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THE EFFECT OF pH ON BIOSURFACTANT PRODUCTION BY BACILLUS SUBTILIS DSM10

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The genus *Bacillus* has long been known for its ability to produce many industrially useful products. These bacteria mostly produce extracellular products like organic acids, enzymes and biosurfactants. In this paper, the production of surfactin using the *Bacillus subtilis* strain DSM10 is investigated. Biosurfactant was produced in a lab-scale 1-liter fermenter. pH control using different bases (NH_4OH and NaOH) was compared to observe whether the amount of produced biosurfactant or the quality of the product was influenced. The formation of the product was followed by measuring the surface tension, and the product formed was analyzed by reversed-phase chromatography. The investigation of the effect of pH control showed that it can be omitted during the fermentation of the biosurfactant. The highest concentration of surfactin (5 g/L) was achieved without pH control in contrast with when the pH was kept constant ($\mathrm{pH} = 7$).

Keywords: biosurfactant, surfactin, fermentation, purification, Bacillus subtilis

1. Introduction

In recent years, the microbial production of tensio-active molecules with various properties, e.g. emulsifying, wetting, foaming, detergency, solubilizing and dispersing, has been gaining interest [1]. Biosurfactants are amphiphilic compounds produced by a variety of microbial communities. Natural surfactants are of great importance in the pharmaceutical, cosmetic, agricultural and food industries due to their beneficial properties including low toxicity, biodegradability, high selectivity and activity under extreme environmental conditions [2, 3]. The market of bio-based surfactants is predicted to be worth $5.52 billion by 2022 [4]. This is unsurprising given our high degree of dependency on kinds of hygiene products, the majority of which include surfactants or emulsifiers.

Biosurfactants are classified based on their chemical structure into the following groups: glycolipids, lipopeptides, fatty acids and lipids, as well as polymeric and particulate biosurfactants [3, 5]. One of the most effective biosurfactants is surfactin. This lipopeptide-type surfactant contains a cyclic peptide linked to a fatty acid chain (Fig. 1) [6]. Bóka et al. reported that the molecular weights of surfactins range from 993 Da to 1049 Da [7]. Several gram-positive *Bacillus* species naturally produce surfactins, which help the bacteria to stabilize their cell membranes and adhere to a surface [8, 9]. The biosynthesis of surfactin occurs through different mechanisms: the conversion of glucose or glycerol as a substrate to glucose 6-phosphate through the glycolytic pathway, providing the main precursor of carbohydrates located in the hydrophilic part and the oxidation of glucose to pyruvate then to acetyl-CoA, which serves as a precursor for the synthesis of lipids and amino acids (Asp, Glu, Leu, Val). However, if the substrate is a hydrocarbon, the metabolism is shifted towards the lipolytic pathway (β-oxidation into acetyl-CoA) and gluconeogenesis (acetyl-CoA involved in the synthesis of the precursor glucose 6-phosphate) [1].

The effectiveness of surfactants is defined by their ability to reduce the surface tension (ST), defined as the cohesive force between molecules which is proportional to the concentration of surfactant in the solution [2]. Their efficiency is measured by the critical micelle concentration (CMC) [1, 11]. Above the CMC, surfactants form micellar structures, but below it, the aggregates dissociate into monomers. Lipopeptides from *B. subtilis* are particularly compelling because their surface activity has been reported to be strong [6, 12, 13]. Powerful surfactants can decrease the surface tension of water (72 mN/m at 20 °C) to less than 30 mN/m [14]. The emulsifying capacity can be monitored by calculating the emulsification index (EI, %) and emulsion stability. As the pH decreases, surfactin becomes less soluble in water because the carboxyl group is protonated [12]. Under neutral or basic conditions, the carboxyl group is in the ionic form, thus its solubility and emulsification capability increases [15]. Moro et al. evaluated the influence of the pH on the stability of the surfactants produced by species of *B. subtilis*, *B. gibsonii* and *B.*

