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In Situ Bubble Fractionation Strategies for Separating Individual Proteins in a Batch Baker's Yeast Fermentation Process

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ABSTRACT

Extracellular proteins produced by yeast have been observed to stratify in the extracellular fluid of a batch bioreactor, thus creating a vertical concentration gradient. We observed that, in the four different experiments conducted, each varied in their protein recovery characteristics. For example, sparging the system with gas accentuates the separation, though even in a nonsparged system, the *in situ* generation of minute carbon dioxide bubbles by yeast cells creates a protein gradient as the bubbles carry proteins upward. Based on these and other observations, we propose possible strategies for recovering the individual proteins from a system containing the four major proteins considered. A simple steady-state mathematical model, based on convective upward protein transport being balanced by downward protein diffusion, has been used to describe the behavior of each of these four extracellular proteins in the fermentation broth.

Index Entries: Fermentation; protein fractionation; baker's yeast; proteins, bubble fractionation of; *in situ* protein separation.

Abbreviations Used: C_b, protein concentration at the bottom of the fermentor; C_L, liquid concentration of an individual protein, mg/cm^3, C_m, protein concentration at the middle of the fermentor; C_t, protein concentration at the top of the fermentor; D, protein dispersion coefficient, cm^2/s; k, 3QK/Dr (πR^2), cm^{-1}; kH, separation efficiency factor; K, proportionality (equilibrium) constant, Γ/C_L, cm; SR, separation ratio, C_t/C_b; Q, gas flow rate, cm^3/s; X, dimensionless protein concentration at position z; X_m, dimensionless concentration at

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the fermentor midpoint (at $z=H/2$); z, column vertical position, cm ($0<z<H$), where $z=0$ is the base position and $z=H$ is the top of the fermentation broth; Γ, bubble protein concentration, mg/cm^2; r, radius of bubble, cm; R, radius of column, cm.

INTRODUCTION

The observation of protein stratification in the broth during a batch Baker's yeast fermentation process has been previously observed (*1–4*). This stratification was noted because measurements of protein concentration in the tall, 1 L beaker, under identical conditions, were difficult to duplicate unless the samples were taken at the same depth. The data analyzed in this paper describe protein stratification in a tall, 1 L graduated cylinder (*4*) Four proteins were selected for this analysis because they were always present on the electrophoresis gels under the four different conditions explored.

The physical system under consideration contained an aqueous solution of proteins in a fermentation growth medium at 32 ± 1°C and 1 atm pressure. The solution may also contain yeast cells and added NaCl. The extracellular protein concentration varies from 40–300 mg/L, measured as total protein. This concentration is low enough that a stable foam does not form at the top of the column and, thus, any such protein carried upward by a bubble is released at the liquid surface and is free to disperse downward. The relatively constant extracellular total protein concentration implies that the fermentation process for each individual protein can be modeled, to a first approximation, by assuming a constant cell level and no protein degradation.

METHODOLOGY AND PROTOCOL

Figure 1 illustrates the physical system. Sample ports at the bottom, middle, and the top of the cylinder allow removal of samples for analysis. The assay for individual proteins was performed using Polyacrylamide-SDS Gel Electrophoresis (*3*). The pH was held constant at 5 ± 0.25 and the total cell mass was also measured. Four different experiments were conducted: experiment 1: Nonsparged; experiment 2: sparged at 0.14 vvm (with air); experiment 3: sparged at 0.14 vvm (with CO_2); and experiment 4: Nonsparged, 0.6*M* NaCl added.

In all cases, samples were withdrawn at $t=0$ h (actually, ca. 10 min), $t=4$ h, $t=7$ h, and $t=10$ h. Four different proteins of molecular weights 39, 62, 78, and 95 kD were monitored for each of these experiments. The individual proteins were identified on the gels using a dye marker with the Wray silver staining procedure (*3*).

