0273-2289/05/121–124/465–474/$30.00

# Surfactin Production from Potato Process Effluent by *Bacillus subtilis* in a Chemostat

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## Abstract

The biosurfactant surfactin has potential to aid in the recovery of energy resources (oil recovery) or subsurface organic contaminants (environmental remediation). However, high medium and purification costs limit its use in these high-volume applications. In previous work, we showed that surfactin could be produced from an inexpensive low-solids potato process effluent with minimal amendments or pretreatments. Previous research has also shown that surfactin can be both produced in *Bacillus subtilis* cultures and recovered by foam fractionation in an airlift reactor. 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 hamper continuous surfactin production. The research reported here features the use of a chemostat operated in batch mode for producing surfactin with concomitant use of antifoam to prevent surfactant loss. The antifoam did not interfere with surfactin recovery by acid precipitation or its efficacy. Initial trials took about 48 h to produce 0.9 g/L of surfactin from potato process effluent. Increasing the oxygen mass transfer by increasing the stirring speed and adding a baffle decreased production time to 12–24 h and produced about 0.6 g/L of surfactin from two different potato-processing facilities.

**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

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of the surface-active material. Previous research at the Idaho National 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 the fact that foam fractionation techniques applied in chemically defined media and optimized bioreactors can improve yields up to 1.67 g/L *(8)*.

We presented results of research to integrate surfactin production with foam fractionation in an airlift reactor with continuous collection of the foam *(9)*. The results using unamended potato process effluent as substrates for surfactin production indicated that the process was oxygen limited and that recalcitrant indigenous bacteria in the potato process effluent hampered continuous surfactin production and recovery using direct fractionation techniques.

In this article, we report initial work to optimize the production of surfactin from potato process effluents within a chemostat. A chemostat was used because there were various ways to eliminate oxygen limitation (bubble size, rpm, and so on) as well as automate the addition of antifoam. Antifoam keeps surfactant in solution, which could prevent growth of the recalcitrant indigenous bacteria, as suggested by previous airlift and shake flask studies.

## Materials and Methods

### Potato Substrates

Low-solids potato process effluent *(1,2)* was obtained from two different southeast Idaho potato-processing facilities. Four liters of effluent was autoclaved 90 min and allowed to stand overnight before use. Autoclaving the material for 90 min ensured that the organisms associated with the relatively high-solids load in the 4-L effluent volume were killed.

### Cultures and Maintenance

*Bacillus subtilis* 21332 was obtained from the American Type Culture Collection. *B. subtilis* was cultured and maintained as previously described *(1,2)*, except that simulated potato effluent (SPE) medium (5.0 g/L of potato starch, 0.5 g/L of glucose, 1.0 g/L of sucrose, 1.0 g/L of maltose, 3.5 g/L of peptone, 3.5 g/L of tryptone, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 0.1 g/L of yeast extract, 0.8 g/L of $(NH_4)_2SO_4$, 0.03 g/L of $FeSO_4$ and 0.0022 g/L of $MnSO_4 \cdot H_2O$) was used instead of Tryptic Soy Broth or Difco Nutrient Broth. For plating, 15 g/L agar was added.

### *Bioreactor*

Surfactin production tests were performed in a New Brunswick BioFlo 3000 stirred-tank bioreactor. Dissolved oxygen (DO) and pH were monitored using sterilizable probes inserted through the head plate. A thermocouple was inserted below the liquid level within a thermowell and used to control the temperature at 30°C. Two liters of potato process effluent was placed in the bioreactor and autoclaved for 60 min owing to the solids content of the effluent. The pH was then adjusted to 7.0 with 5 *N* KOH. The potato process effluent was inoculated with 200 mL of *B. subtilis* grown on SPE medium. Foam was suppressed by the addition of AF antifoam. The pH was automatically controlled by the addition of 5 *N* KOH and 3 *N* $H_2SO_4$. A batch run was conducted for at least 72 h. Air was supplied at 0.5 vvm (1 L/min). Early batch runs were conducted at 250 rpm without a baffle. Later batch runs were conducted at 400 rpm with a baffle in place. Two Rushton impellers provided the agitation. Samples were collected over time and analyzed for cell numbers, starch, surfactin concentration, and surface tension.

