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Bubble and Foam Concentration of Cellulase

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ABSTRACT

Experiments were conducted to determine the effect of pH and sparging-gas composition on the bubble and foam separation of an aqueous protein solution. Cellulase was chosen as a model system that provided its own foam, unlike the previously studied invertase and amylase systems, which did not produce significant foaming. A 1-L graduated cylinder was used as the column into which the sparging gas (CO_2 or air) was introduced. Contact between the rising sparging gas and the bulk solution was espected to lead to a protein concentration gradient (bubble fractionation) within the solution, as it did previously for yeast invertase (*1*). A concentration gradient in the cellulase bulk solution was not observed with either air or CO_2, however, but a high concentration was observed in the foam layer above the liquid solution (foam fractionation). With CO_2 sparging, the bulk foam concentration reached a peak of eight times the solution's concentration at pH=5. When foam-top samples were collected, moreover, the concentration was as high as 220 times the bulk solution's concentration, suggesting a concentration gradient within the foam.

Exposure to the air seems to reduce the viability of the cellulase harvested in the foam, presumably because of cellulase oxidative deactivation. Oxidative deactivation, if found to be controlling, may perhaps be minimized through the use of an inert sparging gas and foam blanketing atmosphere.

Index Entries: Foam fractionation; bubble fractionation; cellulase; protein separation.

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INTRODUCTION

In recent years, the high cost of protein separation and concentration in commercial practice has led to greater interest in less costly purification alternatives. Unfortunately, the sensitivity of proteins to heat, pH, solvents, and shear places severe constraints on any candidate separation technique.

Two possible alternatives to conventional bulk purification methods that may not deactivate the separated proteins significantly are bubble and foam fractionation. Bubble fractionation allows the separation of surface-active compounds in solution by adsorption to gas bubbles being sparged through the medium. The compound is swept to the surface of the medium by the rising bubbles and is returned to solution on bubble collapse. The result is a concentration gradient with a higher solute concentration near the surface of the solution than at the bottom of solution (*1*). The separation is a function of surface activity, and mixtures of solutes with different activities can be separated using bubble fractionation (*2*).

Foam fractionation is similar to bubble fractionation, since it relies on sparged gas bubbles to carry surface-active solutes to the surface of bulk solutions. Foam that results from bubbles that do not collapse tends to retain the adsorbed solute, so that foam can be removed from the system and then collapsed into a concentrated solution. Since many gas-sparged aqueous protein solutions generate foam without the addition of surfactant, foam fractionation may also be a viable separation technique.

Because the surface activity of the solute is essential to bubble and foam fractionation and surface activity is a function of pH, one objective of this research is to determine an effective pH range for the concentration of proteins in an aqueous bulk solution. Cellulase was chosen as a model protein, because its high purification costs currently discourage its use in biomass-ethanol production processes. In addition, cellulase solutions form stable foams for pHs above 5, without added surfactant.

Our criteria for selecting the optimal pH range is formulated from the maximization of the protein recovery ratio and the biological activity of the recovered product. The activity of proteins after fractionation is a concern because of possible oxidative denaturization (*3*).

MATERIALS AND METHODS

The cellulase used during the reported experiments was a sample of Lot No. MVA. 1284, Maxazyme Cl (*Trichoderma reesei*), from Gist-Brocades. nv. Protein assays were carried out following the Bio-Rad Protein Assay with Coomassie Blue (*4*) purchased from Bio-Rad Laboratories, Richmond, CA. (Catalog No. 500-0006).

