0273-2289/05/121–124/541–552/$30.00

Enhancing Cellulase Foam Fractionation with Addition of Surfactant

Vorakan Burapatana, Ales Prokop, and Robert D. Tanner*

Department of Chemical Engineering, Vanderbilt University, Nashville, TN 37235, VU Station 351604, E-mail: rtanner@vuse.vanderbilt.edu

Abstract

Foam fractionation cannot be used to recover cellulase from an aerated water solution effectively because cellulase by itself can produce only a small amount of foam. The addition of a surfactant can, however, increase the foamate volume and enhance the concentration of cellulase. We studied three detergents individually added to a 200 mg/L cellulase solution to promote foaming. These detergents were anionic, cationic, and nonionic surfactants, respectively. Although contributing to foam production, it was observed that nonionic surfactant (Pluronic F-68) barely concentrated cellulase, leaving the enrichment ratio unchanged, near 1. With anionic surfactant, sodium dedecyl sulfate, and cationic surfactant, cetyltrimethylammonium bromide (CTAB), the enrichment ratio became much larger, but cellulase denaturation occurred, reducing the activity of the enzyme. When CTAB was used to help foam cellulase, β-cyclodextrin was subsequently added to the foamate to help restore the enzyme activity.

Index Entries: Foam fractionation; surfactant; cellulase; cyclodextrin; denaturation.

Introduction

Presently, cellulase itself is expensive because its purification and concentration are both costly and time-consuming *(1)*. Foam fractionation is a technique for separating and concentrating proteins, many of which are enzymes. Foam fractionation can operate in either a semibatch or a continuous mode, depending on whether the feed is introduced intermittently (pulse addition) or continuously. In most cases of single-protein system, the performance of foam fractionation is optimal (regarding enrichment, mass recovery, and activity recovery) when the protein solution pH equals the protein isoelectric point *(2,3)*. A high initial feed concentration often results in a low enrichment because at that protein level the available sites for proteins on a foam bubble tend to be saturated and the resulting

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

equilibrium does not favor further concentration. Thus, protein enrichment by a foam fractionation process generally works best for dilute protein feed streams. Changing the air superficial velocity can also affect the subsequent protein enrichment and mass recovery in the column. At a low air velocity, water drainage can take place over a longer period of time and, thus, more water can be removed from the foam to concentrate the remaining portion of protein.

There are two significant drawbacks to foam fractionation. First, foam fractionation has a limited ability to separate an individual protein from a mixture of proteins. It has been shown, however, that the addition of a surfactant can increase the selectivity of this process *(4)*. Foam fractionation is most likely to be effective with one or more proteins in solution in an early downstream protein recovery process because it works best (high enrichment) with dilute solutions. In an early recovery step dealing with highly diluted protein solutions, selectivity is generally not the main issue but, rather, water removal. Second, there is the potential for protein damage, which can be caused by protein oxidation, bubble shear, and the presence of a protein at a gas-liquid interface within the foam *(5,6)*.

Proteins in their native state are not very stable *(7)*. Common industrial processes such as filtering, storage, agitation, freezing/thawing, lyophilization, nebulization, and spray drying can cause proteins to denature *(8)*. Protein renaturation plays an important role in the success of an efficient production process for the manufacture of therapeutic protein drugs *(9,10)*. Renaturation of degraded proteins can occur with the help of molecular chaperones, such as heat-shock proteins. Examples of heat-shock proteins are GroEL (Hsp 60) and GroES (Hsp 10) in *Escherichia coli (11)*. However, renaturation using heat-shock proteins is not economically feasible because such proteins tend to be expensive. Thus, using an expensive product to recover the activity of another expensive product (e.g., a therapeutic protein) would lead to a significant increase in the price of the desired product. A low-cost renaturation process would therefore be a desirable part of a protein recovery process for the therapeutic drug or enzyme industries. One renaturation method that is relatively inexpensive uses "artificial" chaperones *(9,10)*.

