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Preserving the Activity of Cellulase in a Batch Foam Fractionation Process

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Abstract

Foam fractionation is one of the low operating-cost techniques for removing proteins from a dilute solution. The initial bulk solution pH and air superficial velocity play an important role in the foam-fractionation process. Denaturation of proteins (enzymes) can occur, however, during the foam-fractionation process from the shear forces resulting from bursting air bubbles. At the extreme bulk solution pHs (lower than 3.0 and higher than 10.0), the enzymatic activity of cellulase in the foamate phase drops significantly. Within these two pH bounds an increase in the air superficial velocity, V_0, and a decrease in the bulk solution pH leads to a decrease in the separation ratio (SR), defined as the ratio of the protein concentration in the foamate to the protein concentration in the residue. On the other hand, an increase in V_0 provides a higher foamate-protein recovery. The process efficiency is defined as the product of foamate-protein recovery times the SR times the cellulase activity. The optimal operating condition of the cellulase foam-fractionation process is taken into account at the maximum value of the process efficiency. In this study, that optimal condition is at an air superficial velocity of 32 cm/min and a bulk-solution pH of 10.0. At this condition, the recovered foamate is about 80% of the original protein mass, the SR is about 12, and the enzymatic activity is about 60% of the original cellulase activity.

Index Entries: Cellulase; cellulase activity; foam fractionation; batch foam fractionation; protein separation.

Introduction

One of the major costs in the enzymatic saccharification of cellulose to produce sugars for ethanol production is the cost of cellulase. Both the recovery and the reuse of cellulase can reduce the production cost of ethanol, both of which can be enhanced by a foam-fractionation process.

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Cellulase is a surface-active enzyme, which can foam easily when air or an inert gas is introduced into the cellulase solution to create bubbles. Foam fractionation seems to be a promising technique for concentrating cellulase from a dilute water solution (a good first step in recovering cellulase product). Cellulase is a complex of at least three major components: endoglucanase, exoglucanase, and cellobiase. This foam-fractionation study with the cellulase complex is not intended to fractionate the individual components, but rather to recover the enzymatically active combined grouping. Further study of the individual components could lead to different degrees of fractionation (as represented by their individual separation ratios) as well as different degrees of deactivation.

Surface tension is a major parameter in foam-fractionation processes. It is typically one of the controlling variables that describe the separation and concentration of active materials such as detergents, soluble proteins, and enzymes from a dilute-water solution. When the surface tension of the dilute solution is lower than the surface tension of water (72 m*N*/m), the surface-active material (surfactant) tends to create foam. Foaming generally occurs when the surface tension is lower than the surface tension of water. The more the surface tension is lowered below that of water, the more foam is formed. Generally, a surfactant is comprised of a hydrophobic and a hydrophilic part. The hydrophobic part is likely to be positioned at an air/liquid solution surface, thus reducing the surface tension of that solution below that of water. It follows, therefore, that the protein concentration at the air/liquid solution interface is much higher than that in the bulk solution. Based on this concentration effect, a foam-fractionation process can separate and concentrate a foaming surfactant from a bulk-water phase to a foam phase. Moreover, when the foaming gas is air, the air bubbles can provide substantial air/liquid solution interfacial area at which surfactant can attach. This interfacial area can be transformed by bubble formation to create foam at the liquid solution surface. Foamate is collected by collapsing the foam to create a high-concentration, liquid surfactant solution. Typically, the foamate concentration is 2–10 times higher than that in the bulk solution *(1,2)*.

Previous works *(3,4)* have shown that foam fractionation can damage the catalytic ability of an enzyme (denaturation of the enzyme) because high shear forces occur when foam bubbles burst. Enzyme denaturation during foam fractionation needs to be countered in order to preserve the enzyme activity. In addition to the surface tension, shear force associated with a gas bubble is another important controlling variable in foam fractionation. That shear force seems to correlate with the gas superficial velocity, which is one parameter varied in this study. The second parameter varied in this study, the bulk solution pH, can also lead to enzyme denaturation. Typically, extreme acidity/alkalinity, the presence of organic solvents, and heat can cause enzyme denaturation. Cellulase generally has high activity when the bulk solution pH ranges between 3.0 and 10.0 *(5,6)*. In this study, the bulk solution pH was varied in that range and the air

superficial velocity was varied between 4 and 32 cm/min. These two controlled variables were investigated to determine the optimum operating condition that leads to a high protein recovery, a high concentration, and a high activity of cellulase in a batch foam fractionation process.

