0273-2289/04/115/0827–0836/$25.00

Characterization of Surfactin from *Bacillus subtilis* for Application as an Agent for Enhanced Oil Recovery

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Abstract

Surfactin produced by *Bacillus subtilis* (ATCC 21332) was used to examine the effect of altering salt concentration, pH, and temperature on surfactin activity (as measured by reductions in surface tension). These parameters are some of the conditions that define oil reservoir characteristics and can affect the application of surfactants. The Biotechnology for Oilfield Operations research program at the Idaho National Engineering and Environmental Laboratory (INEEL) has successfully produced surfactin from potato process effluents for possible use as an economical alternative to chemical surfactants for improved oil recovery. Surfactants enhance the recovery of oil through a reduction of the interfacial tension between the oil and water interfaces, or by mediating changes in the wettability index of the system. We investigated changes in surfactin activity under a range of conditions by measuring surface tension. Surface tension was determined using video image analysis of inverted pendant drops. Experimental variables included NaCl (0–10%), pH (3.0–10.0), and temperature (21–70°C). Each of these parameters, as well as selected combinations, resulted in discrete changes in surfactin activity. It is therefore important to consider the exploration of the studied surfactin as an enhanced oil recovery agent.

Index Entries: *Bacillus subtilis*; biosurfactant; surfactin; improved oil recovery; oil recovery agent.

Introduction

Surface-active molecules produced by microorganisms, called biosurfactants, could possibly replace costly and potentially toxic chemical

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surfactants in several industries *(1)*. Industries that can use biosurfactants include textile, environmental bioremediation, and fossil fuel recovery *(2)*. Utilization is limited, however, by the cost of producing biosurfactants. This is determined by the process for growing the microorganisms that produce the surfactant, and the cost of purifying the biosurfactant from the culture fluid *(3)*. The Biotechnology for Oilfield Operations research program at Idaho National Engineering and Environmental Laboratory has successfully produced surfactin, the biosurfactant from *Bacillus subtilis* (ATCC 21332), from potato process effluents for possible use as an economical alternative to chemical surfactants for improved oil recovery *(3,4)*. Surfactants reduce interfacial tension between oil and water, thus decreasing the energy required to extract trapped oil in the matrix and displacing it into the mobile liquid phase. *B. subtilis* grown in low-solids (LS) potato process effluents produced surfactin with surface tensions of 25.5–26.5 mN/m *(3)*. The purpose of the present study was to evaluate the changes in surfactin activity of surfactin containing culture supernatant from *B. subtilis* grown on LS potato process effluents by altering pH, salt concentration, and temperature of the cell-free supernatant. We carefully considered typical oil reservoir conditions and the ultimate exploitation of this surfactin as an enhanced oil recovery agent.

Materials and Methods

Production of Surfactin

Surfactin was produced from *B. subtilis* (ATCC 21332) in a continuous stirred tank reactor (CSTR) utilizing LS potato process effluent, which was obtained from a southeast Idaho potato processor, as previously described by Noah et al. *(4)*. At the completion of a run, the culture fluid was centrifuged at 6084*g* for 30 min to remove cells and insoluble material. The supernatant containing surfactin was then stored in a sterile container at 4°C. The same supernatant from one single run was used throughout all experiments to provide continuity for comparison of each study.

Experimental Parameters and Procedures

Three experimental parameters and combinations thereof were investigated: (1) pH, (2) salt concentration, and (3) temperature. The effects on surfactin activity were measured by surface tension ($n = 5$).

Effects of pH

For each experiment, the supernatant was placed in 15-mL conical tubes, and the pH was adjusted with either 1 *N* KOH or 1 *N* HNO_3. The pH of the supernatant after the bioreactor run was 7.0. Simulated potato effluent (SPE) medium was used as a surfactin-free control and the pH adjusted likewise. SPE contained the following per liter of nanopure water: 5 g of potato starch, 3.5 g of peptone, 3.5 g of tryptone, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of yeast extract, and 0.8 g of $(NH_4)_2SO_4$.