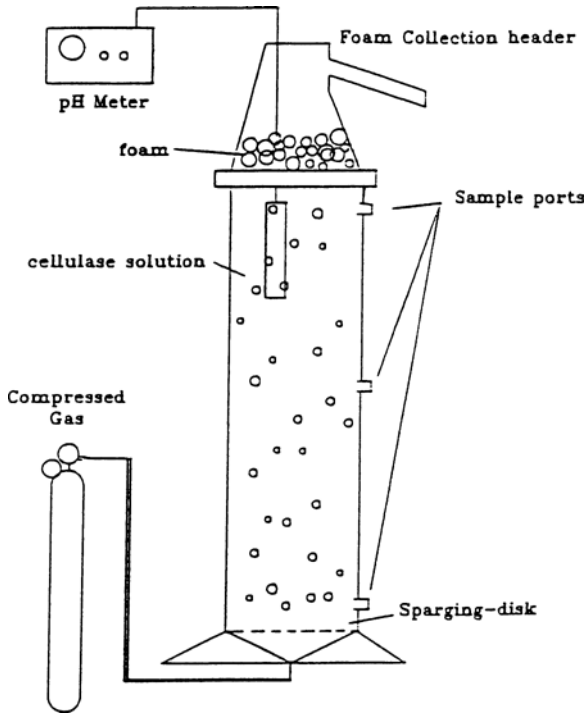

Fig. 1. Schematic diagram of the experimental apparatus. Height/diameter = 5.65. Gas rate: 0.14 L/min. Column vol.: 1L. Aqueous cellulase soln.

Apparatus

The experimental apparatus (Fig. 1) consisted of a vertical 1-L Nalgene polycarbonate graduated cylinder (height/diameter = 5.65), modified and fitted with a sintered-glass sparging disk mounted flush to the bottom. A hole was cut into the bottom of the cylinder to allow the introduction of gas through the disk. The sparging disk gave a bubble size of approx 1 mm in diameter and increased the bubble surface area-to-volume ratio relative to gas injection without a sparging disk. The smaller bubble size from the sparging disk leads to increased liquid-to-gas contact, thereby increasing protein adsorption to the rising bubbles.

The column is equipped with three sample collection ports located at the top, middle, and bottom of the column. Each port is covered with a rubber septum to allow hypodermic-needle sample collection. A 3-mL syringe, with an 18-gage disposable needle was used to collect samples through the septums by inserting the needle until it just entered the solution. All samples were drawn from the same radial depth.

The top of the column was fitted with a plastic header (*see* Fig. 1) to collect any foam generated and to serve as a mount for the pH electrode.

The pH of the solution was monitored with a Beckman Zeromatic pH meter, and was maintained by titration using various aqueous HCl and NaOH solutions injected through the bottom sample port, by using a 3-mL syringe, with an 18-gage disposable needle. The sparged gases usedwere high-pressure cylinder CO_2 and cotton-filtered air from the in-house air line.

Assay Methods

The modified Bradford assay method was used to determine cellulase concentrations in the various sample solutions (*4,5*). The Coomassie Blue Protein Assay was used along with a Bausch and Lomb Spectronic 20 spectrophotometer at 595 nm to evaluate the samples. The samples were assayed 2 min after the introduction of the Coomassie Blue, and were evaluated against a deionized water and Coomassie Blue blank under the same conditions. The standard curve for the system had the equation:

$$\text{Absorbance@595} = 0.002262^* (\text{Concentration, mg/L}).$$

The activity of cellulase was examined by the "filter-paper test" outlined in the paper by Mandels et al. (*6*), in which the activity of a cellulase solution sample is determined by its ability to reduce enzymatically a 1×6 cm piece of filter paper to reducing sugars. A measure of activity is available by comparing the result to an activity vs glucose-produced standard curve.

Experimental Procedure

The solutions that were fractionated were developed by adding 100 mg of solid cellulase powder to 1 L of deionized water and allowing several hours of gentle stirring to dissolve the solute. Undissolved particles were removed by passing the solution through a cotton plug. After filtration, no solid particles were visible in the solution.