Materials and Methods

1. Crude cellulase (Maxazyme CL, Gist-Brocades nv, Delft, Holland, Lot No. MVA. 1284) from the fungus, *Trichoderma reesei*. In addition to the three components endoglucanase, exoglucanase, and cellobiase, the sample probably also contains noncellulase proteins.
2. Coomassie Brilliant Blue G-250 (Bio-Rad, Richmond, CA, Lot No. 23242).
3. 95% ethanol solution (Midwest Grain Products, Atchison, KS).
4. Dinitrosalicylic acid (Aldrich, Milwaukee, WI, Lot No. 09026LW).
5. Phenol (Fluka, Milwaukee, WI, Lot No. 250G).
6. Sodium sulfite (Fisher Scientific Co., Fair Lawn, NJ, Lot No. 785778).
7. Citric acid (Fisher Scientific, Lot No. 795790).
8. Glucose (Fisher Scientific, Lot No. 793019).
9. Sodium hydroxide (Sigma, St. Louis, MO, Lot No. 873487).
10. Sodium citrate (Sigma, Lot No. 66F-0345).

Experimental Procedure

One gram of crude cellulase powder was dissolved into 500 mL of deionized water and then centrifuged at 1073*g* for 10 min. The supernatant was collected and then used to prepare a 1 L stock solution (ca. 1 g solid cellulase/L). The stock solution was stored in the refrigerator at 8°C for a subsequent experiment within the next week. The experimental cellulase concentration (ca. 200 mg solid cellulase/L) was prepared by diluting the stock solution with deionized water. The actual protein concentration of cellulase solution was determined using the Coomassie Blue total protein assay *(7)*.

The effect of pH on foam fractionation was studied at a fixed initial cellulase concentration of 200 mg of solid cellulase/L (the actual protein concentration in solution became 120–160 mg/L) and a fixed air superficial velocity of 16 cm/min. Solutions of sodium hydroxide and hydrochloric acid were used for adjusting the pH of the initial cellulase solution in the range between 2 and 11. The surface tension of the initial cellulase solution for all experimental cases was measured using the SIGMA 701 automatic tensiometer (KSV Instruments Ltd., Helsinki, Finland). Both the cellulase activity and the protein content were measured for the initial bulk solution, the residual bulk solution, and the foamate (collapsed foam liquid).

The effect of air superficial velocity on foam fractionation was studied for air superficial velocities ranging between 4 and 32 cm/min, and at initial bulk solution pHs of 4.0, 7.0, 9.0, and 10.0. Both the total protein content and the enzymatic activity were determined for the initial solution, the residue solution, and the foamate. The enzyme activity of cellulase was

determined using the modified filter-paper method *(8)*, whereas the sugar level was determined by a reducing sugar assay *(9)*.

Total Protein Assay

The Coomassie Blue method *(7)* was used to determine the total protein content in the cellulase solution. In all of these experiments, 2 mL of sample and 3 mL of Coomassie Blue reagent were used for the total protein determination with a Bausch and Lomb Spectronic 20 spectrophotometer at 595 nm. The optical absorbance of the sample was measured at 5 min after adding the Coomassie Blue reagent.

Cellulase Activity Assay

The modified filter paper activity method *(8)* was used to determine the cellulase activity. Whatman No. 1 filter paper (Whatman International Limited, Kent, UK) was cut into 1×6 cm strips to create the substrate. This assay does not tell, however, what happens to the individual enzymes within the cellulase complex. Instead, this empirical method gives an overall view of how the complex is behaving under nearly ideal conditions. Sodium citrate solution of 0.05 *M* was used as a buffer solution (pH 4.7). The enzyme solution of 1 mL and the buffer solution of 2 mL were used and mixed with the substrate (filter paper strip) and then incubated in a water bath at 50°C for 2 h. After incubation, the enzymatic reaction of cellulase on cellulose was stopped by cooling the incubated solution in an ice-water bath. The digested filter paper solution was then left for the reducing sugar test. The cellulase activity was then expressed in terms of the rate of glucose released per mass of cellulase/h (filter paper units or FPUs).

