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Development of Continuous Surfactin Production from Potato Process Effluent by *Bacillus subtilis* in an Airlift Reactor

Karl S. Noah,* Sandra L. Fox, Debby F. Bruhn, David N. Thompson, and Gregory A. Bala

Biotechnology Department, Idaho National Engineering and Environmental Laboratory, PO Box 1625, Idaho Falls, ID 83415-2203, E-mail: karl@inel.gov

Abstract

The biosurfactant surfactin has the potential to aid in the recovery of subsurface organic contaminants (environmental remediation) or crude oils (oil recovery). However, high medium and purification costs limit its use in these high-volume applications. In previous work, we showed that surfactin can be produced from an inexpensive low-solids (LS) potato process effluent with minimal amendments or pretreatments. Previous research has also shown that 95% or more of the surfactin in *Bacillus subtilis* cultures can be recovered by foam fractionation. In this work, we present the results of research to integrate surfactin production with foam fractionation. Experiments were performed in an airlift reactor, with continuous collection of the foam through a tube at the top of the column. Preliminary results using both purified potato starch and unamended low-solids potato process effluent as substrates for surfactin production indicate that the process is oxygen limited and that recalcitrant indigenous bacteria in the potato process effluent may hamper continuous surfactin production.

Index Entries: *Bacillus subtilis*; biosurfactant; surfactin; alternate feedstock; enhanced oil recovery.

Introduction

Utilization of biologically produced surfactants for numerous applications is limited by cost. The costs of these surfactants are primarily determined by the price paid for media, and the cost of purification or isolation of the surface-active material. Previous research at the Idaho National

*Author to whom all correspondence and reprint requests should be addressed.

Engineering and Environmental Laboratory has demonstrated the production of surfactin by *Bacillus subtilis* when cultivated on purified potato starch, as well as on effluent from the potato-processing industry *(1,2)*. Surfactin is a cyclic lipopeptide antibiotic biosurfactant produced by *B. subtilis (3)*. Purified surfactin has an aqueous critical micelle concentration of 25 mg/L and lowers the surface tension of air/water systems to 27 mN/m *(3)*. Production yields of surfactin from glucose and other monosaccharides have been reported in amounts ranging from 0.1 to 0.7 g/L *(4–7)*. Recent literature supports that foam fractionation techniques applied in chemically defined media and optimized bioreactors can improve yields up to 1.67 g/L *(8)*.

In this article, we report initial work to combine the production of surfactin from potato process effluents with direct foam fractionation techniques. This combination has the potential to result in biologically produced surfactants with the appropriate cost profile for applications in high-volume, low-price markets such as environmental remediation or enhanced oil recovery.

Materials and Methods

Potato Substrates

Low-solids potato process effluent *(1,2)* was obtained from a southeast Idaho potato processor, adjusted to pH 7.0 with 5 *N* KOH, and autoclaved before use. In separate experiments, purified potato starch (cat. no. S-2004; Sigma, St. Louis, MO) medium was autoclaved and used at pH 7.0 as previously described *(1,2)*. For potato process effluent and media compositions, refer to Thompson et al. *(1,2)*.

Cultures and Maintenance

B. subtilis 21332 was obtained from the American Type Culture Collection. *B. subtilis* were cultured and maintained as previously described *(1,2)*, except that tryptic soy broth (TSB), (Difco, Detroit, MI) was used in place of Difco nutrient broth. Difco tryptic soy agar was used for plate counts.

Airlift Reactor

Surfactin production tests were performed in an airlift reactor (91.4 cm long × 9.52 cm id) with a 33.02 cm long × 2.54 cm id draft tube, both fabricated from Lexan™ (Fig. 1). The top and bottom plates were fabricated from 316 stainless steel. Aeration was provided from the bottom at 1.5 L/min through a 3.18 mm od stainless steel tube with 16 0.051-mm holes. Effluent collection and level control were achieved using an overflow tube. Dissolved oxygen (DO) and pH were monitored using steri-lizable probes (0.61-m Ingold DO probe and 0.61-m Mettler Toledo pH probe, respectively), inserted through the head plate, which fixed the headspace height at 0.48 m and the liquid volume at 3 L. A thermocouple was inserted

