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Removing Proteins from an Aerated Yeast Fermentation by Pulsing Carbon Dioxide

Replacing Salting-Out as a Method of Protein Precipitation

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Abstract

Salting-out is a common technique used for precipitating proteins and other materials from fermentation and tissue culture processes. It leaves a salt residue in the system. Foam fractionation can also be used to remove proteins by protein precipitation from a dilute solution. In doing so, there is usually a trade-off between enrichment and recovery. An increase in the airflow rate will increase the recovery, but only at the expense of the enrichment. A new method for increasing the recovery in foam fractionations and in yeast fermentations is to add a burst of CO_2 to the process and then restore the air. This CO_2 acts like a temporary salt, but it does not leave behind a residue. The recovery increases as a result of the joint use of these gases, perhaps by more than 10-fold, without sacrificing the enrichment. Chicken egg albumin in a foam fractionation column can serve as a simple, experimental model for the proposed recovery process in lieu of the fermentation process.

Index Entries: Salting-out; foam fractionation; yeast fermentation; protein concentration; chicken egg albumin.

Introduction

Yeasts are important microorganisms in biotechnology processes. Their fermentation is well documented and has several advantages over that of other cells. They grow fast and hardy while secreting proteins; therefore, they do not have to be ruptured to access the proteins for recovery *(1)*. Yeasts also have the ability to glycosylate excreted proteins. This is something that bacterial cells cannot do *(2)*. Glycosylation is often important in activating proteins and enzymes that are made for human drugs. Recombinant DNA synthesis can implant a specific protein for yeast to secrete. One problem has always been reco-vering the product. Another problem is the low concentration of the reco-vered product. Because the

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yeast fermentation process is inexpensive, there are significant motivations for solving these problems.

Salting-out is a valuable and simple precipitation technique commonly used to recover many biologic products including antibiotics, biopolymers, and proteins. It does have a few drawbacks: it often leaves a salt impurity that contaminates the system, the recovered volume and concentration may be too low to be an economically viable process, and recovering a target protein still requires a further and more expensive precipitation scheme. Moreover, the salt brine solution and heavy metals left in the system may create a waste disposal problem. Although the mechanism behind salting—out is not totally clear, it is believed that the salt reduces a protein's solubility. A high concentration of salt is added to the protein mixture, where it retains water. The partitioned water solution, now more concentrated in protein, releases some of the proteins as a precipitate to be extracted from the system *(3)*.

Coupling foam fractionation and a fermentation process presents a powerful technique for effectively concentrating proteins in foam, created from the fermentation. Foam fractionation is an inexpensive, first step in purifying proteins. Simultaneously concentrating proteins while they are synthesized in a fermentation process makes foam fractionation even more attractive as a separation method. The foam readily collapses in the open atmosphere, which makes the resulting concentrated protein solution available for further separation. However, there is still the problem with producing small amounts of protein at low concentrations. Engineers have countered this with purification techniques, but there are always new problems created from them. In this article, we explore a proposed alternative method for salting-out. With this method, salts are not left in the fermentation and the protein recovery increases 2- to 10-fold, without sacrificing protein concentration.

Foam from a fermentation-fractionation process enters the attached foam fractionation column anywhere from 6 to 9 h after the start of the fermentation. CO_2 has been known to increase the separation ratio of cellulase in a foam fractionation process *(4)*. An added burst of CO_2 gas, at the same flow rate as the air at the start of foaming, will simulate a salting-out effect when the aeration is turned off. The total gas flow rate is nearly constant over time. After a short time, CO_2 gasification is terminated and aeration is resumed. The volume of foam in the fractionation column becomes 2 to 10 times what was there before CO_2 gasification. Interestingly, the protein concentration in the foam does not decrease with the increased foam volume. Until now, increasing the airflow rate in a foam fractionation process would increase the recovered volume, but decrease the protein concentration. This intensity would also increase the shear stress applied to the protein, which may denature the proteins. The new procedure can be repeated at later times in the same fermentation to increase the protein recovery continually. It also maintains the recovered volume and possibly improves on it. The new approach does not use higher flow rates, so the shear stress

from gasification has less chance of denaturing the proteins. This makes for a very powerful technique in separating and concentrating proteins from a yeast fermentation. CO_2 has usually been viewed as a contaminant to yeast fermentations but now may be a solution to continual dilemmas. This advancement makes a yeast fermentation seem a much more viable option for growing specific proteins and foam fractionation much more viable for separating the proteins.