The renaturation mechanism of artificial chaperones is similar to that of the GroEL chaperone system, in which GroEL binds with a nonactive protein to prevent protein aggregation. The binding causes the protein to refold to its native state *(12)*. In this two-step process, a detergent is first added to the protein solution, where it forms a complex with the denatured protein. In the second step, cyclodextrin, acting as a detergent-stripping agent, is added to the protein-detergent solution. Cyclodextrin takes away the detergent from the detergent-protein complex, so that the protein can properly orient itself to its native structure. After protein renaturation,

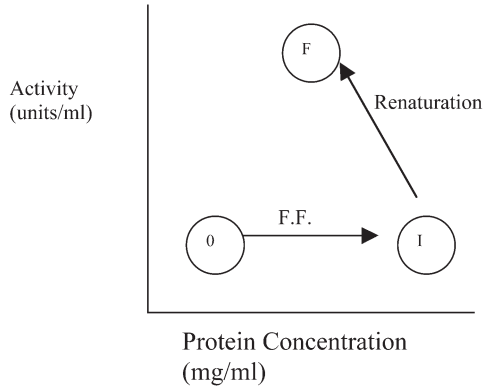

Fig. 1. Schematic representation of strategy proposed to concentrate cellulase by a modified foam fractionation (F.F.) process: 0, I, and F: the initial, intermediate, and final processing states, respectively. From states 0 to I, the solution is foam fractionated. From states I to F, the foamate is renatured.

both the cyclodextrin and the low molecular weight detergent can be removed by ultrafiltration *(12)*. Artificial chaperones have been successfully used to renature the following proteins (which were previously degraded by chemical denaturation): carbonic anhydrase B *(12,13)*, lysozyme *(14–17)*, citrate synthase *(13,18)*, MM-creatine kinase *(19)*, xylanase *(20)*, insulin *(15)*, and human growth hormone *(21)*.

For a foam fractionation process to be applicable to industrial downstream processes recovering proteins from a water solution, protein denaturation occurring in a foam column must be either eliminated or reduced. The use of artificial chaperones coupled with the foam fractionation process may be the way to intrinsically reverse the resultant denaturation. If detergent is added to a protein solution prior to foaming, then the detergent-protein will be carried through the foam fractionation process. Detergent stripping with cyclodextrin then follows in the subsequent processing step. Because the detergent itself is very surface active, it has high foaming potential, which leads naturally into foam fractionation. The renaturation step with the addition of cyclodextrin is usually carried out at a low protein concentration, which also fits well following protein foam fractionation because foam fractionation generally works best at low protein levels. Without added detergent, foam fractionation does not usually work well with relatively hydrophilic proteins (those that barely foam when aerated). In those cases in which a detergent and also a detergent-removing chemical such as cyclodextrin are added, both additives can be removed using ultrafiltration after concentrating the desired protein product in a foam fractionation column.

The work described in this article integrates the use of artificial chaperones into the foam fractionation process, as depicted in Fig. 1. Ideally,

that integration can create a single operating process of only two steps. The addition of a detergent with a foam fractionation of a protein, along with renaturation, is rather easy to implement simply by diluting the foamate product with the cyclodextrin. The fractionation apparatus can even be set up with the detergent-stripping agent (the cyclodextrin) in the foamate collector prior to foam formation in the column. Cellulase is used here as the model protein/enzyme because its enzymatic activity can be determined easily and rapidly and it is an enzyme of significant industrial importance in the production of sugars from cellulose (the intermediate chemicals used as a yeast substrate for making bioethanol). This bioethanol can thus be produced from agricultural waste materials.

Materials and Methods

Chemicals

Cellulase from *Trichoderma reesei* (cat. no. C-8546), Brij30, dinitrosalicylic acid (DNS), and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO.) Bicinchonimic acid (BCA) protein assay reagents, Whatman No. 1 filter paper, Triton X-100, Tween-20, polyvinylpyrrolidone (PVP), and β-cyclodextrin were purchased from Fisher (Pittsburgh, PA). Sodium carboxymethylcellulose (CMC) was purchased from Hercules (Wilmington, DE). Cetyltrimethylammonium bromide (CTAB) and tetradecyltrimethyl ammonium bromide (TTAB) were purchased from Fluka (Buchs, Switzerland). Pluronic F-68, Pluronic F-108, Pluronic L-61, Pluronic L-81, and Tetronic 1107 were purchased from BASF (Mount Olive, NJ). All chemicals were reagent grade.