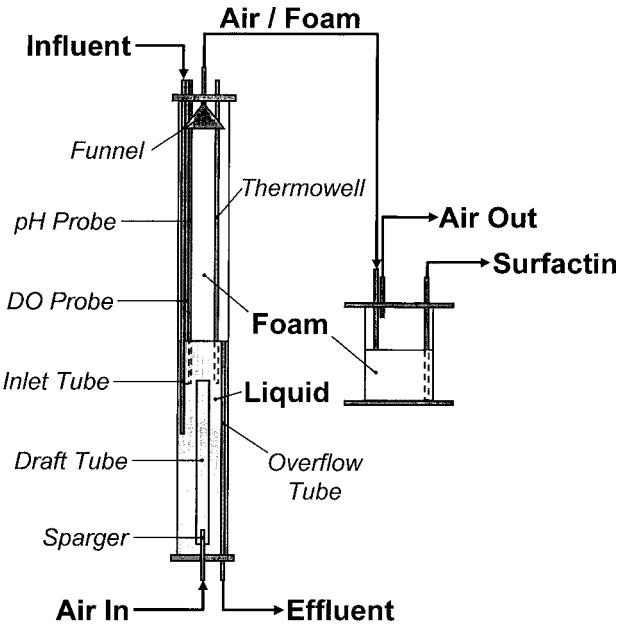

Fig. 1. Airlift reactor schematic.

below the liquid level within a thermowell and used with a temperature controller and heating tape to maintain the reactor at 30°C. Three liters of medium (either potato process effluent or potato starch) was inoculated in the reactor with 50 mL of *B. subtilis* grown on TSB. Foam collection from the top of the column was achieved using an inverted funnel. During continuous operation, fresh, autoclaved medium was pumped from a refrigerated (4°C) 50-L carboy into the column at various dilution rates.

Analytical Methods

General Methods

Cell numbers were determined after sampling as previously described *(1)*. Glucose was measured as previously described *(1)*. Soluble starch was estimated as previously described *(1)*, using the phenol-sulfuric acid assay *(9)*. Surface tensions were measured on cell-free broth and broken-foam supernatants by video image analysis of inverted pendant drops as previously described *(10)*.

Surfactin Concentrations

Surfactin was measured by high-performance liquid chromatography as described by Lin and Jiang *(11)*, using a Supelco LC-18 column (250 × 4.6 mm, 5-µm particle size). Separation was achieved by elution on a gradient of 10 mM KH_2PO_4 (pH 6) and 100% methanol at 0.5 mL/min as follows: (a) 0–30 min, 70–73.4 vol% methanol; and (b) 30–80 min, 73.4–95.4 vol% methanol. Samples were centrifuged for 10 min at 3500*g* and filtered

through a 0.22-μm syringe filter prior to analysis. The injection volume was 500 μL. Surfactin was measured by absorbance at 210 nm. Purified surfactin (cat. no. S-3523, Sigma) was used as a standard. All surfactins eluted from 34–80 min; thus, total surfactin was quantified in samples as the sum of the peak areas eluting in that time period.

Results

Stripping Experiments

The use of an airlift reactor to combine the production of surfactin from potato process effluents with direct foam fractionation techniques was suggested from data obtained from three potato starch runs conducted using a New Brunswick BioFlo 3000 fermentor. Each run had a working volume of 2.5 L and was agitated with two Rushton impellers. The first run was conducted at 2.5 L/min of air and 200 rpm, the second run at 1 L/min of air and 100 rpm, and the third at 0.5 L/min of air and 100 rpm. For all three runs, the starch was utilized within 48 h, and the foam/surfactin produced overflowed the effluent air filter. The surface tension of the liquid in the reactor decreased with decreasing airflow rate, suggesting that the air was stripping the surfactin.