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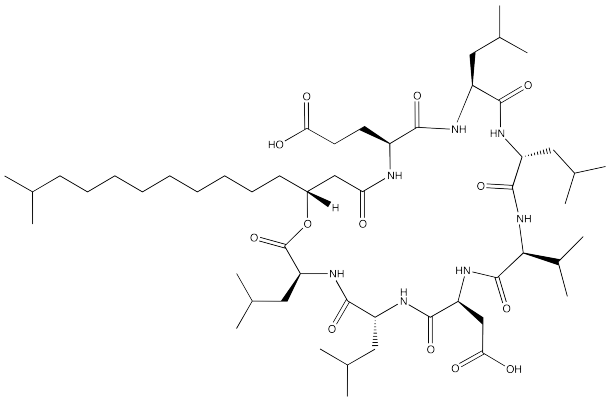

Figure 1: Chemical structure of surfactin: peptide loop of amino acids: five L-amino acids (Val, Asp, Leu, Glu and Leu) two D-amino acids (Leu and Leu), and a α, β-hydroxy C13-C15 fatty acid chain [10]

amyloliquefaciens [9]. All isolates exhibited surface tensions below 30 mN/m. In strongly acidic conditions, the emulsifying activity significantly decreased for both *B. subtilis ODW02* and *B. subtilis ODW15*. As the pH was increased from 7 to 12, the stability of the surfactant produced by *B. subtilis ODW02* decreased even further, but the one by *B. subtilis ODW15* remained stable. In the case of *B. amyloliquefaciens MO13*, a significant increase was observed both under acidic and basic conditions. This different pH-responsive behavior makes surfactin applicable in a variety of industrial fields.

The choice of fermentation cultivation media (i.e. of average composition, carbon source, nitrogen source and trace elements) as well as fermentation strategies (i.e. temperature, pH, aeration and agitation) also need to be considered in relation to the type of applications. Based on a literature review, the best combination among the fermentation conditions was 1.5 vvm at 300 rpm, resulting in a maximum yield of surfactin of 6.45 g/L [16]. *B. subtilis ATCC 21332* was grown on an iron-enriched minimal salt (MSI) medium including glucose (40 g/L). In a recent study, Yang et al. used nanoparticles (NPs) to improve the total yield of surfactin. 5 g/L of Fe NPs were added to the fermentation medium of *B. amyloliquefaciens MT45*, which increased the titer of surfactin from 5.94 to 9.18 g/L. Modifying the biosynthesis of surfactin with metabolic engineering tools can further increase production and titers of surfactin in *B. subtilis*, as demonstrated by Wu et al. (12.8 g/L) [17]. Few studies have focused on individual characteristics and relative amounts of surfactin variants in the extracts of lipopeptides. Many surfactin variants exist with various lengths of fatty acid chains and different amino acid sequences [5]. Akpa et al. analyzed the replacement of L-glutamic acid with four other amino acids, namely L-leucine, L-valine, L-isoleucine and L-threonine, in the culture medium. The presence of Thr was found to be favorable for the synthesis of longer (C15-C16) fatty acid chains in *B. subtilis S499* [18]. Supplementation with Mn^{2+}, Cu^{2+} and Ni^{2+} ions can also promote the production of novel variants of surfactin with different components of fatty acids (C16-C18) and amino acids (central aspartic acid methyl ester residue instead of aspartate) [19]. This approach can be useful for studying specific biological activities.

The objectives of our study were to explore the effect of pH control on the production of biosurfactants (i.e. on titers and productivity) by the *B. subtilis* strain *DSM10* and evaluate the properties of biosurfactants, i.e. type, surface tension reduction and emulsifying activity.

2. Materials and methods

2.1 Cultivation conditions

Bacillus subtilis DSM10 (NCAIM B.02624T) was used for biosurfactant fermentation in this study. Cultivation was performed at 37 °C in 250 ml Erlenmeyer flasks containing 100 ml of an inorganic medium based on the composition used by Joshi et al. (2013): 34 g glucose, 1.0 g NH_4NO_3, 6.0 g KH_2PO_4, 2.7 g Na_2HPO_4, 0.1 g $MgSO_4 \bullet 7\,H_2O$, $1.2 \cdot 10^{-3}$ g $CaCl_2 \bullet 2\,H_2O$, $1.65 \cdot 10^{-3}$ g $FeSO_4 \bullet 7\,H_2O$, $1.5 \cdot 10^{-3}$ g $MnSO_4 \bullet 4\,H_2O$ and $2.2 \cdot 10^{-3}$ g Na-EDTA [20].