Reducing Sugar Assay

The Dinitrosalicylic Acid (DNS) Reagent was employed for determining the reducing sugar *(9)*. Glucose solutions were used to develop a standard reducing sugar calibration curve. The DNS reagent of 3 mL was added to 3 mL of digested filter paper solution. The mixture was placed in a boiling water bath for 5 min. Next, the mixture was cooled in cold running water for 2 min to adjust to ambient temperature. The optical absorbance of the solution was determined at 575 nm wavelength using a Bausch and Lomb Spectronic 20 spectrophotometer within 5 min.

Results and Discussion

The surface tension of cellulase solution was determined at a fixed initial concentration of 200 mg of solid cellulase/L of distilled water while the bulk solution pH ranged between 2.0 and 11.0, as shown in Fig. 1. The local minimum surface tensions occurred at the bulk solution pH values of 3.0 and 10.0. The surface tensions at the bulk solution pH 2.0 and 11.0 rise abruptly from the bulk solution pH values of 3.0 and 10.0. These jumps in

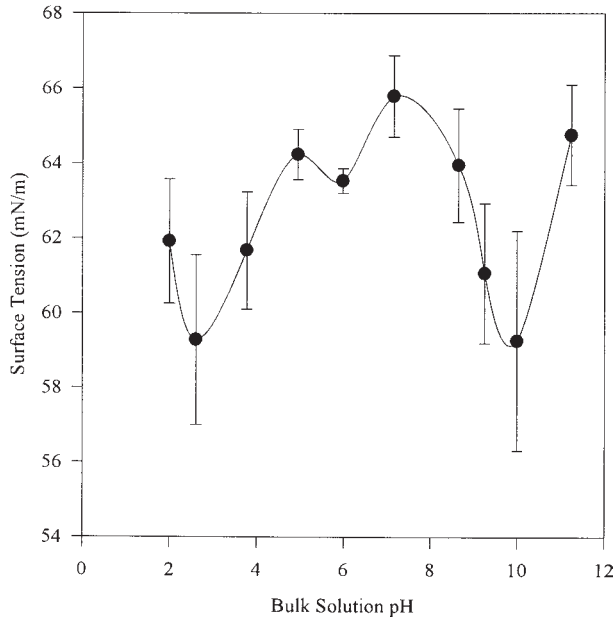

Fig. 1. The surface tension is a function of pH at a crude cellulase concentration of 200 mg solid "cellulase"/L of distilled water.

surface tension may indicate that the molecular structure of cellulase changes when the bulk solution pH is below 3.0 and above 10.0, perhaps leading to denaturation of this enzyme. Between the bulk solution pH values of 3.0 and 10.0, the local maximum surface tension occurs at the bulk solution pH of 7.0. Since the surface tension is a function of the rearrangement of molecules at an air/liquid solution interface, a higher surface tension (one that approaches the surface tension of water at 72 mN/m) indicates that less-surfactant molecules (like cellulase) collect at the surface. The response curve of surface tension between pH of 3.0 and 10.0 in Fig. 1 indicates a strong change in molecular rearrangement at the air/liquid solution interface owing to a change in the bulk solution pH.

Varying the bulk solution pH causes a change in surface tension, which in turn affects the amount of recovered foamate protein. Figure 2 shows the protein recovery (the ratio of the foamate protein mass to the initial bulk protein mass) as a function of the bulk solution pH at the fixed initial cellulase concentration of 200 mg of solid cellulase per liter and at an air superficial velocity of 16 cm/min. It is observed that the protein recovery increases as the bulk solution pH becomes more acidic or more basic, starting from a low cellulase recovery at pH 6.0. The maximum protein recoveries are at the pH bounds of 2.0 (70%) and 11.0 (80%). The minimum protein recovery at pH of 6 is about 25%. The pattern of Fig. 1 is seen to be the reciprocal function of Fig. 2, as shown by the cross-plot between cellulase recovery and surface tension in Fig. 3. Within the pH range of 7.0 and 10.0, the highest protein recovery occurs at the lowest surface-tension val-