Materials and Methods

Organism and Medium

Saccharomyces cerevisiae from Fleischmann's baker's yeast was used in all the batch experiments at an inoculum of 2.5 g/L. The yeast was purchased locally. Maxon and Johnson's synthetic Medium C was used in all experiments. Its composition is 10% by weight glucose and 1% by weight monobasic ammonium phosphate. All ingredients were combined prior to fermentation. The medium was further enriched with vitamins, metals, and salts following the Medium C formulation *(5)*.

Fermentation and Fractionation

Aerobic fermentations were done in a 1-L aerated "sealed" glass beaker. The temperature for each batch was approx 23°C and the initial pH was 4.9. Both were uncontrolled during the fermentation. The liquid operating capacity was 800 mL. The aeration rate was at 1.2 L/min (1.5 vvm). The moderate air rate was chosen in keeping with previous work and is consistent with a low shear environment to minimize damage to the developed proteins *(6)*. The fermentations were agitated with a 2.54-cm magnetic stir bar. A glass column, 61 cm in length and 5.1 cm in diameter, was inserted into the top of the fermentor such that there were no air leaks except at the top of the column. Samples were taken from the bulk fluid, the foam at the base of the column, and the top of the foam in the column. Two (0.635-cm-od) plastic tubes were sealed between the column and the fermentor, one extending to the 400-mL mark in the bulk fluid and the other kept level with the base of the column. Samples from the tubes were obtained with a 50-mL syringe, and vacuum suction was used to collect samples in the column. Samples were taken at the start of fermentation and before, during, and immediately after charging the process with CO_2. A filter was added to the top of the glass column to help prevent spores from releasing into the atmosphere. The exit gas was bubbled through a bleach solution to kill any spores that got through the filter. The air inlet was changed to CO_2 to charge the fermentor. The fermentor was charged for 10–15 min every hour for 3 h. To compare with the salting-out procedure for protein recovery in the foam, 10 mL of 3 *M* NH_4Cl was added to the liquid broth with a 50-mL syringe for one of the fermentations. To create a simple experimental model of the method's ability to replace the salting-out technique, 0.1 g/L of

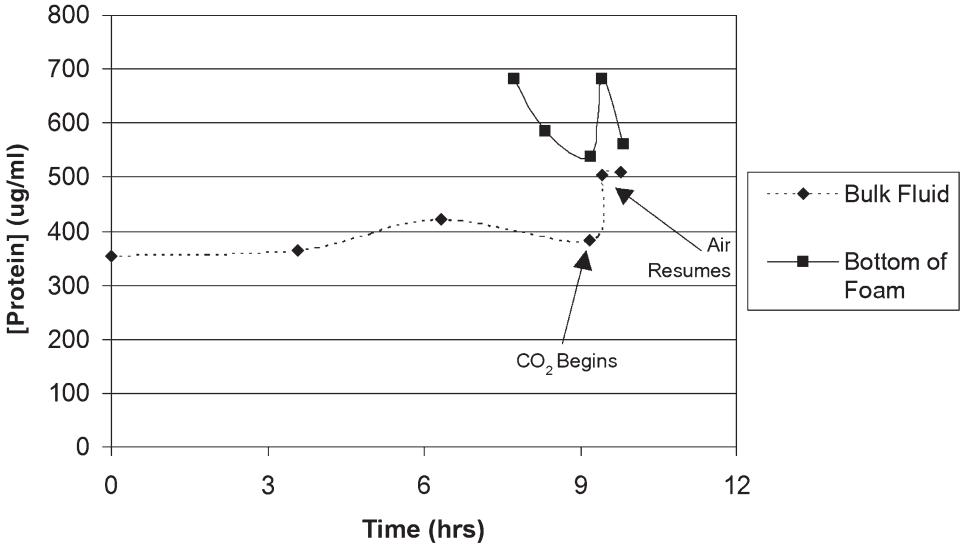

Fig. 1. Protein concentration profiles from experiment 1 for bulk fluid and bottom of foam for yeast fermentation with a CO_2 burst.

chicken egg albumin was foam fractionated with the new technique in a separate glass column, 37.5 cm tall. The column was tall enough to see a change in the amount of foam produced during CO_2 gasification.

