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Enhanced Production of Surfactin from *Bacillus subtilis* by Continuous Product Removal and Metal Cation Additions

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The lipopeptide, surfactin, is produced by *Bacillus subtilis*. A study has been made on large-scale production of this surfactant. A good yield was obtained from a glucose substrate fermentation by continuously removing the product by foam fractionation. The surfactin could be easily recovered from the collapsed foam by acid precipitation. The yield was also improved by the addition of either iron or manganese salts. Hydrocarbon addition to the medium, which normally increases biosurfactant production, completely inhibited surfactin production by *B. subtilis*.

Bacillus subtilis produces a lipopeptide, called surfactin, with exceptional surface activity (1, 2). This compound inhibits fibrin clot formation and lyses erythrocytes and several bacterial spheroplasts and protoplasts. Surfactin also lowers the surface tension of water from 72 mN/m to 27 mN/m. This is significantly lower than any biosurfactant surface tension data reported in the literature (4, 5, 9; S. Inoue and S. Ito, in *Proceedings of the 6th International Fermentation Symposium,* in press). Normally, even the most effective biosurfactants do not reduce the surface tension of water below 30 mN/m.

The yield of surfactin grown in a nutrient broth medium for 24 h was about 0.1 g/liter (1). The compound has been characterized as a cyclic lipopeptide containing a carboxylic acid (3-hydroxy-13-methyl tetradecanoic acid) and seven amino acids (6-8). The peptide is glutamic acid (*N*-bonded to the carboxylate of the fatty acid)-L-leucine-D-leucine-L-valine-L-aspartic acid-D-leucine-L-leucine (bonded to the 3-hydroxyl function).

Biosurfactants have received considerable attention for uses as oil recovery agents, emulsifiers, etc., because they are biodegradable and generally less toxic than synthetic surfactants (4, 11-13, 16-18). Surfactin is a powerful biosurfactant which can be easily isolated in pure form. We have undertaken a study to maximize the production of surfactin by *B. subtilis*.

MATERIALS AND METHODS

Growth studies. *B. subtilis* ATCC 21332 was initially grown in a nutrient broth (Difco Laboratories, Detroit, Mich., 8 g/liter) medium used by earlier workers (1). In subsequent studies, 4% glucose in mineral salts medium was used. The basic mineral salts medium was NH_4NO_3 (0.05 M), KH_2PO_4 (0.03 M), Na_2HPO_4 (0.04 M), $MgSO_4$ (8.0×10^{-4} M), $CaCl_2$ (7.0×10^{-6} M), $FeSO_4$, (4.0×10^{-6} M), and Na_2 ethylenediaminetetraacetic acid (4.0×10^{-6} M). In some experiments this medium was supplemented with various metal salts. Iron and manganese salts were added in a range of concentrations up to 1.4×10^{-2} M. Other salts were added at a concentration of 3×10^{-3} M or slightly higher.

Other additions to the medium included nutrient broth (0.1%), yeast extract (Difco, 0.1%), or hexadecane (Humphrey Chemical Co., North Haven, Conn.; 2 or 4%). In another study, 0.5 mg each of D,L-valine, D,L-leucine, D,L-aspartic acid, and L-glutamic acid were added to the glucose and mineral salts medium.

Large-scale fermentations were carried out in fermentors (New Brunswick Scientific Co., New Brunswick, N.J) using the basic glucose and mineral salts medium. A 28-liter fermentor was used initially. The working volume was 20 liters, the temperature was 30°C, the agitation rate was 200 rpm and the aeration rate was 0.5 vol/vol per min. Later experiments were done with a 14-liter fermentor (working volume 12 liters) which had been adapted with a collection vessel in the air-exhaust line to trap the foam overflow. Sterile collecting vessels could be changed throughout the fermentation. The aeration rate was 0.9 vol/vol per min; but all other parameters were the same as for the 28-liter fermentations. In some experiments, concentrated, sterile metal salt solutions (ca. 5 mg in 200 ml) were added to the 12-liter vessel at various times after the exponential growth phase.