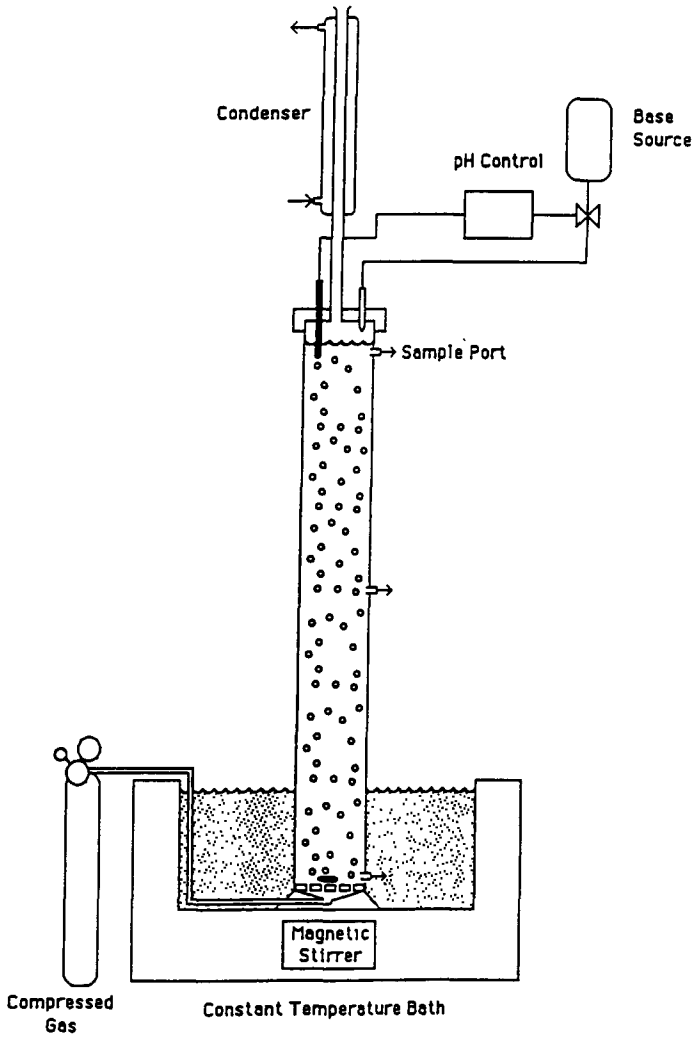

Fig. 1. Bubble fractionation/fermentation apparatus diagram (from (3)).

THE STEADY-STATE MATHEMATICAL MODEL

The model developed by Potter et al. (5) to describe the total protein concentration gradient in a tall, 1 L graduated cylinder, is extended to apply to individual proteins. The assumptions made in developing the original model for the total protein concentration are also assumed applicable here for the individual proteins: (a) The bulk liquid is stagnant (in other words its movement is small relative to the bubble movement), (b) the bubble rise velocity is constant, (c) Quasi steady-state behavior is reached rapidly, compared to the protein production rate, and (d) The proteins

have already been synthesized by the yeast and only need to be separated. (This assumption has been found to be valid in practice to within 50%, for these four individual proteins, as well as for the total protein level.)

At quasi steady-state, the rate of individual soluble proteins transported upward equals their rate transported downward. Thus, Eq. (1) is developed for quasi steady-state operation

$$\frac{d^2C_L}{dz^2} - k\frac{dC_L}{dz} = 0 \qquad (1)$$

where the terms are defined in the "Abbrreviations Used" section. Applying the boundary conditions at the bottom and top of the fermentor

$$C(O) = C_b \text{ and } C(H) = C_t$$

to the analytical solution of Eq. (1), we obtain the dimensionless concentration profile. This equation can be used to compare data from any condition only on the basis of kH, separate from the boundary conditions C_b and C_t since time does not appear, explicitly owing to the quasi steady-state nature of the system.

$$X = \frac{C(z) - C_b}{C_t - C_b} = \frac{(e^{kz} - 1)}{(e^{kH} - 1)} = \frac{(e^{kH(z/H)} - 1)}{(e^{kH} - 1)} \qquad (2)$$

We observe that Eq. (2) can also be expressed in an alternative form, using SR, the separation ratio as follows

$$X = \frac{C(z) - C_b}{C_t - C_b} = \frac{C/C_b - 1}{SR - 1}$$

The parameter kH is indicative of the degree of separation. A high value of kH means that the X vs (z/H) plot is steep, which implies that the X values in the lower portion of the column are significantly lower than in the higher portion, indicative of a higher separation. Reference to this factor will follow throughout this paper, and kH will be used as a means of discriminating between the separation ability of each of the four separation methods (in short, the higher the value of kH, the better the separation ability).

We can analytically determine kH from the protein concentration measurement at the middle of the fermentor (C_b and C_t are already incorporated into X) as shown below. This confirms and elaborates on what we can estimate from typical graphical plots of Eq. (2), such as that shown in the Fig. 2, except with more precise resolution.

At $z=H/2$, $C=C_m$. Thus, from Eq. (2), we calculate

$$X_m = \frac{C_m - C_b}{C_t - C_b} = \frac{(e^{kH/2} - 1)}{(e^{kH} - 1)} = \frac{1}{(e^{kH/2} + 1)} \qquad (4)$$

