PARTIAL REMOVAL OF PROTEINS FROM LACTIC ACID FERMENTATION BROTH AND RECOVERY OF PROTEINS FROM BREWERY WASTES BY FOAM FRACTIONATION TECHNIQUE

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ABSTRACT

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Foam separation is a simple and economic method for separation of surface-active molecules such as proteins and enzymes from aqueous solutions.

In this study, lactic acid broth, spent brewer's yeast extract and residual beer was used to investigate the applicability and efficiency of foam separation technique in partial purification of fermentation products and recovery of valuable components from industrial waste streams.

The effects of the process variables initial feed concentration, air flow rate, foaming time, liquid pool height and temperature on separation performance were studied and optimum conditions for removal of proteins from lactic acid broth was determined.

Highest enrichment (172.2) and separation ratio (314) with a high protein recovery (45.2 %) were obtained by foaming 200 ml of lactic acid broth with an initial feed concentration of 0.018 mg/ml at an air flow rate of 38.5 cm^3/min.

Selectivity of foam separation in protein purification, and its effect on protein structure was investigated in brewery wastes using SDS-PAGE and native PAGE, respectively.

Keywords: Foam Separation, Protein, Lactic Acid, Brewery Wastes

ÖZ

KÖPÜKLE AYRIŞTIRMA TEKNİĞİ İLE LAKTİK ASİT FERMENTASYON ORTAMINDAN PROTEİNLERİN KISMEN AYRILMASI VE BİRA ENDÜSTRİSİ ATIKLARINDAN PROTEİNLERİN GERİ KAZANIMI

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Köpükle ayırma proteinler ve enzimler gibi yüzey-aktif molekülleri sıvı çözeltilerden basit ve ekonomik şekilde ayırmak için kullanılabilecek bir tekniktir.

Bu çalışmada, laktik asit fermentasyon ortamı, atık bira mayası özütü ve atık bira, köpükle ayırma tekniğinin fermentasyon ürünlerinin kısmı saflaştırılmasında ve endüstriyel atıklardan değerli bileşenlerin geri kazanımında uygulanabilirliğini ve verimini araştırmak için kullanılmıştır.

Yapılan deneylerde ilk çözelti konsantrasyonu, hava akış hızı, köpürtme süresi ve ilk sıvı yüksekliği gibi proses değişkenlerinin ayırma performansına etkisi araştırıldı ve proteinlerin laktik asit ortamından ayrılması için uygun koşullar belirlendi.

Laktik asit fermentasyon ortamından proteinlerin ayrılmasında en yüksek performans değerleri (zenginleştirme katsayısı: 172.2, ayrıştırma katsayısı: 314 ve gerikazanılan protein miktarı: %45.2) 0.018 mg/ml protein içeren 200 ml örneğin 38.5 cm^3/min hava hızında köpürtülmesi ile elde edildi.

Köpükle ayırma tekniğinin protein saflaştırmada seçiciliği ve protein yapısına etkileri sırasıyla SDS-PAGE ve native-PAGE teknikleri kullanılarak araştırıldı.

Anahtar Kelimeler: Köpükle Ayrıştırma, Protein, Laktik Asit, Bira Endüstrisi Atıkları

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CHAPTER 1

INTRODUCTION

Historical Perspective

Waste process streams in many food, dairy and biochemical industries contain small amounts of valuable components like proteins. Economic and efficient recoveries of these components not only reduce the waste stream biological oxygen demand (BOD), but also create a source of high value products from low-value waste. Foam separation offers a simple and effective method for separating surface-active molecules from aqueous solutions (Monvar *et al*. 2001).

The application of foam fractionation for purification of proteins, including enzymes, has been considered for many years (Andrews and Schutz, 1945; Davis et al., 1949; London *et al.*, 1954; Charm *et al.*, 1966; Holmstrom, 1968; Ahmad, 1975a and 1975b; Sarkar et al., 1987). Renewed interest in recent years reflects the continued need for relatively cheap, selective methods for purification of a wide range of biological products.

The potential application of this method lies in the early stages of a downstream purification regime, where the separation and concentration of a dilute protein from a large volume of crude starting material is required (Brown et al., 1999a). Substances to be recovered can be proteins, enzymes or compounds such as

biosurfactants which, it has been shown, can be recovered directly into the foam phase (Phae and Shoda, 1991). It may also be possible to use foam separation to remove surface active contaminants (Varley et al., 1996; Suzuki and Maruyama, 2002; Suzuki *et al*., 2003) and surface inactive constituents from industrial wastewater by using a surfactant (Huang *et al*., 1982 and 1988).

1.1 ADSORPTIVE BUBBLE SEPARATION TECHNIQUES

All techniques or methods of separation, whether physical or chemical, are based on differences in properties. The adsorptive bubble separation techniques are based on differences in surface activity. Material, which may be molecular, colloidal, or macro-particulate in size, is selectively adsorbed or attached at the surfaces of bubbles rising through the liquid, and is thereby concentrated or separated. A substance which is not surface active itself can often be separated by the addition of an appropriate surfactant which either combines with this substance or simply adsorbs it at the bubble surface. This can occur through the formation of a chelate, electrostatic attraction, or some other mechanism (Lemlich, 1972; Okamoto and Chou, 1997).

1.1.1 Classification of Adsorptive Bubble Separation Techniques

There are a number of individual adsorptive bubble separation techniques that are divided into two main groups: *foam separation* which requires the generation of a foam or froth to carry off material, and *nonfoaming adsorptive bubble separation*, which does not. Figure 1.1 shows the schematic classification of these techniques.

Bubble fractionation is the transfer of material within a liquid by bubble adsorption or attachment, followed by deposition at the top of the liquid as the bubbles exit.

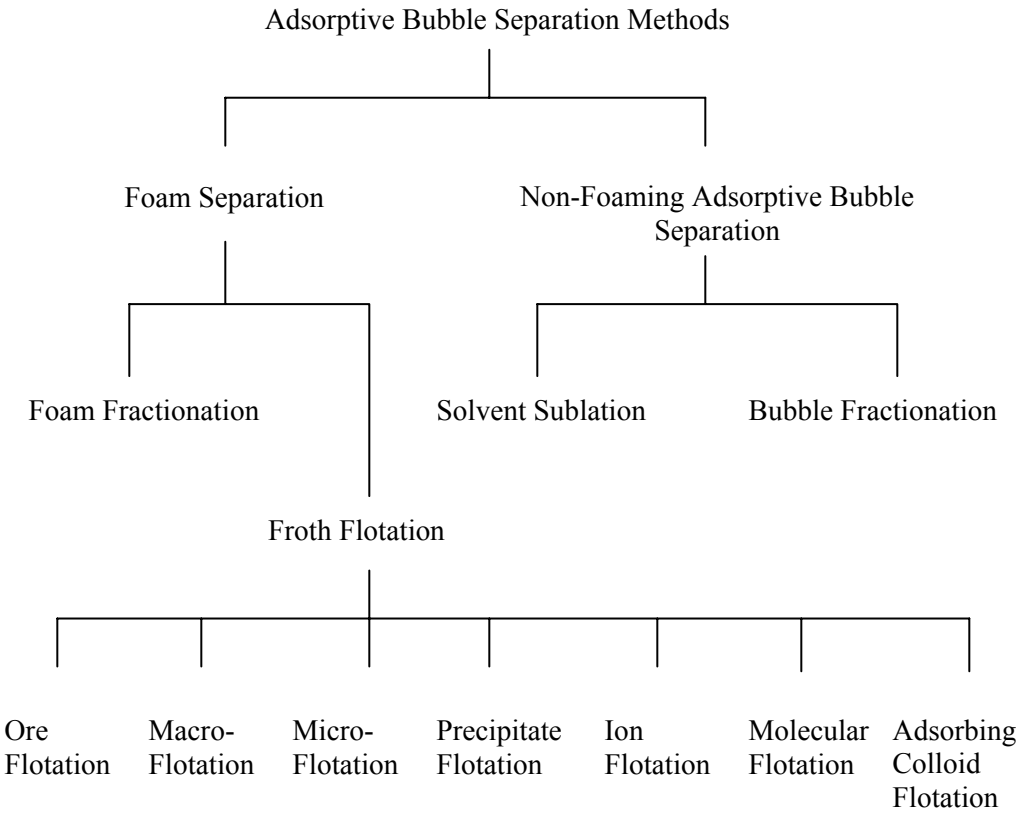


Figure 1.1 Schematic classification of the adsorptive bubble separation techniques (Taken from Lemlich, 1972).

Solvent sublation is the transfer of material to an immiscible liquid placed at the top of the main liquid.

Foam fractionation is the foaming off of dissolved material from a solution by adsorption at the bubble surfaces.

Froth flotation, or simply flotation is the removal of particulate material by frothing (foaming). Froth flotation has many subdivisions. *Ore flotation* is the separation of minerals. *Macroflotation* is the separation of macroscopic particles. *Microflotation* is the separation of microscopic particles, especially colloids or microorganisms. In *precipitate flotation*, a precipitate is formed and then foamed

off. *Ion flotation* is the separation of surface inactive ions by foaming with a collector which yields an insoluble product, particularly if the product is removed as a scum. Similarly, *molecular flotation* is the separation of surface inactive molecules by foaming with a collector which gives an insoluble product. Finally, *adsorbing colloid flotation* is the separation of a solute through adsorption on colloidal particles which are then removed by flotation (Lemlich, 1972).

1.1.2 Adsorption

Under equilibrium conditions, adsorption of surface-active materials from bulk solution at a gas-liquid interface can be quantitatively described by the Gibbs equation:

$$d\gamma = - \mathbf{R}T \sum \Gamma_i \, d \ln a_i \qquad (1)$$

where γ is the surface tension, a_i is the activity of the adsorbed solute, **R** is the gas constant, T is the absolute temperature, and Γ is the surface excess of the adsorbed solute (i.e., concentration at the surface).

This equation is often difficult to apply in practice since it requires the knowledge of activity coefficients. Therefore, in practice it is applied only in certain special cases. In the case of sufficiently low concentrations of surface-active solute, the activity can be assumed to be unity, and the above equation simplifies to equation (2) (Perry, 1968; Okamoto and Chou, 1997):

$$\frac{\Gamma}{C} = - \frac{1}{RT} \frac{d\gamma}{dC} \qquad (2)$$

The value of $d\gamma/dC$ may be determined from the slope of γ-C plots. A hypothetical surface tension vs. surface-active species concentration curve is shown in Figure 1.2.

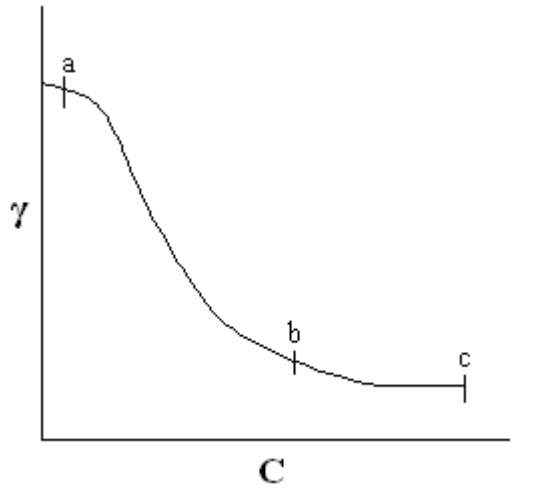


Figure 1.2 Hypothetical surface tension versus concentration for a surface-active agent in a solution

In the very low concentration region, i.e. below *a* in Fig.1.2, little adsorption can occur, since very few surface-active species are present and the surface tension is close to that of the solvent. The surface excess is then close to zero and the separation occurs only to a small extent. At intermediate concentrations (between *a* and *b*), γ decreases with increasing concentration of the surface-active material; i.e., a negative slope occurs. Γ/C becomes constant and separation of surface-active material from the bulk solution can be achieved. The average concentration of surface-active material in this region is 10^{-7} to 10^{-3} M depending on various conditions. In the region above concentration *b*, the slope becomes close to zero.

This is the region in which micelles form, and the point at which the curve levels off is called the *critical micelle concentration* (CMC). According to equation (2) Γ/C should become close to zero and no removal should occur by foam separation processes. Practically, foam nevertheless forms in the micelle region and separations can be successfully carried out; however, better separations would occur below the CMC (Okamoto and Chou, 1997).