Isolation of surfactin. Crude surfactin was isolated by adding concentrated hydrochloric acid to the broth after removing biomass by centrifugation. A precipitate formed by pH 2 which could be collected, dried, and extracted with dichloromethane. The solvent was removed under reduced pressure to give an off-white solid.

Further purification was achieved by recrystalliza-

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tion. The dichloromethane extract was dissolved in distilled water containing sufficient NaOH to give pH 7. This solution was filtered through Whatman no. 4 paper and reduced to pH 2 with concentrated HCl. The white solid was collected as a pellet after centrifugation.

Analyses. Biomass was determined by passing a sample of the broth through a prewashed, preweighed micropore filter (0.22 μm pore size). This sample was dried at 105°C and reweighed. When necessary, values were corrected for the weights of precipitates, such as iron oxide, measured for uninoculated media.

Surface tension measurements were made with a Fisher Autotensiomat (Fisher Scientific Co., Pittsburgh, Pa.). Relative surfactin concentrations were determined by diluting the broth until the critical micelle concentration (CMC) was attained (5). The dilution necessary to reach this point, where the surface tension starts to rise dramatically, was designated the CMC^{-1} and was proportional to the amount of surfactant present in the original sample. A known weight of recrystallized surfactin was used to determine its CMC, and this value was used to estimate the amount of surfactin present (in grams per liter) from the CMC^{-1} data.

Thin-layer chromatography studies were done with commercially prepared silica gel plates ($CaSO_4$ binder) activated at 110°C. Analytical studies used Fisher Rediplates developed in two dimensions. The first solvent was chloroform-methanol-28% NH_4OH (65:25:4, vol/vol). The second solvent was chloroform-methanol-acetic acid-water (25:15:4:2, vol/vol). Components were visualized by spraying the plates with one of the following: concentrated H_2SO_4 (plus charring at 150°C); α-napthol, and then concentrated H_2SO_4 (plus heating at 110°C); ninhydrin solution (Supelco, Bellefonte, Pa.); or phospray (Supelco). Preparative thin-layer chromatography was done with Analtech plates (Fisher Scientific Co., Pittsburgh, Pa.) developed in the first of the above solvent mixtures.

Infrared spectra were obtained on a Beckman IR-20 spectrometer (Beckman Instruments Inc., Fullerton, Calif.). Iron concentrations were determined with an atomic absorption spectrometer (model 157, Instrumentation Laboratory Inc., Lexington, Mass.).

RESULTS

When *B. subtilis* was grown in the nutrient broth medium, the production of the biosurfactant was relatively poor ($CMC^{-1} < 10$). The best substrate found for surfactin production was 4% glucose in a mineral salts medium. Typical CMC^{-1} values were 20 to 40, and the biomass was 1 to 2 g/liter. The minimum surface tension was 27 mN/m, and the interfacial tension against hexadecane was 1 mN/m.

Supplementing the glucose medium with nutrient broth, yeast extract, or amino acids did not improve the yield of biosurfactant. Although the addition of hexadecane increased the biomass, it inhibited the production of biosurfactant.

Surfactin was isolated from the basic glucose medium by acid precipitation. The dichloromethane extract contained all of the surface activity of the original broth.

Acidification of the supernatant increased the surface tension to 62 mN/m. If this was neutralized without removing the precipitate, the surface tension dropped to the original value of 27 mN/m and the original CMC^{-1} was obtained after serial dilution. Figure 1 shows the pH dependence of the surfactant properties of surfactin.

The dichloromethane-soluble portion of the precipitate could be redissolved in water by adding sufficient NaOH to achieve neutral pH. This solution has the unusually low surface tension of 27 mN/m typical of surfactin. By adding a known weight of the recrystallized surfactin to water, the CMC was determined to be 0.025 g/liter.

Two-dimensional thin-layer chromatography of the chloroform extract resulted in one major component with an R_f of 0.37 in the chloroform-methanol-NH_4OH mixture and an R_f of 0.76 in the chloroform-methanol-acetic acid-water mixture. There were also trace amounts of a few phospholipids and glycolipids which were not studied further.