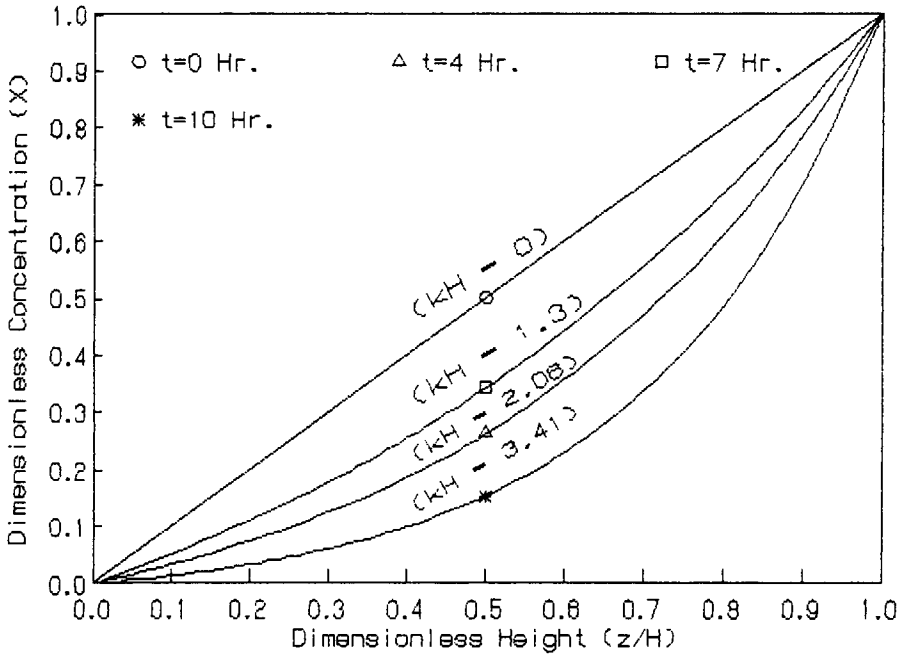

Fig. 2. Sample concentration profile within the bioreactor, as predicted by the model.

where: X_m = dimensionless concentration at the fermentor midpoint (at $z = H/2$). Thus, we can estimate kH directly using the simplified model and the midpoint protein concentration measurement

$$kH = 2 * \ln\left(\frac{1}{X_m} - 1\right) \tag{5}$$

Therefore, it is seen that a lower value of X_m implies a higher kH and, consequently, a greater separation.

PROTEIN SEPARATION STRATEGIES

Each of the four experiments conducted with our system displayed different separation effects for the four individual proteins. Based on these experiments, and assuming that each protein separation is an independent event, we propose a different time sequence of methods (strategy) to optimize the recovery of each individual protein. Each of these strategies consists of rerunning the four experiments, either singly or in combination, over a 10 h time interval to achieve maximum recovery for each polypeptide. The underlying assumption needed to develop each strategy is that a change from one experiment to another (say from no sparging to air sparging), with the exception for the case of added salt, can be run inde-

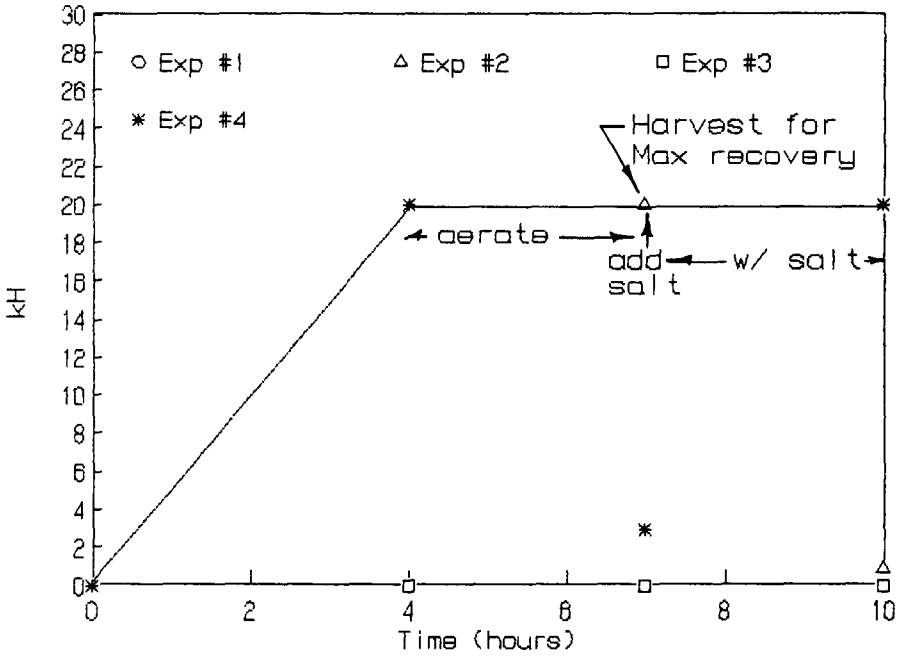

Fig. 3. Variation of kH with time for the 39 kD protein.

pendently of each other (that is in either order and without a "built-in" history), which of course must be verified by subsequent experiments. The strategies to maximize the concentration of each individual protein (to keep the kH values as high as possible for that desired protein) are highlighted in Figs 3–6 which in turn were developed from individual figures, like Fig. 2. It is expected that there will be interactions between the various experiments, so in practice the proposed ideal strategies will need to be modified accordingly. What is important, however, is the approach proposed here.