Protein Assay

A Coomassie Blue total protein assay was performed on 0.1-mL samples and was adjusted for yeast protein *(5).* The samples taken from the process were centrifuged for 2 min to remove the yeast cells prior to determination of protein concentration. The supernatant was used for the assay, and the centrifuged solids were bleached and discarded. The assay was performed with a Bausch & Lomb Spectronic 20 spectrophotometer at 595 nm. The total protein concentration was measured initially and before, during, and immediately following the CO_2 burst.

Results and Discussion

When CO_2 was added to the yeast fermentation, it caused a dramatic 25% increase in the protein concentration in the bulk fluid, as shown in Fig. 1. Presumably, this is owing to cell secretion, but possibly to cell rupture. Future work should include a DNA assay to determine whether the cells are rupturing or secreting proteins. There was a corresponding resultant effect in the foam, meaning that the protein concentration in the foam also increased by 25%. Once the system had the air restored, the protein concentration in the foam decreased back down to its concentration before adding CO_2. The protein concentration in the bulk fluid stayed at the higher level. This is expected because proteins do not return to the yeast cells after they are secreted.

Table 1
Foam Height for Yeast Fermentation in Experiment 1 for Third CO_2 Burst[a]

Time (h)	Gas	Foam height (cm)	Density of foam	Transparency
8.3–9.2	Air	30.5	Very low	Very transparent
9.2–9.4	CO_2	23	Moderate to high	Slightly transparent
>9.4	Air	61+	Moderate	Moderately transparent

[a]The foam height corresponds to the end of each time range. The "+" denotes that the foam exceeded the height of the column. Density and transparency were visual observations.

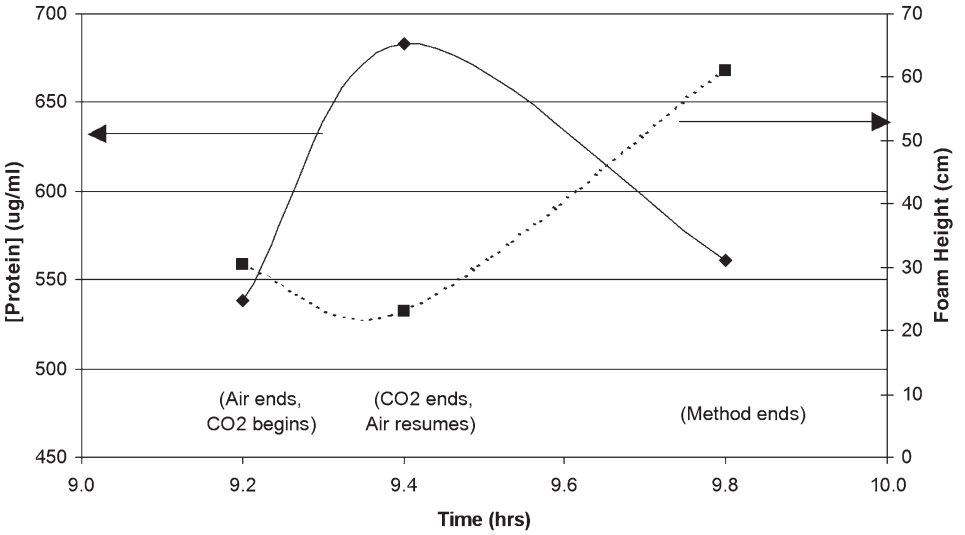

Fig. 2. Changes in foam height and protein concentration for third CO_2 burst during experiment 1. The times are noted with the corresponding gases. Protein increased during CO_2 gasification and foam height increased when aeration resumed. The maximum foam height was 61 cm, the height of the foam fractionation column. (■) Represents the foam height and (♦) represents the protein concentration.

The main benefit for this technique is the increase in foam without sacrificing the protein concentration. This new method kept the same flow rate, but the recovered volume increased significantly. As shown in Table 1, the foam height increased after the CO_2 burst from 30.5 to 61 cm. The air was stopped to keep the foam from exceeding the height of the glass column.