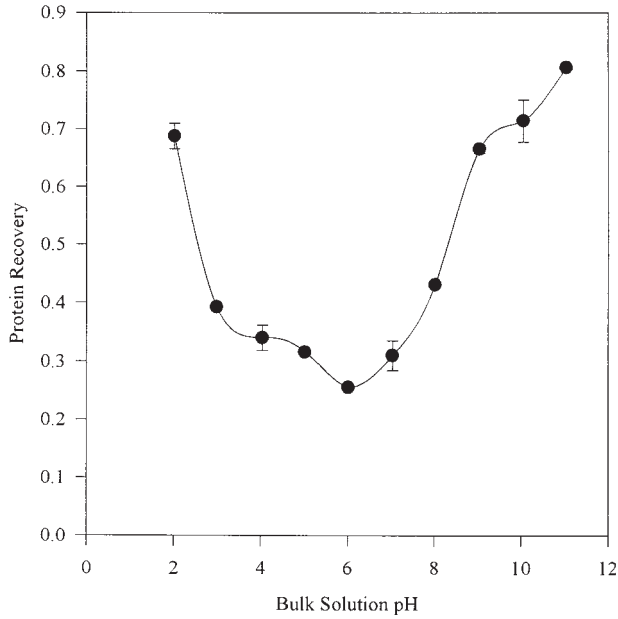

Fig. 2. The effect of bulk solution pH on the protein recovery at a crude cellulase concentration of 200 mg solid "cellulase"/L and an air superficial velocity of 16 cm/min.

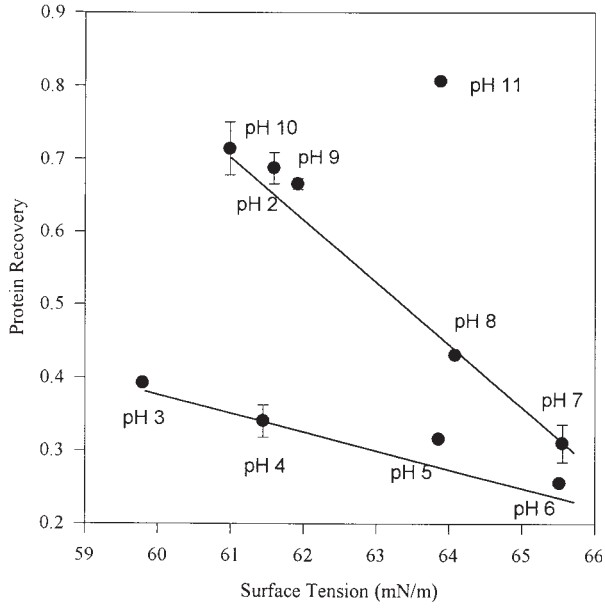

Fig. 3. The effect of surface tension on the protein recovery at a crude cellulase concentration of 200 mg solid "cellulase"/L and an air superficial velocity of 16 cm/min.

ues. A lesser protein-recovery effect occurs in the acidic pH range of 3.0–6.0. This reciprocal relationship thus occurs in two branches: first the pH between 3.0 and 6.0, and second between 7.0 and 10.0. The hysteresis curve,

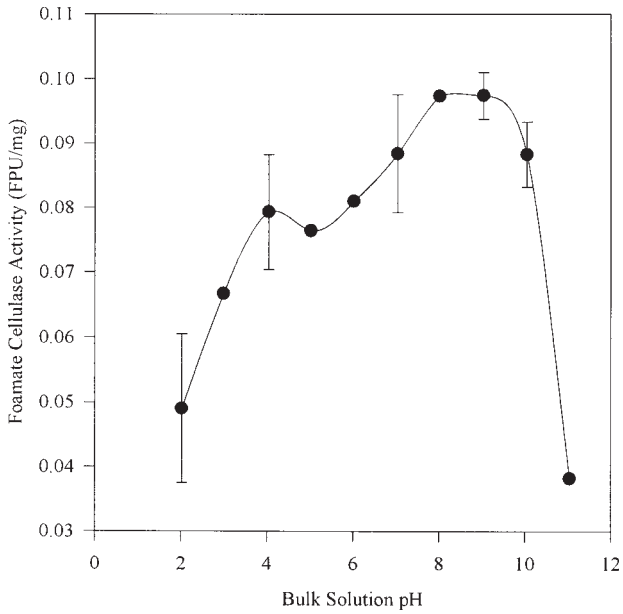

Fig. 4. The effect of bulk solution pH on the foamate cellulase activity at a crude cellulase concentration of 200 mg solid "cellulase"/L and an air superficial velocity of 16 cm/min.

depicted by these two branches, starting from pH 3.0 and ending with pH 10.0, does not cover the high recoveries for the bulk-solution pH values of 2.0 and 11.0, where the cellulase may be denatured.