A set of experiments was performed in the airlift to evaluate the effect of airflow rate on stripping of the surfactin into the foam. Three liters of grown culture broth (potato starch in shake flasks for 48 h) containing cells and surfactin was placed in the airlift. The airflow rate was set at either 1.5 or 3.0 L/min. Samples of foam were collected approximately every 20 min over a period of 160 min. For the 1.5 L/min runs, the surface tension of the liquid averaged 25.5 mN/m. The surfactant concentration in the foam increased to 3.5 from 1.8 g/L in the liquid in the first 20 min, and averaged 3.74 g/L in the foam over the remaining 160 min. For the 3.0 L/min runs, the surface tension of the liquid averaged 26.5 mN/m. Surfactant concentration peaked at 2.8 g/L in the foam from 1.8 g/L in the broth over the first 20 min, then declined steadily to 2.3 g/L over the remaining 160 min. In total, 241 g of foam was collected at 1.5 L/min and 1011 g was collected at 3.0 L/min by 120 min. As expected, there was higher liquid entrainment in the foam at the higher airflow rate, hence the lower concentration of surfactin at the higher airflow rate. This experiment demonstrated that surfactin could be stripped and concentrated into the foam. An airflow rate of 1.5 L/min was chosen for future work.

Batch Experiments Utilizing Purified Starch

Batch experiments utilizing purified potato starch medium were performed first to test the airlift reactor. Both medium and foam were sampled over time and analyzed as described in the previous section. Figure 2 shows the results from a typical run. As the starch was utilized, the pH dropped and the cell numbers increased. Appreciable foam production from the top of the column did not occur until the DO approached 0%. The surface

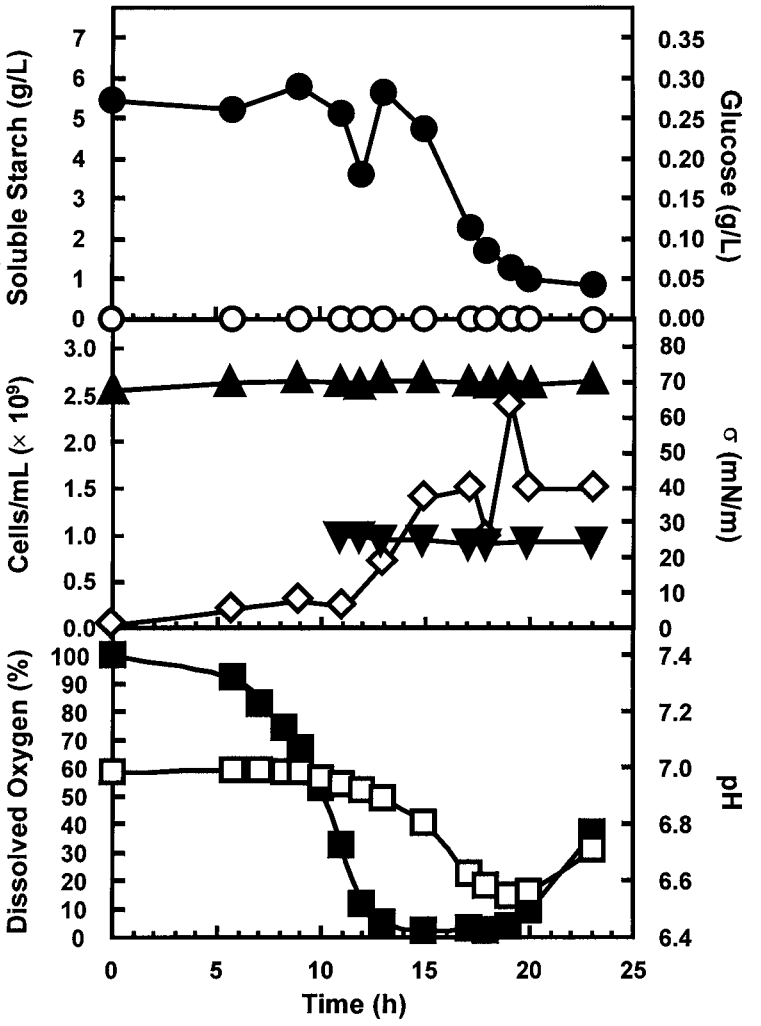

Fig. 2. Typical batch purified starch run data. (●), soluble starch; (○), glucose; (◇), cells/milliliter in liquid; (▲), surface tension of liquid; (▼), surface tension of broken foam; (■), DO; (□) pH.

tension of the foam was about 25 mN/m, indicating that there was good stripping of the surfactin into the foam. The run was stopped because there was little starch left and the volume of liquid was approaching the bottoms of the probes.

