0273-2289/01/91–93/0487/$13.75

The Effect of Pretreatments on Surfactin Production from Potato Process Effluent by *Bacillus subtilis*

David N. Thompson,* Sandra L. Fox, and Gregory A. Bala

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

Abstract

Pretreatments of low-solids potato process effluent were tested for their potential to increase surfactin yield. Pretreatments included heat, removal of starch particulates, and acid hydrolysis. Elimination of contaminating vegetative cells was necessary for surfactin production. After autoclaving, 0.40 g/L of surfactin was produced from the effluent in 72 h, vs 0.24 g/L in the purified potato starch control. However, surfactin yields per carbon consumed were 76% lower from process effluent. Removal of starch particulates had little effect on the culture. Acid hydrolysis decreased growth and surfactant production, except 0.5 wt% acid, which increased the yield by 25% over untreated effluent.

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

Introduction

Numerous investigators have examined chemical surfactants to enhance *in situ* removal of hydrocarbons, pesticides, and polychlorinated biphenyls *(1–3)*. Chemically synthesized surfactants, however, are not always environmentally benign *(4,5)* and are frequently expensive. Biosurfactants have been suggested as replacements for synthetic surfactants in environmental remediation, as well as for food emulsifiers, detergents, and use in tertiary oil recovery *(6)*. Biosurfactants are potentially desirable vs synthetic surfactants on the basis of biodegradability, lowered toxicity, and the potential use of renewable substrates for their production *(6)*.

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

Surfactin is a powerful cyclic lipopeptide antibiotic biosurfactant produced by *Bacillus subtilis (7)*. Purified surfactin has an aqueous critical micelle concentration of 25 mg/L and lowers the surface tension to 27 mN/m *(7)*. Surfactin has been produced from glucose and other monosaccharides in amounts ranging from 0.1 to 0.7 g/L *(8–11)*. Foam fractionation techniques and the addition of iron or manganese can improve yields to 0.8 g/L *(7)*. High medium and separation costs limit surfactin's use in lower-value applications such as *in situ* bioremediation and enhanced oil recovery *(7,12)*. However, high-purity surfactants are not needed for environmental or enhanced oil recovery applications, and thus substrate costs and product yields become overriding constraints *(12)*.

In a previous study, we showed that *B. subtilis* ATCC 21332 produces surfactin from a low-solids (LS) potato-processing effluent *(13)*. In the present study, we examined the effects of several pretreatments on surfactin production from LS potato process effluent. Tested were the effects of autoclaving, removal of particulates, and dilute-acid hydrolysis at several levels of severity. The data show that although it is necessary to initially kill contaminating vegetative cells in the effluent, no significant benefit in surfactin production is gained over the unamended effluent by further pretreatments. However, surfactin yield was increased slightly by dilute-acid hydrolysis of the substrate before use.

Materials and Methods

Potato Substrates

LS potato process effluent was obtained from a southeast Idaho potato processor. An average composition of the effluent is presented in Table 1. Effluent slurry was diluted 1:10 by volume with nanopure water before use. Diluted effluent was pretreated and tested for surfactin production. Control experiments utilized both the diluted effluent and a purified potato starch obtained from Sigma (St. Louis, MO).

Pretreatments

Pretreatments included autoclaving, filtration, and dilute-acid hydrolysis. Autoclaving was done at 121°C for 20 min. To prepare the filtered effluent, diluted LS was centrifuged for 10 min at 1180*g*. The slurry in the lower half of the tube was discarded, and the supernatant slurry was filtered through P8 filter paper (average pore size of 20 μm; Fisher). The filtrate (FLS) was used as the final substrate after autoclaving. Dilute-acid hydrolysis of diluted LS was done by autoclaving after adding 1.42, 2.85, or 5.69 mL of concentrated H_2SO_4 to the undiluted effluent and adding sufficient nanopure water to give 500 mL of 1:10 diluted LS containing 0.5, 1.0, or 2.0 wt% H_2SO_4. The acid-hydrolyzed substrates were neutralized with 10 *N* NaOH before use. Acid-hydrolyzed substrates were designated ALS½, ALS1, and ALS2, respectively. Untreated and pretreated media are summarized in Table 2 with their initial data.