The protein recovery assay includes both the inactive (denatured) and active cellulase in the value for total protein content. In order to identify active cellulase, the cellulase activity is determined directly using the modified filter-paper method *(8)*. Figure 4 shows the foamate cellulase activity as a function of the initial bulk-solution pH at a fixed cellulase concentration of 200 mg solid cellulase/L and a superficial air velocity of 16 cm/min. When the bulk solution approaches both acid and base regions, the cellulase activity decreases from the highest value at pH 8.0 and 9.0.

Figure 5 shows the separation ratio (the ratio of foamate protein concentration to the residual protein concentration) as a function of the bulk solution pH. This figure describes the result for a fixed cellulase concentration of 200 mg solid cellulase/L and an air superficial velocity of 16 cm/min. The separation ratio ranges between 4 and 20. The shape of the curve is similar to that of the protein recovery shown in Fig. 2. The highest protein recovery in the studied region occurs at the highest measured bulk solution pH of 11.0.

The air superficial velocity is the second parameter in this controlled study. Again, the experiments were conducted at a fixed cellulase concentration of 200 mg solid cellulase per liter and at bulk-solution pH values of 4.0, 7.0, 9.0, and 10.0. It is noted in Fig. 6 that the separation ratio decreases

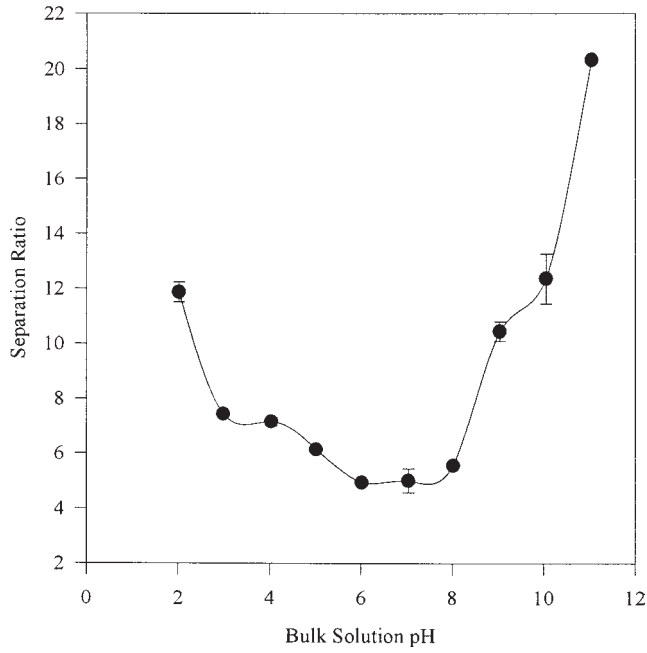

Fig. 5. The effect of bulk solution pH on the separation ratio at a crude cellulase concentration of 200 mg solid "cellulase"/L and an air superficial velocity of 16 cm/min.

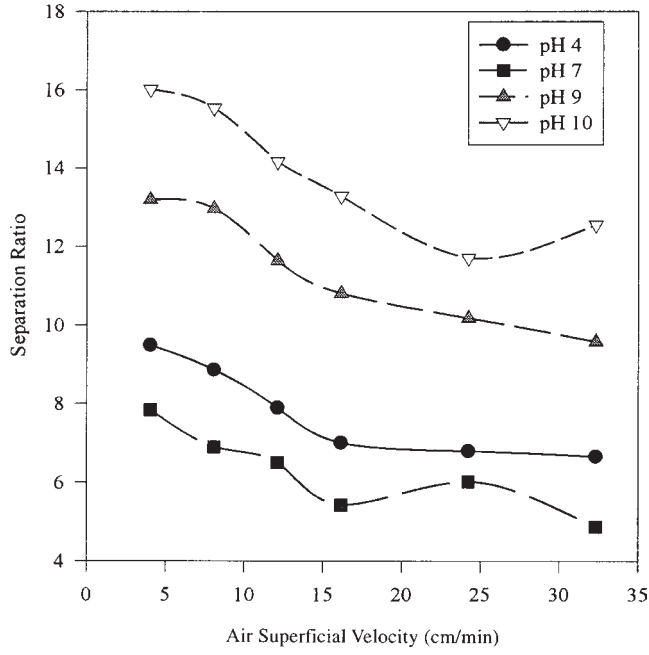

Fig. 6. The effect of air superficial velocity on the separation ratio at a crude cellulase concentration of 200 mg solid "cellulase"/L and at bulk solution pHs of 4.0, 7.0, 9.0, and 10.0.