For example, for the 39 kD protein, the optimal strategy depicted in Fig. 3 consists of running Experiment 2 up to $t=7$ h with aeration. Then, for the next 3 h, Experiment 4 is brought into operation (aeration is discontinued, and salt is added). When data points for certain experiments do not appear on the figures, it is because they coincide with data points for the other experiments.

It is interesting to note that Experiment 1, *per se* does not contribute toward an optimal recovery strategy for any of the four desired proteins. This perhaps can be attributed to the fact that the rate of carbon dioxide bubbles generated *in situ* is insufficient to transport an appreciable amount of protein. The greater separation exhibited during Experiments 2 and 3 indicate that sparging the system (with either CO_2 or air) provides a suitable method for significant protein separation, at least for part of one of the proposed 10-h runs.

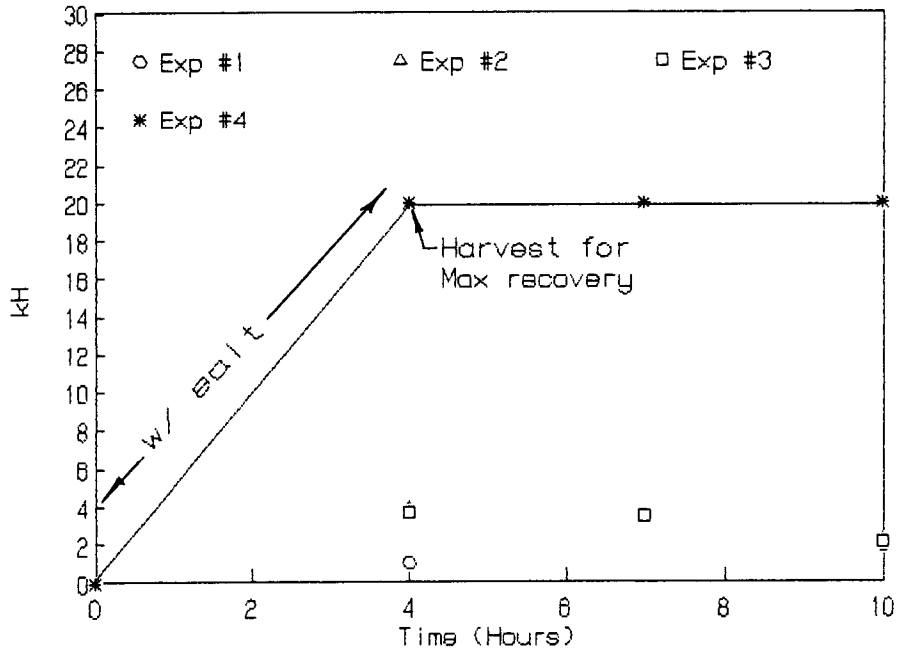

Fig. 4. Variation of kH with time for the 62 kD protein.

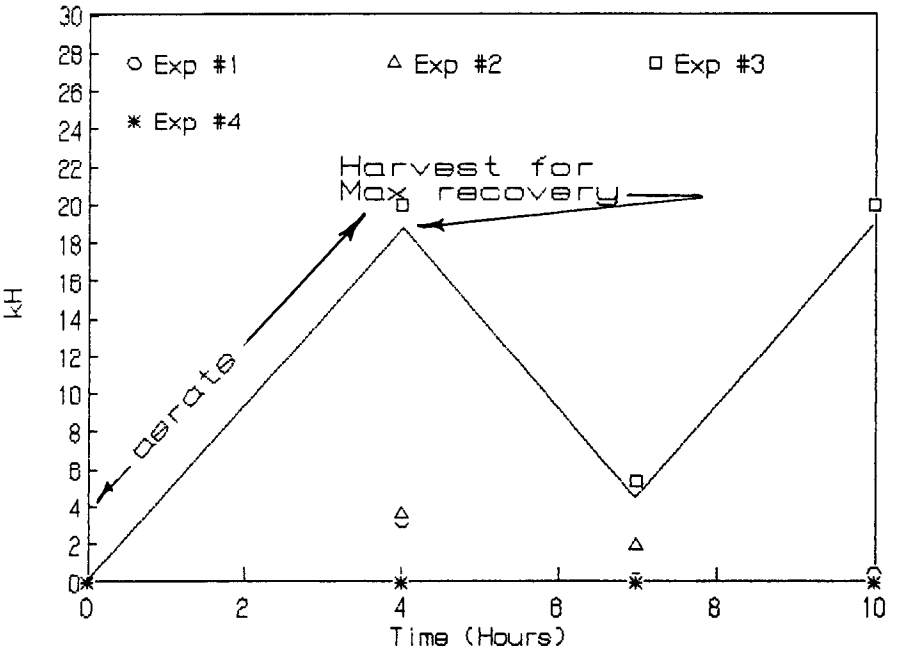

Fig. 5. Variation of kH with time for the 78 kD protein.