The experiments were carried out in a 1 L benchtop bioreactor, with a working volume of 0.8 L (Biostat Q fermenter, B. Braun Biotech International, Germany) and a 10% v/v inoculum. For biosurfactant production, the temperature was adjusted to 37 °C with an agitation speed of 300 rpm and an aeration rate of 0.25 vvm. The pH was controlled by 25% H_2SO_4 and two different bases, namely 25% NH_4OH and 25% NaOH. Biosurfactant fermentation without external pH control served as a controlled experiment. A cyclone separator for reducing foam was connected to the outlet airstream of the fermenter (Fig. 2). The foam could overflow from the fermenter via the air outlet, through the cyclone separator to the collector flask.

2.2 Analysis of biomass

Bacterial growth was monitored by measuring the optical density of the fermentation broth at 600 nm using a Pharmacia LKB Ultrospec Plus spectrophotometer in comparison with that of the centrifuged supernatant of the sample.

The biomass concentration (g cell dry weight/L) was determined by using a calibration curve ($R^2 = 1$):

$$\text{Biomass}\,[\text{g/L}] = 0.4283 \cdot \text{OD}_{600} + 1.4568 \quad (1)$$

The sampled broth was centrifuged at 6,000 rpm for 15 mins. The cell pellets were collected and dried at 105 °C to constant weight by a Sartorius MA35 moisture analyzer to measure the cell dry weight.

Figure 2: Fermentation setup with foam-separating glass cyclone

2.3 Analysis of glucose consumption

Glucose consumption was determined using the Waters Breeze 2 HPLC System. The mobile phase was 5 mM H_2SO_4 and the rate of elution was 0.5 mL/min. A BIO-RAD Aminex HPX-87H (300 × 7.8 mm, 9 μm) column (65 °C) was applied with a Refractive Index detector (40 °C).

The glucose concentration was calculated from the peak area by the following calibration curve equation ($R^2 = 1$):

$$\text{Glucose [g/L]} = 4 \cdot 10^{-6} \cdot \text{PeakArea} + 0.0147 \quad (2)$$

2.4 Analysis of biosurfactants

Surface tension measurement

The surface activities of biosurfactants produced by the bacterial strains were determined by measuring the surface tension of the samples of cell-free broth using the stalagmometric method with a Traube Stalagmometer (2.5 mL, Wilmad-LabGlass LG-5050-102 Stalagmometer Tube for samples of low viscosity) at room temperature (25 °C). To increase the accuracy of the surface tension measurements, the averages of triplicates were used in this report. The surface tension can be determined based on the number of drops that fall per unit volume, the density of the sample and the surface tension of a liquid reference, e.g. deionized water.

The actual number of drops was calculated using

$$N = N_0 + \frac{x - y}{c} \quad (3)$$

where N denotes the number of drops of the sample calculated to the nearest tenth of a drop; N_0 represents an integer of drops counted between capillary-scale readings x and y; x and y stand for capillary-scale readings based on the maximum data point as 0 and the minimum data point as 40; x and y refer to the distances in millimeters from the beginning of each scale; and c is the capillary-scale calibration in millimeters per drop.

The surface tension (ST in mN/m) was calculated according to

$$\text{ST} = \frac{\text{ST}_\text{w} \cdot N_\text{w} \cdot D}{N \cdot D_\text{w}} \quad (4)$$

where ST_w denotes the surface tension of water at 25 °C (72 mN/m); N_w represents the number of water drops (20 drops); D stands for the density of the sample in g/mL; N refers to the number of drops of sample, and D_w is the density of water at 25 °C.

Emulsifying activity

The emulsifying activity was determined by the addition of 2 mL of sunflower oil to the same volume of cell-free sample or surfactin solution in a test tube, which was vortex-mixed vigorously for 2 mins. [21]. The tubes were incubated at 25 °C and the emulsification index (EI) determined after 24 hours according to:

$$\text{EI}_t = \left(\frac{H_\text{e}}{H_\text{t}}\right) 100 \quad (5)$$

where H_e and H_t are the height of emulsion and total height of the liquid in the tube, respectively.