The major component was isolated using preparative thin-layer chromatography. This had an infrared spectrum identical to the published spectrum of surfactin (2).

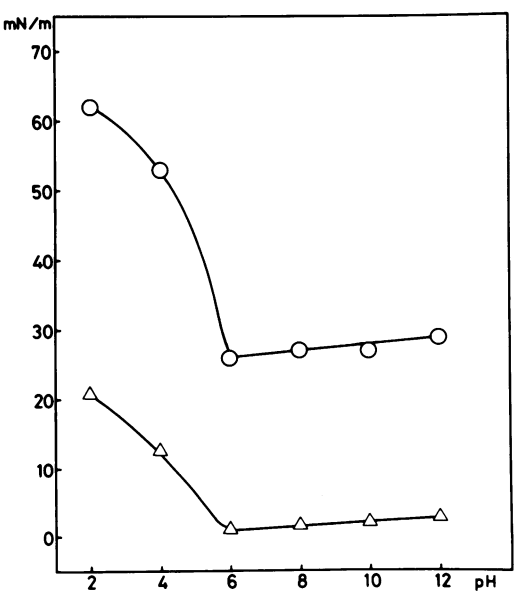

FIG. 1. *Surface tensions* (○) *and interfacial tensions against hexadecane* (△) *of a sample of B. subtilis broth adjusted to various pH values with hydrochloric acid or sodium hydroxide.*

The first large-scale fermentations were done in a 28-liter fermentor without removing the foam. At the end of these experiments, there was very little surfactin in the medium. Subsequent studies were done in a New Brunswick 14-liter fermentor as this could be easily adapted to collect the overflowing foam. The volume of the collapsed foam and the CMC^{-1} were measured and used to estimate the yield of surfactin (CMC of 0.025 g/liter).

In these fermentations, most of the surfactin was in the foam. Very little was found in the media remaining in the vessel. Figure 2 shows data for the cumulative amount of product in the foam, the biomass in samples from the vessel, and the amount of soluble iron remaining in these samples after removing the solids, for a typical fermentation. In all of these fermentations, the organism quickly reached a stationary phase of growth, with a biomass of about 1.3 g/liter in the fermentor and 3 to 4 g of surfactin collected in the foam.

If sterile aqueous solutions of $FeSO_4$ were added to the fermentor after the stationary period had been reached, there was a second dramatic growth phase and production of surfactin (Fig. 2). The salt could be added immediately after the first exponential growth was over or up to at least 2 days later with the same result. The effect was also observed after the addition of $FeCl_2$. The estimated total yield of the biosurfactant in these fermentations could be as high as 9 g.

Figure 1 also contains data for the concentration of soluble iron present in the medium throughout the fermentation. There was no appreciable change in the amount of iron in solution until after the addition of the extra salt.

A similar study with $MnSO_4$ resulted in a second burst of growth and surfactin production after the addition of the metal salt. Another experiment with $Ca(NO_3)_2$ addition had no effect on either growth or biosurfactant production.

The study of the effect of the additions of other metals was done on a smaller scale. To 500-ml shake flasks were added 100 ml of the basic media and about 10^{-4} mol of various metal salts. The flasks were incubated at 30°C for 6 days and compared to control flasks.

Only three salts, $MnSO_4$, $FeSO_4$, and $Fe_2(SO_4)_3$ caused significant enhancement of CMC^{-1}. Other salts such as $MgSO_4$, $CaCl_2$, Na_2HPO_4, KH_2PO_4, $NaNO_3$, $ZrOCl_2$, $UO_2(C_2H_3O_2)_2$, or $VOSO_4$ had virtually no effect on either biomass or surfactin concentration. One salt, $ZnSO_4$, suppressed growth of *B. subtilis* and several others [$CuSO_4$, $NiSO_4$, $CoSO_4$, and $Al_2(SO_4)_3$] completely inhibited growth.