Table 1
Average Composition
of Low-Solids Potato Process Effluent
as Received from the Processor[a]

Effluent component	Concentration (g/L)
Soluble starch	128.
Insoluble starch/fiber	14.
Glucose	5.2
Fructose	3.0
Galactose	<0.30
Sucrose	9.7
Maltose	9.1
Lactose	<0.30
Protein	72.
Ca	0.98
Cu	0.0021
Fe	0.012
Mg	1.1
Mn	0.0044
P	2.0
K	23.
Na	1.3
Zn	0.011
Total NH_3-nitrogen	12.
Total ash	52.

[a]Effluent media for the experiments were prepared using 1:10 (v/v) dilutions of this effluent.

Controls

Controls for surfactin production included abiotic and biotic controls using purified starch in an optimized medium. In each case, the pH 7.0 medium (PS) contained 5.0 g/L of potato starch (Sigma), rendered soluble by boiling in distilled water for 30 min, and trace minerals as previously described *(13)*. All media were autoclaved at 121°C for 20 min. Abiotic controls (A-PS) were not inoculated, and biotic controls (B-PS) were inoculated to 1 vol% with *B. subtilis* seed inoculum.

Because indigenous spore formers were found to survive autoclaving of diluted LS effluent *(13)*, controls employing diluted LS medium, pH 7.0, were included. The first of these controls was an "abiotic" control (A-LS) that was autoclaved but not inoculated with *B. subtilis*; it was called abiotic to indicate that it was not inoculated with *B. subtilis*. The second of these controls was a "biotic" control (B-LS) that was not autoclaved but was inoculated to 1 vol% with *B. subtilis* seed inoculum; it was called biotic to indicate that *B. subtilis* was added to the culture. The abiotic control was used to show the growth of germinated bacteria in autoclaved samples and

Table 2
Substrate Characterization Data for Purified Potato Starch Control and Pretreated Potato Media[a]

Medium[b]	Pretreatment	Inoculated with *B. subtilis*?	Glucose (g/L)	Soluble starch (g/L)	Insolubles (g/L)	Specific growth rate (h^{-1})
Uninoculated controls						
A-PS	Autoclaved	No	0.009	4.88	0.00	0
A-LS	Autoclaved	No	1.29	18.9	12.2	0.084 ± 0.029
Inoculated controls						
B-PS	Autoclaved	Yes	0.009	4.88	0.00	0.177 ± 0.014
B-LS	None	Yes	1.41	14.0	5.50	0.419 ± 0.007
Pretreated substrates						
LS	Autoclaved	Yes	1.13	16.5	10.8	0.348 ± 0.012
FLS	Filtered and autoclaved	Yes	1.15	20.3	11.2	0.369 ± 0.006
ALS½	0.5 wt% H_2SO_4 and autoclaved	Yes	2.37	14.3	10.2	0.402 ± 0.048
ALS1	1.0 wt% H_2SO_4 and autoclaved	Yes	2.52	12.6	10.2	0.362 ± 0.011
ALS2	2.0 wt% H_2SO_4 and autoclaved	Yes	5.37	11.3	10.3	0.333 ± 0.005

[a]The B-LS control, which was not autoclaved, is the untreated 1/10-diluted process effluent. Some variation among batches was seen in this stream. The final column is the observed specific bacterial growth rates at 12 h seen in experiments using each substrate (A-LS and B-LS are at 24 h). The growth rates are for total bacteria in the cultures, and include the 95% confidence intervals (±2.5σ) assuming a normally distributed population.

[b]A-PS, abiotic purified starch control; B-PS, biotic purified starch control; A-LS, abiotic low-solids effluent control; B-LS, Biotic low-solids effluent control; LS, low-solids effluent medium; FLS, filtered low-solids effluent medium; ALS½, ½ wt% acid-hydrolyzed low-solids effluent medium; ALS1, 1 wt% acid-hydrolyzed low-solids effluent medium; ALS2, 2 wt% acid-hydrolyzed low-solids effluent medium. Unless otherwise indicated, all media were autoclaved.

the lack of surfactin production in the absence of *B. subtilis*. The biotic control was used to determine the competitiveness of *B. subtilis* and the production of surfactin in the presence of high numbers of vegetative contaminating bacteria. Initial substrate data for diluted LS and its controls and for the purified potato starch (PS) controls are also presented in Table 2.

Cultures and Maintenance

Bacterial Strains

B. subtilis 21332 was obtained from the American Type Culture Collection and cultured as previously described *(13)*. Freezer stocks were prepared from cells grown in maintenance broth *(14)*. Seed cultures containing $4.0 \pm 0.6 \times 10^8$ cells/mL were prepared from the freezer stocks and were used to inoculate surfactin production tests. The seed inocula were grown on Difco nutrient broth *(15)* as previously described *(13)*.