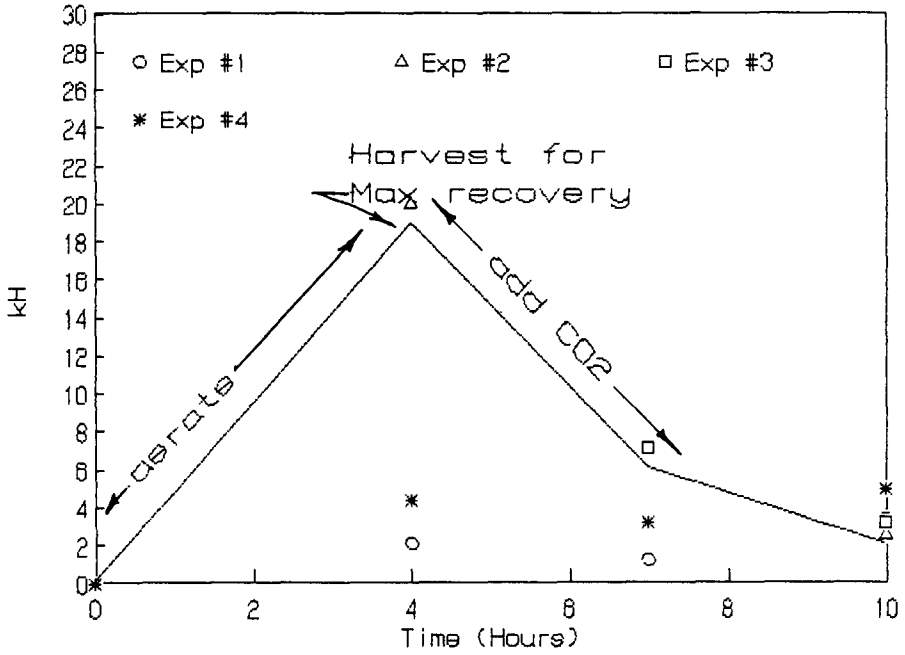

Fig. 6. Variation of kH with time for the 95 kD protein.

39 kD Protein

For Experiment 1, using a Figure like that shown for the 95 kD Protein (Fig. 2), along with Eq. (5), it is noted that $kH=0$ for all times, which implies minimal separation. The raw data used in this and the subsequent analyses were taken by William T. Effler, Jr. and are given in Table 1, and implicitly (for Ct/Cb) in Table 2.

For Experiment 2, when $t=4$ and 7 h, $kH=\infty$, which implies a very good separation. At $t=10$ h, $kH=0.81$, which means that the separation has dropped sharply in the final sampling interval. For Experiment 3, the results are the same as Experiment 1 ($kH=0$). For Experiment 4, when $t=4$ and 10 h, $kH=\infty$, which implies a very good separation. In between these times at $t=7$ h, $kH=2.9$, which means that there is a reversion to relatively poorer separation, compared to the 4 and 10 h sampling times. The above discussion is summarized in Table 2.

In the composite summary graph (Fig 7) for the optimal recovery of the 39 kD protein only, separation is highest in Experiment 2 at 4 and 7 h and in Experiment 4 at 4 and 10 h (all at $kH=\infty$). If the experiments could be considered as independent of their time histories, then shutting off the air and adding salt at $t=7$ h may lead to a $kH=\infty$ at $t=10$ h to maximize the recovery of the 39 kD protein. Since the addition of salt cannot be reversed in practice (in contrast to adding air), the salt is added at the last step (after 7 h) instead of earlier at 4 h. Again, confirmation by experiment is needed to verify this possibility. Note that for display purposes, $kH=20$ represents $20 \leq kH \leq \infty$ on all of the graphs.