1.2 FOAM FRACTIONATION

1.2.1 Foam Structure

Foam is a coarse dispersion of gas in liquid in which volume fraction of liquid in foam is very small: typically 0.1 and reduces to 0.05 or less with drainage. Foams can be divided into two extreme types: wet foams and dry foams. In wet foams, liquid volume fraction is high and bubbles are separated from each other by liquid layer of considerable thickness and the bubbles are nearly spherical in shape. Except for very small bubble sizes or very viscous liquids, wet foams do not normally exist at distances higher than one bubble diameter above the liquid pool. Concentrated or "dry" foams are mostly gas phase, and consist of polyhedral gas cells separated by thin liquid films which may develop from more dilute foams as a result of liquid drainage, or directly from a liquid of relatively low viscosity (Leonard and Lemlich, 1965; Shaw, 1992, Bhattacharjee, 1994).

The physicist Plateau (1873) first investigated the geometrical aspects of foams. Based on the structural stability and minimization of surface energy, he proposed that,

- three and only three films meet at an edge, and the angle between the adjacent films are 120º

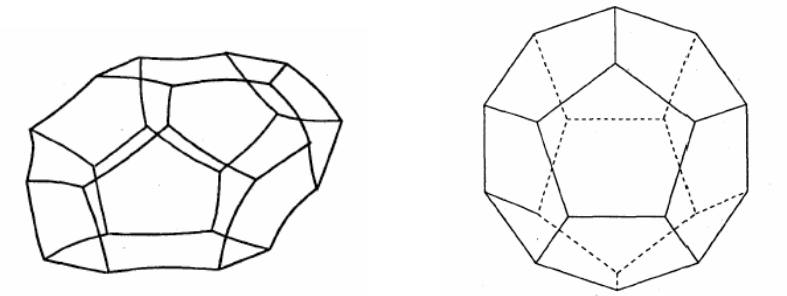
- four of these edges meet at a point, and the angle at which they meet is the tetrahedral angle (109º28˙16”).

These edges are referred to as Plateau borders (Bhattacharjee, 1994).

1.2.1.1 Bubble Shape

Close observation of polyhedral foam bubbles in the foam reveals that foam comprises of square, pentagonal and hexagonal films.

Several predictions were made on the shape of bubbles, i.e. tetrakaidecahedron, pentagonal dodecahedral, and β-tetrakaidecahedron. Based on these predictions, it was concluded that β-tetrakaidecahedron is the correct shape of close packed cells. It has 14 faces: eight pentagonal, four hexagonal and two square (Figure 1.3a). However, since it is difficult to model, pentagonal dodecahedron (Figure 1.4b) is found more suitable in modeling (Bhattacharjee, 1994).



(a) (b)

Figure 1.3 (a) β-tetrakaidecahedron bubble; (b) Pentagonal dodecahedron bubble

1.2.1.1.1 Pentagonal Dodecahedron

A single pentagonal dodecaghedron bubble consists of twelve films and thirty Plateau borders. The shapes of the films are pentagonal and each film is shared by two bubbles, whereas each Plateau border is shared by three films. Plateau borders are classified into two kinds: horizontal Plateau borders and vertical Plateau borders. The horizontal Plateau borders make an angle of 72º and the vertical Plateau borders make an angle of 36º with the vertical.

1.2.1.1.2 β-Tetrakaidecahedron

β-Tetrakaidecahedron bubbles consist of fourteen films: eight pentagonal, four hexagonal and two square. Similar to the pentagonal dodecahedral bubble the type of Plateau border are also decided by the angle it makes with the vertical. Properties of the Plateau border for β-Tetrakaidecahedron bubble are given in Table 1.1.

Table 1.1 Properties of the Plateau border for β-Tetrakaidecahedron bubble (Bhattacharjee, 1994).

Angle with the vertical	*Name of the Plateau border*	*Number of Plateau border*	*Number of Plateau border per bubble*
90º	Horizontal	6	2
0º	Vertical	6	2
60º	Oblique	24	8

1.2.1.2 Geometry of Plateau Borders

Most of the liquid present in the foam is in the Plateau borders. The Plateau borders form a network along the length on the foam column. The actual shape of a Plateau border is very complex. As it is difficult to calculate the flow through such a complex geometry, the shape was modeled by simple structures, such as circular, equilateral triangle, three circular cylinders touching each other, etc. Later the Plateau border was idealized as a pipe of triangular cross sectional area (Figure 1.4) (Bhattacharjee, 1994).

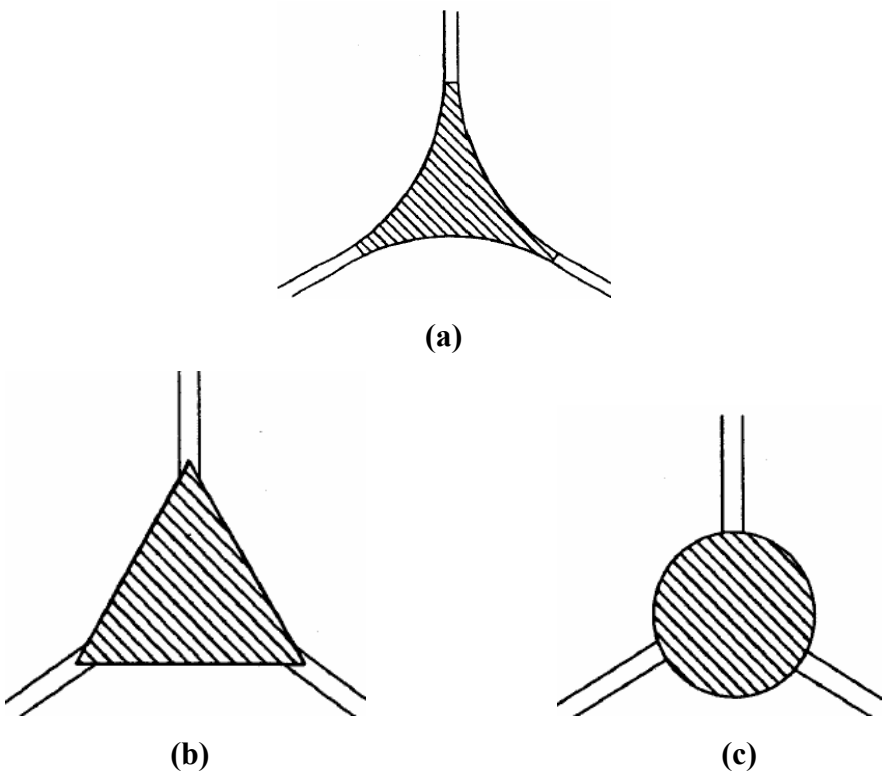


Figure 1.4 Schematic diagram of different shapes of Plateau borders

1.2.2 Foam Drainage

Initially formed liquid films of foam are relatively thick and drainage takes place mainly due to gravitational forces. At this stage, surface active components play an important role in restricting liquid flow to a level where local disturbances and consequent film rupture is minimized. When the films have attained a thickness of the order of micrometers, gravitational flow down the laminar regions becomes extremely slow. The predominant drainage mechanism then involves liquid discharged locally at the Plateau borders, where the liquid capacity is relatively high. Subsequent drainage then takes place through the network of Plateau borders that exists throughout the foam.

The discharge of liquid from the laminar part of a thin film is governed by the pressure of the liquid in this region compared with that of the liquid in the Plateau borders. At least three factors are likely to be involved, *van der Waals* attractive forces that favor film thinning, and the overlapping of similarly charged *electric double layer* which opposes film thinning. The other important factor is the *capillary pressure*, which favors film thinning. This arises because the pressure of the adjacent gas phase is uniform and, therefore, the pressure of liquid in the Plateau borders, where the interface is curved, is less than that of liquid in the laminar film. Depending on the balance of these forces, a film may either thin continuously and eventually rupture, or attain an equilibrium thickness (Shaw, 1992).

1.2.3 Foam Stability and Film Rupture

Only transitory foams can be formed with pure liquids, and a surface active component – a foaming agent – is necessary to achieve any reasonable degree of stability.

The stability of a foam depends upon two principle factors: the tendency for the liquid films to drain and become thinner, and their tendency to rupture as a result of random disturbances. Other factors which may significantly influence foam stability include evaporation and gas diffusion through the liquid films. Owing to their high interfacial area (and surface free energy), all foams are unstable in the thermodynamic sense. Some distinction can be made, however, between *unstable* and *metastable* foam structure. *Unstable* foams are typified by those formed from aqueous solutions of short-chain fatty acids or alcohols. The presence of these mildly surface-active agents retard drainage and film rupture to some extent, but does not stop these processes from continuously taking place to the point of complete foam collapse. *Metastable* foams are typified by those formed from solutions of soaps, synthetic detergents, proteins, saponins, etc. The balance of forces is such that the drainage of liquid stops when a certain film thickness is reached and, in the absence of disturbing influences (such as vibration, draughts, evaporation, diffusion of gas from small bubbles to large bubbles, heat, temperature gradients, dust and other impurities), these foams would persist almost indefinitely.

In addition to film drainage, the stability of a foam depends on the ability of the liquid films to resist excessive local thinning and rupture which may occur as a result of various random disturbances. A number of factors may be involved with varying degrees of importance, depending on the nature of the particular foam in question.

One of them is the *Gibbs-Marangoni surface elasticity effect*, which is an important stabilizing effect in foams that are formed from solutions of soaps, detergents, etc. If a film is subjected to a local stretching as a result of some external disturbance, the consequent increase in surface area will be accompanied by a decrease in the surface excess concentration of foaming agent and therefore, a local increase in the surface tension (Gibbs effect). Since certain time is required

for surfactant molecules to diffuse to this surface region and restore the original surface tension (Marangoni effect), this increased surface tension may persist for long enough to cause the disturbed film region to recover to its original thickness.

An absence of the Gibbs-Marangoni effect is the main reason why pure liquids do not foam. Foams from moderately concentrated solutions of soaps, detergents, etc., tend to be less stable than those formed from more dilute solutions. With the more concentrated solutions, the increase in surface tension which results from the local thinning is more rapidly nullified by diffusion of surfactant from the bulk solution. The opposition to fluctuations in film thickness by corresponding fluctuations in the surface tension is, therefore, less effective.

The mechanical properties of the surface films often have a considerable influence on foam stability. Several considerations may be involved. A high bulk liquid viscosity simply retards the rate of foam collapse. High surface viscosity, however, involves strong retardation of bulk liquid flow close to the surfaces and, consequently, the drainage of thick films is considerably more rapid than that of thin films, which facilitates the attainment of a uniform film thickness. Surface elasticity facilitates the maintenance of a uniform film thickness; however, the existence of rigid, condensed surface films is detrimental to foam stability, owing to the very small changes in area over which such films show elasticity (Shaw, 1992).

1.2.4 Mechanism of Foam Fractionation

Foam Fractionation is an adsorptive bubble separation technique in which soluble, surface-active substances are partially removed from the solution by preferential adsorption at the gas-liquid interface generated by a rising ensemble of bubbles through the solution. As the foam thus formed rises up the column, the liquid hold-up decreases because of drainage. Collapse of the rising foam at the top of the

column leads to the concentration/separation of the surface-active solute, as a result of recovery of the adsorbed solute from the gas-liquid interface (Uraizee and Narsimhan, 1990).

Two major physical processes are responsible for separation of surface-active substances in the foam fractionation technique: (1) adsorption of the objective substance onto the bubble surface in a dispersed bubble bed, and (2) enrichment in foam bed due to coalescence of bubble cells and liquid drainage. The former is well characterized by the physicochemical properties of the objective substance, which influence the adsorption density at the bubble-liquid interface and the production rate of bubble liquid interfacial area. The latter is common in all foam separations and would be a function of liquid properties, operating conditions, and liquid content in foam layer (Maruyama, 2000).

1.2.5 Modes of Operation in Foam Fractionation

A foam fractionation column can be operated in a number of ways, which are schematically shown in Figure 1.5 and Figure 1.6. Some of these include batch-mode, semi-batch mode, continuous mode and stripping, enriching and combined modes for continuous type of operation.