Experimental Procedure

The surfactin production tests were performed in 250-mL Erlenmeyer flasks on a gyratory shaker at 30°C, 150 rpm, for 72 h, as previously described *(13)*. Media used in the tests are listed in Table 2. All media were adjusted to pH 7.0 before autoclaving except for the acid-pretreated substrates, which were adjusted just before use.

Analytical Methods

Cell Numbers

Cell numbers were determined immediately after sampling using direct visual microscopic count techniques, as previously described *(13)*.

Glucose and pH

Glucose was measured, after removing cells and particulates by centrifugation for 3 min at 13,214*g*, using a YSI Model 2700 Glucose Analyzer (Yellow Springs Instrument, Yellow Springs, OH). Culture pH was measured using a standard pH probe.

Soluble Starch

Soluble starch was estimated as previously described *(13)*, after removing cells and particulates by centrifugation for 3 min at 13,214*g*, and using the phenol-sulfuric acid assay for total carbohydrates *(16)*. The estimates of soluble starch included all reducing sugars (see Table 1) except for the measured amounts of free glucose, which were subtracted from the total.

Insolubles

Frozen samples saved for surface tension analyses were thawed and centrifuged for 8 min at 4811*g*. Insolubles were estimated as previously described *(13)*, using the lyophilized pellet weight and the estimated weight of cells in each sample assuming an average per-cell mass of 10^{-12} g *(17)*.

As defined here, "Insolubles" includes not only insoluble starch, but also cellulose, insoluble proteins, ash, etc. (but not cells). The supernatant was used for surface tension measurement.

Surface Tension

Surface tensions were measured by video image analysis of inverted pendant drops as previously described *(18)*. All measurements were made on cell-free supernatants obtained by centrifugation.

Isolation of Surfactin and Critical Micelle Concentration

Crude surfactin was isolated by precipitation *(9)*, as previously described *(13)*. The crude lyophilized powder was then used to estimate the critical micelle concentration, in nanopure water, as previously described *(19,20)*.

Results

Pretreatments

The effects of the pretreatments on initial substrate composition are summarized in Table 3. The B-LS control (unautoclaved) represents the LS medium without pretreatment. There were some small variations in the initial glucose and starch levels of undiluted LS effluent as obtained from the processor. Initial glucose in the B-LS medium was 1.41 g/L, with soluble starch and insolubles at 14 and 5.5 g/L, respectively. Autoclaving the effluent lowered the initial glucose by about 20%, increased the soluble starch by 18%, and nearly doubled the insolubles content. As expected, filtering the diluted LS before autoclaving had little effect on glucose content. However, the soluble starch content increased by 45%, and the total insolubles content was essentially unchanged. Acid hydrolysis increased the glucose content from hydrolysis of the starch, thereby decreasing the soluble starch content. Again, the insolubles content increased relative to the unautoclaved effluent.

Cell Growth

In all cases except the B-PS and A-LS controls, log phase growth was complete by 12 h. For the B-PS control, 24 h were required. The specific growth rates for the log phase are presented along with the initial substrate data in Table 2. For LS-based media, growth cannot be specifically attributed to *B. subtilis*, since contaminating bacteria were present (germination and growth of these bacterial spores was observable in the A-LS control). Thus, the growth rates in Table 2 represent the sum of contaminant bacteria and *B. subtilis*.

Removal of particulates by filtration had little effect on growth. The specific growth rate averaged over 12 h for the FLS culture was slightly higher than that for the LS culture. The addition of acid to the LS medium and autoclaving increased the specific growth rate when 0.5 wt% H_2SO_4

Table 3
Effect of Pretreatments
on Relative Substrate Concentrations in Pretreated Diluted LS Effluent[a]

Pretreatment	Medium	Fraction of initial concentration (after pretreatment)		
		Glucose	Soluble starch	Insolubles
None	B-LS	1.00	1.00	1.00
Heat	LS	0.801	1.18	1.96
Filtered + heat	FLS	0.816	1.45	2.04
0.5 wt% H_2SO_4 + heat	ALS½	1.68	1.02	1.86
1.0 wt% H_2SO_4 + heat	ALS1	1.79	0.900	1.86
2.0 wt% H_2SO_4 + heat	ALS2	3.81	0.807	1.87

[a]All pretreatments included autoclaving the substrate at 121°C for 20 min. The B-LS substrate was not autoclaved. Note that "Soluble Starch" includes all reducing sugars other than free glucose.

was added. To 95% confidence, there was a very slight overlap of ALS½ and ALS1 growth rates. However, doubling the acid concentration decreased the specific growth rate relative to 0.5 wt%, although this was about equal to the 0% acid medium (LS). The addition of acid to 2 wt% also had a detrimental effect on cell growth, decreasing the specific growth rate to below that for the LS medium.