Three salts with a positive effect were added in various concentrations. The biomass data were corrected for the increasing amounts of iron oxide precipitate. In several sets of experiments, as the concentration of iron was increased, the biomass and CMC^{-1} also increased (Fig. 3). The absolute numbers varied, but consistently both the biomass and the CMC^{-1} were increased fivefold by about 1.3×10^{-3} M added iron salt. Above this concentration there was little or no further improvement and the CMC^{-1} actually decreased at the highest iron concentra-

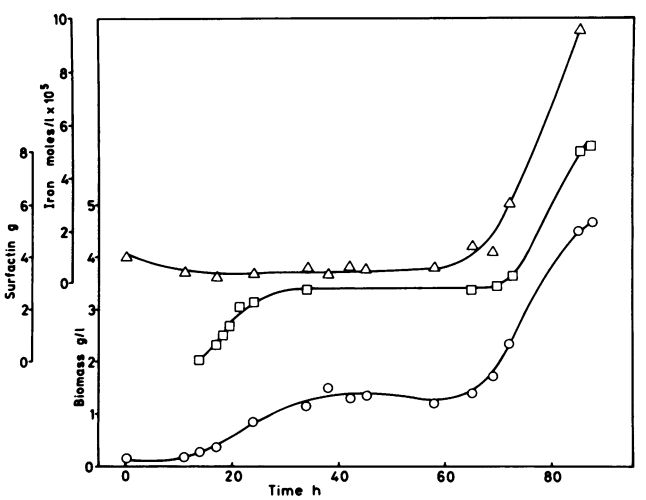

FIG. 2. *Data from a typical fermentation of B. subtilis. The curves include biomass in the fermentor* (○), *cumulative surfactin collected in the foam* (□), *and iron in solution in the fermentor* (△). *At 62 h,* 1.7×10^{-3} *mol of* $FeSO_4$ *was added to the vessel.*

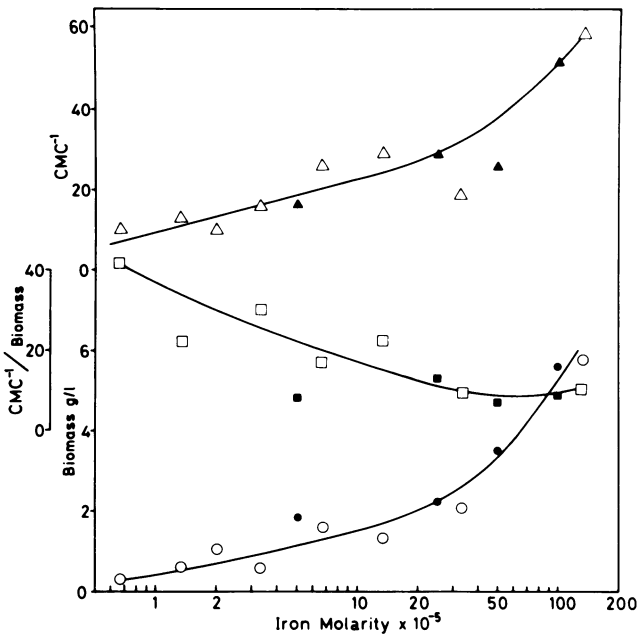

FIG. 3. *Effect of various amounts of added $FeSO_4$ or $Fe_2(SO_4)_3$ on biomass and surfactin production by B. subtilis. Data include biomass [Fe(II) ○, Fe(III) ●], CMC^{-1} [Fe(II) □, Fe(III) ■], and the CMC^{-1} per biomass [Fe(II) △, Fe(III) ▲] for a series of 100-ml, 6-day fermentations.*

tions used (>3×10^{-3} M). The data were very similar for both the ferric and ferrous salts. This was probably due to the oxidation of the ferrous salt in aqueous solutions in contact with air (3). Figure 3 also contains a plot of the ratio of CMC^{-1} to biomass. As the iron concentration was increased, the yield of surfactant per cell decreased.