To study the emulsion stability, the same protocol was used. The emulsification index (EI, %) was determined after 1 h and the EI measured after 24 h (EI_{24}, %), the tubes were incubated at 25 °C. The emulsion stability was expressed as a function of the changes in EI over the 24 h.

High-performance liquid chromatography (HPLC)

The surfactin concentration was measured by HPLC using a Waters Alliance 2695 Separations Module, which is a high-performance liquid chromatographic system equipped with a Waters 2996 photodiode array detector, at 205 nm and a Symmetry C18 Column (4.6 × 150 mm, 5 μm - Waters, Ireland). The mobile phase consisted of 20% v/v trifluoroacetic acid (TFA) (3.8 mM) and 80% v/v acetonitrile. The elution rate was 1 mL/min at 25 °C and the sample volume was 10 μL. The purified surfactin was identified by using commercially available surfactin (Wako Chemicals) as the authentic compound [22].

2.5 Isolation of the biosurfactant

The method for purifying the biosurfactant was adapted from the one outlined by Joshi et al. (2008) [23]. The cell-free broth was obtained by centrifuging the fermentation broth at 4,000 rpm for 20 mins. at 4 °C using a Janetzki MLW K23D centrifuge. The cell-free broth was used for further purification steps. The biosurfactant was recovered from the supernatant by acid precipitation: the

Table 1: Summary of the results of the fermentations

	without pH control	25% NH_4OH	25% NaOH
Biomass yield [g/g]	0.06±0.02	0.06±0.02	0.08
Biosurfactant yield [g/g]	0.120±0.04	0.073±0.07	0.123
Glucose conversion [%]	78.90±22	71.40±4	58.35
Final biosurfactant concentration [g/L]	3.36±2.3	2.00±1.5	2.34
Minimum surface tension [mN/m]	51.1±1	54.3±17	68.4
Biomass productivity [g/l·h]	0.060±0.03	0.089±0.05	0.064
Biosurfactant productivity [g/l·h]	0.100±0.05	0.034±0.02	0.064

pH was adjusted to 2.0 using 6 N HCl and kept at 4 °C overnight. The precipitate was collected by centrifugation at 4,000 rpm for 20 mins. at 4 °C, then resuspended in distilled water. The pH was adjusted to 7.0 using 6 N NaOH and the solution lyophilized by a Christ Alpha 2-4 LSC freeze dryer. The concentration of biosurfactant was determined gravimetrically from the resulting yellowish white powder. The concentration of biosurfactant was determined gravimetrically from the lyophilized powder. The identity of the purified biosurfactant was checked by HPLC.

2.6 Calculation of fermentation parameters

To compare the results of the fermentation, the following parameters were determined.

Substrate (glucose) conversion was calculated according to:

$$\Delta S\ \% = \frac{S_0 - S_\mathrm{f}}{S_0} \tag{6}$$

where S_0 and S_f denote the initial substrate and final glucose concentrations, respectively.

The biomass yield on glucose ($Y_{\frac{x}{S}}$, g/g) was defined by:

$$Y_{\frac{x}{s}} = \frac{x_f - x_0}{S_0 - S_f} \tag{7}$$

where x_f and x_0 are the final and initial biomass concentrations, respectively.

The biosurfactant yield on glucose ($Y_{\frac{P}{s}}$, g/g) was defined by:

$$Y_{\frac{P}{s}} = \frac{P_\mathrm{f} - P_0}{S_\mathrm{f} - S_0} \tag{8}$$

where P_f and P_0 are the final and initial biosurfactant concentrations, respectively.

The volumetric productivities

$$J_x = \frac{x_\mathrm{max}}{t_{x_\mathrm{max}}} \tag{9}$$

and

$$J_P = \frac{P_\mathrm{max}}{t_{P_\mathrm{max}}} \tag{10}$$

(g/l · h) were calculated as the quotients of the maximum biomass concentration (x_max, g/l) or the maximum biosurfactant concentration (P_max, g/l) and the fermentation time (t_{x_max} or t_{P_max}, h) when the maximum concentration was achieved, respectively.

