 mL/min and 205 nm, respectively. Surfactin (Sigma, St. Louis, MO, USA) was used as a standard and was quantified on the basis of a standard curve.

2.4. Determination of Dry Cell Weight

Ten milliliters of culture broth was centrifuged at $16,000 \times g$ (5810R, fixed-angle type; Eppendorf, New York, NY, USA) for 5 min. Cell precipitates were washed 3 times with 10 mL of 0.9% saline solution, centrifuged, and placed in a plastic dish. The dishes were placed in a dry oven (VS-120203; Vision, Seoul, Korea) at 75 °C for 24 h. Dry cell weight was calculated as the difference between the weight of the dishes before and after drying.

2.5. Determination of Reducing Sugars

The concentration of reducing sugars was determined by a dinitrosalicylic acid assay [12]. The reagent was prepared as follows: 0.25 g of 3,5-dinitrosalicylic acid (Sigma-Aldrich, St. Louis, MO, USA), 75 g Rochelle salts (sodium potassium tartrate; Sigma-Aldrich, St. Louis, MO, USA), and 4 g NaOH (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 300 mL of distilled water. The reagent was purged with nitrogen gas prior to use. The culture broth of *B. subtilis* Y9 was centrifuged at $16,000 \times g$ for 5 min to remove the cells, and the supernatant was filtered through a 0.45 µm PTFE syringe filter (Whatman, Pittsburgh, PA, USA). One hundred microliters of filtered supernatant was added to 1 mL of the reagent. The reaction mixture was boiled in a water bath for 10 min, transferred to an ice bath to cool down, and placed at room temperature. Absorbance at 570 nm was determined using a spectrophotometer (UV-1800; Shimadzu, Tokyo, Japan).

2.6. Endospore Staining

Based on the Schaeffer-Fulton staining method [13], spores were stained to investigate the effect of agitation speed on the spore ratio. The culture broth of *B. subtilis* Y9 was serially diluted. *B. subtilis* Y9 broth (5 µL) was poured in a glass slide, fixed with heat, and covered with blotting paper. The paper was saturated with 0.5% (*w/v*) malachite-green stain solution (Kanto Chemical, Tokyo, Japan) for 5 min, steamed over a container of boiling water, and washed with distilled water. The glass was counterstained with 0.25% (*w/v*) safranin (Sigma, St. Louis, MO, USA) for 30 s and washed with distilled water. Spores (bright green) and vegetative cells (pink) were distinguished by microscopic observation (IX73; Olympus, Tokyo, Japan).

2.7. Determination of the Volumetric Mass Transfer Rate (K_La) and Oxygen-Uptake Rate (OUR)

K_La is one of the important factors in aerobic fermentation. The material balance for dissolved oxygen in a liquid phase can be established with Equation (1) [14]:

$$dC_L/dt = K_La(C^* - C_L) - q_{O_2}X \quad (1)$$

where dC_L/dt is the rate of change in dissolved oxygen concentration, K_L is the liquid film oxygen-transfer coefficient (cm/h), a is the gas-liquid interfacial area per unit volume of liquid (cm^2/cm^3), C^* is the saturated dissolved oxygen concentration in the broth (mg/L), C_L is the actual dissolved oxygen concentration in the broth (mg/L), q_{O_2} is the specific oxygen-consumption rate ($\text{mg O}_2/\text{g dry cell weight} \cdot \text{h}$), and X is the cell concentration ($\text{g dry cell weight/L}$). Dissolved oxygen tension (DOT), which is the partial pressure of oxygen molecules dissolved in the broth, was measured by using a dissolved-oxygen meter. When $dC_L/dt = 0$, the material balance changes according to Equation (2):

$$q_{O_2}X = K_La(C^* - \bar{C}_L) \quad (2)$$

where $\bar{C}_L$ is the saturated dissolved oxygen concentration during reoxygenation (mg/L). Substituting Equation (2) with Equation (1) gives:

$$dC_L/dt = K_L a (\bar{C}_L - C_L) \quad (3)$$

Assuming that $K_L a$ is constant over time, Equation (3) can be integrated between t_1 and t_2 :

$$K_L a (t_2 - t_1) = \ln((\bar{C}_L - C_{L1})/(\bar{C}_L - C_{L2})) \quad (4)$$

where C_{L1} and C_{L2} are the actual dissolved oxygen concentration during reoxygenation (mg/L) at t_1 and t_2 , respectively. $K_L a$ can be estimated from the slope when $\ln((\bar{C}_L - C_{L1})/(\bar{C}_L - C_{L2}))$ is plotted against $(t_2 - t_1)$.