The new method is better demonstrated in Fig. 2, where the protein concentration at the bottom of the column and the corresponding foam height are compared for the same time intervals. The foam height decreased slightly while transferring the air to CO_2. The protein concentration of the start and end points was about the same, but the foam height doubled over that period. By observation, the bubbles in the foam were smaller and more compact after the gasification than those at the start of the gasification. The increased density indicates, perhaps, that the actual increase in "liquid

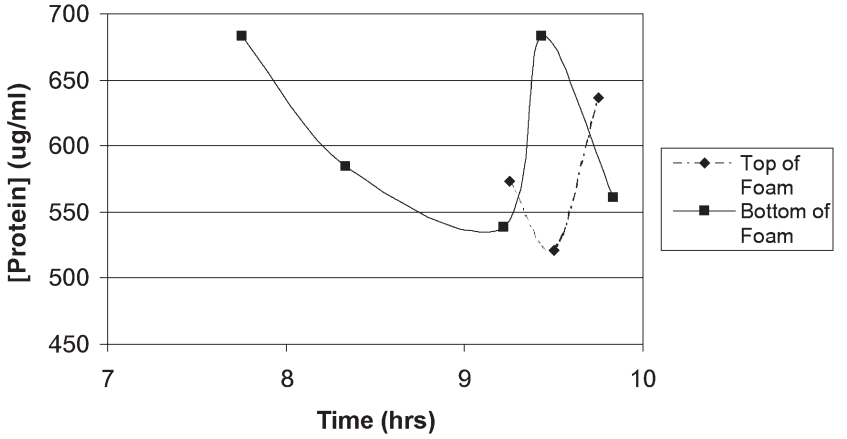

Fig. 3. Protein concentration profiles for experiment 1 at top and bottom of foam.

volume" was much higher than the twofold increase that was measured in the foam height. It also appears that the system lost its protein precipitation effect when a few minutes elapsed before the air was restored. An additional 30-s burst of CO_2 replenished the system's ability to recover additional volume.

Figure 3 shows that the protein in the foam appeared to move from the top of the foam toward the bottom of the foam. This is an expanded time scale for experiment 1, with profiles for protein concentration in both the top and bottom of the foam. The protein concentration at the top of the foam increased after 9.4 h when the air was returned to the system.

Figure 4 shows the effect of ammonium chloride in experiment 2. The salt pushed protein into the foam, similar to what occurred in experiment 1; however, the salt was left behind in the system as a residue and the CO_2 left, just like the air. In the CO_2 burst system, the bicarbonate and carbonate salts in equilibrium with CO_2 are thus flushed out of the system with the air as CO_2.

Yeast fermentations are complex processes to set up and can be tedious to run with consistency. It would be beneficial to have a simple, nonfermentation working model to study the new gasification method. Chicken egg albumin was selected here to use as a model protein in the simpler process, experiment 3. The gasification method used in experiment 3 was the same as that used in experiment 1; however, experiment 3 was only a foam fractionation process, and not coupled with a fermentation process. The goal of this experiment was to show that the protein in the foam, as well as the foam height, would increase using the CO_2 gasification method developed in experiment 1. Figure 5 shows a similar CO_2 effect on the albumin as was previously seen with the yeast protein (Fig. 3). The foam height increased after the CO_2 gasification, the same effect seen with the yeast fermentation (Fig. 2).

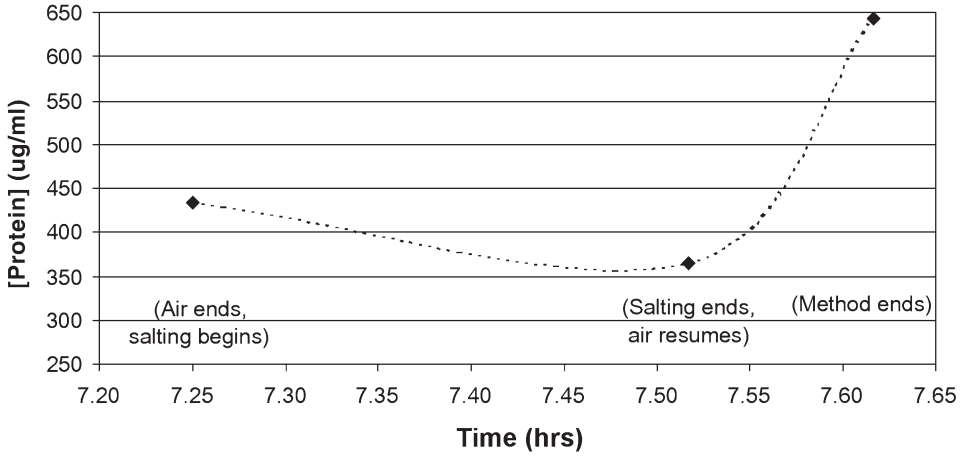

Fig. 4. Effect of ammonium chloride on yeast fermentation in experiment 2. The times are noted with the corresponding gases. The result is similar to that seen with CO_2. Foam height is not included because the total gas flow rate was not constant.