Table 1
Experimental Data

Experiment no.	Polypeptide mol. wt., kD	(Cm/Cb) vs time			
		0 h	4 h	7 h	10 h
1	39	1	1	1.2	1.1
	62	1	1.2	2.5	1.6
	78	1	1.2	2.2	1.8
	95	1	1.5	3.3	1.4
2	39	1	1	1	1.1
	62	1	1.1	1	1.5
	78	1	1.2	1.8	1
	95	1	1	1.5	2
3	39	1	0.9	0.9	0.9
	62	1	1.1	1.3	2
	78	1	1	1.1	1
	95	1	1.1	1.9	2
4	39	1	0.9	1.2	1
	62	1	1	1	1
	78	1	1.5	1.1	1
	95	1	1.1	1.3	1.2

Table 2
Variation of kH with Time

Molecular Wt., kD	Time, h,	Experiment			
		1	2	3	4
39	4	0	∞	0	∞
	7	0	∞	0	2.9
	10	0	0.81	0	∞
62	4	1.07	3.67	3.67	∞
	7	∞	∞	3.47	∞
	10	1.94	2.2	2.14	∞
78	4	3.22	3.59	∞	0
	7	0.16	1.93	5.42	0
	10	0.45	∞	∞	0
95	4	2.06	∞	4.39	4.39
	7	1.3	3.37	7.2	3.22
	10	3.41	2.51	6.69	4.97

62 kD Proteins

Following the procedure for the 39 kD protein, the kH values for Experiments 1–4 are developed and summarized in Table 2. With reference to Fig. 8, for the 62 kD protein, Experiment 4 gives the best separation at all times, and thus should be the preferred vector to isolate the 62 kD protein above from the mixture of proteins.

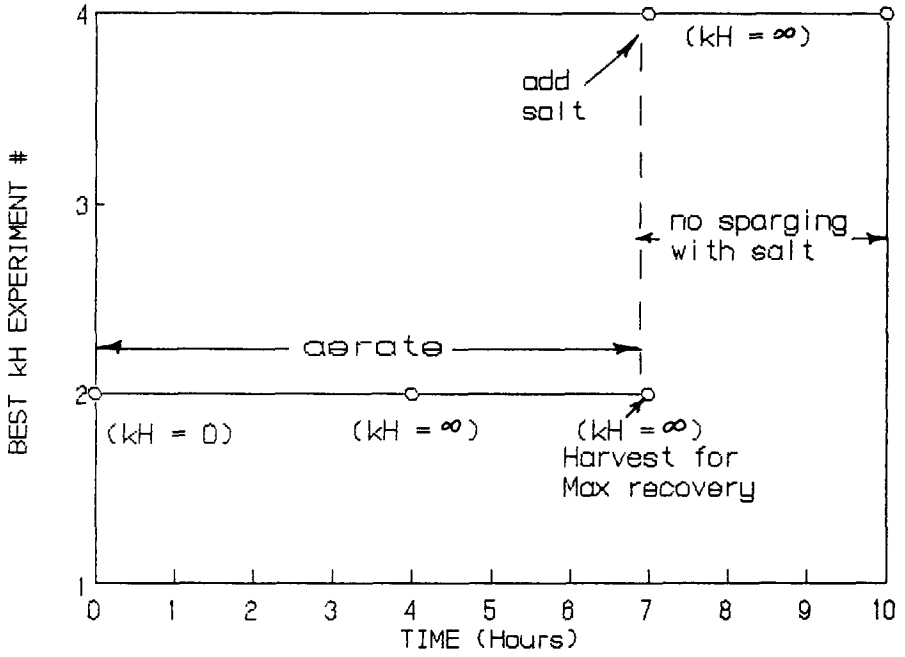

Fig. 7. Individual optimal strategy for 39 kD protein recovery.

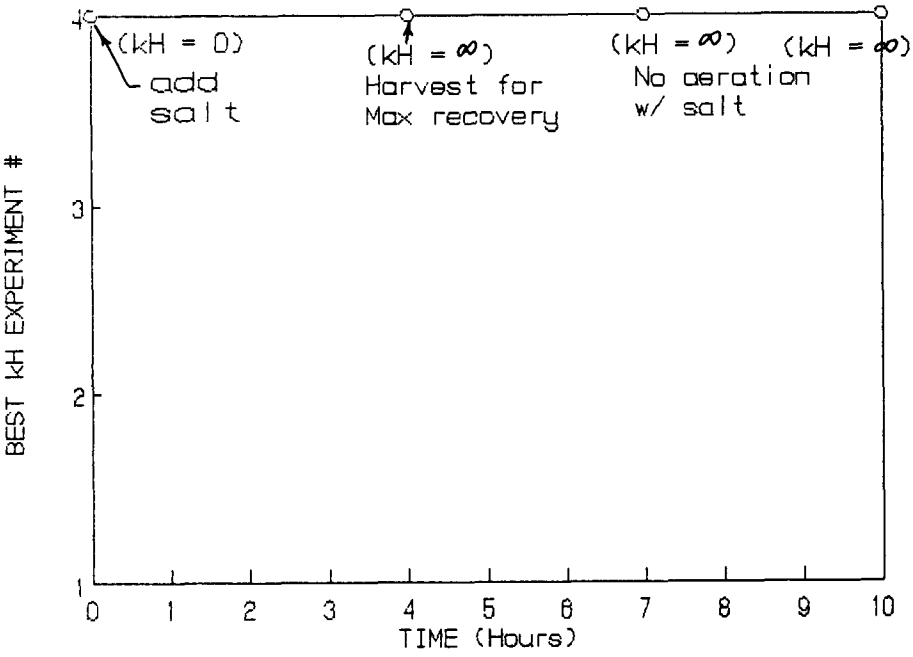

Fig. 8. Individual optimal strategy for 62 kD protein recovery.