Effects of Salt Concentration

For each experiment, up to 10% (w/v) NaCl was placed in 15-mL conical tubes. The supernatant was added and the tubes were gently stirred on a laboratory rotator for 2 h. With concentrations above 3%, the supernatant became cloudy with a precipitate that interfered with surface tension measurements. The supernatant was therefore left overnight in an upright position to allow the precipitate to settle out, and readings of surface tension were taken the following day. The SPE medium was used as a surfactin-free control and NaCl added likewise. When pH was included as a parameter in this experiment, NaCl was added first and the supernatant was stirred for 2 h. Then the pH of the supernatant was adjusted with either 1 *N* KOH or 1 *N* HNO_3.

Effects of Temperature

For each experiment, the supernatant was placed in 15-mL conical tubes and then incubated at temperatures up to 70°C. The supernatant was incubated for a minimum of 1 h. When experiments included NaCl and pH adjustments, the supernatant was incubated overnight, or longer, to allow for precipitate to settle out. For the surfactin stability experiment, supernatant was incubated at either 4 or 70°C for over 95 d. Surface tension was measured at elevated temperature using a special heated cell. The cell was made from stainless steel with channels drilled in the cell to accommodate attachment to a heated circulating water bath (Polystat Water Circulator; Cole-Parmer, Vernon Hills, IL). Density was determined for the supernatant used in all experiments by placing a little more than 1 mL in a 1-mL volumetric flask and incubating at 4, 21, 37, 51, and 70°C for 1 h. Afterward, the excess supernatant was removed to the 1-mL mark. The flasks were then weighed to determine the density of the fluid. The average density ($n = 2$) from this experiment was used in a quadratic equation to extrapolate density for each temperature in the range of 21–70°C.

Analytical Methods

Surfactin in the supernatant was confirmed and the concentration determined by high-performance liquid chromatography analysis as described by Noah et al. *(4)*. Surface tension was measured using video image analysis of inverted pendant drops as previously described *(5)*. All data points are an average of five measurements taken of the cell-free supernatant.

Results

Effects of pH

Figure 1 shows the effects of altering the pH of the supernatant containing surfactin. The starting pH of the supernatant produced from potato process effluents, without any pH adjustments, was 7.0 and the surface tension was 28.3 ± 0.1 mN/m. The effects of altering the pH were found to alter surface tension of the surfactin at pH values <6.0. The sharpest tran-

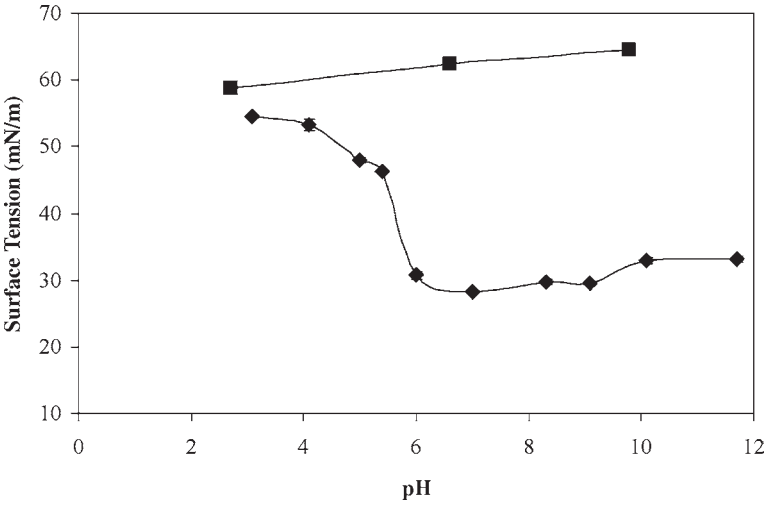

Fig. 1. Effects of pH on surfactin activity. The pH of the supernatant without any alteration in pH was 7.0. (◆) Surfactin; (■) surfactin-free control.