Effect of Various Detergents

Foam fractionation experiments were carried out in a small glass column (2-cm diameter, 10-cm height). The initial liquid volume of 12 mL contained a 200 mg/L cellulase solution at pH 4.8. The following surfactants were individually added to the cellulase solution in the glass column: TTAB, Pluronic F-68, Pluronic F-108, Pluronic L-61, Pluronic L-81, SDS, Brij 30, Tetronic 1107, Triton X-100, Tween-20, CTAB, and PVP. The concentration of the surfactants in the initial bulk solution was 100 mg/L. Foam fractionation was conducted for each surfactant-cellulase mixture at an airflow rate of 15 cc/min.

Effect of Airflow Rate

SDS, CTAB, Pluronic F-68, and Triton X-100 were used in the airflow rate experiments. The initial cellulase concentration was 200 mg/L and the added surfactant concentration was 100 mg/L. The solution mixture of cellulase and surfactant (at pH 5.0) was tested at several airflow rates.

Table 1
Effect of Various Detergents on Cellulase Foam Fractionation at pH 4.8 and Airflow Rate of 15 cc/min on Mass and Activity Ratios[a]

Surfactant	Foam volume collected (mL)	Mass		Activity	
		ER	MR	AE	AR
TTAB	2.1	1.06	0.23	0.90	0.16
F-68	5.2	1.01	0.48	0.95	0.41
SDS	1.3	1.81	0.22	1.32	0.14
Brij 30	1.0	1.24	0.10	1.10	0.09
L-81	0.0	N.A.	N.A.	N.A.	N.A.
L-61	2.9	0.99	0.24	0.93	0.23
Tetronic 1107	5.7	0.98	0.46	1.18	0.56
Triton X-100	5.0	1.02	0.42	1.05	0.44
F-108	4.2	0.97	0.34	1.10	0.39
CTAB	3.8	1.82	0.49	1.02	0.27
Tween-20	5.7	1.05	0.50	0.90	0.43
PVP	0.0	N.A.	N.A.	N.A.	N.A.

[a]N.A., not available.

Renaturation of Cellulase After Foam Fractionation

After foam fractionation of a cellulase and CTAB mixture, 350 μL of collected foamate was diluted with 150 μL of 13 m*M* β-cyclodextrin solution. The resulting solution was stored overnight before checking the cellulase activity.

BCA Assay for Protein Concentration

Twenty microliters of the sample was put in a 96-well microplate in triplicate. Then, 180 μL of BCA reagent was added to each well of the plate. The microplate was scanned after 30 min at 562 nm for determination of absorbance.

Cellulase Activity

Both filter paper *(22)* and CMC assays *(23)* were used to test for cellulase activity. A DNS reducing sugar assay was used to measure the amount of sugar produced. A filter paper assay was used to determine the activity of the foam-fractionated solutions of cellulase is added when screening various detergents in order to find the ones that performed the best. These detergents are provided in Table 1, along with the associated mass and activity recoveries. The best of the three different classes of detergents (each with a different charge)—SDS (anionic), Pluronic F-68 (nonionic), and CTAB (cationic)—were then studied. These follow-up studies used the CMC assay because it could be done with smaller samples, which resulted from the very small volume (at low airflow rate) follow-up experiments.

Results and Discussion

From our previous studies, 200 mg/L of cellulase was established to be an effective concentration for foam fractionation experiments *(24)*. Therefore, this concentration was used as the base point throughout the present study. The following surfactants were individually added to the base point (200 mg/L) cellulase solution: TTAB, Pluronic F-68, Pluronic F-108, Pluronic L-61, Pluronic L-81, SDS, Brij 30, Tetronic 1107, Triton X-100, Tween-20, CTAB, and PVP. The concentration of detergent after mixing with the cellulase in solution was preset at 100 mg/L (at pH 5.0). After the detergent was added in one dose, 12 mL of the resulting detergent-cellulase solution was introduced into the foam column and subjected to an airflow rate of 15 cc/min, and several mass and activity recoveries were determined from the resulting foamate.

The mass enrichment ratio (ER) is defined as the protein concentration in the foamate divided by the initial protein concentration. The mass recovery ratio (MR) is defined as the amount of protein in the foamate divided by the initial amount of protein. The activity enrichment ratio (AE) is defined as the activity per unit volume in the foamate divided by the initial activity unit per volume. The activity recovery ratio (AR) is defined as the amount of activity in the foamate divided by the initial amount of activity. The ER, MR, AE, and AR ratios were all calculated from the mass concentration and activity concentration experimental results. The determined ratios are displayed in Table 1.