78 kD Protein

Following the procedure for the 39 kD protein, the kH values for Experiments 1–4 are developed and summarized in Table 2. With reference to Fig. 9, for the 78 kD protein, separation is high in Experiment 2 at 10 h and Experiment 3 at 4 and 10 h. For maximizing the recovery of the 78 kD protein, using only the strategy described in Experiment 3 seems to be the best for all times, with optimal recovery at either 4 or 10 h (which are the times at which we obtain the most 78 kD protein).

95 kD Protein

In Fig. 2, it is seen that, for Experiment 1, the separation is highest at $t = 10$ h ($kH = 3.41$), with separation at $t = 4$ h ($kH = 2.06$), slightly better than at $t = 7$ h ($kH = 1.3$). This is summarized in Fig. 6. Following the procedure for the 39 kD protein, the kH values for Experiments 1–4 are developed and summarized in Table 2.

With reference to Fig. 10, for the 95 kD protein, Experiment 2 gives the best separation at $t = 4$ h. Assuming that the experiments are independent of time history, the optimal strategy would consist of running Experiment 2 for 4 h and then shutting off the air. Then, CO_2 is bubbled into the fermentor, and Experiment 3 is run up to $t = 10$ h.

POSSIBLE GLOBAL STRATEGY FOR SEPARATING THE FOUR MAJOR PROTEINS

Based on the experience presented here, and assuming that the effects are independent of each other, we formulate a global strategy for harvesting the four proteins. This strategy is depicted in Fig. 11:

Run Experiment 2 for 4 h. At 4 h, remove the 95 kD protein from the upper layer of the broth. At the end of 7 h, skim off the 39 kD protein from the upper broth layer and stop bubbling air. Start bubbling CO_2, thus bringing Experiment 3 into operation. Recover the 78 kD protein at the end of 10 h, leaving the 62 kD protein in the broth.

The effectiveness of this strategy in separating the individual proteins from the system could be tested experimentally.

CONCLUSIONS

The model originally developed for bubble fractionation of total proteins can be extended to apply to individual proteins as well. Comparing the data, using dimensionless graphs, points the way to optimal recovery strategies. The assumption of independent events (independent of time history) and reversible effects can now be tested with further experiments.

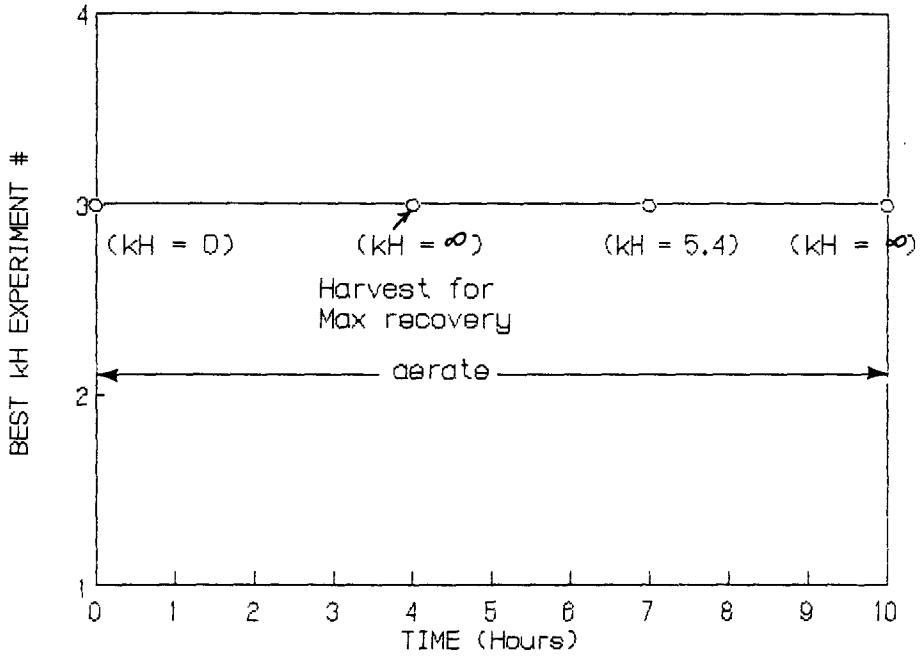

Fig. 9. Individual optimal strategy for 78 kD protein recovery.