Each trial was begun by placing 1 L of an aqueous 100 mg/L protein solution in the column and taking a sample from the bottom port. The gas (either CO_2 or air) was then sparged through the column at a rate of 0.14 L/min. The pH of the solution was then set, monitored, and maintained with injections of either aqueous NaOH or HCl solutions. The experiment was run for 30 min. Samples were drawn from the three ports by extracting 2.4 mL of the solution with a 3-mL hypodermic syringe. Foam, if formed, was either stirred and sampled for a bulk foam sample, or sampled from the top of the foam without stirring. In both cases, the foam samples were removed from the system and collapsed into liquid. All samples were assayed for overall protein concentration using the modified Bradford assay. Concentrations are reported in the form of separation ratios (SRs), where the separation ratio is defined as the concentration of the sample from the top port, the middle port, or the foam

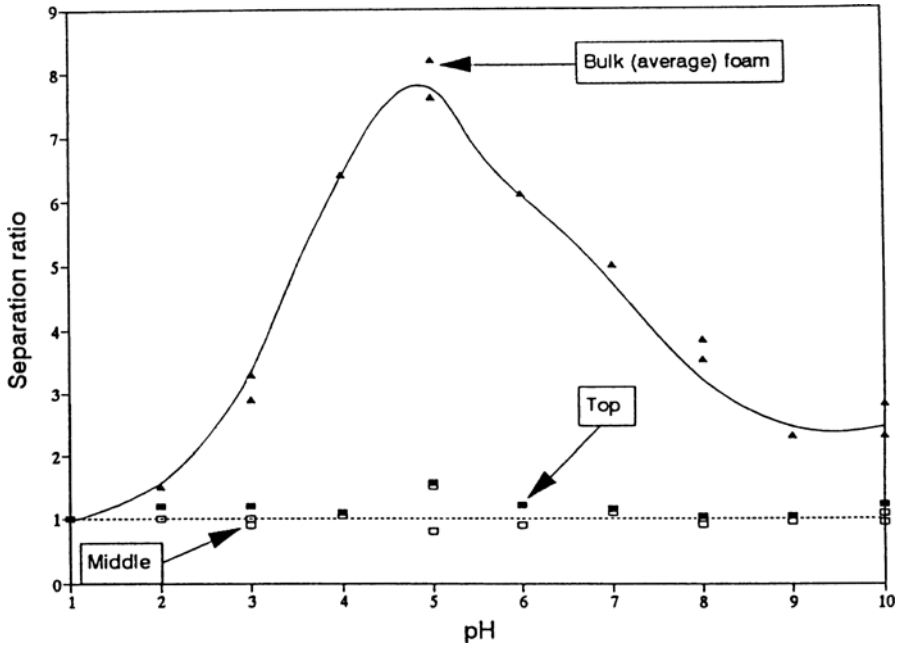

Fig. 2. Concentration of an aqueous cellulase solution with carbon dioxide as the sparging gas.

divided by the concentration of the solution at the bottom port. The experiment was repeated for bulk solutions at various pHs in the range of one to ten.

RESULTS AND DISCUSSION

When carbon dioxide is used as the sparging gas, bulk foam separation ratios (SRs) were found to increase from one at pH=1 up to a maximum of eight at pH=5 (Fig. 2). Following the peak, the separation ratios continued to drop until they reached two at pH values of 9 and 10.

Figure 2 also shows that the SRs for the bulk liquid measured at the top and middle ports remained near one across the entire pH range. The results indicate little or no bubble fractionation, except at pH=5, where the two SRs increased to about 1.5. Greater separation ratios were not reached, presumably, because most of the protein from the bubbles entered the foam phase and did not recycle back into the bulk fluid phase. Instead, a well-mixed residual liquid remains with a negligible solution concentration gradient, except around pH=5. Those bubbles that do not burst on reaching the surface tend to retain any adsorbed cellulase and enter into the foam phase. Because of the high foam stability in the pH 5–9 range, the foam tends to "strip" the bulk aqueous solution