After a run was completed, the surfactin was recovered by centrifugation and acid precipitation *(5)*. The contents of the reactor were collected and the solids and cells removed by centrifuging at 10,000*g* for 14 min at 4°C. The supernatant was subjected to acid precipitation to recover the surfactin. Concentrated HCl was added until a pH of about 2.0 was obtained. The supernatant was then refrigerated for at least 24 h. The precipitate contained the surfactant. The contents were shaken and then centrifuged at 11,000*g* for 20 min at 4°C to recover the surfactin pellet. To place the surfactin back into solution, a known amount of nanopure water was added to resuspend the pellet and the suspension pH was raised to 7.0. The surfactin suspension was analyzed for starch as well as surfactin quantity and quality (surface tension).

### *Antifoam Screening*

Three different antifoams, T-H (Thompson Hayward, Kansas City, KS), AF (Dow Corning, Midland, MI), and B (Dow Corning) were screened in 500-mL chemostats. Three hundred milliliters of low-solids potato effluent was inoculated with 30 mL of *B. subtilis*. Antifoam was added manually by syringe when the foam reached the head plate. The volume of antifoam needed to suppress foaming and the effect of surfactin production were used for screening criteria.

### *Oxygen Mass Transfer Study*

The purpose of this study was to determine the oxygen mass transfer coefficient ($k_la$) as a function of revolution per minute with and without the baffle in place in the BioFlo 3000. Two liters of 3 *N* KCl was placed in the reactor. The air was turned on and the DO followed over time until it reached 100%. The air was then turned off, the nitrogen gas was turned on,

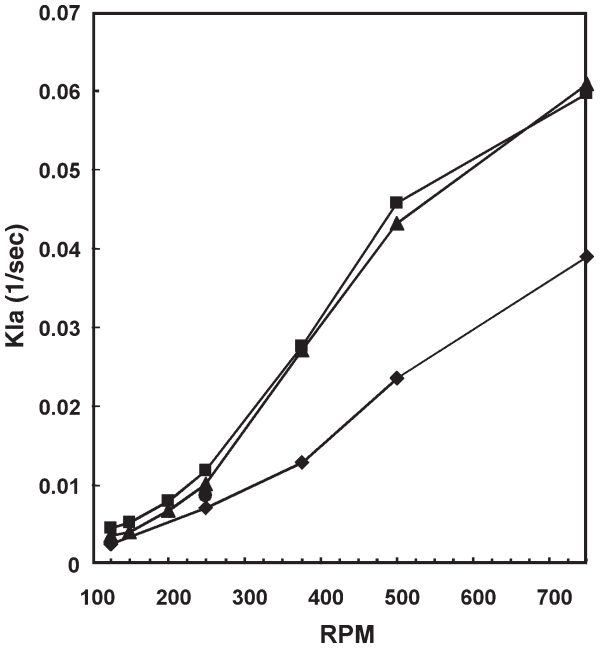


Fig. 1. Determination of $k_la$ in batch chemostat with and without baffle at various stirrer speeds. The medium was 3 *N* KCl. (◆) Without baffle; (▲) with baffle; (■) with baffle and $N_2$; (●) 2L/min of air without baffle.

and the DO was followed over time until it reached 0%. This was done at an airflow rate of 1 L/min at 125, 150, 200, 250, 375, 500 and 750 rpm with and without the baffle (Fig. 1). One run was at 2 L/min of air and 250 rpm without the baffle.

## *Analytical Methods.*

### General Methods

Cell numbers were monitored by plating on solid SPE medium. Contaminants were differentiated from *B. subtilis* based on colony morphology. Soluble starch was estimated as previously described *(1)* using a phenol–sulfuric acid assay *(10)*. Surface tensions were measured on cell-free broth by video image analysis of inverted pendant drops as previously described *(11)*.

### Surfactin Concentrations

Surfactin was measured by high-performance liquid chromatography using a modified method of Lin and Jiang *(12)* as previously described *(9)*. Surfactins eluted from 34 to 80 min; thus, total surfactin was quantified in samples as the sum of the peak areas eluting in that time period.

## Results and Discussion

### *Antifoam Screening*

Only 400 ppm of surfactin was produced when adding antifoam B whereas 1585 and 1793 ppm of surfactin were produced when adding antifoam T-H and AF, respectively. AF antifoam was chosen over T-H antifoam because less AF antifoam was needed to control the foaming.

### *Oxygen Mass Transfer*

Increasing the flow rate to 2 L/min did not significantly increase the $k_1a$. However, the $k_1a$ increased with both higher revolutions per minute and the addition of a baffle. At 500 rpm, the $k_1a$ was about 3.25 times greater than at 250 rpm without the baffle. By adding the baffle at 500 rpm, the $k_1a$ approximately doubled. At 750 rpm, the chemostat entrains air from the head space and appears to be violently mixed. By running the chemostat at 400 rpm with the baffle in place, the $k_1a$ was approx 4.3 times greater than the previous runs at 250 rpm without the baffle, 0.0308 vs 0.0072 1/s.