The additions of $MnSO_4$ were made to media prepared without the $FeSO_4$ usually added. However, no attempt was made to rigorously exclude either metal. A control flask with no added iron or manganese had almost normal growth but produced very little surfactin. The addition of a small amount of $MnSO_4$ (7×10^{-7} M) caused a dramatic increase in CMC^{-1} to about 200. By 4×10^{-6} M Mn(II), the CMC^{-1} was 240, but addition of more salt up to 4×10^{-3} M did not result in further improvement. The experiments with added manganese gave the best yield of surfactin observed in this study. However, $MnSO_4$ had no appreciable effect on the biomass. Thus, unlike the iron addition, there is a large increase in the yield of product per cell.

DISCUSSION

The lipopeptide surfactin is an exceptionally potent biosurfactant (1, 2, 4, 5, 9; Inoue and Ito, in press). When *B. subtilis* was grown on a nutrient broth medium, the yield of surfactin was about 0.1 g/liter (1). This yield could be improved significantly by growing the organism in a glucose and mineral salts medium. Augmenting this with nutrient broth or yeast extract did not improve surfactin production. There was also no improvement when a mixture of all of the amino acids in the peptide (6) was added to the medium.

The addition of 2 or 4% hexadecane to the medium eliminated the production of surfactin even though the bacterium grew well. This was surprising because it is usually postulated that hydrocarbons in a medium enhance the production of biosurfactants by bacteria (4, 5, 9, 11).

Large-scale surfactin production was dramatically improved by removing the product throughout a fermentation. When fermentations were carried out without removing the foam, there was a very poor yield. If the foam was collected during the fermentation, this fraction was found to contain most of the surface-active product. The removal of the foam stimulated the production of more surfactin, and the continuous collection of the overflowing foam resulted in very good yields of product (0.8 g/liter). Although the foam also contained unidentified protein and biomass, the surfactin could be easily separated by reducing the pH to 2 and ex-

tracting the precipitate with dichloromethane. This crude product contained only trace amounts of lipid impurities and could be recrystallized from water by pH adjustments.

The yield from the fermentations could be further increased by adding iron or manganese salts. A large number of metal salts added to culture media in shake-flask experiments either had no effect on, or inhibited, growth and surfactant production. Only iron and manganese salts caused significant enhancement of surfactin production. The addition of iron also increased the biomass, and the ratio of surfactin to biomass remained constant or decreased. Manganese sulfate caused a much larger increase in CMC^{-1} than the iron salts without increasing the biomass. Furthermore, only a small amount of $MnSO_4$ was necessary for the maximum effect. There appear to be two different mechanisms of enhancement by the two metals.

Manganese is necessary at about 10^{-6} M for a high yield of surfactin from *B. subtilis*. The trace amounts of manganese present as impurities in the original mineral salts medium are sufficient to support cell growth, and excess manganese does not influence biomass measurements. It is well established that manganese is a "key" metal for the production of other secondary metabolites by *Bacillus* species without having an effect on cell growth (14, 15).

The effect of iron salts is unusual. The organism appears to need excessive amounts of iron for heavy growth, but the additional iron does not improve the yield of surfactin per cell. In the fermentation study (Fig. 2), there is a significant amount of soluble iron present throughout the fermentation (0.3 mg/liter), but the addition of more iron causes a second pulse of growth. One possibility is that this strain of *B. subtilis* has a defective iron transport system. Another possibility is that the bacterium produces a sequestering compound which keeps the iron unavailable. *B. subtilis* is known to produce iron-chelating compounds (10), but these aid growth by passing the ferric ions to the cells. A molecular model shows that surfactin is a potential chelating agent. The peptide ring could provide a roughly octahedral arrangement of the two carboxyl residues and four other oxygen- or nitrogen-containing functional groups. It is possible that surfactin sequesters the ferric ions in the media from the cells. This would explain why removing the surfactin from the medium or adding a large excess of iron to the menstrum (possibly saturating the chelator) stimulates growth. The interaction of surfactin with metals is being studied in more detail.

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