Both Pluronic L-81 and PVP generated a lot more foam volume than cellulase solution without the detergent, but still not enough to fill the column space and overflow from the column into the foamate receiver. The other detergents provided sufficient foam volume to fill the column for subsequent overflow and foamate collection. Significant ERs often resulted in the foamate but usually with low mass recoveries. Even with significant ERs, there was little, if any, improvement in the AE over the original solution. Ideally, an increase in ER would result in an equal increase in the AE if the activity per unit mass held constant (no protein denaturation). However, it was noted that significant activity loss/denaturation occurred during the foam fractionation. Only in the case of SDS was the AE significantly enhanced.

The ER was also influenced by the airflow rate. Two nonionic detergents, Pluronic F-68 and Triton X-100 (at 100 mg/L levels), were used to determine whether the enrichment could be increased by adjusting the airflow rate. When the airflow rate was reduced from 45 to 5 cc/min, it was observed (see Fig. 2A,B) that Triton X-100 could enrich (in both the mass and activity) the protein solution by a factor of 1.4. Most of this improvement in enrichment occurred below the 15 cc/min airflow rate. Triton X-100, therefore, could protect the cellulase from gas-liquid interface denaturation, although the overall mass and activity recoveries (Fig. 2C,D) were reduced as much as ninefold.