sition in surfactin quality, as indicated by an increase in surface tension, occurred between pH 6.0 (30.7 ± 0.5 mN/m) and 5.0 (47.9 ± 0.4 mN/m). Between pH 6.0 and 10.0 surface tensions remained almost unchanged and had a range of 28.3 ± 0.1 to 33.0 ± 0.4 mN/m. Since isolation procedures utilize the precipitation of surfactin under acidic conditions *(4)*, it was expected that a precipitate would form and surface tension would increase at pH <3.0. Indeed, a precipitate formed and surface tension increased to 54.5 ± 0.3 mN/m. When the sample was centrifuged and the resulting pellet resuspended in nanopure water, the surface tension returned to 32.2 ± 0.3 mN/m.

Effects of Salt Concentration

Figure 2 shows the effects of the addition of salt (NaCl) to the supernatant. The surface tension of the surfactin without any additions of NaCl was 29.4 ± 1 mN/m. Experiments found NaCl concentrations above 30 g/L to increase surface tension of surfactin. Between 30 and 50 g/L of NaCl, surface tension increased from 29.4 ± 0.1 to 46.2 ± 0.4 mN/m. Between 60 and 100 g/L of NaCl, surface tension remained at about 50 mN/m.

Effects of Temperature

Figure 3 shows the effects of incubating surfactin from 21 to 70°C. Although there was an apparent decrease in surface tension of surfactin at higher temperature, there was also a decrease in surface tension of water as

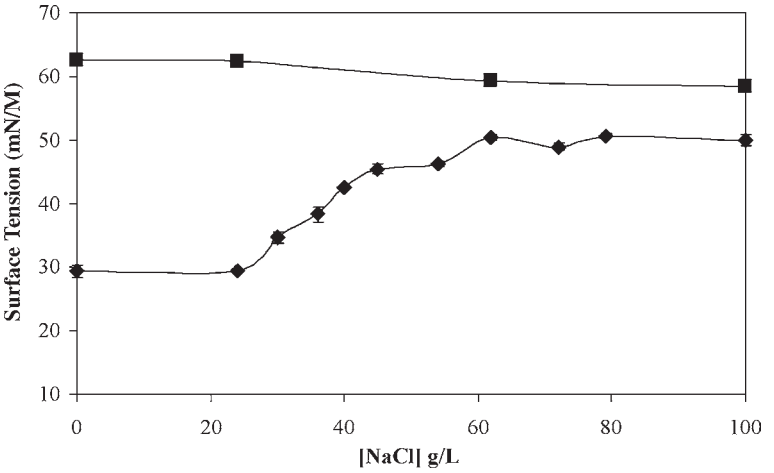

Fig. 2. Effects of salt concentration on surfactin activity. (◆) Surfactin; (■) surfactin-free control.

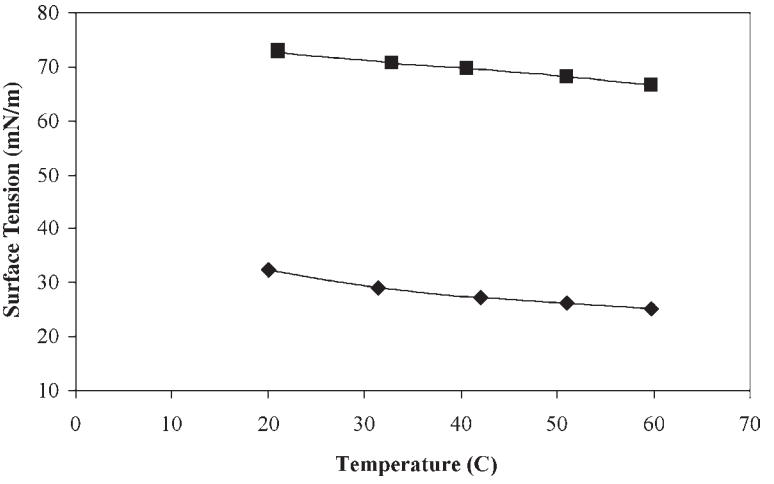

Fig. 3. Effects of temperature on surfactin activity. (◆) Surfactin; (■) water.

temperature increased. The difference in surface tension between water and surfactin remained the same, showing that there was no effect of temperature on surfactin. Furthermore, a stability experiment indicated no change in surface tension of surfactin when surfactin was incubated at 70°C for over 95 d (Fig. 4). There was also no change in surface tension of surfactin incubated at 4°C for 95 d.