3. Results and Discussion

To evaluate the effect of pH on surfactin production, a series of batch fermentations were performed either with or without pH control. The biosurfactant solution was analysed quantitatively and qualitatively using the HPLC method reported by Mubarak et al. [24].

An overview of the calculated parameters of the batch runs can be seen in Table 1. In the absence of pH control, the maximum biomass concentration achieved was 4.00 g/L after 35 h (Fig. 3). Without pH control, the increased acidity of the medium inhibited further growth at pH 4.4. The maximum biosurfactant concentration was 4.99 g/L, which resulted in the surface tension decreasing to 50.1 mN/m. In pH-controlled fermentations, the biomass yields (0.06 and 0.08 g/g - pH adjusted with 25% NH_4OH and 25% NaOH to 7.0, respectively) were similar to that in the absence of pH control (0.06 g/g) (Table 1), while the production of biosurfactants was unable, with a few exceptions, to reduce the surface tension significantly (66.5 mN/m with 25% NH_4OH, Table 1; Figs. 4 and 5). This may account for the presence of residual glucose concentrations of 8 to 12 g/L (Figs. 4 and 5). The maximum surfactant concentrations were 3.50 and 2.34 g/L by adjusting the pH using NH_4OH and NaOH, respectively. Although these results are similar to the average yield of surfactants (3.36 g/L) in the absence of pH control, a significant drop in productivity of approxi-

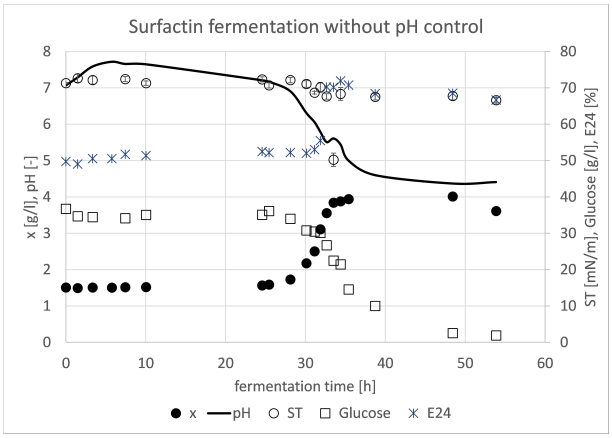

Figure 3: Fermentation of Surfactin - without external pH control

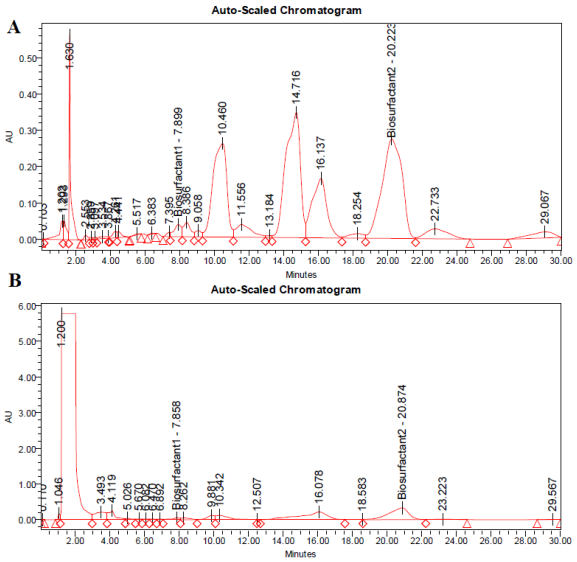

Figure 4: A) HPL chromatogram of a 1.25 g/L surfactin standard, B) HPLC chromatogram of the isolated biosurfactant fraction from the foam out sample

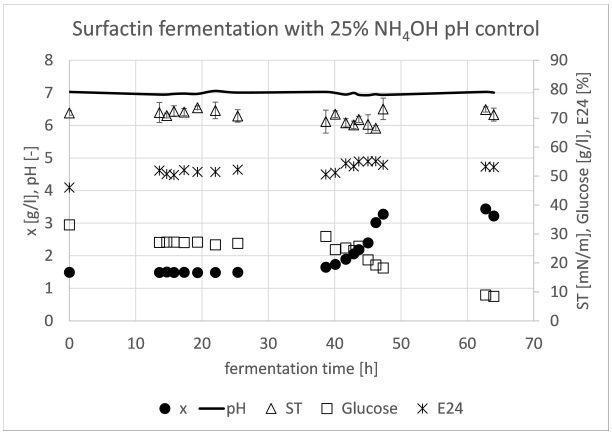

Figure 5: Fermentation of Surfactin - pH controlled by 25% NH_4OH.

mately 50% was observed (Table 1).