OUR was calculated as the slope of the plot of dissolved oxygen concentration during temporary interruption of air supply to the bioreactor.

2.8. Statistical Analysis

Data were analyzed using PASW software (Ver. 17; SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA), followed by the Bonferroni test, were used to determine significant differences between treatments at $p < 0.05$.

3. Results

3.1. Batch-Fermentation Characteristics at Different Agitation Speeds

Surfactin-production profiles varied with varying agitation speeds of 300–500 rpm, which directly affected DOT throughout the culture (Figure 1). DOT levels at 300–500 rpm decreased sharply in the exponential stage of the culture, with less than 5% of DOT at 12 h. At 500 rpm, DOT recovered to over 50% after 28 h, while a negligible DOT level was maintained throughout the culture at 300–400 rpm. However, variability in surfactin production under different agitation speeds differed from the patterns in the DOT levels. The highest concentration of surfactin of 734 mg/L was observed at 400 rpm, followed by 619 mg/L at 500 rpm, and 453 mg/L at 300 rpm. Both the concentration and volumetric productivity of surfactin were determined by agitation speed in the first half of the culture. Surfactin concentration increased with the increment of agitation speed, especially from 12 h to 24 h. Volumetric productivities were maintained above 16 mg/L·h during that time. After 24 h, the productivities sharply decreased in order of agitation speed, with rates barely being over 10 mg/L·h until 32 h at 300 rpm, 28 h at 400 rpm, and 20 h at 500 rpm, respectively.

Figure 1. Surfactin production depends on agitation speed. Cultivations were carried out in 5 L jar fermenters for 48 h. Filled triangle, surfactin; filled square, dissolved oxygen tension (DOT).

OUR was positively correlated with volumetric surfactin productivity (Figure 2). When the OUR value increased from 0 to 250 s^{-1} , volumetric productivity increased up to $45 \text{ mg/L}\cdot\text{h}$; however, no further increase was observed in proportion to volumetric productivity when the OUR value was above 255 s^{-1} . K_La increased with increasing agitation speed ($F = 133.5$, $df = 2.15$, $p < 0.001$) (Figure 3). The highest K_La , of 0.0235 s^{-1} , was observed at 500 rpm, followed by 0.0177 s^{-1} at 400 rpm, and 0.0138 s^{-1} at 300 rpm.

Figure 2. Relationship between oxygen uptake rate (OUR) and volumetric surfactin productivity.

Figure 3. The volumetric mass transfer coefficient, K_La , depends on agitation speed.

3.2. Spore Ratio at Different Agitation Speeds

As shown in Figure 4, spore production of *B. subtilis* Y9 was correlated with agitation speed. Increasing agitation speed resulted in early spore production. When agitated at 500 rpm, spores of *B. subtilis* Y9 were produced from 24 h, while no spore production was observed throughout the culture at 300–400 rpm. Volumetric productivity of surfactin sharply decreased after spore appearance at 500 rpm, and remained low until the end of culture. Volumetric productivity from 12 h to 24 h was higher than 24.0 mg/L·h at 500 rpm, and was maintained at less than 5.0 mg/L·h after spore production. Under agitation at 300–400 rpm, volumetric productivity was maintained for longer than at 500 rpm. Surfactin was produced until 44 h at 300 rpm, and until 36 h at 400 rpm.

Figure 4. Profiles of volumetric surfactin productivity and spore ratio. Closed and open symbols indicate volumetric productivity and spore ratio, respectively. Cultivations were performed in 5 L jar bioreactors at 30 °C for 48 h.

3.3. Batch-Fermentation Characteristics with a Mesh-Type Sparger

Instead of an original-type sparger, a mesh-type sparger was installed to increase K_La by generating micro-sized air bubbles. Under agitation at 400 rpm with the mesh-type sparger, the K_La was 0.0258 s^{-1} , which was higher than of 0.0177 s^{-1} obtained with the original sparger at 400 rpm (Figure 5). Volumetric productivity of surfactin from 12 h to 24 h was higher than that with the original sparger at 400 rpm (Figure 6), resulting in the highest surfactin concentration of 875 mg/L (Figure 7). Productivity was maintained above $42.3 \text{ mg/L}\cdot\text{h}$ until 24 h. Once spores were produced as of 36 h, productivity dramatically dropped to $3 \text{ mg/L}\cdot\text{h}$. When the spore ratio went up to 18.8% at 48 h, productivity even dropped to $2 \text{ mg/L}\cdot\text{h}$. Thus, the mesh-type sparger promoted K_La , leading to an increase in volumetric productivity of surfactin in the early stage of culture.