Culture pH

In all autoclaved media inoculated with *B. subtilis*, culture pH remained essentially constant at about 7.0 over the first 48 h of the experiment (data not shown). After 48 h, all but the B-PS control culture showed an increase in pH to near 8.0; the B-PS control culture pH remained constant at 7.0 for the entire experiment. With autoclaving but without inoculation of *B. subtilis* (A-LS), the pH stayed essentially constant at about 7.0 for 8 h and then decreased to 6.5. Finally, the inoculated, unautoclaved culture pH decreased to 4.5–5.0 over the first 8 h of culture and remained low over the rest of the experiment.

Glucose Consumption

Glucose consumption in the controls and pretreated media is presented in Fig. 1. The B-PS control culture initially used all glucose released from the added starch but began to accumulate glucose to a small degree after 8 h. The A-LS culture showed an 8 h lag before use of the 1.3 g/L of free glucose but eventually began utilizing much of the free glucose. Glucose in the B-LS culture quickly dropped to about 0.25 g/L over 8 h and then remained relatively constant. The LS and FLS media essentially mirrored one another, first accumulating glucose over 12 h and then slowly utilizing the glucose over the remainder of the culture. Finally, the acid-pretreated LS media showed 8- to 12-h lag times before utilization of the free glucose,

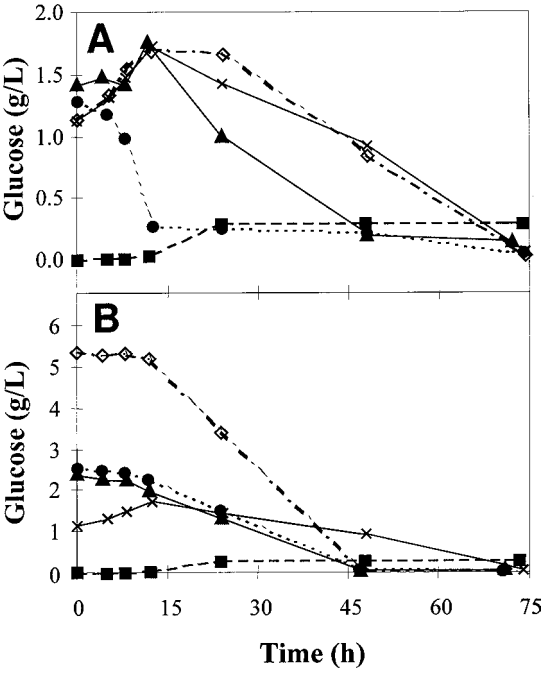

Fig. 1. Glucose consumption vs time for growth on control and pretreated potato effluent. **(A)** Controls, autoclaving, and filtration pretreatments: (■) B-PS control; (▲) A-LS; (●) B-LS; (◇) FLS; (×) LS. **(B)** Potato starch control and dilute-acid pretreatments: (■) B-PS control; (×) LS (0% acid); (▲) ALS½; (●) ALS1; (◇) ALS2.

but after the lag, each culture utilized the glucose at an essentially linear rate until it was gone.

Soluble Starch

Soluble starch consumption for all media is presented in Fig. 2. In the B-PS control medium, there was a short lag of 4–8 h in degradation of soluble starch, with apparently linear degradation thereafter. The A-LS medium showed a slight increase in soluble starch over the first 4 h of culture and again showed linear degradation afterward. The B-LS control displayed an initial increase in soluble starch, and then nonlinear degradation. The LS and FLS medium behaved similarly, peaking at 8 h. However, acid-pretreated LS media showed essentially a linear degradation of the starch over the entire culture.