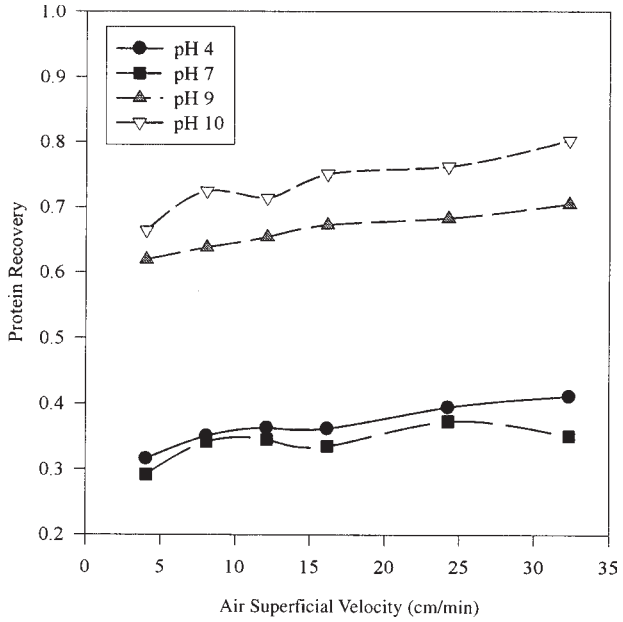

Fig. 7. The effect of air superficial velocity on the protein recovery at a crude cellulase concentration of 200 mg solid "cellulase"/L and at bulk solution pHs of 4.0, 7.0, 9.0, and 10.0.

when the air superficial velocity is increased. Presumably, this decrease in the result of increased bulk-liquid entrainment when the air superficial velocity is increased.

Figure 7 shows the protein recovery as a function of air superficial velocity at the bulk-solution pH values of 4.0, 7.0, 9.0, and 10.0. As the air superficial velocity increases, the protein recovery increases about 10–20%. The low value of the surface tension of cellulase solution at pH 10.0 (Fig. 1) corresponds to the highest value of protein recovery (Fig. 7). Conversely, the highest surface tension at the bulk-solution pH 7.0 (Fig. 1) corresponds to the lowest protein-recovery protein (Fig. 7). It is observed in Fig. 7 that the protein recovery was about double in the basic region of pH 9.0–10.0 that of the acidic region of pH 4.0–7.0.

The cellulase activities in the foamate for the bulk solution pH of 4.0, 7.0, and 10.0 are grouped together, whereas the activity at the bulk solution pH 9.0 is clearly the highest, as shown in Fig. 8. In general, the foamate cellulase activity increases significantly when the air superficial velocity increases. A higher air superficial velocity normally entrains more of the bulk solution within the foam, thereby protecting the amount of active cellulase in the foamate with the additional mass.

The process efficiency here is defined as the product of the foamate cellulase activity times the protein recovery times the separation ratio. Figure 9 shows the process efficiency as a function of the air superficial velocity. For all of the bulk solution pH's cases studied, the process efficiency