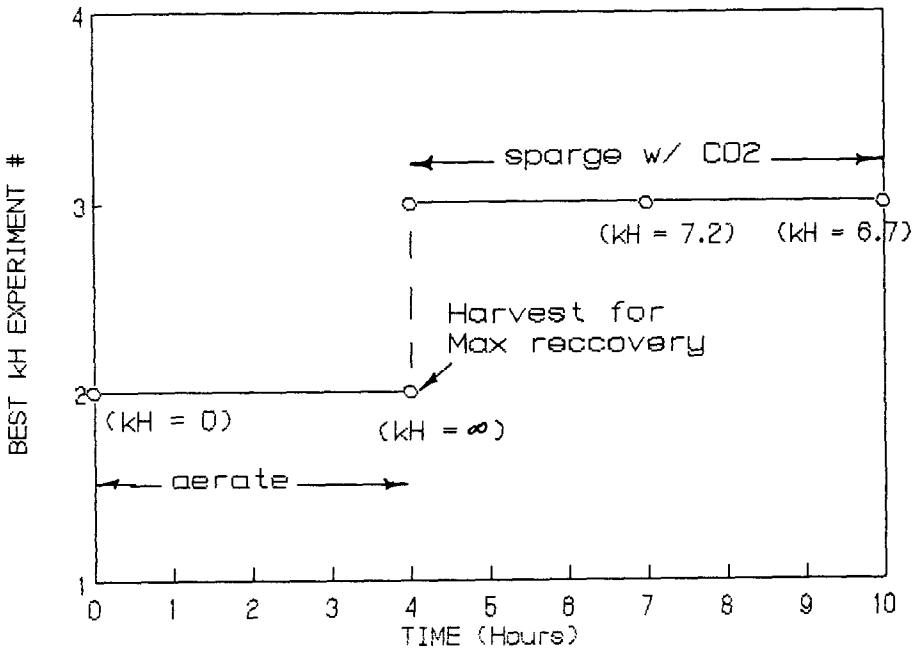

Fig. 10. Individual optimal strategy for 95 kD protein recovery.

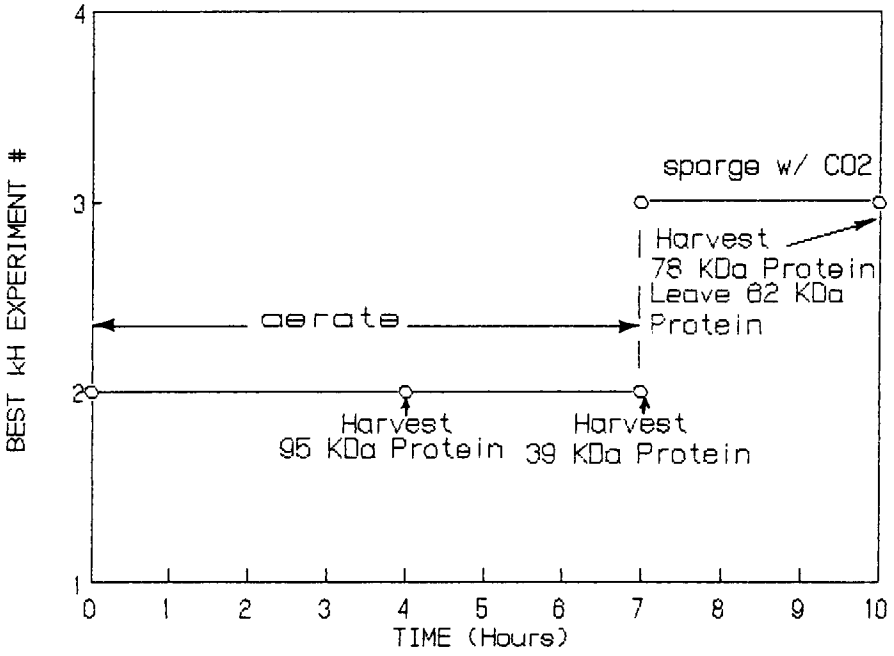

Fig. 11. Global protein separation strategy.

The different modes of operation, such as the addition of salt, all cause different responses to the tracked four key proteins, and together, may lead to strategies for preconcentrating and recovering them individually online.

Once the individual proteins are identified, the proteins must then be tested to see if they still retain their biological activity. Previous workers (*1*) have shown that certain enzymes did not exhibit any appreciable loss of catalytic activity when subjected to foaming/bubble fractionation. If such catalytic activity is retained in the suggested bubble fractionation procedures, for those proteins which are enzymes, then an inexpensive method for collecting individual proteins online may be applicable to typical fermentation systems. Removing the proteins online minimizes their possible degradation by extracellular proteases present in the system. This preconcentration method purifies proteins by removing much of the bulk fluid and may be an important first step in a multiple step sequence to isolate a purified, biologically-active polypeptide product.

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