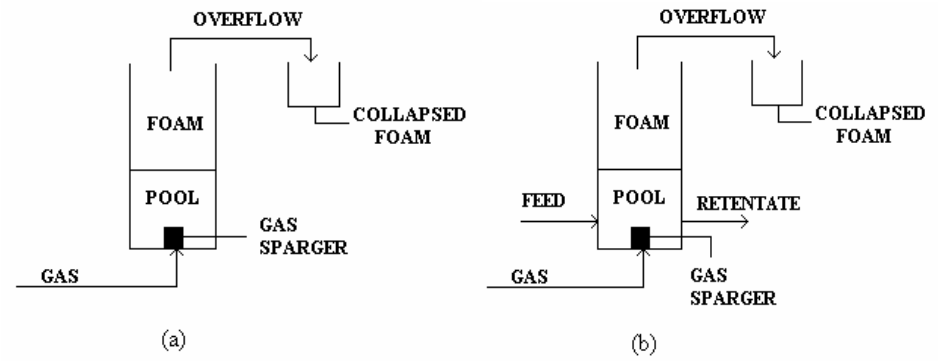


Figure 1.5 Foam fractionation in the simple mode
(a) batch operation, **(b)** continuous flow operation,

Batch column: A batch of liquid is fed into the column once for all. Then the gas is passed into the column. As soon as the foam reaches the desired height the gas supply is shut off. The liquid in the foam column continues to drain with time until an equilibrium hold-up is established.

Semi-batch Column: Here the foam continuously forms at the foam liquid interface and breaks at the foam gas interface. The gas released escapes, whereas the liquid drains back to the liquid pool. At the top, some liquid may get entrained and some make up liquid in the pool may be required.

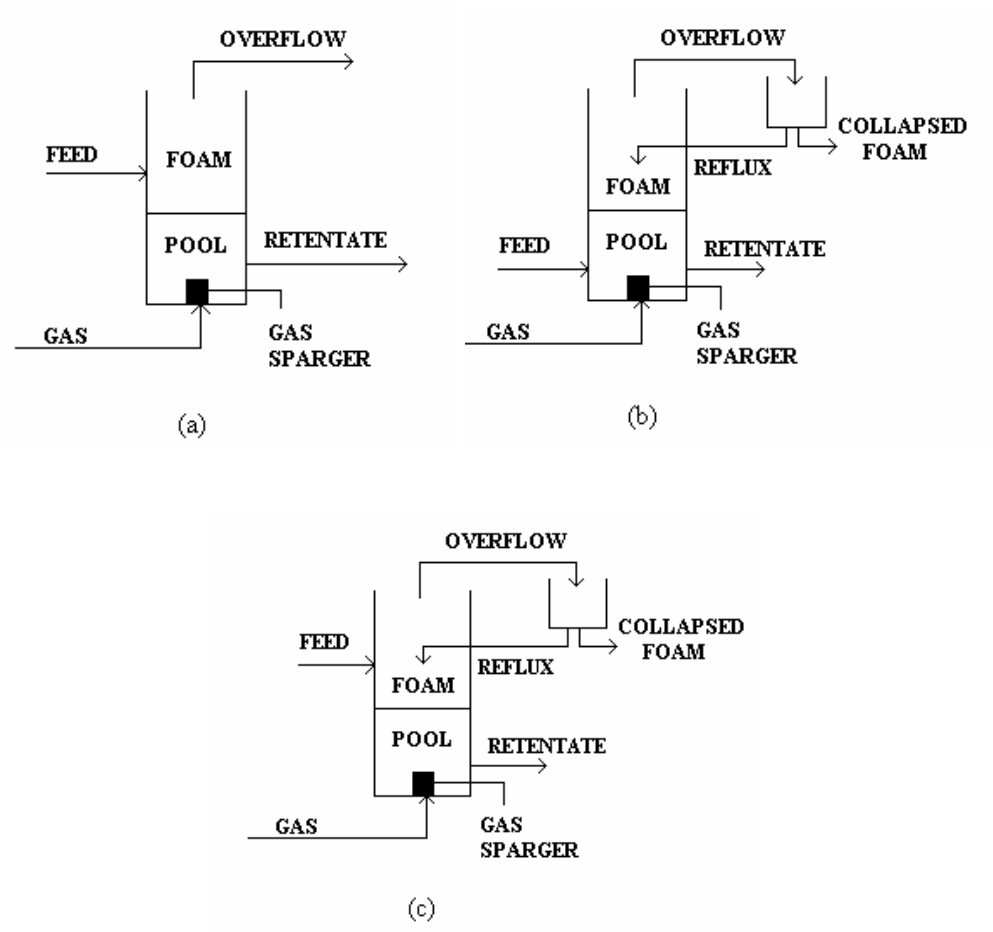


Figure 1.6 Foam fractionation in continuous mode

(a) Stripping mode, **(b)** enriching mode, **(c)** combined mode

Continuous Column: Continuous column has inlet and exit of the liquid from the pool and foam continuously forms at the foam liquid interface and collected from the foam-gas interface.

In the stripping mode, feed enters the foam some distance above the liquid pool and trickles down counter-currently through the rising foam. This tends to replace

interstitial liquid pool composition with interstitial liquid of feed composition. The overall result is an improvement in the degree of stripping.

The enriching mode involves the use of reflux. A portion of the collapsed foam is fed back to the top of the column. From the top of the column, it trickles back down through the rising foam. Since the reflux is much richer than the rising interstitial liquid, the mass transfer resulting from this counter-current action can greatly cascade the enrichment and yield a much higher concentration in the foamate than would be obtainable without the reflux (Perry, 1968).

1.2.6 Factors Affecting Foam Fractionation Efficiency

1.2.6.1 Initial Feed Concentration

Initial feed concentration is an important factor that affects separation efficiency significantly. When the concentration is very low, little adsorption can occur since very few surface-active constituents are present. Therefore, separation occurs only to a small extent since surface excess is close to zero. On the other hand, when the feed concentration is very high (higher than the critical micelle concentration) proteins form aggregates with the hydrophilic regions in contact with the surrounding solvent, and sequestering the hydrophobic regions in the micelle center. As a result of this, protein molecules have less contact with the bubble surfaces and this retards the adsorption of proteins on the interface of bubbles (Okamoto and Chou, 1997).

As the initial protein concentration in the feed is increased –keeping the other process variables constant- the enrichment ratio decreases and the foam-flow rate increases. This increase in the foam flow rate is attributed to the formation of a more stable foam at higher values of initial protein concentration in the feed, which leads to increased amount of bulk liquid taken into the foam phase with a

consequent reduction in the protein concentration in the foam and also enrichment (Brown *et al.*, 1999a).

1.2.6.2 Feed Flow Rate

Feed flow rate is an important factor in continuous foam fractionation and it varies the protein concentration of liquid pool and residence time of proteins in liquid pool.

High feed flow rates result in lower enrichments. When feed flow rate is increased while keeping the other variables constant, the rate of mass of protein supplied to the system is increased. However, despite a higher mass of protein being taken into the foam phase, lower enrichments are obtained since the volume of foam collected, i.e., foam flow rate, is also higher at increased feed flow rates.

Generally observed effect of increasing the feed flow rate is a decrease in protein recovery. Although high feed flow rates cause the liquid hold-up in the foam to increase, protein removal efficiency form the liquid pool is actually decreased since the fraction of protein recovered in the foam is reduced (Brown *et al.* 1999a).

1.2.6.3 Gas Flow Rate

Gas flow rate is another factor that affects separation efficiency significantly since it varies the retention time of gas bubbles inside the liquid pool, residence time of foam in the column and liquid drainage.

Enrichment of protein in foam phase decreases as gas-flow rate is increased. High levels of gas-flow rate cause greater amounts of liquid to be taken into the foam, and reduced residence time of foam in the column, which limits liquid drainage and internal reflux. Both these factors result in an increase in foam-flow rate, which leads to a reduction in protein concentration in the foam, and hence, to

lower enrichments. Conversely, at lower gas-flow rates, enrichment is greater because less liquid is taken into the foam, and the residence time of foam in the column is greater, allowing more time for drainage, and resulting in an increase in coalescence.

High levels of gas-flow rate increase protein recovery. High gas-flow rates increase the interfacial area available for protein adsorption and hence, the mass of protein entering the foam (Ahmad, 1975a). Furthermore, high levels of gas-flow rate increase the amount of interstitial liquid taken into the foam, which also contributes to the mass of protein entering the foam and results in a high foam-flow rate. Thus, these effects have the overall effect of causing the protein recovery to increase (Brown *et al*. 1999a).

1.2.6.4 Bubble Size

Studies on the effect of bubble size on foam fractionation efficiency have resulted in conflicting evidence. Some researchers (Brown *et al*., 1990; Bhattacharya *et al.*, 1991; Uraizee and Narsimhan, 1996) found that larger bubble sizes promoted enrichment, whereas others found no dependence between enrichment and bubble size (Gehle and Schugerl, 1984). However, Gehle and Schugerl (1984) suggested that an increase in gas-liquid interfacial area with smaller bubbles would reduce the protein concentration in the retentate and therefore increase the separation efficiency.

Previous studies on the effect of bubble size on recovery of proteins revealed that smaller bubbles promoted protein recovery (Brown *et al.*1990; Brown *et al.*1999a; Bhattacharya *et al.* 1991; Uraizee and Narsimhan 1996). The increase in protein recovery was explained by the increase in interfacial area available for protein adsorption, higher amount of protein entering the foam phase and formation of foam with a greater liquid hold-up.

1.2.6.5 Liquid Pool Height

An increase in the liquid pool height promotes the enrichment of protein in the foam phase. By extending the liquid height, the contact time between the bubbles and the liquid is increased and more time for the protein to diffuse onto the surface of the rising gas bubbles is allowed (Ahmad, 1975a).

1.2.6.6 pH of the Feed Solution

The pH of the feed solution is an important variable that affects the performance of the foam fractionation of proteins. Some researchers found that the effect of pH on enrichment is small (Brown *et al.*, 1990; Uraizee and Narsimhan, 1996) in the pH ranges studied, while others found that the effect of pH is significant (Montero *et al.,* 1993; Tanner *et al*., 2004). For example, the maximum enrichment was obtained at the isoelectric point of BSA (Ahmad, 1975a; Schnepf and Gaden, 1959; Lockwood *et al.*, 2000). However, an exception to the pI being the pH point where maximum enrichment is obtained was observed by Brown *et al.* (1990) and Uraizee and Narsimhan (1996). Both groups observed minimum enrichment for BSA at the pI. Generally, it is expected that at the pI of a protein, the surface adsorption is enhanced as a result of both the decreased electric repulsive forces and the minimum solubility of the protein in the solution. Generally, foam stability was observed to be maximized at the pI. The conflicting enrichment results regarding the pI may result from the interaction between the pH and the other factors such as the bubble size, feed concentration, and the foam column characteristics (Tanner *et al.*, 2003). For example, Brown *et al.* (1990) and Uraizee and Narsimhan (1996) observed smallest bubble size at the isoelectric point pH of BSA.

1.2.6.7 Foam Height

The foam height was found to have a significant effect on the separation, and the effect was very pronounced near the foam-liquid interface.

Higher foam heights result in an increase in the foam protein concentration as a result of longer drainage times and increased coalescence, both of which reduce foam flow rate. The lower liquid amount in the foam, therefore promotes a greater protein concentration in the foam phase, resulting in higher enrichments (Ahmad, 1975a).

1.2.6.8 Temperature

There are few results on the effect of temperature on foam fractionation performance. It was reported that a decrease in temperature, results in a reduction in liquid drainage from the foam which diminishes the foam protein concentration (Schügerl, 2000). Gehle and Schügerl (1984) found higher separation and enrichment for BSA can be attained at 40ºC than at lower temperatures.

1.2.6.9 Ionic Strength

The influence of ionic strength on the foaming behavior depends on the type of the adsorbed protein and the concentration of salt (Zhang *et al*., 2004).

Lockwood *et* al. studied the effect of ionic strength on the binary mixture of lysozyme and BSA and found that as the ionic strength is increased, the recovery of surface active BSA in the foam phase increases, while the less surface active lysozyme is recovered in the retentate phase (Lockwood *et al*., 2000).

Zhang *et al*. observed improvement in foamability of skim milk powder when the ionic strength is increased and an increase in the foamability of whey protein isolates and a reduction with further increase in ionic strength (Zhang *et al*., 2004).

Webb *et al.* observed that enrichment of BSA decreases and recovery remains constant when the ionic strength is increased, and reported that as the ionic strength increases, the increased interaction between the charged solution and charged interface could decrease the rate at which liquid flows back to the solution pool. This would increase the rate at which liquid is collected as foamate. As a result the foam concentration would decrease, reducing the enrichment (Webb *et al*., 2003).