Batch Experiments Utilizing Potato Process Effluent

Figure 3 shows data from a typical batch potato process effluent run. Results for this run were similar to those from the potato starch run. Glucose was utilized first, followed by starch. The pH dropped, and the cell numbers appeared to increase and then declined as the glucose and soluble

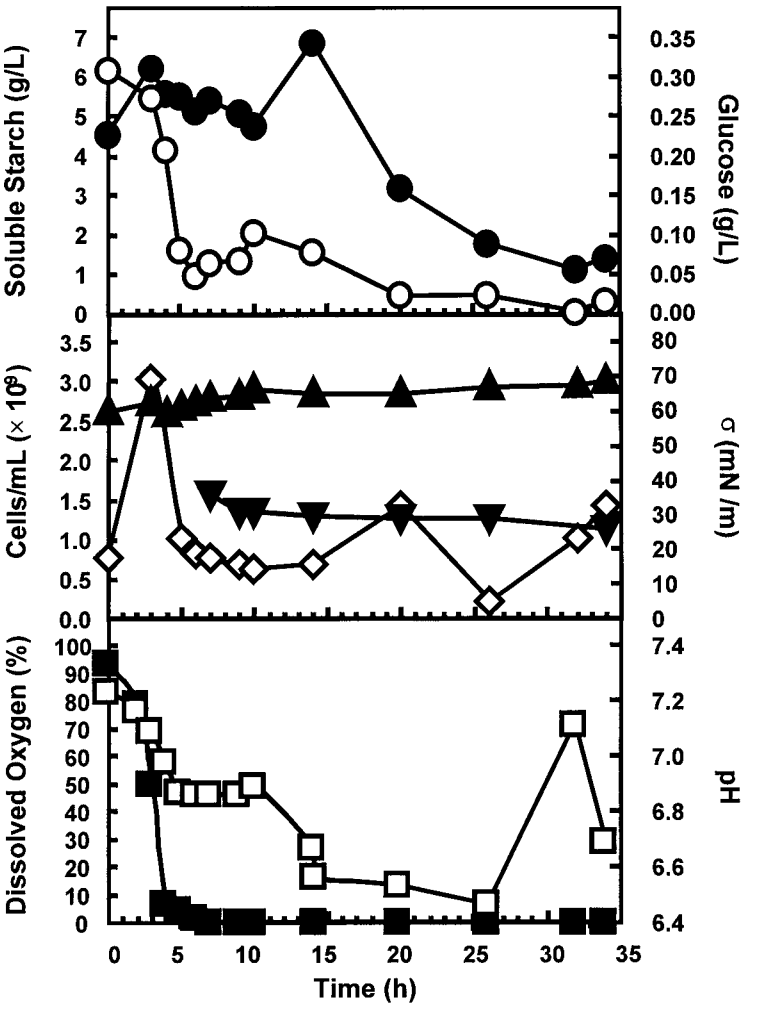

Fig. 3: Typical batch potato process effluent run data. (●), soluble starch; (○), glucose; (◇), cells/milliliter in liquid; (▲), surface tension of liquid; (▼), surface tension of broken foam; (■), DO; (□) pH.

starch were utilized. Foam production usually started when the DO approached 50%, but foam did not overflow the headspace until the DO approached 0% (6-h mark). There was good stripping of the surfactin from the liquid to the foam. The surface tension of the liquid was about 70 mN/m, whereas the surface tension of the foam was about 30 mN/m. The experiment was stopped at 35 h because liquid levels were approaching the bottoms of the probes.

Continuous Experiments Utilizing Purified Starch

Since the potato process effluent contains solids, proteins, and indigenous bacteria *(1,2)* that interfere with cell counts and potentially lower