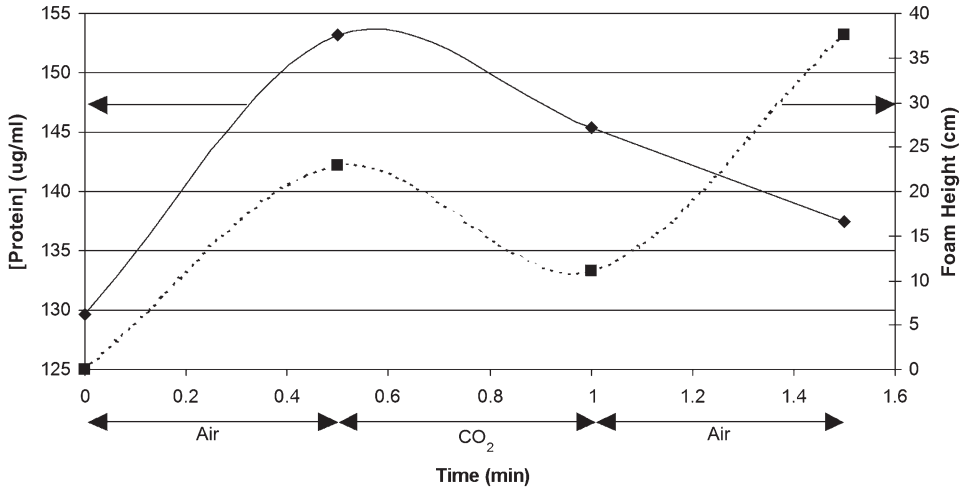

Fig. 5. Protein concentration and foam height of chicken egg albumin during a 0.5-min CO_2 burst in experiment 3. The double-headed arrows show the time ranges corresponding to the gas used. The maximum foam height was 37.5 cm, the height of the foam fractionation column. The foam height increased after CO_2 gasification, similar to what was previously seen in Fig. 2. (■) Represents foam height and (♦) represents protein concentration.

The protein concentration level in the albumin test was lower than that of the yeast in this initial comparison with yeast proteins. This model system may simplify the development of the CO_2 burst and should be examined for a more selective removal scheme for the yeast proteins in future work. After the first CO_2 burst, the foam, not removed from sampling, was allowed to collapse and the method was repeated twice. Removing a sample also removed proteins from the system, limiting the ability of air to produce any

Table 2
Albumin Foam Height in Experiment 3 with Density and Transparency Observations for Changes in Sparged Gas[a]

Gas	First burst (cm)	Second burst (cm)	Third burst (cm)	Density of foam	Transparency
Air	23	0	0	Low	Highly transparent
CO_2	11	20	18	High	Almost opaque
Air	37.5+	37.5+	37.5+	High	Almost opaque

[a]The foam was depleted either from removing a sample or from being allowed to collapse; thus, the foam height for the second and third bursts started at 0 cm.

more foam. The second and third CO_2 bursts were used to precipitate more proteins from the bulk fluid so that the air would be able to produce more foam. Samples were not taken of these bursts; the observation of more foam shows that CO_2 did precipitate more proteins within the bulk fluid.

The foam height of albumin in the column exceeded the height of the column (37.5 cm) for all three CO_2 bursts, each about 5 min apart. Table 2 shows the foam heights and how the consistency of the foam changed with each stage in the process. Visual observations of density and transparency, described in Table 2, supplement the foam height data.

The results of experiment 3 were similar to what was previously seen with the yeast fermentation process. The albumin seems to be a good standalone, nonfermentation model for studying the method in further investigations and for use in foam fractionation processes themselves.

Conclusion

CO_2 may be used in conjunction with air to remove proteins from a yeast fermentation as well as in foam fractionation. Foam fractionation of albumin can serve as a simple model for studying the CO_2/air protein precipitation method. The foam fractionation of albumin also shows that the method extends the range of protein that can readily precipitate with air, even after samples have depleted the total protein concentration. Plainly, it helps to get more out of the system. The new process appears to have the same precipitation effect as salting-out; however, it has advantages over a salting system. A salt is not left behind with the technique, and the recovered volume may be more than 10-fold that observed in a coupled fermentation/foam fractionation process. Using CO_2 in place of a salt could potentially save the cost of the salt, as well as eliminate contamination of enzymes by heavy metals in the salt and the brine cleanup problem following the fermentation process. It may also be useful as a technique for recovering dilute protein samples at selected times from a fermentation process, merely by pulsing the CO_2 into the system.

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