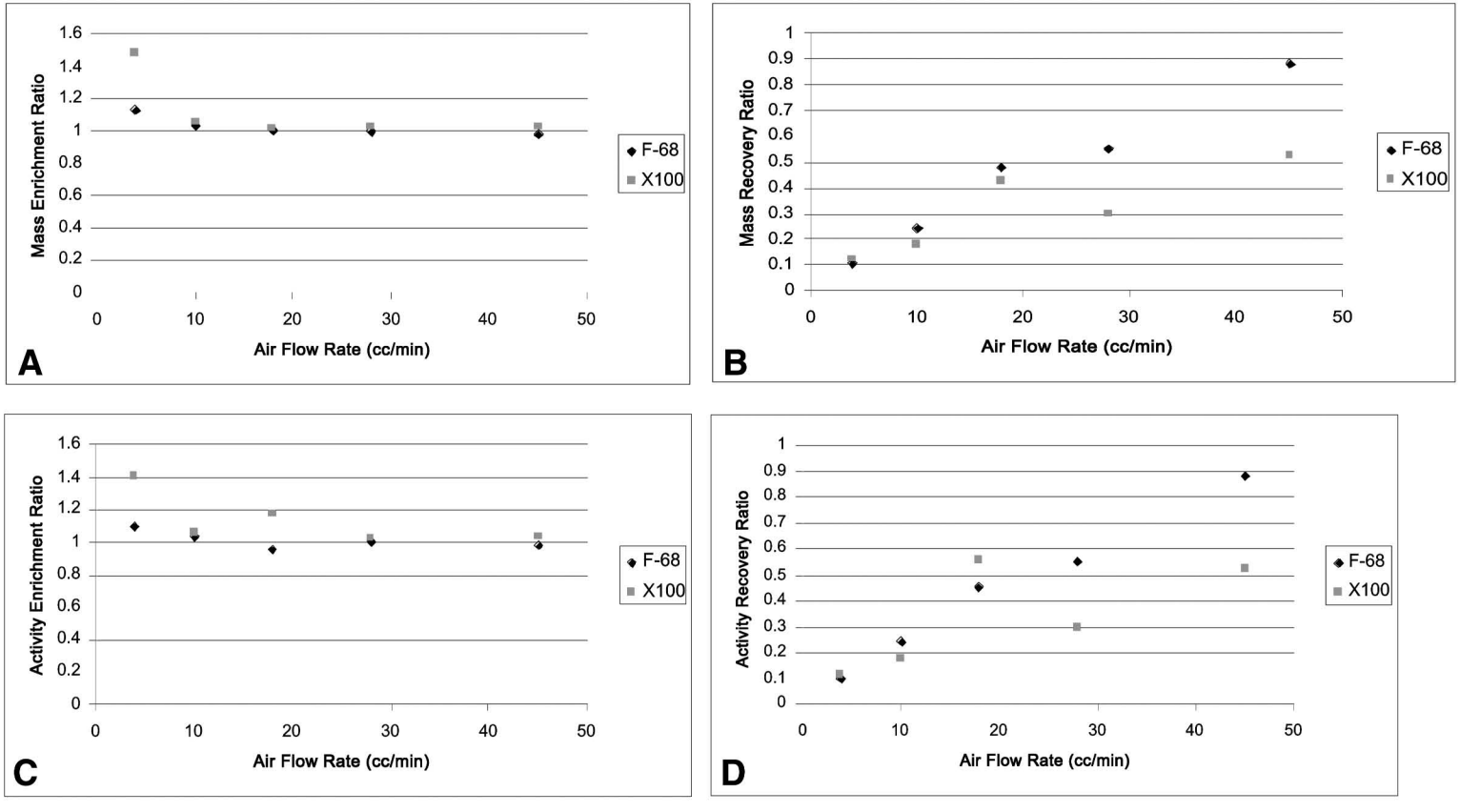

Fig. 2. **(A)** AE, **(B)** ER, **(C)** MR, and **(D)** AR vs airflow rate for foam fractionation experiments conducted at pH 5.0 with 200 mg/L of cellulase and 100 mg/L of nonionic detergent solution (either Pluronic F-68 or Triton X-100). A CMC assay was used to determine cellulase activity.

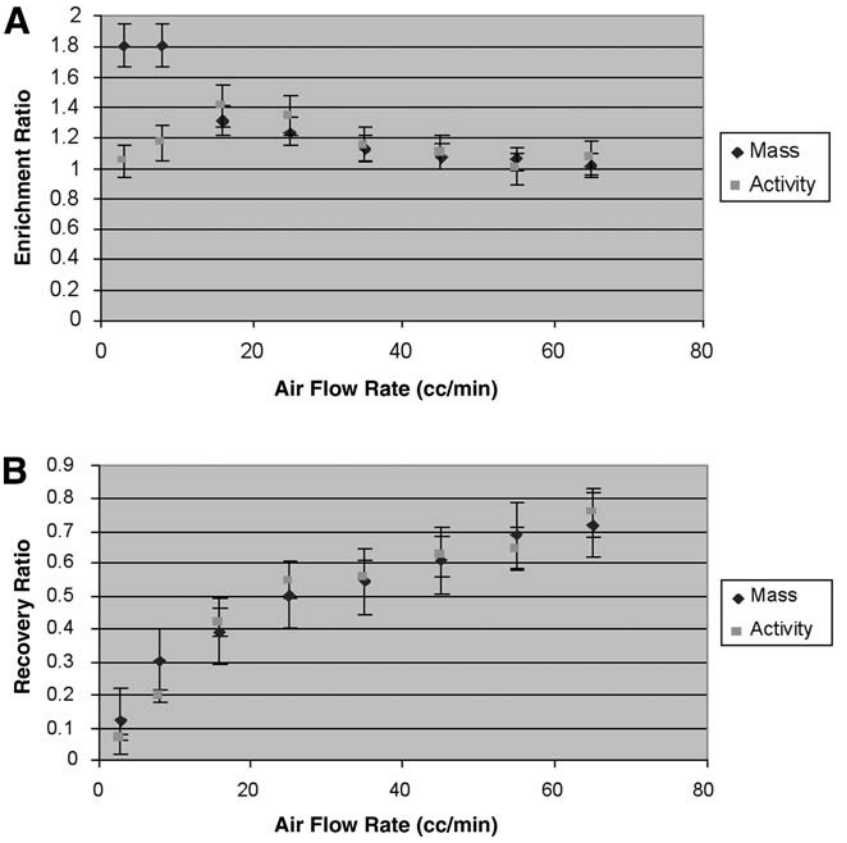

Fig. 3. (A) AE and ER and **(B)** AR and MR vs airflow rate for foam fractionation experiments conducted at pH 5.0 with 200 mg/L of cellulase and 100 mg/L of anionic detergent (SDS) solution. A CMC assay was used to determine cellulase activity.

Assuming that the detergent and a cyclodextrin detergent-stripping agent (added after the foam fractionation experiment) did not affect the hydrolysis of cellulose in the subsequent activity measurement, it was not possible to increase the AE without increasing the ER because the enzymatic activity per mass cannot increase above the initial activity per mass. This is based on the assumption that foaming probably causes denaturation and decreases enzymatic activity.