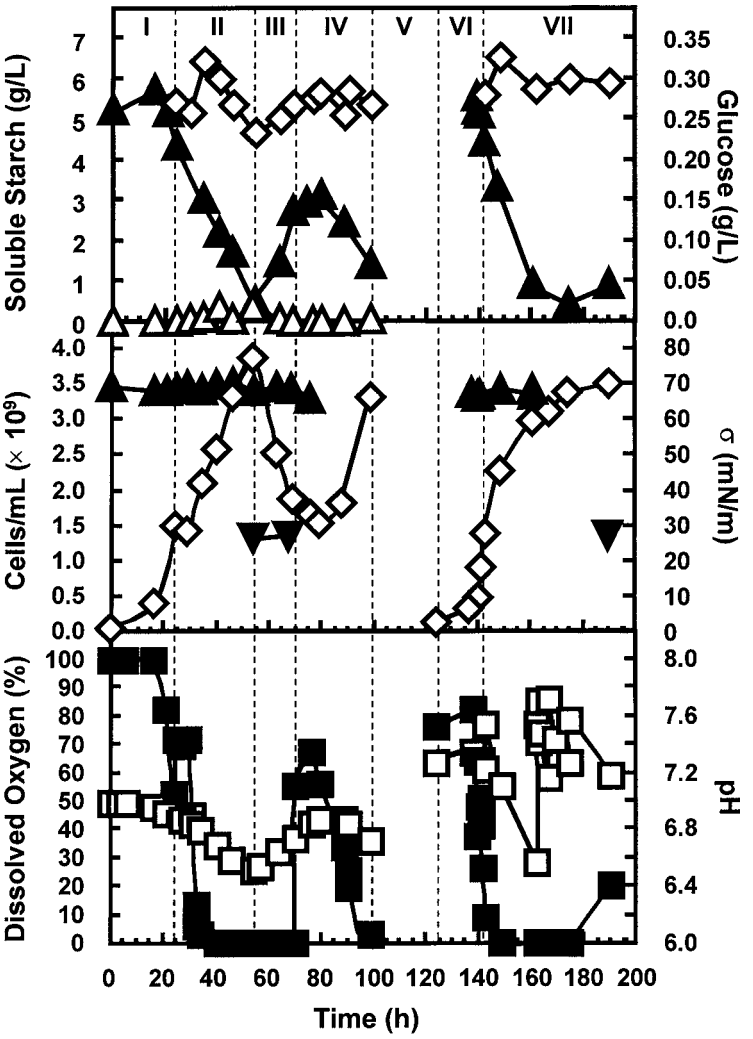

Fig. 4: Continuous purified starch run data. (**Top**) (▲), reactor soluble starch; (◇), inlet soluble starch; (Δ), reactor glucose. (**Middle**) (◇), cells/milliliter in the liquid; (▲), surface tension of liquid; (▼), surface tension of broken foam. (**Bottom**) (■), DO; (□), pH. Operation ranges were as follows: (I) batch, 1.5 L/min of air; (II) continuous, 4.5 mL/min, 1.5 L/min of air; (III) continuous, 11.5 mL/min, 1.5 L/min of air; (IV) continuous, 11.5 mL/min, 3.0 L/min of air; (V) shut down for cleaning; (VI) batch, 1.5 L/min air; (VII) continuous, 4.5 mL/min, 1.5 L/min of air, manual pH control at 7.5.

surfactin production, a continuous run was performed using purified potato starch medium in an attempt to find the highest dilution rate before washout occurred. The data from this control run would provide a reference point for the continuous potato process runs. For the first 25 h, the airlift was operated in batch mode to allow cell growth and initial surfactin production (Fig. 4). At 25 h, flow was started at 4.5 mL/min. Cell production increased, starch utilization continued, DO decreased to 0%, pH decreased,

and surfactin production continued. However, a microbial contaminant appeared in the reactor during this time frame. The contaminant was believed to be germinated from spores carried over from previous potato process runs. It was decided to change several process variables to determine whether the *B. subtilis* could outcompete this contaminant with the end goal of using this information to learn ways to do the same for the continuous potato process effluent runs. First, the flow rate was increased to 11.5 mL/min to washout the contaminant. At this flow rate, the total cell numbers decreased, the soluble starch concentration in the reactor increased, the DO remained at 0%, the pH increased, and the surface tension of the foam was about 26 to 27 mN/m. Next, the airflow rate was increased to 3 L/min to evaluate the impact on cell numbers (an increase was expected). The total cell numbers (*B. subtilis* and contaminant) rebounded, the soluble starch decreased, the DO increased and then dropped below 5%, and the pH stayed about the same. Since foaming ceased, which meant that no surfactant was being produced, the experiment was stopped and the reactor was shut down, rinsed with sterile hot water, and soaked overnight in 30 μg/mL of tetracycline.