The highest value of the emulsifying activity (EI_{24}) was in excess of 70% at the end of the exponential phase of the growth curve (at 35 h, Fig. 3). The EI_{24} values obtained from samples extracted from pH-controlled experiments increased from 45 to 55% (Figs. 5 and 6, respectively). These inconsistencies can be explained in part by the fact that different *Bacillus* species and strains are capable of producing numerous surfactin variants with distinct properties. It is highly likely that the production of surfactants is independent of cell growth as EI_{24} decreased and ST increased during the stationary phase perhaps as a result of degradation by enzymatic hydrolysis or uptake under substrate-limiting conditions [25, 26].

Since the spectrum of lipopeptide-type biosurfactants is broad, the profiles of the extracts obtained after the purification process were compared to the surfactin standard. Fig. 4 presents two representative chromatograms:

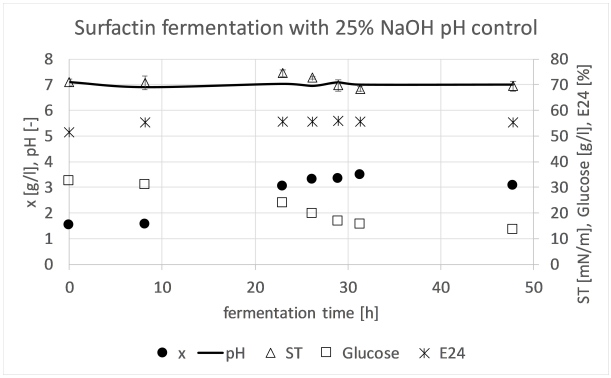

Figure 6: Fermentation of Surfactin - pH controlled by 25% NaOH

(A) surfactin standard and (B) purified culture broth of *B. subtilis DSM10*. Comparatively speaking, the samples from our study exhibited similar peaks (number of peaks, retention time). The intense peak at the beginning of the chromatogram indicates the presence of some non-retained impurities, namely contaminants and inorganic salts such as NH_4NO_3, which are often co-extracted with the targeted biosurfactant, that may need to be separated. Overall, based on the separation of peaks and retention time, the isolated biosurfactant was identified as surfactin.

The surfactin titer of *B. subtilis DSM10* was compared with the results from relevant studies (Table 2). Even though the fermentation strategies differ to some extent, the surfactin concentration from our work compares well with the values previously reported in the literature.

4. Conclusion

The aim of this study was to assess the biosurfactant-producing capability of *Bacillus subtilis DSM10* and establish an economically feasible fermentative process. This paper investigated the effect of pH control on the amount of biosurfactant production. The maximum amount of biosurfactant (approximately 5 g/L) was recovered from fermentation experiments in the absence of pH control at 37 °C. Furthermore, a preliminary characterization of the surface-active compounds produced during fermentation was conducted. HPLC analysis confirmed the presence of surfactin in the purified product.

Table 2: Surfactin production by *Bacillus* species

Strain	Surfactin titer [g/L]	Ref.
B. subtilis ATCC 21332	6.45	[16]
B. subtilis	2.00	[27]
B. subtilis SPB1	4.92	[28]
B. subtilis DSM10T	3.99	[29]
B. subtilis #573	4.80	[6]
B. subtilis CN2	7.15	[30]
B. subtilis DSM10	4.99	this work

The minimum surface tension was 50 mN/m. The emulsifying activity achieved using sunflower oil was approximately 70%. These results represent an initial step towards large-scale production of this biosurfactant. From a technical and economic standpoint, the fermentative process of surfactin carried out in the absence of pH control in a mineral salt medium using glucose as the sole carbon source seems to be an effective strategy for pilot- and industrial-scale production.

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