1.2.6.10 Feeder Position

It was found that a change in the feed position into the solution resulted in a change in the retentate concentration. The introduction of the feed at the foam-liquid interface reduced the retentate concentration slightly and increased the retentate volume. On raising the feed position to higher levels within the foam, the drainage concentration decreased continuously, but the efficiency of the process increased.

A decrease in the drainage concentration and an increase in the enrichment ratio are found when the liquid feed is introduced in the foam at any height (Ahmad, 1975a).

1.3 SEPARATION OF PROTEINS BY FOAM FRACTIONATION

The separation of proteins from a culture medium or from industrial process streams is usually carried out by adsorption, ion exchange, chromatography,

ultrafiltration and precipitation. Foam fractionation offers several advantages over these methods (Saleh and Hossain, 2001).

These include its mechanical and operational simplicity and therefore, low capital and maintenance costs (Monvar *et al*. 2001) and high separation efficiency for dilute feeds. Foam fractionation works best at low concentrations (<1 wt %) while other separation methods such as ultrafiltration are economical only at higher concentrations (Brown. *et* al., 1990). Furthermore, the process can be carried out in either batch or continuous mode and it requires no thermal energy, which can denature biologically active constituents (Monvar *et al*. 2001). The possible denaturing effect of foaming on sensitive proteins is a drawback of this method (Brown *et al.*, 1999b).

1.3.1 Protein Adsorption at Gas-Liquid Interface

Proteins have a hydrophobic/hydrophilic nature, for this reason, they tend to orient themselves so that the exposure of the hydrophobic portion of the protein to the aqueous solution is minimized. Thus, in systems containing air-water interfaces, proteins will tend to accumulate at these interfaces, forming a surface layer of protein oriented in such a way that only their hydrophilic regions are exposed to water.

If the surface tension of the air-water interface is greater than the internal tension in a protein molecule, then the surface area of the protein increases until two tensions equal, i.e. the protein molecule unfolds. If the process of surface adsorption and unfolding of protein stops after the formation of an equilibrium monolayer, the amount of adsorbed protein would be very small. However, depending on the degree of surface hydrophobicity and the characteristics of protein in question, additional processes such as gas-to-liquid surface phase transitions, surface precipitation, and the formation of surface sub-layers can occur

in the adsorbed films. In many cases, the adsorbed protein molecules can be rapidly released from the surface and surface sub-layers, refold, and may exchange with the bulk protein molecules. Alternatively, because of slow refolding kinetics proteins can become irreversibly adsorbed at the interface. These processes of adsorption and release of structurally-perturbed protein molecules into the bulk solution are thought to be one of the main causes of protein aggregation and denaturation during processing.

In adsorption of proteins at interfaces, globular proteins are characterized in terms of structural flexibility and hydrophobicity. Flexible hydrophobic proteins attain monolayer coverage of the air-water interface in a short period of time and are generally more surface active at hydrophobic surfaces than less flexible hydrophilic proteins. The driving force for the protein adsorption is the decreased entropy of the water molecules that are ordered around the hydrophobic protein domains when the protein is in the bulk solution. Thus, the role played by the relative degree of proteins only differing in their hydrophobicities, the more hydrophilic one will interact more strongly with the surface and will form a monolayer more quickly than does the less hydrophobic one.

Protein flexibility is important in protein spreading that occurs at the interface. A flexible protein can expose additional non-polar residues, leading to an increased binding strength to the surface. The protein flexibility therefore dictates the number of protein molecules that can adsorb at the interface, and their spreading rate (Führling, 2004).

Adsorption at an air-liquid interface can be described by three consecutive or competitive processes (Figure 1.7):

(1) diffusion of protein molecules from the bulk to subsurface region;

(2) adsorption of protein molecules from the bulk to the subsurface to the air-liquid interface; and
(3) molecular rearrangement of the adsorbed protein molecules

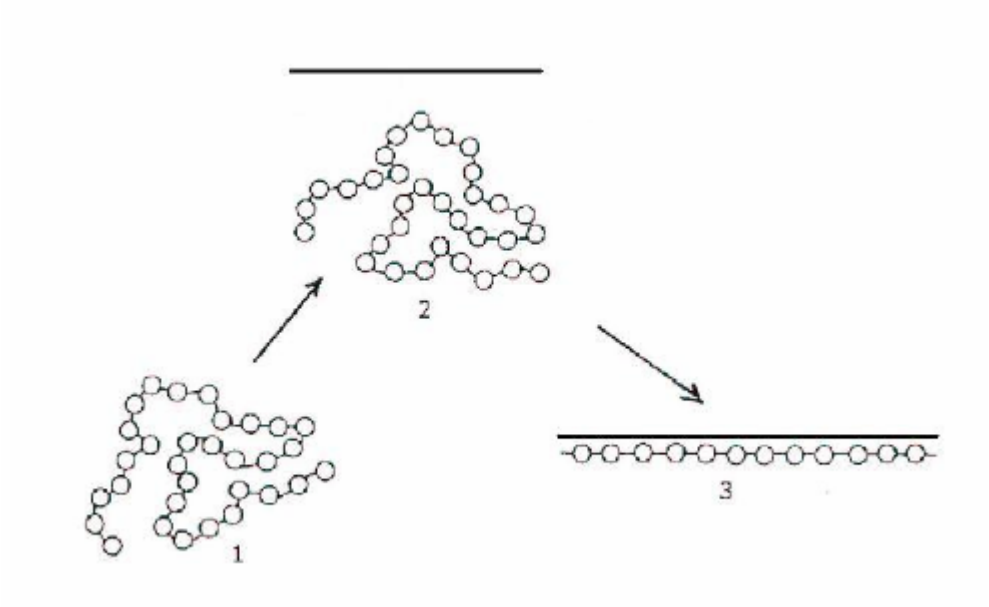


Figure 1.7 Schematic illustration of protein molecules in the solution state (1), after diffusion to the subsurface region (2), and the reoriented molecules in the adsorbed state (Taken from Führling, 2004)

1.3.2 Effect of Foam Fractionation Technique on Protein Structure

1.3.2.1 Protein Denaturation in Foam

Foaming is a common occurrence in the processing of protein solutions and substantial damage is known to occur to protein solutions in this environment (Phillips, 1995). Protein foams are formed in a wide range of operations in both

biotechnology and food and beverage industries. Protein denaturation due to foaming may mean a significant reduction in the value of the end product.

There have been several studies of on the effect of foaming on protein structure. Most of these concentrate on the structural alterations that occur to the protein molecules, and some of them focus on the mechanisms by which this damage occurs.

In a spectroscopic study of conformational properties of bovine serum albumin (BSA), foamed BSA was compared with native BSA. Far-UV circular dichroism spectra revealed only minor changes in the protein secondary structure, while the biggest differences in conformation were found to be at the tertiary structure, which were detected by intrinsic fluorescence (Clark *et al*, 1988).

In another study, structural characteristics and foaming properties of β-lactoglobulin were investigated under various conditions of shear, pH and temperature. The results as established by hydrophobicity change, circular dichroism and gel filtration studies demonstrated that although slight changes in secondary structure were observed following foaming, these changes were reversible. Furthermore, very little, if any, oxidation, free thiol-disulfide interchange, aggregation, or denaturation was observed for β-lactoglobulin solutions whipped under the conditions used (Phillips *et al.*, 1995).

1.3.2.2 Mechanisms of Protein Denaturation in Foam

In literature, three possible mechanisms are suggested by which protein molecules may be damaged in foam: (1) the unfolding at the gas-liquid interface, which may not be reversible, (2) damage due to high shear rates, which cause a change in structure through stress differences across the protein molecule, (3) chemical

damage that may occur by oxidation, as suggested by foam fractionation studies (Clarkson, Cui, and Darton, 1999).

1.3.2.2.1 Surface Denaturation

In a foam, proteins adsorbed at the surface experience local expansion and compression of the surface, due to fluid motion. The Marangoni stress at the surface, which resists excessive local thinning of the surface, is balanced by shear stress in the bulk liquid. Thus, both at the surface and in the bulk, the proteins are in an environment subject to fluid deformation (Clarkson, Cui and Darton, 1999).

Changes in conformation of proteins upon adsorption at a gas-liquid interface have been demonstrated using a variety of experimental techniques.

The kinetics of protein adsorption have shown that the reduction in surface tension is slower than the adsorption of molecules as measured by radio-tracers which suggests that once protein molecules are adsorbed at the surface they undergo a change in structure which further reduces surface tension (Graham and Phillips, 1979).

Experimentally determined areas per molecule at the interface have been shown to be much larger than the molecular dimensions of the protein, thus indicating unfolding of the molecules at the interface (Cho *et al*., 1996).

Ellipsometry (Graham and Phillips, 1979) and neutron reflectivity (cited in Clarkson, Cui and Darton, 1999) have been used to probe the protein structure at the interface and have shown that the molecules form a close packed hydrophobic layer at the interface, with more diffuse hydrophilic layer extending into the aqueous phase.

It has been demonstrated that flexible, hydrophobic proteins will adsorb faster at the interface, with less flexible proteins experiencing an energy barrier corresponding to the unfolding process (cited in Clarkson, Cui and Darton, 1999).

1.3.2.2.2 Shear Stress

Studies on the effect of shear stress on protein structure suggest that shear will only cause significant denaturation to proteins in the presence of gas-liquid interface. In this case, shear will increase the level of denaturation due to an enhancement of the surface renewal rate, causing more proteins to adsorb and unfold at gas-liquid interface (Clarkson, Cui, and Darton, 1999).

Maa and Hsu examined the combined effect of shear and gas-liquid interface on the stability of two model proteins, recombinant human growth hormone (rhGH) and recombinant human deoxyribonuclease (rhDNase). They observed that rhGH denatured at the air-liquid interface, especially under high shear whereas rhDNase was relatively stable. This was explained by the fact that rhGH was more prone to be adsorbed to the gas-liquid interface than rhDNase. Also, rhGH was found to be aggregated, particularly at high protein concentrations and a large gas-liquid interfacial area. As a part of this study, they examined denaturation in foam citing this as a low shear environment. Since levels of denaturation were found to be high they concluded that shear plays only a minor role with the damage being mainly due to interaction with gas-liquid interfaces (Maa and Hsu, 1997).

1.3.2.2.3 Oxidation

In literature, there are conflicting ideas on oxidative denaturation of proteins in foam fractionation.

Some researchers thought oxidation to be the major cause of protein damage in foam (Schnepf and Gaden, 1959; Montero *et al*., 1993). Montero *et al*., reported that the use of air as the sparging gas and foam exposure to atmosphere seemed to cause losses in cellulase activity and recommended use of an inert and oxygen free gas (i.e., nitrogen gas) to avoid protein oxidation (Montero *et al*., 1993). Furthermore, Zheng *et al.* reported enzyme denaturation due to oxidative deactivation in the foam fractionation of trypsin and catalase and suggested that suitable choice of sparging gas composition can reduce the loss of enzyme activity to 10% in case of trypsin and to nearly zero in case of catalase (cited in Liu *et al*., 1998)

There is a thermodynamic barrier to organic molecules with C-H bonds, such as proteins, becoming oxidized by molecular oxygen. The two unpaired electrons on the O_2 molecule have parallel spin rates, while organic molecules have no unpaired electrons. In order for the O_2 molecule to accept a pair of electrons from the organic molecule, the spin of one of the O_2 electrons or of one of the electrons on the organic molecule, would have to be inverted. This process entails a high activation energy, so that proteins are rarely oxidized directly by molecular oxygen (Clarkson, Cui, and Darton, 1999).

Proteins may be oxidized by the reactive oxygen species, superoxide (O_2^-), the hydroxyl free radical ($OH^\bullet$) and hydrogen peroxide (H_2O_2). These reactive oxygen species can be formed from molecular oxygen by trace metal ion catalysis. The transition metals which are often implicated in this process are Cu^{2+} and Fe^{3+}. The amino acids which can be oxidized by these free radicals are lysine, arginine, histidine, tryptophan, tyrosine, proline, methionine, and cysteine.

For this reason, it was concluded that contact with molecular oxygen in foams is not the cause of irreversible damage to the proteins though it may contribute to the

slow stabilization of aggregates. There remains the possibility of oxidative damage arising from active species formed from dissolved oxygen. However, there is no evidence of significant rates of denaturation in protein solutions caused in this way. From these considerations of possible mechanisms, it seems unlikely that oxidation will contribute significantly to protein damage in foam (Clarkson, Cui, and Darton, 1999).