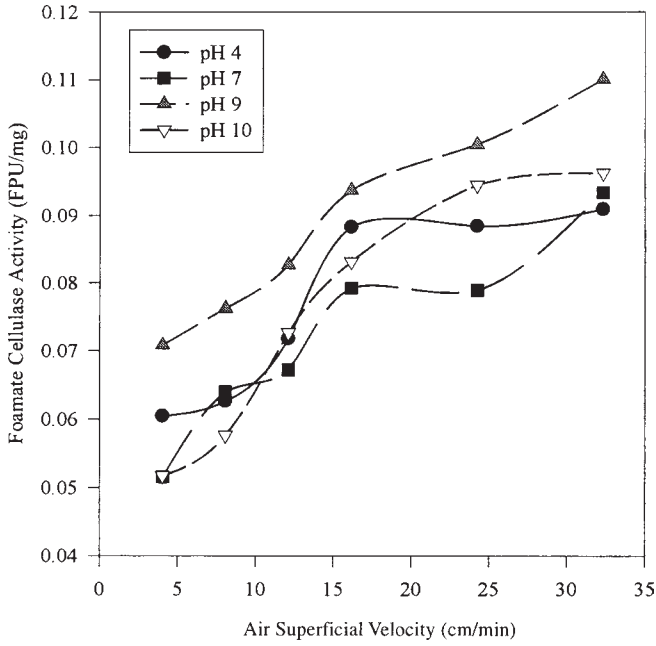

Fig. 8. The effect of air superficial velocity on the foamate cellulase activity at a crude cellulase concentration of 200 mg solid "cellulase"/L and at bulk solution pHs of 4.0, 7.0, 9.0, and 10.0.

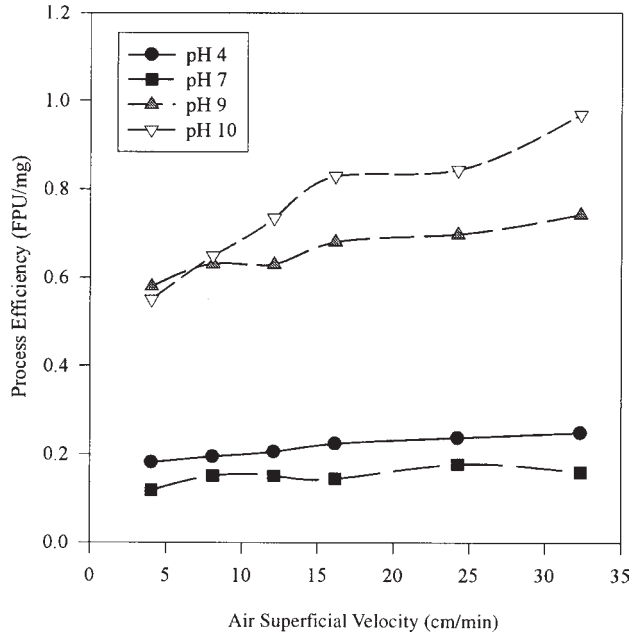

Fig. 9. The effect of air superficial velocity on the process efficiency at a crude cellulase concentration of 200 mg solid "cellulase"/L and at bulk solution pHs of 4.0, 7.0, 9.0, and 10.0.

increased as the air superficial velocity increased. The bulk solution pH of 10.0 case provides the highest values, in spite of the observation that the foamate cellulase activity is much lower than that in the bulk solution. It is noted that the standard working pH for determining the cellulase activity is at pH 4.7 *(8)*. It is seen in Fig. 9, that under the conditions studied the batch foam fractionation of cellulase seems to prefer the operating condition at pH 10.0 and superficial air velocity of 32 cm/min. The optimal operating conditions are now determined by defining an objective function, which combines the described high protein recovery, enzymatic activity, and SR. Although this objective function gives equal weight to the three components with the given units (as shown in Fig. 9), it does serve as a point of departure for optimizing the process with respect to the bulk-solution pH and air superficial velocity control variables.

Conclusions

The maximum cellulase activity occurred when the bulk-solution pH ranged between 8.0 and 9.0. Above pH 7.0, the SR increased when the bulk-solution pH increased. On the other hand, the SR decreased as the air superficial velocity increased. The protein recovery increased about 10–20% when the air superficial velocity increased from 4–32 cm/min. The highest protein recovery (about 80%) was at bulk-solution pH 10.0, which was about double that for bulk solution pH values of 4.0–7.0. The highest foamate cellulase activity occurred when the bulk solution pH was 8–9. An increase in air superficial velocity enhanced the foamate cellulase activity for all of the pHs studied. The process efficiency consisting of the product of the cellulase activity multiplied by the total protein recovery and the SR was maximized when the optimum operating condition was at bulk solution pH 10.0 and air superficial velocity at 32 cm/min.

The surface tension seems to be correlated to the amount of foamate protein recovery in the foam-fractionation process. The foamate protein recovery is an inverse function of the surface tension in the working range of bulk pH 3.0–6 and separately from 7.0 to 10.0. The bulk-solution pH is an important parameter for the surface tension of cellulase solution, which may be related to the cellulase molecular configuration.

Acknowledgment

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