The reactor was then recharged with 3 L of potato starch medium and reinoculated with *B. subtilis*. The reactor was operated in batch mode at 1.5 L/min air until the DO approached 0% and foaming was robust (124–142.5 h), (Fig. 4). During continuous operation at 4.5 mL/min, 5 N KOH was added manually in an attempt to control the pH at 7.5. After approx 19 h of continuous operation, about half of the cells, as seen during cell counts, appeared to be a bacterium other than the inoculated *B. subtilis* strain. Even though the total cell count increased, somewhere between 19 and 32 h of continuous operation foam production stopped. By plate counts, it was estimated that 60% were cells other than *B. subtilis*. After approx 48 h of continuous operation, the contaminant cells believed to be an indigenous potato waste effluent organism had again taken over the culture.

These data indicate that after approx 24 h of continuous operation at a DO near 0% *B. subtilis* was outcompeted by an unknown organism, if the surfactin was stripped from the broth.

Continuous Experiments Utilizing Potato Process Effluent

Continuous experiments utilizing potato process effluent were performed to evaluate the ability of *B. subtilis* to outcompete indigenous bacteria in the potato process effluent. The airlift was operated in batch mode until the DO approached 0% (Fig. 5). During continuous flow at 8 mL/min, the DO remained at 0%, the total cell numbers increased, glucose was utilized, the soluble starch decreased to about 1 g/L, the pH dropped to about 6.3, and foam was produced. At 26 h, the flow was increased to 13 mL/min. Five hours later, even though starch and glucose were being utilized, there was little or no foam production. By this time, >90% of the cells in the reactor were indigenous bacteria from the potato