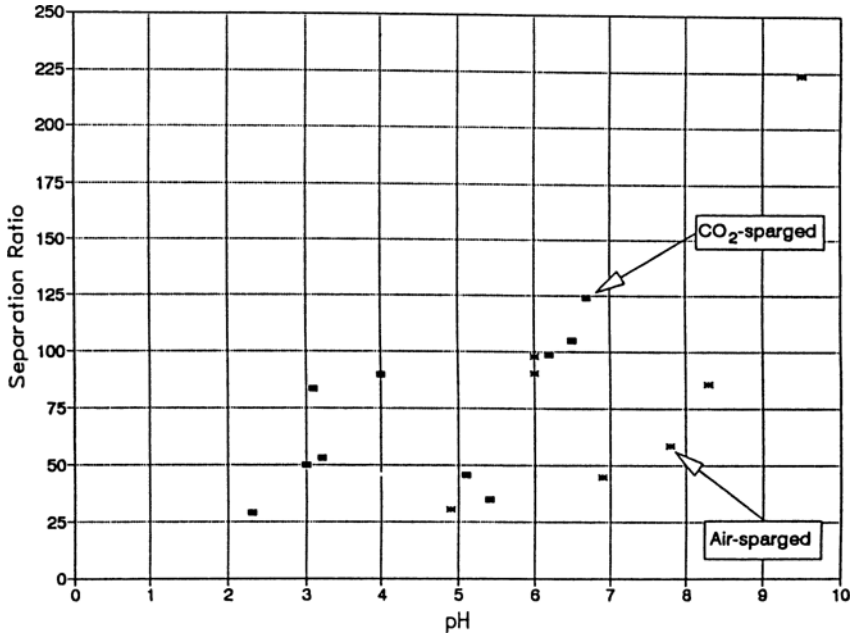

Fig. 3. Foam surface separation ratios.

of cellulase instead of concentrating it in the upper portion of the solution, as it would if foam were not present (*1*).

Within the foam, there is a considerable axial cellulase concentration gradient. Repeated bulk (average) foam measurements consistently give separation ratios up to eight, at pH=5. By measuring the cellulase concentration in the top of the foam, however, separation ratios can reach values of between 30 to 125, as shown in Fig. 3 (with the exception of one point at 225). In the case of the uppermost position foam separation (Fig. 3), the effect of pH is not as marked for cellulase, as it appeared to be for the bubble fractionation or bulk foam separation experiments (Fig. 2).

The apparent random nature of the data in Fig. 3 may indicate the dependence of the foam SRs on the sample depth in the foam. A reliable measure of the sample-point foam depth was difficult to obtain because of the differing nature of the foam. At low pHs (approx 1–5), cellulase foam was unstable and collapsed back into the bulk solution. At pHs >5, however, the foam layer became increasingly stable and maintained a sharp concentration gradient. The foam gradient gave a solution-foam interface concentration near the bulk solution concentration and a foam-top concentration up to two orders of magnitude greater than the bulk solution concentration. The high-foam SRs, shown in Fig. 3, obtained at pHs >5 seem to indicate that by the time the foam reaches the top, it dries out somewhat. This dewatering process may be caused by water in the foam falling back into the bulk solution. Since the cellulase tends to remain in the foam, the resulting water loss magnifies the protein concentration.

The unstable foam (lower pHs) gave foam-top samples closer to bulk foam concentration, since the foam was better mixed by constant bubble collapse. This lower separation ratio result further suggests that foam water removal may cause the foam protein concentration gradient rather than an axial change in protein content for a fixed water content.

The sodium hydroxide necessary to achieve pHs greater than the solution's "natural" pH (the solution's pH before the addition of acid or base; pH=4.5 and pH=6 for CO_2 and air-sparged systems, respectively) may also enhance separation ratios by "salting-out" the protein. By adding salt (or NaOH) to the aqueous solution, the solubility of cellulase decreases and provides greater protein availability for cellulase adsorption to rising gas bubbles. The increased adsorption may also be contributing to higher separation ratios in the foam. In support of this premise, Fig. 3 shows an exponential increase in foam SR at more alkali pHs ($7 \leq pH \leq 9.5$). Since pH is a logarithmic measure, an exponential rise in SR is expected in response to the exponential increase in salt/NaOH addition.