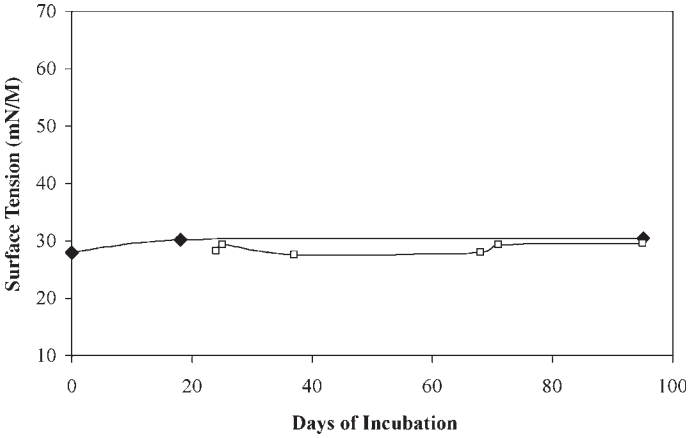

Fig. 4. Temperature stability of surfactin over time. (◆) Surfactin incubated at 70°C; (□) surfactin incubated at 4°C.

Effects of Temperature and pH

Temperature and pH effects were examined together to determine whether there were synergistic interactions that were not seen when each was tested alone. Figure 5 shows theresults from this experiment. Temperature alone did not alter surface tension of surfactin, nor did it alter the results seen from pH alone. The combination of temperature at 31 and 56°C and pH 5.0 did increase precipitate in the supernatant, which interfered with measurement of surface tension. Attempts to centrifuge did not remove the precipitate and still did not allow measurement of surface tension. However, visual observation of the pendant drop size indicated that surface tension had increased relative to the supernatant that had not been altered with KOH or HNO_3. At an incubation temperature of 70°C, pH 3.0 and pH 5.0 supernatants were cloudy, which interfered with surface tension measurements. Visual observation indicated that the surface tension was relatively high for these samples as well, since the pendant drop size was large compared to surfactin with surface tensions in the range of 27 to 28 mN/m.

Effects of Temperature and Salt Concentration

Figure 6 shows the results of the experiment on the effects of temperature and salt concentration. The supernatant was prepared as described in the Materials and Methods section, except that there was a 9-d incubation instead of overnight owing to the replacement of a burned-out lamp in the interfacial tension instrument. There were no significant differences among all salt concentrations at 21 and 45°C. However, at 70°C, the higher salt concentrations did not increase surface tension of surfactin, as did those at the lower temperatures. At 50 g/L of NaCl and 70°C, surface tension was 31.5 ± 1.3 mN/m compared with 50.9 ±0.3 and 53.4 ± 0.2 mN/m at 50 g/L of NaCl at 21 and 45°C, respectively.