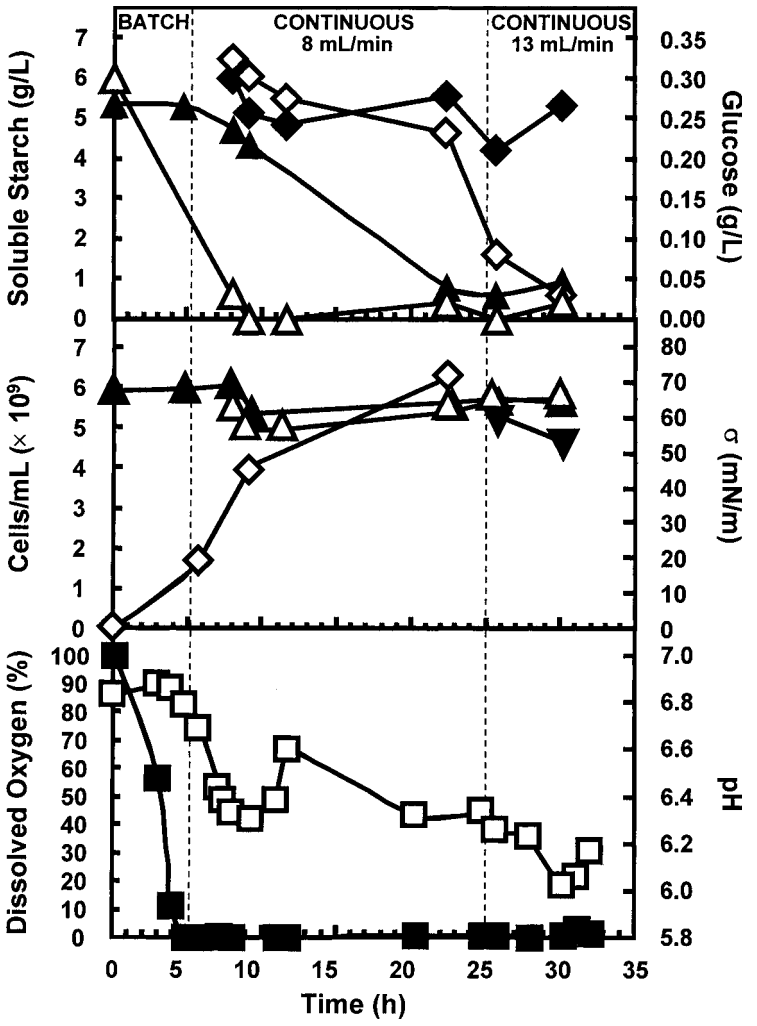

Fig. 5. Continuous potato process effluent run data. (**Top**) (▲), reactor soluble starch; (◇), inlet soluble starch; (Δ), reactor glucose; (◆), inlet glucose. (**Middle**) (◇), cells/milliliter in liquid; (Δ), surface tension of influent; (▲), surface tension of liquid; (▼), surface tension of broken foam. (**Bottom**) (■), DO; (□) pH.

process effluent. The foam that was produced was of low quality with a surface tension between 50 and 60 mN/m.

Discussion

Experiments Utilizing Purified Starch

All batch experiments utilizing purified starch medium produced surfactant with a surface tension of about 25 mN/m. Foaming usually started when the DO was about 50%, but no foam/surfactin was recovered

from the top of the column until the DO approached 0%. In the continuous purified starch medium runs, surfactin was produced in batch mode (6–24 h) but stopped 24–36 h after continuous operation was started. As shown earlier, these purified starch runs were apparently becoming contaminated during the continuous phase, believed to be from spores of the indigenous bacteria from previous potato process effluent runs. Increasing the dilution rate, increasing the airflow rate, and controlling the pH did not allow the *B. subtilis* to outcompete the contaminant.

Experiments Utilizing Potato Process Effluent

Potato process effluent usually produced surfactin with a surface tension ranging from 30 to 60 mN/m. When using potato process effluent, the reactor will produce a small amount of foam owing to the foaming of proteins in the potato process effluent. These nonsurfactin proteins in the potato process effluent added to the quantity of foam and also increased the amount of liquid carryover in the foam, thus diluting the surfactin concentration in the foam, accounting for the high surface tension measurements. This could be corrected by adding an additional column on top of the airlift.

There are known indigenous bacteria in the potato process effluent *(1,2)*. When the medium was obtained directly from the potato-processing plant, autoclaved, and used immediately, very few indigenous bacteria could be seen on plate counts for the first 36 h. However, when the medium was used in continuous runs, the indigenous bacteria would appear 24–48 h after flow was started even though the feed carboy was placed in a refrigerator. If the potato process effluent was autoclaved and stored at 4°C for any significant length of time, indigenous bacteria would appear prior to use.

B. subtilis could not outcompete the indigenous bacteria during continuous operation. During batch operation in shake flasks, there is minimal foam formation and surfactin accumulates in the liquid, potentially assisting *B. subtilis* in overtaking indigenous bacteria *(1,2)*. When the batch cultures are performed in the airlift reactor, the surfactin is stripped into the foam. However, there is better transfer of O_2 into the liquid phase, allowing *B. subtilis* to grow and consume resources faster than the indigenous bacteria, which are fermentative *(1,2)*, and which grow from slowly germinating (8–12 h) spores that survive the autoclaving *(2)*. By the time the spores are germinated, the glucose and soluble starch levels in the batch airlift cultures are near zero. During continuous operation in the airlift reactor, surfactin is stripped into the foam as in the batch airlift cultures. However, now there is continuous addition of new substrate and additional indigenous bacteria into the reactor, in which the DO is near zero and the *B. subtilis* has moved into late log phase/early stationary phase growth rates. These conditions favor the indigenous potato process effluent bacteria, and *B. subtilis* can no longer compete. Work that is currently under way to minimize the indigenous bacteria competition includes a larger inocu-

lum of *B. subtilis* grown on potato starch, pH control, a pressurized reactor operation, the growth and recovery of surfactin in a semibatch reactor system, and evaluation of effluent population dynamics with respect to reactor operations.

Conclusions

An airlift reactor can be used to combine the production and recovery of surfactin from purified starch medium with direct fractionation techniques in good yield. When producing surfactin from potato process effluent in the airlift reactor, which contains recalcitrant indigenous bacteria and significant amounts of protein, both production and recovery are hampered. It is likely that removal of the foam, which contains the surfactin, may allow the indigenous bacteria to outcompete the inoculated *B. subtilis*.

Acknowledgment

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