### *Batch Chemostat Runs With Potato Effluent From Plant 1*

Three trial runs were required to establish the correct parameters for automated pH and antifoam control. These runs confirmed that AF antifoam kept the foam under control and did not interfere with surfactin production or isolation. Complete starch utilization was noted at about 72 h.

Runs were conducted at 250 rpm without the baffle in place. The results were similar, so only data from one experiment are presented. The potato effluent was 3.25% solids. Surfactin concentration reached 0.8 g/L at 30 h and 0.9 g/L at 72 h (Fig. 2). By 42 h 90% or more of the starch was utilized. DO dropped to 0% by about 7 h and stayed at 0% the rest of the run (72 h). In addition, during these runs other cell types appeared after 52 h.

In an effort to maintain DO above 0% and decrease run time, reactor runs at an increased stirrer speed (400 from 250 rpm) and a baffle in place were evaluated. This was hypothesized to prevent contaminants and decrease run time. The results were similar, so only data from one experiment are presented. The potato effluent was 3.8% solids. The soluble starch was also utilized in 17 h (Fig. 3), producing about 1.1 g/L of surfactin. DO never dropped to zero and, in fact, started to rise when all the starch was utilized. In addition, for these runs no other cell types appeared. In essence, all runs were finished between 12 and 17 h. The increased oxygen mass transfer cut the run time from about 48 h to between 12 and 17 h and prevented contamination.

### *Batch Chemostat Runs With Potato Effluent From Plant 2*

In April 2003, we were no longer able to obtain potato effluent from the original plant (plant 1). We therefore obtained permission to use effluent

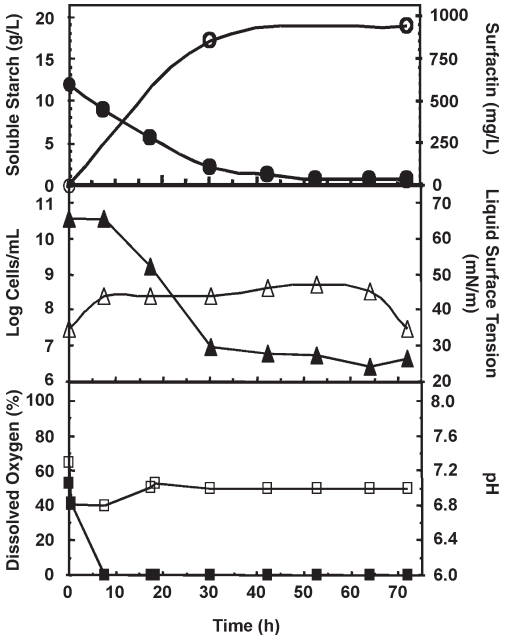


Fig. 2. Batch chemostat run with potato effluent from plant 1 (3.25% solids). (●) Soluble starch; (○) surfactin; (△) cells/mL in the liquid; (▲) surface tension of the liquid; (■) DO; (□) pH.

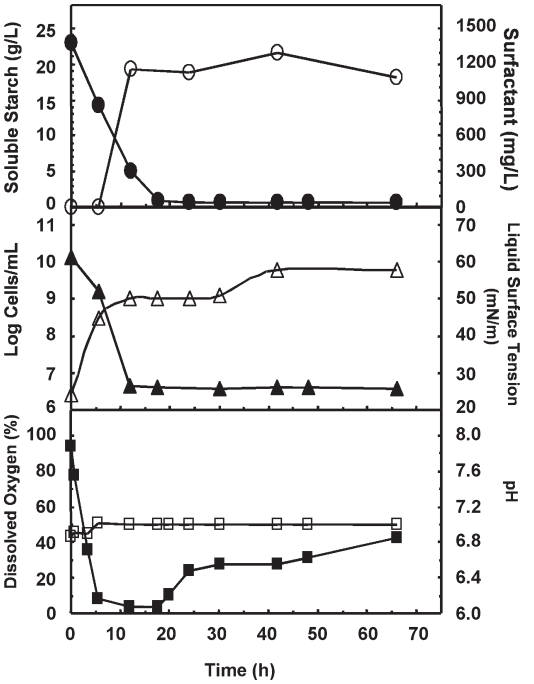


Fig. 3. Batch chemostat run with potato effluent from plant 1 (3.84% solids). During this run, the baffle was in place and the stirrer speed was 400 rpm. (●) Soluble starch; (○) surfactin; (△) cells/mL in the liquid; (▲) surface tension of the liquid; (■) DO; (□) pH.