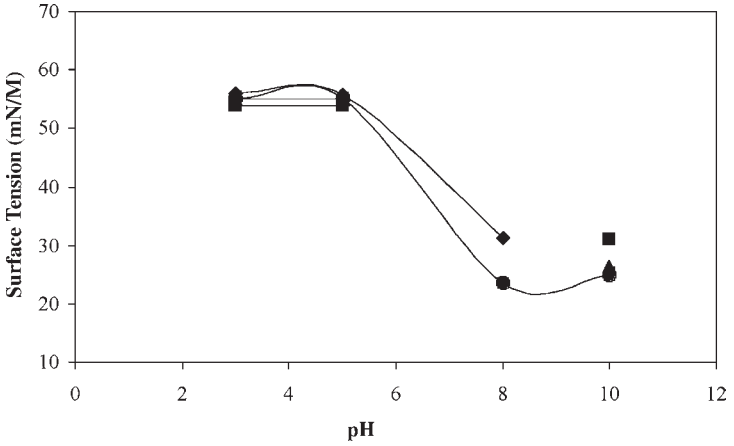

Fig. 5. Effects of temperature and alterations in pH on surfactin activity. (◆) Surfactin, 21°C incubation; (■) surfactin, 31°C incubation; (▲) surfactin, 56°C incubation; (●) surfactin, 70°C incubation.

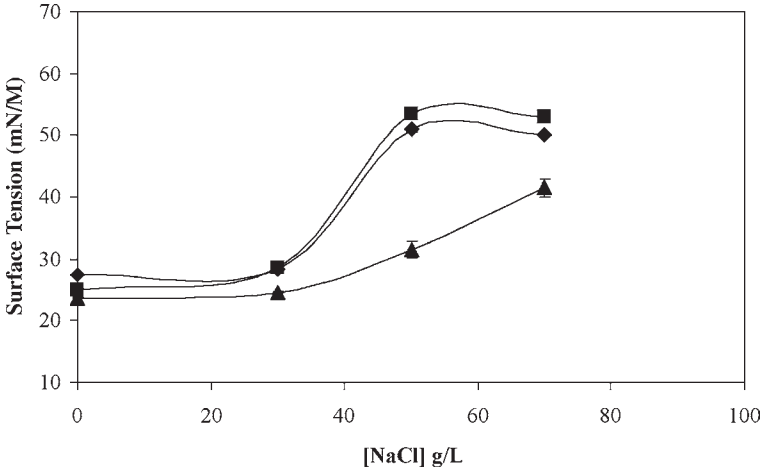

Fig. 6. Effect of temperature and salt concentration on surfactin activity. (◆) Surfactin, 21°C incubation; (■) surfactin, 45°C incubation; (▲) surfactin, 70°C incubation.

Effects of Temperature, Salt Concentration, and pH

Ther effects of the addition of NaCl (3–8%), alterations in pH (3.0–10.0), and temperature (21 and 70°C) were tested together to examine the effects of all three parameters combined. Figure 7 compares two experiments at 21 and 70°C. Samples were incubated overnight except for those at 70°C that were too cloudy to measure surface tension. These samples were incubated for 6 d until measurements could be taken. Incubation time was increased

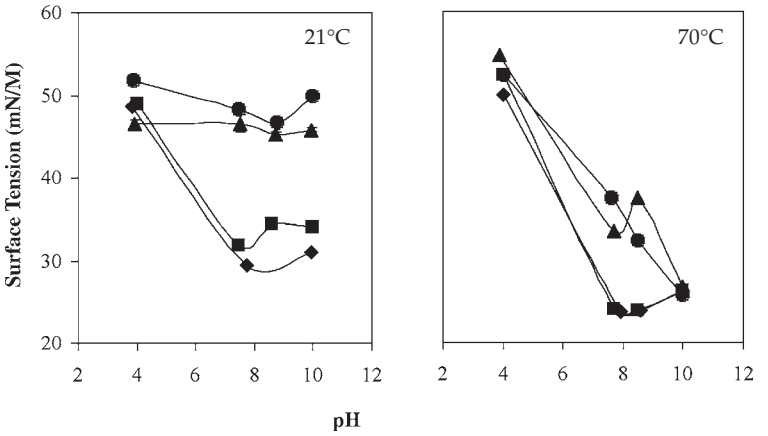

Fig. 7. Effect of temperature, salt concentration, and pH on surfactin activity. (◆) 0 g/L of NaCl; (■) 30 g/L of NaCl; (▲) 50 g/L of NaCl; (●) 70 g/L of NaCl at 21°C, 80 g/L of NaCl at 70°C.