1.3.3 Effect of Solution Conditions on Protein Damage in Foam

There are few reports in literature dealing with the influence of solution conditions on the amount of protein damage in foam. Varley and Ball found that increasing the bulk protein concentration and reducing the residence time in the foam favored the retention of enzyme activity in foam fractionation. Two mechanisms were suggested by which increasing the bulk liquid concentration would reduce the amount of damage. First, it was suggested that at concentrations below those which produce a saturated film at the interface, the more dilute the adsorbed layer, more fully the protein molecules will unfold at the interface, because they have more space. Second, it was suggested that at bulk concentrations above those required for saturated film coverage, an increased protein concentration would lead to a decreased residence time in the foam phase. This would allow a shorter time for changes in the protein structure to occur (cited in Clarkson *et al*. 2000). Clarkson *et al.* also found that at lower bulk concentrations, the percentage of damaged molecules were increased, however, for most of the experiments, the total amount of damaged protein was found to be the same at different bulk concentrations. The one exception was the result for pepsin at a bulk concentration of 0.7 mg/mL, where less damage was detected. However, this bulk liquid concentration was much lower than the apparent CMC, so that the amount of surface adsorption is less than that at surface saturation, and fewer molecules would be exposed to surface-induced damage. From these results, it was concluded that damage did not seem to occur to a greater extent in non-saturated

films, as suggested by Varley and Ball, neither did it appear that above the CMC further increases in protein concentration reduce the amount of protein damaged (Clarkson *et al.*, 2000).

1.3.3.1 Effect of pH

The pH of the solution may have an important effect on the amount of denaturation occurring, although most previous studies of this parameter (Ahmad, 1975a,b; Tanner *et al.*, 2003; Tanner *et* al.,2004; Lambert *et al.*, 2003; Narsimhan and Uraizee, 1996) have concentrated on the change in foam volume, and separation efficiency in foam fractionation, rather than on denaturation. Studies on the effect of pH have mostly shown both foam volume and foam stability to be greatest at the isoelectric point of the protein (cited in Clarkson *et al.*, 2000; Kim and Kinsella, 1985; Uraizee and Narsimhan, 1996). This is due to enhanced surface adsorption as a result of decreased repulsive force at the interface and lower solubility. However, for certain proteins coagulation (aggregation) occurs at the pI, and in this case the amount of foaming is reduced.

In the pH studies of Clarkson *et al.* the concentration of IgG in the foam was found to be highest at the isoelectric point of the protein since surface adsorption is enhanced at this pH and the results of fluorescence spectroscopy showed that damage is also higher at the pI, thus, it was concluded that the amount of damage occurring in the foam correlates with the protein concentration is foam. Also, it was found that foam levels were lower at the pI due to extensive aggregation of the protein (Clarkson *et al.*, 2000).

1.3.3.2 Effect of Ionic Strength

The effect of salt on foam stability is complicated. Several mechanisms may be important, including variation in solubility, inhibition of intermolecular

interactions and change in the disjoining pressure between the two interfaces in the thin liquid film (cited in Clarkson *et al*., 2000).

Clarkson *et al.* examined the effects of ionic strength on protein denaturation in foam using the proteins pepsin, BSA, and Human IgG. The enzyme assays showed that the level of denaturation for pepsin decreases with increasing ionic strength towards a minimum at ionic strength of about 0.4 M. Also, the amount of denaturation was found to decrease as the protein concentration in the foam decreases, which has a minimum value at this ionic strength. The fluorescence spectroscopy studies showed a decrease in fluorescence intensity with no shift in peak wavelength, which is a characteristic of the unfolding of the pepsin molecule. Circular dichroism spectra of pepsin showed an increase in the random coil content. No aggregation of the pepsin molecules was detected in the foam sample, suggesting that the intermolecular interactions of this protein at the gas-liquid interface were minimal. The fluorescence spectroscopy analysis of BSA indicated a decrease in damage at higher ionic strengths, and no changes in the secondary structure were detected by circular dichroism. Furthermore, aggregation was detected by fluorescence spectroscopy in BSA foam samples except at the ionic strengths of 0.4 and 0.5 M. Ionic strength had little effect in experiments with IgG, and aggregation was detected in all the foam samples, but occurred to a little extent at higher ionic strengths (Clarkson *et al.*, 2000).

1.3.3.3 Effect of Sugar Additives

The addition of polyhydric compounds such as glycerol and sucrose has been found to stabilize the native conformation of proteins in solutions. The sugar trehalose has been found to be more effective in stabilizing protein conformation than other carbohydrates. The mechanism of stabilization for all these compounds has been shown to be preferential exclusion. These compounds are preferentially excluded from the vicinity of the protein molecule, since the interaction with the

protein surface is thermodynamically less favorable than the interaction between protein and water. Unfolding of the protein molecule, which leads to a larger surface area, is therefore thermodynamically unfavorable (cited in Clarkson *et al.*, 2000).

Clarkson *et al.* examined the effect of sugar on protein damage in foam, and found that, the addition of trehalose to the solution reduced the pepsin concentration in the foam and the level of damage occurring, the effect being more pronounced at higher trehalose concentrations. The analysis of foam samples from sugar experiments with IgG showed that sucrose provided better protection from damage by foaming than trehalose for this protein (Clarkson *et al.*, 2000).

Careful choice of solution conditions can stabilize the native structure of the dissolved protein, however leads to a smaller amount of surface adsorption in foam. In turn, this leads to a reduction in the amount of protein denaturation occurring.

1.3.4 Selectivity of Foam Fractionation Technique

Most process streams which require purification are complex multi-component mixtures (i.e., fermentation broths). Therefore, for foam separation technique to be a viable recovery technique, it is important that high enrichments and recoveries can be achieved together with selectivity of partition from multi-component mixtures (Brown *et al*., 1999b).

Selective separation between different molecules is likely to be a result of a result of differences in behavior at the gas-liquid interface, i.e., differences in surface activities, the most surface active component being adsorbed at the interface. The surface activity of any protein is dependent on its physicochemical characteristics, e.g., size, charge, and hydrophobicity, and the environmental conditions such as

ionic strength, pH, and the presence of detergents, salts, sugars, and other additives (cited in Brown et al., 1999a).

Although surface activity depends intrinsically on protein structure, in practice, surface activity is measured in terms of surface tension, the lowest surface tension indicating highest surface activity. Surface tension is also a function of protein concentration, solvent conditions, and time of exposure at the interface (Brown *et al*., 1999b).

Previous studies (Varley *et al*., 1996; Brown *et al.* 1999b; Lockwood *et al*., 2000; Zhang *et al*., 2004) show that selective adsorption of proteins from binary mixtures can be achieved between the bulk phase and the foam phase. Whilst it is possible to obtain a relatively pure bottom phase, the purity of the foam phase is limited since liquid carried up between the foam cells (at a concentration close to that in bulk liquid) will contain species present in the bulk liquid (Varley *et al*., 1996). When foam heights are high, however, liquid carry-up could be reduced by maximizing drainage in the foam and hence reducing the liquid content which in turn may lead to improved partition.

Brown *et al*., studied the selective partition of proteins from two protein mixtures using β-casein, BSA and lysozyme. High selectivity of partition of β-casein and BSA were found simultaneously with high enrichments and recoveries in binary mixtures of β-casein-lysozyme, and BSA-lysozyme, indicating that for these mixtures, foam fractionation is a particularly attractive separation technique. In these mixtures, very low values of enrichment ratios were obtained for lysozyme indicating little adsorption of this protein at the interface. This was expected since lysozyme is known to be a protein with low surface activity. In binary mixture of β-casein and BSA, high enrichments and recoveries were achieved for each protein, however, there was very little separation of one protein from the other and

this was attributed to the fact that both proteins have high surface activities (Brown *et al*.,1999b).

1.4 LACTIC ACID

Lactic acid (2-hydroxypropionic acid), $CH_3CHOHCOOH$, was first discovered by the Swedish chemist Scheele in 1780 as a sour milk component. It is present in many foods, either naturally or as a product of microbial fermentation as in sauerkraut, yoghurt, buttermilk, sourdough breads, and many other fermented foods. Lactic acid is also a major metabolic intermediate in most living organisms - from anaerobic prokaryotes to human beings.

1.4.1 Lactic Acid Production

Lactic acid production has a global market in excess of 100,000 tons per year and predictions of increasing demand are quickly becoming reality. Much of this growth is attributable to two emerging products, poly-lactic acid for biodegradable plastics and the environmentally safe solvent ethyl lactate. Both of these rely on lactic acid as a renewable raw material (Skory, 2003).

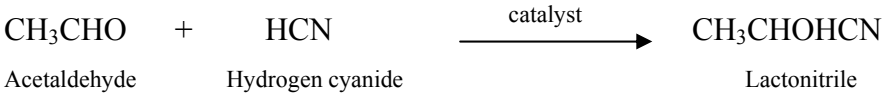
Lactic acid can be manufactured by either (1) chemical synthesis or (2) carbohydrate fermentation; both are used for commercial production (Datta *et al*., 1995)

1.4.1.1 Chemical Synthesis

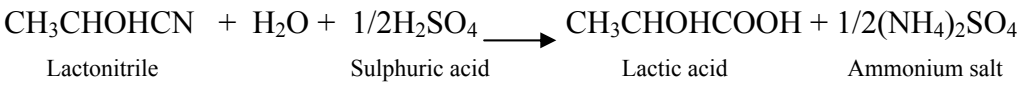
The chemical synthesis routes produce only the racemic lactic acid. The commercial process for chemical synthesis is based on lactonitrile, which used to

be a by-product from acrylonitrile synthesis. It involves base catalyzed addition of hydrogen cyanide to acetaldehyde to produce lactonitrile. This is a liquid-phase reaction and occurs at atmospheric pressures. The crude lactonitrile is then recovered and purified by distillation and hydrolyzed to lactic acid, by using either concentrated hydrochloric acid or sulfuric acid, producing the corresponding ammonium salt as a by-product. This crude lactic acid is then esterified with methanol, producing (1) methyl lactate, which is recovered and purified by distillation and hydrolyzed by water under acid catalyst to produce lactic acid and (2) methanol, which is recycled. Reactions representing this process are given in Figure 1.8.

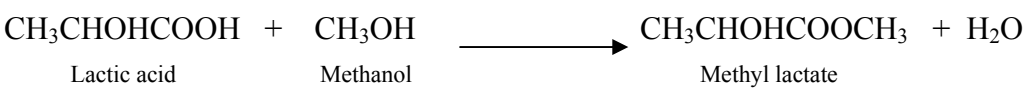
(1) Addition of Hydrogen Cyanide



(2) Hydrolysis by H_2SO_4



(3) Esterification



(4) Hydrolysis by H_2O

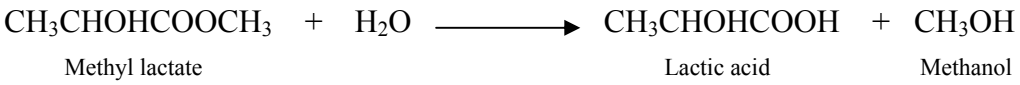


Figure 1.8 Reactions involved in chemical synthesis of lactic acid.

Other possible routes for lactic acid include base catalyzed degradation of sugars; oxidation of propylene glycol; reaction of acetaldehyde, carbon monoxide and water at elevated temperature and pressures; hydrolysis of chloropropionic acid, and nitric acid oxidation of propylene, among others. None of these routes have lead to technically and economically viable processes (Data *et al.*, 1995).

1.4.1.2 Carbohydrate Fermentation

Carbohydrate fermentation allows stereo specific lactic acid to be produced depending on the strain being used. The existing commercial processes often use ne için adam *Lactobacillus* spp. such as *Lactobacillus delbrueckii*, *L. bulgaricus*, *L. leichmanii* (Datta *et al*., 1995), which are fastidious in their growth requirements and unable to produce significant lactic acid below pH 4 (Skory, 2003). Excess calcium carbonate is added to the fermenters to neutralize the acid produced and produce a calcium salt of the acid in the broth, which can be easily separated from the cell biomass and other insoluble. The broth containing calcium lactate is filtered to remove cells, carbon treated, evaporated, and acidified with sulfuric acid to convert the salt into lactic acid and insoluble calcium sulfate, which is removed by filtration. The filtrate is further purified by carbon columns and ion exchange and evaporated to produce technical and food-grade lactic acid, but not a heat-stable product, which is required for the stearyl lactylates, polymers, and other value-added applications. The technical-grade lactic acid can be esterified with methanol or ethanol, and the ester is recovered by distillation, hydrolyzed by water, evaporated, and the alcohol is recycled. This separation process produces a highly pure product, which is white and heat stable, like the synthetic product (Datta *et al*., 1995). The reactions involved in this process are given in Figure 1.9 (Narayanan *et al*., 2004).