Insolubles

The time courses of insolubles concentration in the cultures are presented in Fig. 3. The B-PS control did not contain initial insolubles and therefore is not included. In all LS-based media, a fraction of the insolubles was quickly solubilized, leveling off after about 8 h. In all but the A-LS and FLS cultures, the final insolubles concentration was 5 to 6 g/L. The final

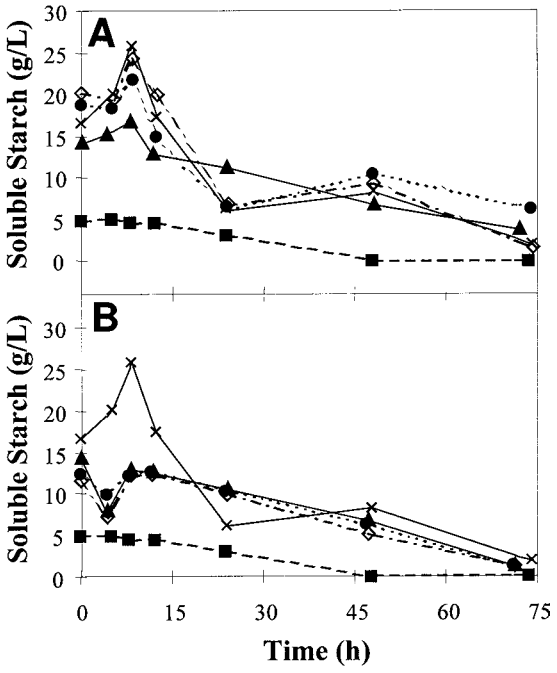

Fig. 2. Soluble starch vs time for growth on control and pretreated potato effluent. **(A)** Controls, autoclaving, and filtration pretreatments: (■) B-PS control; (▲) A-LS; (●) B-LS; (◇) FLS; (×) LS. **(B)** Potato starch control and dilute-acid pretreatments: (■) B-PS control; (×) LS (0% acid); (▲) ALS½; (●) ALS1; (◇) ALS2.

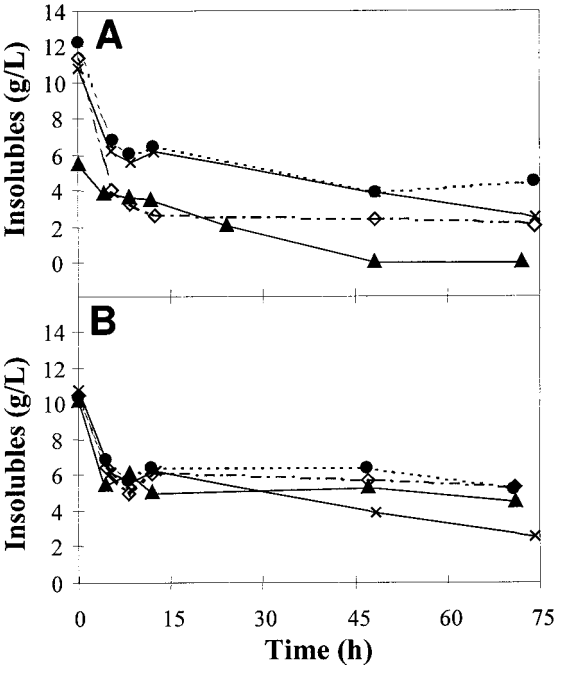

Fig. 3. Insolubles vs time for growth on pretreated potato effluent. **(A)** Controls, autoclaving, and filtration pretreatments: (▲) A-LS; (●) B-LS; (◇) FLS; (×) LS. **(B)** Potato starch control and dilute-acid pretreatments: (×) LS (0% acid); (▲) ALS½; (●) ALS1; (◇) ALS2.

A-LS insolubles concentration was <1 g/L, and that in the FLS culture was 2 to 3 g/L. Most of the decrease in insolubles took place while soluble starch was increasing, indicating solubilization of the starch fraction of the insolubles.

Surface Tension

The surface tensions of the effluent-based control media were initially in the range of 60–65 mN/m. The surface tensions of the LS and acid-hydrolyzed effluent media were in the range of 50–55 mN/m. The B-PS control medium began with a surface tension of 72 mN/m. The B-PS control culture reached 31 mN/m in 24 h but did not change much thereafter. The surface tension in the A-LS culture remained essentially constant, with a small drop after 24 h to about 55 mN/m. In the B-LS control, the surface tension increased slightly from 60 to 65 mN/m. The LS and FLS cultures again behaved identically, reaching 29 mN/m in 24 h and 25–27 mN/m after 72 h of culture. The behavior of the acid-pretreated media was slightly different from that of the LS culture, slowly increasing from the initial surface tension of 55 to 62 mN/m, and then decreasing to 29 mN/m at 48 h; no change in surface tension was seen after 48 h of culture.