because a high-temperature centrifuge was not available to remove the precipitate that had formed in the supernatant. It was observed that the effect of increasing surface tension by increasing salt concentration was moderated at higher temperature and basic pH. The surface tension of the supernatant at salt concentrations >50 g/L at 21°C remained between 45.2 ± 0.4 and 51.8 ± 0.3 mN/m. The surface tension of the supernatant at salt concentrations >50 g/L and 70°C, however, was lower. This was observed most at pH 10.0, where surface tension of the supernatant with 50 g/L of NaCl was 26.6 ± 0.2 mN/m, and the surface tension of the supernatant with 80 g/L of NaCl was 25.8 ± 0.1 mN/m. The surface tension of the supernatant with no addition of NaCl and no alterations in pH at 70°C was 23.7 ± 0.2 mN/m, and 29.4 ± 0.6 mN/m at 21°C.

Discussion

Supernatant containing surfactin, produced from LS potato process effluents in an airlift reactor, was characterized under various environmental parameters to better define appropriate conditions for its use as an agent for enhanced oil recovery. By itself, pH was found to increase surface tension of the surfactin below 6.0. Concentrations of NaCl a>30 g/L were seen to increase surface tension, with the highest surface tension occurring at about 50 g/L. When the effect of temperature was added to the pH experiment, there were no significant changes, and again, surface tension, at any temperature, increased at pH <6.0. Temperature alone, up to 70°C, did not alter surface tension of the surfactin. However, when temperature was added to experiments with salt concentration, increases in surface tension seen at 50 and 70 g/L of NaCl appeared to be somewhat moderated at 70°C.

This was further verified when all three parameters were combined in one experiment and no increase in surface tension was observed at 80 g/L of NaCl, pH 10.0, and 70°C. Temperature experiments did not go beyond 70°C, so the high temperature limit of the surfactin has yet to be determined.

Surfactin is an anionic, amphiphilic, lipopeptide compound. These properties are the reason for its ability to lower surface tension so effectively *(6)*. Surfactin is also an effective antimicrobial and antiviral, able to induce the formation of ionic pores in phospholipid bilayers *(7)* and transport cations across membranes *(8)*. Its cation-complexing property, owing to two negative charges on the aspartyl and glutamyl residues *(8)*, is probably fully utilized in our system containing ubiquitous amounts of Ca^{+2} and Na^{+} ions in the LS potato process effluent *(9)*. The increase in surface tension of surfactin with higher NaCl concentration and lower pH is more likely owing to a precipitation process commonly seen with proteins and used in protein purification. Precipitation of proteins can be achieved by adding salts, adding organic solvents, altering the pH, or altering the temperature *(10)*. In our study, we altered three of these variables. Furthermore, an increase in temperature to 70°C and an increase in pH to 10.0 probably decreased the hydrophobic effects caused by high salt concentration on the surfactin in the solution so that protein aggregation by association of hydrophobic surfaces did not occur.

These are favorable results for the application of this surfactin as an agent of enhanced oil recovery since high salt concentration, high temperature, and high pH describe the conditions of many oil reservoirs. This surfactin was also produced cheaply with potato process effluents, another attractive feature. Previous experiments with surfactin produced in minimal salts medium containing potato starch have shown similar results. However, note that changes in the process from which the feedstock is derived could have an impact on these results.

Future production of surfactin from potato process effluents will be used in core floods to characterize further its potential application as an agent for enhanced oil recovery.

Acknowledgments

We thank Jerry Casteel, Rhonda Lindsey, Suzanne M. Mehlhoff, Purna Halder, and Daniel Gurney of the US Department of Energy (DOE) National Petroleum Technology Office and Patricia St. Clair of the US DOE Idaho Field Office for their support. We also express our gratitude for the generous help and support from Basic American Foods, Blackfoot, ID. This work was supported by the US Department of Energy, Assistant Secretary for Fossil Energy, Office of Fossil Energy under contract no. DE-AC07-99ID13727.

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