(1) Fermentation and Neutralization:

$$C_6H_{12}O_6 + Ca(OH)_2 \xrightarrow{\text{fermentation}} (2CH_3CHOHCOO^-)Ca^{2+} + 2H_2O$$

Carbohydrate — Calcium hydroxide — Calcium lactate

(2) Hydrolysis by H_2SO_4:

$$2(CH_3CHOHCOO^-)Ca^{2+} + H_2SO_4 \longrightarrow 2CH_3CHOHCOOH + CaSO_4$$

Calcium lactate — Sulfuric acid — Lactic acid — Calcium sulfate

(3) Esterification:

$$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O$$

Lactic acid — Methanol — Methyl lactate

(4) Hydrolysis by H_2O:

$$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH$$

Methyl lactate — Lactic acid — Methanol

Figure 1.9 Reactions involved in the production of lactic acid through fermentation.

A wide variety of carbohydrate sources can be used (molasses, corn syrup, whey, dextrose, cane, or beet sugar). The use of a specific carbohydrate feedstock depends on its price, availability, and purity (Datta *et al*., 1995).

1.4.2 Properties of Lactic Acid

Lactic acid is a three carbon organic acid: one terminal carbon atom is part of an acid or carboxyl group; the other terminal carbon atom is part of a methyl or

hydrocarbon group; and a central carbon atom having an alcohol carbon group. Lactic acid exists in two optically active isomeric forms: L(+) lactic acid and D(-) lactic acid. Lactic acid is soluble in water and water miscible organic solvents but insoluble in other organic solvents. It is odorless and has a low volatility the physical properties of lactic acid are summarized in Table 1.2 (Narayanan, 2004):

Table 1.2 Physical Properties of Lactic Acid (Narayanan, 2004)

Molecular weight	90.08
Melting Point	16.8ºC
Boiling point	82ºC at 0.5 mm Hg 122ºC at 14 mm Hg
Dissociation constant, K_a at 25ºC	1.37×10^{-4}
Heat of combustion, ΔH_c	1361 KJ/mole
Specific heat, C_p at 20ºC	190 J/mole/ºC

1.4.3 Uses of Lactic Acid

Lactic acid has a wide area of applications, i.e. in the food industry, beverage production, pharmaceutical and chemical industries and medicine.

The major use of lactic acid is in food and food-related applications. Lactic acid is used as a food acidulant and has a mild acidic taste in contrast to other food acids. It is nonvolatile odorless, and classified as GRAS (generally regarded as safe) for use as a food additive by the FDA in the US and other regulatory agencies

elsewhere. It is a very good preservative and pickling agent and used in sauerkraut, olives, and pickled vegetables. It is also used as acidulant/flavoring/pH buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods, such as candy, breads and bakery products, soft drinks, soups, sherbets, dairy products, beer, jams and jellies, mayonnaise, and processed eggs.

Another use for lactic acid or its salts is the disinfection and packaging of poultry and fish, where the addition of aqueous solutions of lactic acid and its salts during the processing increased shelf life and reduced the growth of anaerobic spoilage organisms such as *Clostridium botulinum* (Datta, 1993; Narayanan, 2004).

A large amount of the lactic acid for food-related consumption is used to produce emulsifying agents for especially bakery products. These emulsifying agents are esters of lactate salts with longer chain fatty acids, and four important products are calcium and sodium, stearoyl-2-lactylate, glyceryl lactostearate, and glyceryl lactopalmitate. The calcium stearoyl lactylate is a very good dough conditioner, and the sodium stearoyl lactylate is both a conditioner and an emulsifier for yeast-leavened bakery products. The glycerates and palmitates are used in prepared cake mixtures and other bakery products and in liquid shortenings. The manufacture of these emulsifiers requires heat-stable lactic acid, therefore; only the synthetic or the heat-stable fermentation grades are used for this application (Datta, 1993).

Lactic acid has been used as an acidulant in leather tanning industry for deliming hides and in vegetable tanning. In various textile finishing operations and acid dying of wool, technical grade lactic acid is used extensively.

Lactic acid is also used in a wide variety of specialized industrial applications where the functional specialty of the molecule is desirable. Some examples are pH adjustment of hardening baths for cellophane that is used in food packaging,

terminating agent for phenol-formaldehyde resins, alkyd resin modifier, textile printing developers, adhesive formulations, detergent formulations, etc.

In pharmaceutical and cosmetic industries, lactic acid and ethyl lactate have been used in topical balms, lotions, parenteral solutions, and biodegradable polymers for medical applications such as surgical sutures, controlled-release drugs, and prostheses. A substantial part of pharmaceutical lactic acid is used as the sodium salt for parenteral and dialysis applications. Its calcium salt is widely used for calcium-deficiency therapy and in toothpastes as an effective anti-caries agent. In cosmetic applications, lactates are used as humectants and ethyl lactate is the active ingredient in many anti-acne preparations. The use of chirality of lactic acid allows the synthesis of compounds such as R (+) phenoxypropionic acid and its derivatives which are used in herbicide production (Datta, 1993).

1.4.4 Down Streaming of Lactic Acid from Fermentation Broth

The process steps required to gain the final product after fermentation are termed down streaming. Depending upon the purity requirements, several process steps are combined that exert a significant influence on the cost. The expenses for the down streaming of a biological product can cost up to 90% of the entire production costs, depending on the value of the product. Therefore, down streaming of fermentation media is of particular importance (Reimann, 2005).

Several unit operations are required to obtain lactic acid with suitable properties for future use from fermentation medium. Conventional processes are based on precipitation steps to generate large amounts of chemical effluents. Consequently the environmental impact and the operating costs of traditional precipitation processes can be reduced by using alternative technologies, such as two-stage electrodialysis. In the first step of desalting electrodialysis, sodium lactate is recovered, purified and concentrated. In the water-splitting or acidification step,

lactic acid is generated from sodium lactate, and sodium hydroxide is recovered and purified.

Membrane operations, for instance microfiltration, nanofiltration, and reverse osmosis, as well as mono-polar and bipolar electrodialysis, are increasingly being used as an alternative to conventional methods such as filtering, separating, crystallizing, vaporization, drying, and ion exchange. These methods provide gentle product treatment and are cost-effective, attractive in terms of energy and safety engineering, as well as being environmentally sound technologies (cited in Reimann, 2005). The fermentation broth is first clarified by cross flow microfiltration from biomass in order to obtain a fluid with appropriate properties for carrying out electrodialysis in proper conditions. Subsequently, multivalent metal ions (Ca, Mg, etc.) are removed from the clarified broth to prevent irreversible damage to the membranes in the bipolar electrodialysis membrane stack. After this, the target product sodium lactate is concentrated by conventional mono-polar electrodialysis. After conversion of sodium lactate into lactic acid by bipolar electrodialysis, the product is finally upgraded by ion exchange resins (Reimann, 2005).

1.5 BREWERY WASTES

Over the last few decades, the adverse impacts of industrial wastes on environment have increasingly become a major concern of regulatory agencies and businesses. Traditionally, waste management efforts have focused primarily on “end-of-pipe” treatment methods which simply transfer pollutants from one medium to another. It was then realized that in order to effectively avoid or minimize the potential adverse environmental impacts associated with pollutants and wastes, industries should not only better control emissions and discharges, but also prevent generation of pollutants through the use of more efficient processes and improved management of resources.

The brewing process involves malting of grain, milling and mashing, wort cooling and fermentation, packaging, and pasteurization (Figure 1.10).

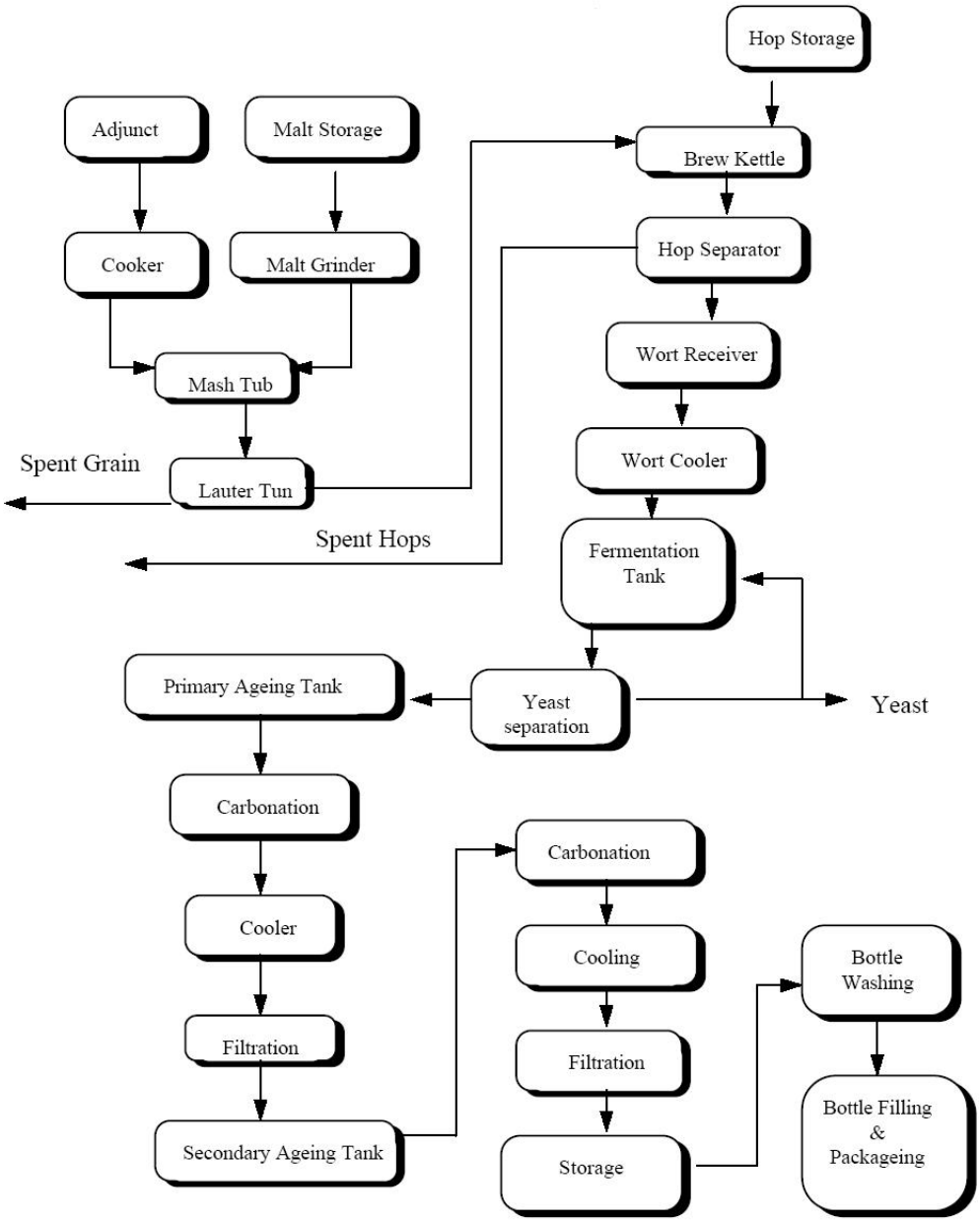


Figure 1.10 Flow chart for a brewery operation (Taken from El-Rayes, 1997)

Brewery wastes generated include:

1. Water treatment wastes, caustic boil-out solutions used for clean-ups in the brew house, and soak solutions and caustic rinses in the bottling area.
2. Organic loading, spent grain and hops, filter cakes in dry form and accidental losses from operational errors and leaking equipment.

1.5.1 Solid Wastes

Solid waste mainly consists of organic material residuals from the process including spent grains and hops, trub, sludge, surplus yeast, şabel sludge, Kieselguhr, powdered carbon and broken glass.

1.5.1.1 Spent Grains

The amount of spent grains is normally 14 kg/hl wort with a water content of 80%.

1.5.1.2 Yeast

Excess yeast is produced during fermentation and only a part can be reused. The amount of spent yeast slurry is 2-4 kg (10-15% dry matter content) per hl beer produced. The BOD value is around 120,000-140,000 mg/ml.