Figure 5. Volumetric mass transfer coefficient, K_La , when an original and a mesh-type sparger were used. Cultivations were performed in 5 L jar bioreactors at 30 °C at 400 rpm.

Figure 6. Profiles of volumetric surfactin productivity and spore ratio when an original and a mesh-type sparger were used. Cultivations were performed in 5 L jar bioreactors at 30 °C at 400 rpm.

Figure 7. Surfactin production when a mesh-type sparger was used. Cultivations were performed in 5 L jar fermenters at 30 °C for 48 h.

4. Discussion

DOT is a critical fermentation parameter for aerobic micro-organisms to produce their metabolites. When microbial metabolite production is correlated to OUR, DOT should be maintained above a certain threshold because sufficient DOT leads to a higher OUR [15,16]. Reductions in microbial metabolite yields have been reported when DOT fell below a critical level [17,18]. To maintain a suitable DOT level, it is important that oxygen transfer takes place faster than oxygen uptake, especially in the log phase, when the microbial population undergoes exponential growth. Both agitation and aeration have been applied to solve this problem, elevating K_La in bioreactors [10,11,19]. In the case of surfactin production using *Bacillus* spp., oxygen-transfer efficacy plays an important role in production kinetics. Increases in agitation speed and aeration rate reportedly resulted in maximum surfactin production [9,10]. However, a reduction in surfactin production was observed at a higher agitation speed due to vigorous foam production [11]. Rapid foam formation caused the culture to overflow, resulting in decreased

cell mass and reduced surfactin production. In our study, an increase in agitation speed led to an increase in K_La , resulting in an increment in surfactin production. Similar to findings by Yeh et al. [11], maximal surfactin concentration was not observed at the highest agitation speed of 500 rpm, but at 400 rpm. However, the reason for avoiding high agitation speed differed in our study. Since foam formation was perfectly controlled by an antifoam agent, there was no foam formation at 500 rpm. However, high agitation speed led to early spore formation, while no spore formation was observed at low agitation speeds. Considering that the volumetric productivity of surfactin was reduced when endospores were produced, sporulation of *B. subtilis* Y9 seems to have a negative effect on surfactin production.

Since sufficient oxygen supply is required for surfactin production, a mesh-type sparger was applied to improve the oxygen-transfer rate without increasing agitation speed. Use of the mesh-type sparger led to an increase in the gas-liquid interfacial area by generating micro-sized bubbles. K_La with the mesh-type sparger was higher than that with the original sparger, showing the highest surfactin production. Surfactin production rate from 12 h to 24 h was substantially higher than that in the case of the original sparger under all agitation speeds. Under agitation at 500 rpm with the mesh sparger, however, endospore appearance was observed from 24 h, resulting in a reduction in surfactin concentration to 584.0 mg/L·h (data not shown).

There are numerous reports that a high DOT level has a positive effect on spore production of *Bacillus* spp. [20–22]. The higher the oxygen supply is, especially K_La , the higher the spore productivity [21,23]. In our study, high DOT resulted from increased agitation speed, leading to fast sporulation. Considering that sporulation might have an adverse effect on surfactin production in the submerged culture of *B. subtilis* Y9, the increase in agitation speed to improve DOT level should be limited. Instead, we attempted to promote K_La by installing the mesh-type sparger. As shown in Figure 5, both the highest volumetric productivity and prolonged production period were observed, although sporulation started in the stationary stage of culture (1.2% of spore ratio at 36 h).

5. Conclusions

In this study, fermentation parameters for surfactin production in a submerged culture of *Bacillus subtilis* Y9 with various oxygen-transfer rates were optimized. In 5 L jar fermenters, surfactin production and OUR were positively correlated; the volumetric productivity of surfactin increased proportionally to increasing the OUR value from 0 to 250 s^{−1}. In addition, K_La was positively correlated with volumetric surfactin productivity. However, a reduction in surfactin production was observed at a vigorous agitation speed, i.e., 500 rpm. The volumetric productivity of surfactin sharply decreased after spore appearance and remained low until the end of culture. A mesh-type sparger, which generates micro-sized air bubbles, promoted K_La without the need for vigorous agitation, leading to an increase in volumetric surfactin productivity in the early stage of the culture. These findings are expected to be useful in building a strategy for scaling up this fermentation process.

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