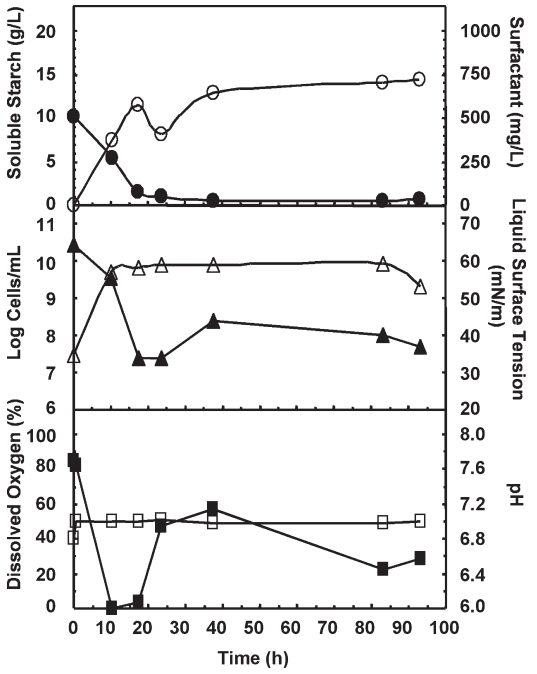


Fig. 4. Batch chemostat run with potato effluent from plant 2 (3.15% solids). During this run, the baffle was in place and the stirrer speed was 400 rpm. (●) Soluble starch; (○) Surfactin; (△) cells/mL in the liquid; (▲) surface tension of the liquid; (■) DO; (□) pH.

from a different location (plant 2). The composition of the streams from the two plants was somewhat different owing to variation in this process. Nonetheless, shake-flask results on surfactin production using material from plant 2 were similar to those of shake-flask studies from plant 1.

Runs were performed at 400 rpm. with the baffle in place. The results were similar, so only data from one experiment are presented. The potato effluent was 3.15% solids. The majority of the starch was utilized in 17–24 h (10–1 g/L) (Fig. 4). In addition, the majority of surfactin was produced in 17–24 h at a concentration of 0.6 g/L. DO dropped to 0–3.2% within 10–17 h and rose to 47% at 23 h. No other cell types appeared during this run. In essence just like the runs using the effluent from plant 1, these runs were finished between 12 and 24 h owing to the increased oxygen mass transfer, which decreased run time and prevented contamination.

## *Acid Precipitation of Surfactin*

Surfactin was recovered by first spinning out the cell mass and solids and adding HCl to the supernatant. The procedure *(5)* calls for the addition of HCl until the pH is about 2.0. We verified that a pH below 2.5 is necessary to obtain full recovery of the surfactin from solution. Following a run, the

Table 1
Effect of pH on Surfactin Recovery by Acid Precipitation of 200-mL Supernatant Fractions

| Fraction no. | pH | 12 *N* HCl (mL) | Surfactin (g/L) |
|---|---|---|---|
| 1 | 1.75 | 2 | 10,000 |
| 2 | 2.10 | 1 | 10,988 |
| 3 | 2.55 | 0.5 | 9944 |
| 4 | 3.14 | 0.25 | 8527 |
| 5 | 3.90 | 0.12 | 5088 |

reactor supernatant was distributed into 5 equal vol of 200 mL. To each fraction, HCl was added to a different pH between 4.0 and 2.0 to determine whether pH made a difference in surfactin recovery (Table 1). Fractions for which the pH was 2.55 or below had greater surfactin recovery. Although the results support that a pH close to 2.0 is required to obtain full surfactin recovery, the additional acid may not be economical on a lager scale.

## Conclusion

A batch chemostat can be used to produce surfactin from potato process effluent using antifoam to prevent loss of surfactin. The antifoam did not interfere with surfactin recovery or its efficacy. Initial chemostat runs were limited by DO. These runs took about 48 h to produce about 0.9 g/L of surfactin and started to become contaminated at 54 h. Improved oxygen mass transfer by increasing the stirrer speed and adding a baffle cut the batch run time from about 48 to 12-–24 h and prevented contamination while producing about the same concentration of surfactin using effluent from two different southeast Idaho potato-processing facilities.

## Acknowledgments

We wish to express our gratitude to Henry Camin, Larry Ferro, and Steve Goodson for their generous help and support.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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