Further complication occurs with the use of carbon dioxide as a sparging gas. Passing carbon dioxide through water causes the formation of bicarbonate ions, as described by the equilibrium expression:

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \quad (2)$$

The acidic product lowers the pH and requires addition of sodium hydroxide to maintain basic pHs. Since the CO_2/H_2O reaction does not reach equilibrium within the trial time (30 min), pH maintenance requires continuous addition (large amounts) of sodium hydroxide during that ½-h transient. The amount of added alkali necessary increases exponentially with increasing pH, and becomes enormous (and presumably impractical) at pHs >9.

The enzymatic activity of cellulase after fractionation is also a primary concern. Preliminary activity trials were run to establish relative cellulase catalytic activities of solutions sparged with air and carbon dioxide to determine the effects of pH and shear. The trials' control samples were bulk solution withdrawn from the fractionating column (bottom port) prior to sparging, but after initial titration. Although these activity trials are in no way exhaustive, the results allow insight into factors thought to affect activity. Trial outcomes (in units of activity) are shown in Table 1.

The similar middle- and top-port activities for the carbon-dioxide-sparged solutions suggest that bubble shear is a small (or negligible) contributor to cellulase denaturization in the bulk solution. At pH=4, carbon-dioxide-sparged system led to a cellulase activity in the foam of approximately one-third of its control activity. For the air-sparged system, pH=4, the cellulase activity in the foam fell to about one-fifth of its control activity, indicating that oxygen in the air may deactivate the cellulase. The higher rate of denaturization in the air-sparged system is attributed to enzymatic oxidation that occurs with stable foam's prolonged exposure to the atmosphere. In comparison, pH=4 carbon dioxide

Table 1
Cellulase Activities (Per Unit Mass Cellulase)

Condition	Carbon dioxide			Air
pH	4.0	4.5	5.0	4.0
Foam	0.23	0.08	0.08	0.09
Top port	0.50	0.22	0.43	0.48
Middle port	0.50	0.20	0.44	0.25
Control (bottom port)	0.62	0.30	0.30	0.50

foam maintains a higher activity than pH=4.5 and pH=5 samples. Presumably, this occurs because the more unstable foam (constant collapse) does not permit the longer atmospheric contact time the more stable foams (pH 4.5 and 5) allow; i.e., the lower atmospheric exposure time at pH=4 seems to limit the extent of oxidation/denaturization.

CONCLUSIONS AND RECOMMENDATIONS

The reported results indicate that cellulase foam fractionation can reach bulk foam separation ratios (SRs) of eight and top foam SRs of around 100 at moderate pHs (at the aqueous solution's equilibrium pH in both air- and carbon-dioxide-sparged systems).

Although bulk foam SRs may reach values as high as eight, the leading edge of the foam (foam top) has SRs ranging from 30 to 220. This result indicates a sharp axial concentration gradient within the foam, starting around SR=1 at the base of the foam. The high SRs achieved using the foam indicate the potential for an inexpensive prepurification concentration step for the recovery of proteins. Foam is easily removed from sparged systems and collapsed into a solution more concentrated than the original bulk solution.

The enzymatic activity of cellulase apparently was not affected by shear in the bulk solution, but seemed to be lowered by oxidation, with exposure to the air. Accordingly, the use of air as a sparging gas and foam exposure to the atmosphere seems to cause losses in activity. Although carbon dioxide may protect the cellulase against oxidation (if used as a blanket around the foam), it leads to pH control problems in basic aqueous systems.

Based on this work, the recommended sparging gas should be both inert and oxygen free (e.g., nitrogen gas). An inert gas may be needed to avoid undesired reactions with the solution (e.g., CO_2 sparged into water at pHs other than 5 leads to pH control problems). Keeping the sparging gas free of oxygen may minimize protein oxidation (deactivation) in both the foam and in the collapsed (recovered) foam.

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