Surfactin Isolation and Critical Micelle Concentration

Surfactin recovery data and critical micelle concentrations are given in Table 4. The B-PS control produced 2200 mg/L of crude surfactant, and the B-LS control produced 870 mg/L. Since the surface tension did not change in the B-LS control, the 870 mg/L is an acid-insoluble fraction present in the LS effluent. This cannot be verified with the A-LS medium because no recovery was done. The FLS medium produced 4000 mg/L of crude surfactant vs 3600 mg/L from the LS medium. Somewhat less solid was recovered from the acid-pretreated media, at about 2700 mg/L for each.

The critical micelle concentrations indicate that there was substantial carryover of nonsurfactant acid-precipitable solids into the crude surfactant. The lowest measured critical micelle concentration was 141 mg/L for ALS½, which indicates that about 17 wt% of the crude surfactant was surfactin. The ALS½ was the best pretreatment, producing nearly 0.50 g/L of surfactin at 72 h. The nearest result was for autoclaved LS, at about 0.40 g/L of surfactin. The B-PS control produced 0.24 g of surfactin/L by 72 h. The B-PS control produced 0.154 g of surfactin/g of carbon consumed, compared with 0.037 g of surfactin for LS and 0.051 g from ALS½. Removing the large particulates from the LS decreased the yield from the effluent to 0.025 g of surfactin/g of carbon, and stronger acid pretreatments also substantially decreased surfactin yields from glucose.

Discussion

PS Medium: Purified Starch Control

Growth of *B. subtilis* on PS medium was poorer than cell growth on LS effluents. The lag observed in cultures with low initial glucose occurs while

Table 4
Crude Surfactant Recovery, Critical Micelle Concentration, and Yield for Each Pretreatment[a]

	Recovered solid (g)	Solid concentration at 72 h (mg/L)	Measured critical micelle concentration (mg/L)	Surfactin yield at 72 h (g/L)	Surfactin yield at 72 h (g/g carbon)
Nanopure H_2O	0	0	∞	—	0
A-PS	0	0	ND	0	0
B-PS	0.46	2200	231	0.238	0.154
A-LS	ND	ND	ND	—	—
B-LS	0.21	870	∞	—	0
LS	0.76	3600	228	0.395	0.037
FLS	0.85	4000	340	0.294	0.025
ALS½	0.58	2700	141	0.479	0.051
ALS1	0.56	2600	265	0.245	0.030
ALS2	0.57	2700	458	0.147	0.017

[a]Crude surfactant was recovered from the combined 72-h culture fluid from the three replicates. ND, not determined.

the amylase system is induced, as verified by glucose and soluble starch data. The surface tension in the B-PS control culture reached 31 mN/m in 24 h but did not change much thereafter. Crude surfactant recovery from the B-PS control was 2200 mg/L, with a critical micelle concentration of 231 mg/L. The concentration of surfactin at 72 h, estimated as previously *(13)* from the critical micelle concentrations of pure surfactin and of the crude precipitate, was 0.238 g/L, which is in the range of 0.1–0.7 g/L previously reported from monosaccharides *(8–11)*. This suggests that much of the glucose consumed went to production of the amylase system. The yield per gram of carbon consumed was the highest from any of the media tested in this study, at 154 mg of surfactin.

B-LS, A-LS, and LS Media: Effect of Autoclaving

The A-LS and B-LS controls both supported growth, indicating significant contaminating microbial activity in the effluent. However, the purpose of autoclaving was not to sterilize the effluent but to minimize cost by simply allowing *B. subtilis* to compete successfully in the culture. After autoclaving, inoculated *B. subtilis* grew well and produced surfactant and thus could apparently compete for resources. The contaminant bacteria were likely fermentative, since pH in the A-LS and B-LS controls quickly dropped to 4.5, well below that required by *B. subtilis* 21332.

Glucose in the B-LS control dropped over 8 h and remained constant thereafter, which correlates with the bottoming out of culture pH. Soluble starch was degraded in all cultures; thus, contaminant cells expressed amylase activity. Soluble starch degradation was slower in the A-LS culture than in the others but was to a greater extent than in either the B-LS or the LS cultures. Both B-LS and LS cultures initially showed increasing soluble starch, corresponding with high rates of degradation of insolubles. As in the other media, insolubles leveled off at 5 to 6 g/L, indicating a recalcitrant or nonstarch fraction.