The excess yeast can be resold as animal feed. On a dry solids basis, the yeast contains 50-60% proteins, 15-35% carbohydrates and 2-12% fat making it another valuable source of protein for animal feed.

1.5.1.3 Trub

Trub is a slurry consisting of entrained wort, hop particles and unstable colloidal proteins coagulated during wort boiling. It is separated prior to wort cooling and

represent 0.2-0.4 % of the wort volume with a dry matter content of 15-20%. Its content of wort and extract depends on how efficiently the wort and trub are separated. The BOD value of this trub is around 110,000 mg/kg wet trub.

Other solid wastes from a brewery are:

- glass cullets from the packaging area;
- Kieselguhr from the filtration process;
- paper pulp from the bottle washer;
- paper, plastic and metal from received auxiliary material (especially packaging materials); and,
- waste oil and grease.

1.5.2 Liquid Wastes

The brewing process requires a significant amount of water and produces wastewater with high biochemical oxygen demand and suspended solid content. Wastewater generated from beer manufacturing amounts to 65-70% of the water intake volume.

The effluent contains: maltose, dextrose, wort, trub, spent grains, yeast, filter slurry (Kieselguhr and lucilite), green beer and bright beer. This effluent will have a high organic pollution load and a relatively high solid pollution load.

1.5.2.1 Weak Wort

Weak wort is 2-6% of the wort volume. This increases the BOD of the wastewater significantly.

1.5.2.2 Rinse Water

Rinse water, which may contain product or raw material, represents 45% of the total water use in a brewery.

1.5.2.3 Residual Beer

Residual beer is the beer lost during the various production stages which include:

- residual amount of beer after emptying of process tanks;
- pre-runs and after-runs in the Kieselguhr filter result in a mixture of beer and water, which is discharged into the sewer;
- using water to clean the process pipe lines, beer is pushed out by water, and a mixture of water and beer results;
- beer rejected in the packaging area due to wrong filling height, quality defects, or incorrect placement of labels;
- returned beer;
- exploding bottles as a result of poor quality, poor bottle inspection, or lack of temperature control in the tunnel pasteurizer; and
- use of solid additions for maturing beer resulting in loss of beer and yeast.

Residual beer equals 1-5 % of total production. Most of it can be collected and reused in brewery process. Any amount not collected is discharged as effluent (El-Rayes, 1997).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Lactic Acid Broth

Lactic acid broth was supplied by H^2BİYOTEK LTD.ŞTİ.,Ankara,Turkiye.

2.1.2 Residual Beer

Waste beer was supplied by Anadolu Efes Biracılık ve Malt Sanayi A.Ş., Kazan, Ankara.

2.1.3 Spent Brewer's Yeast Extract

Spent brewer's yeast was supplied by Anadolu Efes Biracılık ve Malt Sanayi A.Ş., Kazan, Ankara.

2.1.4 The Chemicals

The chemicals used in all experiments were all of analytical grade and commercially available from Sigma, Merck, Eastman, and Codex.

2.2 Methods

2.2.1 Preparation of Feed Solution

Lactic acid broth:

The feed solution was prepared batch-wise. Pure water (Millipore Elix 10 UV, USA) was used to dilute lactic acid broth for all the feed samples and the feed temperature was kept in the temperature range of 20-25ºC.

Spent yeast extract solution:

Spent yeast extract was dried and powdered. Samples were dissolved in pure water and centrifuged at 11000rpm for 5 minutes (Sorvall RC5C, USA). For pH adjustment of spent yeast extract solutions, either 0.1M HCl or 0.1 M NaOH was added.

2.2.2 Foam Separation of Proteins

Figure 2.1 shows the apparatus used for semi-batch foaming of sample solutions. It comprises of a glass column (height: 62-118 cm; inner diameter: 2.8-3.6 cm) with a rectangular wooden air sparger (dimensions:1.7x1.7x6.5 cm), an air pump, a humidifier, a flowmeter, a vacuum pump for foam collection and a foam collection vessel. Air was passed through the base of the column through the air sparger, and any extractable foam produced was collected at the column exit by utilizing the vacuum pump. The process variables were: gas flow rate, initial feed volume, initial feed concentration, column height, air velocity, foaming time.

All experiments were conducted at room temperature (20-25ºC). Collected foam samples were stored at 4ºC overnight for foam collapse. Samples of brewery waste

experiments were mixed with 85% glycerol solution in a ratio of 4:1 and stored at -20ºC for electrophoretic analysis. All initial feed, retentate, and foam samples were assayed for protein concentration, as described in Appendix A-B.

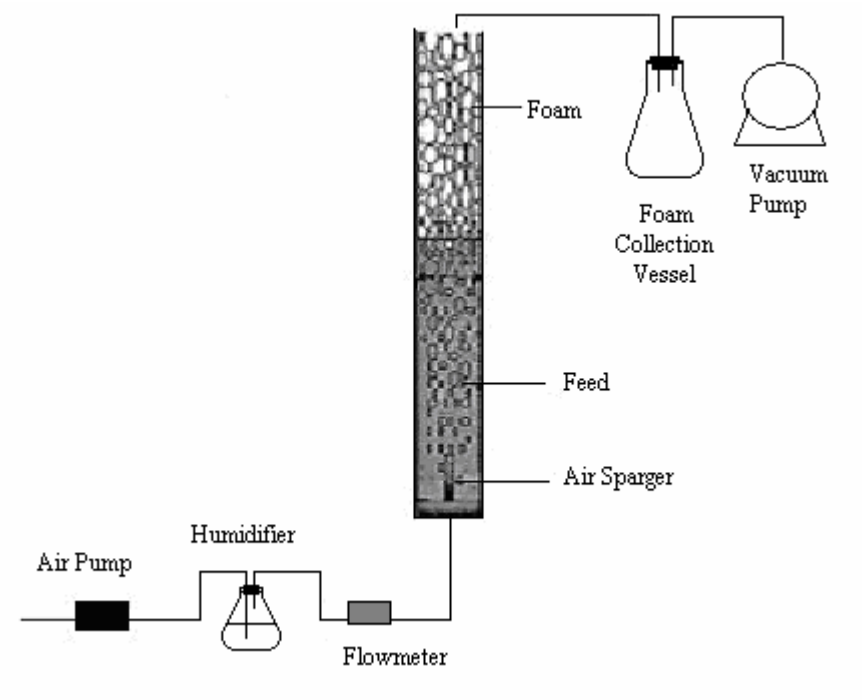


Figure 2.1 Foam Fractionation Column

The foaming experiment was conducted by introducing the feed into the glass column and producing gas bubbles by introducing pre-humidified air into the feed solution from the bottom of the column through wooden air sparger. As the gas bubbles rise through the feed solution, proteins adsorb to the gas-liquid interface. Bubbles leave the surface of the feed solution entraining both adsorbed proteins and bulk liquid into the rising column of foam. The interstitial liquid drains slowly through the lamella between the individual bubbles returning unadsorbed proteins

to the feed solution. Drainage results thinning of the lamella, promoting gas diffusion between the bubbles and as a result bubbles rupture. At the exit of the column, foam that is enriched in proteins is collected via vacuum pump into a vessel and collapsed.

2.2.3 Determination of Protein Content

Protein determination was done by measuring the optical density of appropriately diluted samples with a spectrophotometer (Shimadzu UV-Vis Spectrophotometer, Japan). Samples from the initial solution, the retentate, and collected foam were analyzed in triplicates with the Bradford protein assay (Bradford, 1976) with optical glass cells at wavelength of 595nm. Bovine serum albumin was used as a standard. Samples for analysis were stored at 4ºC, foam samples were allowed to collapse before analysis (generally within 24 hours). The concentrations of the samples were calculated from the slope of the standard curve which relates linearly the UV absorbance and concentration.

2.2.4 Surface Tension Measurement

Surface tension of samples was measured in triplicates at 20ºC on a Kruss surface tensiometer using the ring method (Kruss Ltd., Germany). The surface tension of water was measured first and was taken as a reference point. The surface tension of water or the sample was measured by increasing the torque of the ring system. The value at which the ring broke free of the surface was recorded as the surface tension of the system in dynes/cm.

2.2.5 Analysis of Foam Fractionation Performance

Performance of foam separation technique was evaluated in terms of four defined parameters: enrichment ratio, separation ratio, and percentage protein recovery.

2.2.5.1 Enrichment Ratio

Enrichment ratios of the experiments were calculated from the protein concentrations of the initial solutions and collected foam samples:

$$E_r = \frac{C_f}{C_i}$$

where E_r is the enrichment ratio, C is protein concentration in samples, and *i* and *f* denotes initial solution and foam, respectively.

2.2.5.2 Separation Ratio

Separation values were calculated from the protein concentrations of retentate solutions and collected foam samples:

$$S_r = \frac{C_f}{C_r}$$

where S_r is the separation ratio, C is the protein concentration in samples, and *r* and *f* denotes retentate solution and foam, respectively.

2.2.5.3 Percentage Protein Recovery

Percentage protein recovery values were calculated from total mass of protein in initial solutions and foam samples:

$$R_p = \frac{M_f}{M_i} x100(\%) = \frac{C_f x V_f}{C_i x V_i} x100(\%)$$

where R_p is the percentage protein recovery, M is the total mass of protein, C is the protein concentration, V is the volume of samples, and *i* and *f* denotes initial solution and foam, respectively.

2.2.6 Electrophoretic Analysis

2.2.6.1 SDS-Polyacrylamide Gel Electrophoresis

For monitoring the progress of separation procedure and its selectivity in recovery of proteins into the foam phase SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used. The initial solution, retentate solution and collected foam samples were analyzed by determining the molecular weight profile of samples by SDS-PAGE. Solutions used in the procedure and their preparation were given in Appendix C.

SDS-PAGE was conducted according to the method of Laemmli (1970) using 10% separating and 4% stacking gels. Samples for electrophoresis were prepared in volumes containing 10µg protein and double strength (2X) sample solubilization buffer in a ratio of 4:1. Sample buffer contained 2% (w/v) SDS, 5% β-Mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl and pH was adjusted to 6.8. Samples were heated for 5 minutes in boiling water prior to loading. The molecular weight standards used were the wide range molecular weight markers (Sigma Chem. Ltd, USA). Separation was conducted at a constant current of 20 mA in stacking and 35 mA in separating gel at 4ºC.

2.2.6.2 Native- Polyacrylamide Gel Electrophoresis

Native-PAGE was used to determine whether the foam fractionation technique caused any damage on the structure of the proteins. Procedure is given in Appendix D.

2.2.6.3 Gel Processing

2.2.6.3.1 Gel Fixing

The gels were processed in the fixer solution containing 50% methanol, 12% glacial acetic acid, and 150 µL of 37% formaldehyde/L by gently shaking for approximately 5 minutes and leaving to stand in solution for more than 1 hr before staining (Appendix E).

2.2.6.3.2 Silver Staining

The gels were silver-stained according to the method of Blum *et al*. (1987). The procedure comprised of 5 steps: washing with 50% ethanol, pretreatment, impegnation with silver nitrate solution, color developing and stopping. Composition of the solutions, their preparations and details of the procedures were given in Appendix E. After silver-staining procedure, gel photograph was taken.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Partial Removal of Proteins from Lactic Acid Broth

Different performance criteria can be used for foam separation to serve different purposes. For stripping of protein out of a process stream, high recovery and low residual concentration are desirable, whereas a high enrichment is necessary for purification of proteins.

In this work, the partial separation of proteins from lactic acid broth was examined using a 3.6 cm inner diameter, 118 cm long glass column fitted with a wooden sparger for bubble generation. The effects of the operating factors, i.e. initial feed concentration, air flow rate, foaming time, and liquid pool height on separation performance were studied and suitable conditions for removal of proteins from lactic acid broth was determined under the conditions studied.

The performance of foam fractionation was expressed in terms of enrichment ratio, separation ratio, and percentage protein recovery (defined in Section 2.2.5), as commonly employed in literature to determine the suitable conditions.

3.1.1 Effect of Feed Concentration on Bulk Surface Tension

The surface tension of lactic acid broth at various concentrations is shown in Figure 3.1. Sample solutions of different concentrations were prepared by diluting the lactic acid broth with pure water.