A similar experiment to that reported in Fig. 2 was run with a mixture of SDS and cellulase; Figure 3 shows the results. In the slow airflow rate region (5–20 cc/min), the anionic SDS gave a better mass enrichment than the nonionic surfactants F-68 and Triton X-100 (shown in Fig. 2). The MR in that flow region was very small, as it was for the nonionic surfactants. SDS offered protection from denaturation for the cellulase enzyme in the airflow range of 15–25 cc/min, whereas Triton X-100 offered protection for

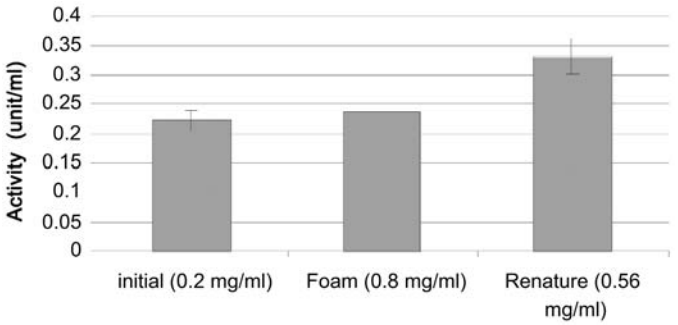

Fig. 4. Amount of activity per milliliter of cellulase before foaming, after foaming, and after 9 h of foamate treatment with β-cyclodextrin. The initial state (before foaming) had a cellulase concentration of 200 mg/L and 100 mg/L of CTAB. The amount of CTAB did not affect the cellulase activity. The foamed state had a cellulase concentration of 800 mg/L, and the renatured state had a cellulase concentration of 560 mg/L following dilution with cyclodextrin solution. Thus, the renatured state had less cellulase per unit volume, but it could produce more sugar (higher activity in CMC; cellulase activity test) than the original foamed state. Therefore, it was partially renatured. The system follows the expectation formulated in Fig. 1. This foam fractionation was carried out at pH 5.0 with a phosphate buffer and an airflow rate of 4 cc/min. A CMC assay was used to determine cellulase activity.

the airflow rates of 4–55 cc/min. These effects need to be clarified with additional experiments.

Figure 4 shows the change in activity at different stages of the process. The increase in activity per liquid volume from the foam step to the renatured state step shows that cellulase (presumably denatured at the gas-liquid interface) could be refolded by an artificial chaperone combination of CTAB and β-cyclodextrin. The renaturation step also diluted the cellulase mass concentration. Calculation of the specific activity confirmed that some of the activity was restored. The specific activity in the initial, foam, and renaturation steps was 1.1, 0.3, and 0.63 U/mg respectively.

It is known that the degree of protein denaturation can depend on the foam fractionation column airflow rate *(25)*. The degree of denaturation decreases as the airflow rate increases, presumably because of the decrease in column residence time. This was clearly shown by the cellulase AR following foaming with CTAB at different airflow rates (see Fig. 5D for the before and after refolding/renaturation case). Besides the decrease in denaturation of the cellulase with an increasing airflow rate, the higher airflow rate also increases the effectiveness of the subsequent refolding process. The percentages active after refolding at airflow rates of 6, 10, and 15 cc/min were approx 58, 85, and 98%, respectively. The percentages are determined by dividing the AR by the MR. By comparison, the percentages active before refolding were 35, 46, and 56%.