Surface tensions in the A-LS control did not change appreciably, verifying that surface tension changes in *B. subtilis*–inoculated cultures were attributable to *B. subtilis*. The surface tension also did not change in the B-LS control nor was there a measurable critical micelle concentration. Since the pH of this culture quickly dropped to 4.5, the *B. subtilis* was not able to produce surfactin and may not have been able to grow significantly.

The LS medium produced 0.395 g/L of surfactin at 72 h, which was within the reported range for monosaccharides *(8–11)*, and above that observed for the B-PS control. However, the estimated yield of surfactin from carbon consumed was only 24% of that from B-PS medium, at 0.037 g of surfactin. It is likely that additions of medium or complete sterilization of the effluent could make up this yield loss. However, because the aim is to keep costs low, it is unclear whether the economics of the process through the final separation step would favor feedstock additions or treatments.

FLS Medium: Effect of Filtration

The principle reasons for the filtration pretreatment were to remove large particulates that may serve as carriers for spores that survive autoclaving, and that could plug oil reservoirs if carried over to the surfactin product. Filtration had little effect on cell growth, as expected. The great majority of the particulates was clearly able to pass through the 20-µm filter paper, evidenced by the essentially unchanged insolubles concentration.

Culture pH, glucose consumption, and soluble starch consumption in the FLS medium all paralleled those of the LS medium. Insolubles consumption in the FLS medium was somewhat higher than that in the LS medium, ending at 2 to 3 g/L of insolubles. Thus, it is likely that some of the recalcitrant insolubles were removed during the filtration. The lack of differences in LS and FLS cultures suggests that removal of particulates from the diluted LS effluent before autoclaving had no effect. This was again seen in the surface tensions, which mirrored one another over the course of the runs. However, the critical micelle concentration of the crude surfactant from the FLS culture was substantially higher than that from the LS medium. The estimated surfactin concentration at 72 h was 25% lower than that observed from the unfiltered LS medium, and the yield of surfactin per gram of carbon consumed was 32% lower than from LS and 84% lower than from B-PS. Thus, it is preferable to leave the particles in the LS medium during surfactin production.

ALS Media: Effect of Dilute-Acid Pretreatments

The addition of a small amount of acid to the medium before autoclaving (0.5 wt%) slightly increased cell growth rates, although higher acid concentrations adversely affected cell growth (Table 2). It is likely that the higher acid concentrations caused some decomposition of the glucose released, forming 5-hydroxymethyl-2-furfuraldehyde, levulinic acid, and formic acid *(21)*. These decomposition reactions are common in acid hydrolysis of cellulosic biomass *(22)*, and acid hydrolysis products of lignocellulose have been shown to be somewhat toxic to yeasts used for ethanol fermentations *(23)*. It is probable that similar decomposition products are formed during starch hydrolysis and that these products could be toxic to *B. subtilis* 21332.

Culture pH in the acid-pretreated LS media again mirrored that in the other LS-based media. The acid-pretreated LS media all showed 8- to 12-h lag times in glucose consumption, but after the lag each culture utilized the glucose at a linear rate. Soluble starch consumption was also linear, indicating balanced glucose consumption and soluble starch degradation. There was no difference in the rates of soluble starch degradation with increasing severity of pretreatment, indicating that initial glucose concentrations from 2.3 to 5.3 g/L had little effect on amylase induction and production. Insolubles consumption in the acid-pretreated cultures again bottomed out near 5 to 6 g/L.

The critical micelle concentration of the crude surfactant increased with pretreatment severity, indicating that more acid-precipitable compounds were present after hydrolysis with higher amounts of acid. The 0.5 wt% acid treatment had the highest estimated surfactin concentration of any of the media tested, at 0.479 g/L, which is 68% of the highest reported value without foam removal (60% of that with foam removal) *(7)*. The 0.5 wt% acid treatment also had the highest surfactin yield from glucose, at 0.051 g of surfactin/g of carbon consumed, as compared with 0.030 and 0.017 g/g for the 1 and 2 wt% pretreatments, respectively. This again suggests that an inhibitory product of the acid hydrolysis limits surfactin production and also suggests that the lowered surfactin production seen in a previous work in which corn steep liquor (CSL) was added to the process effluent *(13)* was owing to an inhibitory compound present in the CSL.