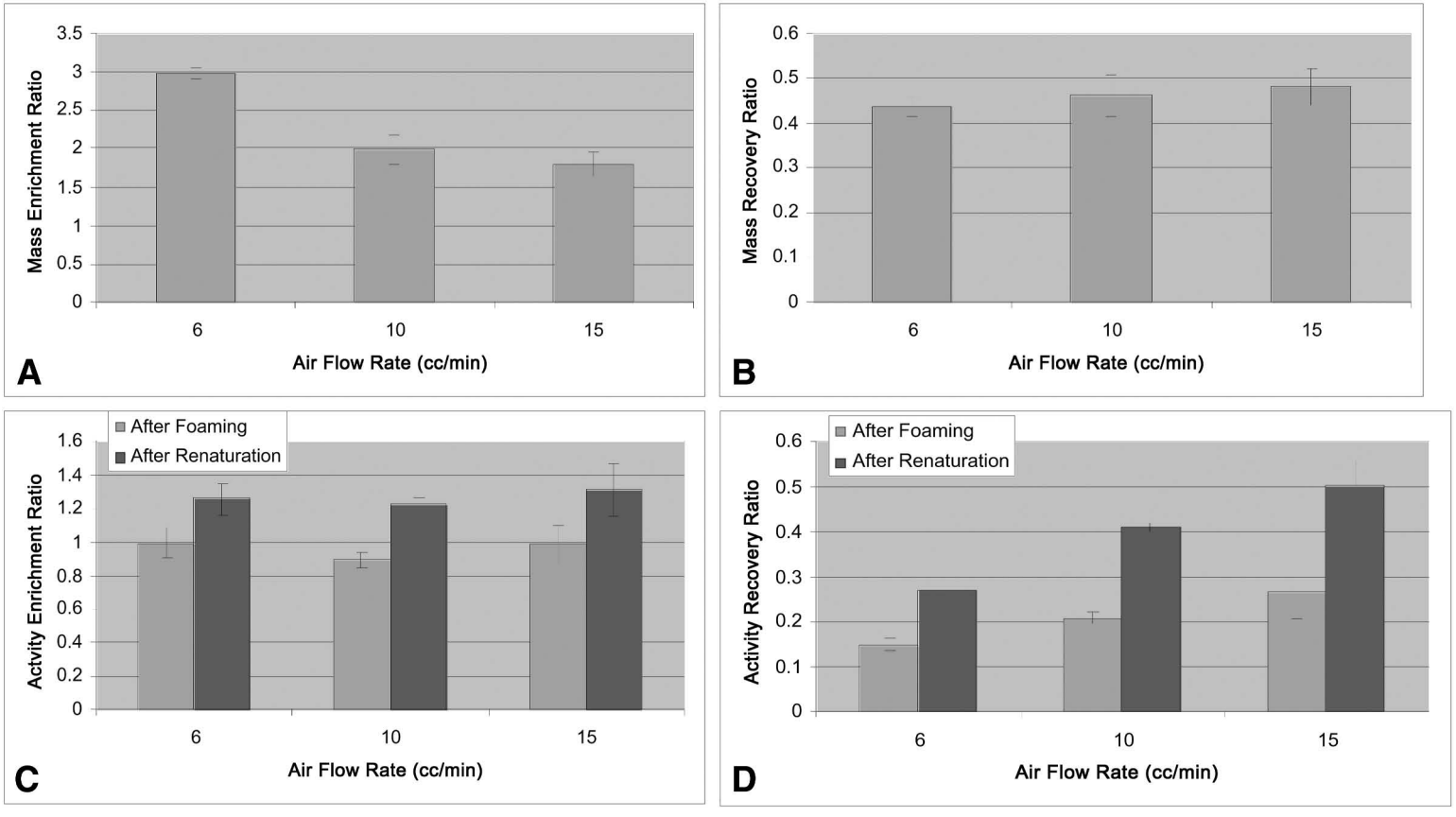

Fig. 5. β-cyclodextrin was added to the foam collected after foam fractionation of a 200 mg/L of cellulase and 100 mg/L of CTAB solution at different airflow rates (pH 5.0). **(A)** effect of airflow rate on ER; **(B)** effect of airflow rate on MR; **(C)** improvement in AE relative to foamate both before and after β-cyclodextrin addition; **(D)** increase in activity recovered after treatment with β-cyclodextrin compared with before addition of β-cyclodextrin. A CMC assay was used to determine cellulase activity.

Conclusion

Integrating artificial chaperones (here, the combination of a detergent and β-cyclodextrin) into a cellulase foam fractionation process could reduce cellulase activity losses caused by denaturation. The addition of a surfactant could also increase the amount of protein foam even when the protein or enzyme was inherently nonfoaming. In addition, such addition of detergent could lead to protein or enzyme concentration in that foam fractionation process. In the case of cellulase, CTAB seemed to work better than the other surfactants tested in enhancing both the ER and the MR. Although apparent cellulase denaturation occurs under many conditions in the foam fractionation process, using the cationic surfactant CTAB could reduce the damage when β-cyclodextrin was added to the foamate, following cellulase concentration. This study shows that artificial chaperones can even be used to restore cellulase that has been damaged in a foam fractionation process. Nonionic surfactants alone, such as Pluronic F-68, may protect the cellulase from degradation during the foam fractionation process. The amount of enriched cellulase solution produced using pluronic F-68 however, is so small that the process becomes infeasible.

Acknowledgment

We greatly appreciate the financial support from the US Department of Agriculture (Grant No, 2001–52104–11476).

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