Conclusion

Autoclaving of the process effluent before use as a substrate for surfactant production is absolutely required. If removal of particulates is necessary, this step would be better placed after surfactant production. Dilute-acid hydrolysis of the diluted LS effluent with 1 wt% acid or higher has a detrimental effect on growth, rate of production, and total amount of surfactant produced. Pretreatment with 0.5 wt% acid modestly increased surfactin yield over untreated LS. All media performed poorly on a yield-per-carbon consumed basis when compared with the optimized control culture. While it is likely that additions of medium or complete sterilization of the effluent could make up this yield loss, the question remains: Do the economics of the process through the final separation step favor feedstock additions or complete sterilization? Further studies that include separation of the surfactin will be necessary to answer this question.

Acknowledgments

We thank Rick Scott at the Idaho National Engineering and Environmental Laboratory for performing the surface tension measurements. This work was supported by the US Department of Energy, Assistant Secretary for Fossil Energy, Office of Fossil Energy, under contract number DE-AC07-99ID13727.

References

1. Abdul, A. S., Gibson, T. L., and Rai, D. N. (1990), *Ground Water* **28,** 920–926.
2. Ang, C. C. and Abdul, A. S. (1991), *Ground Water Monitor. Rev.* **11,** 121–127.
3. Ellis, W. D., Payne, J. R., and McNab, G. D. (1985), EPA/600/S2-85/129, United States Environmental Protection Agency, Office of Research and Development, Washington, DC.
4. Rudolph, P. (1989), in *Aquatic Toxicity Data Base*, Federal Environmental Agency, Berlin.
5. Schröder, H. F. (1993), *J. Chromatogr.* **647,** 219–234.

6. Zajic, J. E. and Seffens, W. (1984), *CRC Crit. Rev. Biotechnol.* **1(2),** 87–107.
7. Rosenberg, E. (1986), *CRC Crit. Rev. Biotechnol.* **3(3),** 109–132.
8. Arima, K., Kakinuma, A., and Tamura, G. (1968), *Biochem. Biophys. Res. Commun.* **31,** 488–494.
9. Cooper, D. G., McDonald, C. R., Duff, S. J. B., and Kosaric, N. (1981), *Appl. Environ. Microbiol.* **42,** 408–412.
10. Besson, F. and Michel, G. (1992), *Biotechnol. Lett.* **14(11),** 1013–1018.
11. Georgiou, G., Lin, S.-Y., and Sharma, M. M. (1992), *Bio/Technology* **10,** 60–65.
12. Lin, S.-Y. (1996), *J. Chem. Technol. Biotechnol.* **66,** 109–120.
13. Thompson, D. N., Fox, S. L., and Bala, G. A. (2000), *Appl. Biochem. Biotechnol.* **84–86,** 917–930.
14. Gherna, P. and Pienta, P., eds. (1989), in *American Type Culture Collection Catalogue of Bacteria and Phages*, 17th ed., American Type Culture Collection, Rockville, MD, p. 403.
15. Atlas, R. M. (1993), in *Handbook of Microbiological Media*, Parks, L. C., ed., CRC Press, Boca Raton, FL, p. 672.
16. Gerhardt, P., Murray, R. G. E., Wood, W. A., and Krieg, N. R., eds. (1994), in *Methods for General and Molecular Bacteriology*, American Society for Microbiology, Washington, DC, pp. 518, 519.
17. Bailey, J. E. and Ollis, D. F. (1986), in *Biochemical Engineering Fundamentals*, 2nd ed., Verina, K. and Martin, C. C., eds., McGraw-Hill, New York, p. 5.
18. Herd, M. D., Lassahn, G. D., Thomas, C. P., Bala, G. A., and Eastman, S. L., (1992), in *Proceedings of the DOE Eighth Symposium on Enhanced Oil Recovery*, SPE/DOE 24206, Tulsa, OK.
19. Gerson, D. F. and Zajic, J. E. (1979), *Process Biochem.* **14,** 20–29.
20. Sheppard, J. D. and Mulligan, C. N. (1987), *Appl. Microbiol. Biotechnol.* **27,** 110–116.
21. Grethlein, H. E. (1978), *Biotechnol. Bioeng.* **20,** 503–525.
22. Marsden, L. M. and Gray, P. P. (1986), *CRC Crit. Rev. Biotechnol.* **3(3),** 235–276.
23. Lee, J. (1997), *J. Biotechnol.* **56,** 1–24.