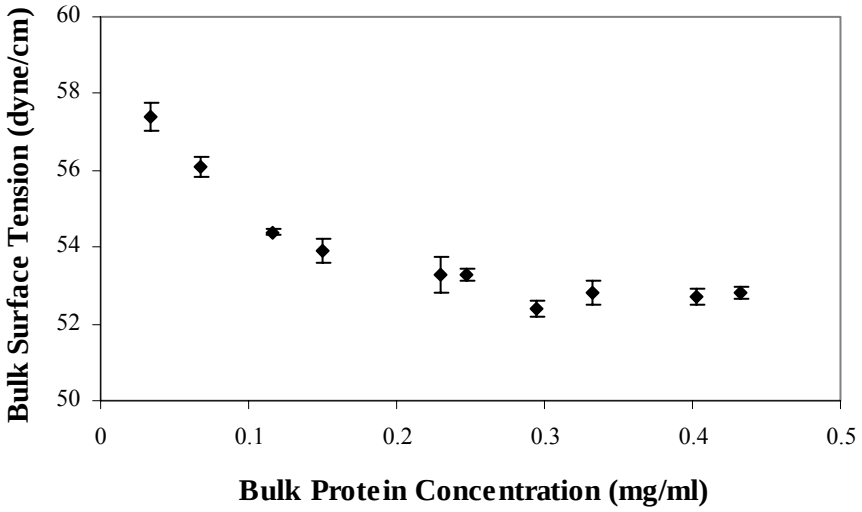


Figure 3.1 Effect of protein concentration (0.03-0.43 mg/ml) on bulk surface tension of lactic acid broth (measured at 20ºC). The points are average of triplicate measurements.

The surface tension of solutions was found to decrease as the protein concentration in the bulk solution increased and the decrease was more sharp at lower concentrations (i.e. 0.1 mg/ml) as reported in literature for some other protein solutions. The surface tension attained a constant value at higher protein concentration of 0.27 mg/ml which is termed as the critical micelle concentration. The value of this CMC is a measure of the affinity of the protein for a gas-liquid

interface. Taking into account the previous studies on foam separation of proteins, it is expected that highest separation values be obtained below this CMC value, and very small separation to be obtained when the protein concentration is above 0.27 mg/ml.

When the concentration is higher than CMC value, proteins assemble in the bulk solution into aggregates (micelles). When the micelles form in water, hydrophobic portion of the proteins form a core and their hydrophilic portions form an outer shell that maintains contact with water. In systems containing air-water interfaces, protein adsorption takes place by accumulation of proteins at these interfaces, forming a surface layer of proteins oriented such that only their hydrophilic regions are exposed to water. At concentrations higher that CMC, formation of these aggregates retards adsorption to the interfaces, because the exposure of the hydrophobic portions of proteins to the aqueous solution is already minimized and separation of these proteins from micelles and reorientation of them at the interfaces to minimize this exposure is not favorable.

3.1.2 Effect of Feed Concentration on Performance of Foam Fractionation

Effect of initial feed concentration on performance of semi-batch foam fractionation is shown in Figures 3.2-3.5. Solutions with different initial protein concentration (0.018-0.168 mg/ml) were prepared by diluting the lactic acid broth with pure water and foamed for 1 hour while other process variables (temperature, feed volume, air velocity, column diameter, etc.) were kept constant.

As the initial protein concentration in the feed increased, enrichment ratio was found to decrease (Figure 3.2).

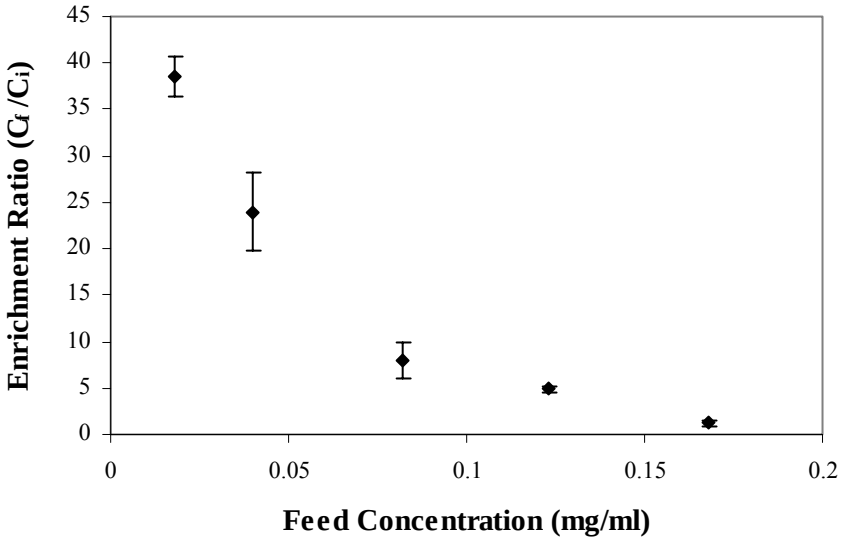


Figure 3.2 Effect of feed protein concentration on enrichment ratio (at room temperature: 20-25 ºC, air velocity: 56±1 cm^3/min, feed volume: 200 ml, feed protein concentration: 0.018-0.168 mg/ml). The points are average of triplicate experiments.

This reduction in enrichment ratio was a result of increased foam flow rate (Figure 3.3) at higher initial protein concentrations. More stable foam was obtained at higher values of feed concentration and bubble rupture decreased. This resulted in higher liquid content in the foam phase. As a consequence, the protein concentration in foam decreased, leading to lower enrichment ratios at higher initial feed concentrations. In the conditions studied, highest enrichment ratio obtained was 38.5 at an initial feed concentration of 0.018 mg/ml and lowest value obtained was 1.2 for a feed protein concentration of 0.168 mg/ml. The enrichment obtained at this condition revealed that enrichment of proteins in foam phase is very low at high feed protein concentration even lower than the CMC. Similar results were obtained in published research.

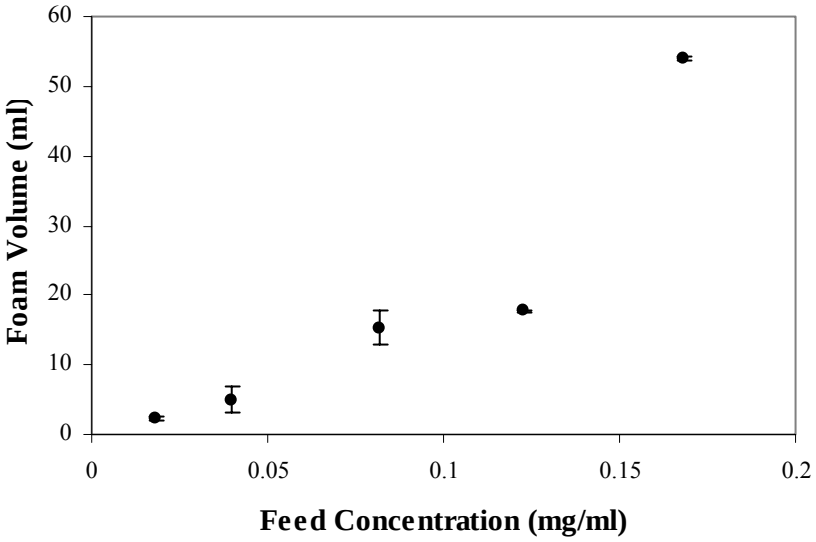


Figure 3.3 Change in volume of the foam collected with respect to initial feed concentration (at room temperature: 20-25 ºC, air velocity: 56±1 cm^3/min, feed volume: 200 ml, feed protein concentration: 0.018-0.168 mg/ml). The points are average of triplicate experiments.

A similar profile was obtained for the effect of initial protein concentration in feed on separation ratio (Figure 3.4). A reduction in separation ratio was obtained when feed protein concentration is increased. As discussed above, low initial feed concentrations promote higher protein concentration in foam phase, by reducing the amount of liquid carried up into the foam phase.

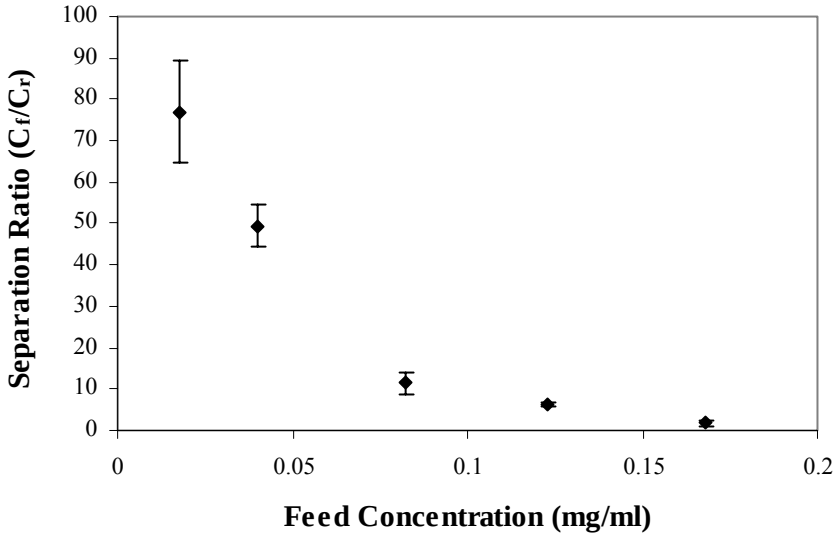


Figure 3.4 Effect of feed protein concentration on separation ratio (at room temperature: 20-25 ºC, air velocity: 56±1 cm^3/min, feed volume: 200 ml, feed protein concentration: 0.018-0.168 mg/ml). The points are average of triplicate experiments.

Effect of feed protein concentration on percentage of total protein recovered in foam is shown in Figure 3.5. Protein recovery first increased and then leveled off as the initial protein concentration was increased. Both protein concentrations of the foam phase and volume of foam collected influence the protein recovery. Although the protein concentration in foam was high at feed concentration of 0.018 mg/ml, low protein recovery was obtained at this feed concentration. This resulted from low foam volume obtained at this condition. As for enrichment ratio and separation ratio, lowest recovery under the conditions studied (31.8 %) was also obtained at highest feed protein concentration (0.168 mg/ml). Highest protein recovery (57.6 %) was obtained at an initial feed concentration of 0.04 mg/ml at which also highest foam concentration was obtained together with a high volume of foam in these conditions. Further increasing the feed concentration decreased

the protein recovery because low foam concentrations are obtained at these feed concentrations although the foam volumes are very high.

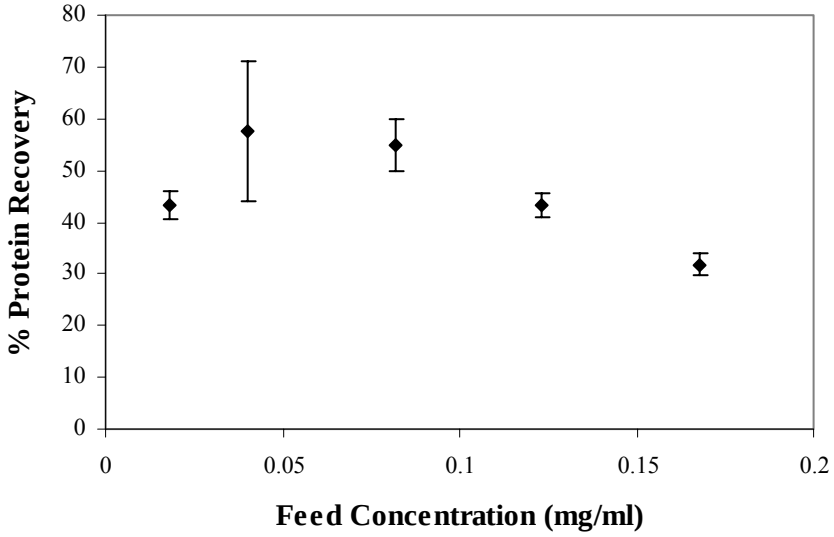


Figure 3.5 Effect of feed protein concentration on percentage of total protein recovered as foam (at room temperature: 20-25 ºC, air velocity: 56±1 cm^3/min, feed volume: 200 ml, feed protein concentration: 0.018-0.168 mg/ml). The points are average of triplicate experiments.

3.1.3 Effect of Air Flow Rate on Performance of Foam Fractionation

An increase in air flow rate could cause change in the following process conditions: (i) average bubble size, (ii) contact time of air bubbles in the liquid pool, and (iii) liquid entrainment in the foam phase (Monwar, 2001).

In order to observe the effect of air flow rate on performance criteria, all process variables other than air flow rate (temperature, feed protein concentration, feed

volume, temperature) were kept constant and air flow rate was changed within a range of 24-101.5 cm^3/min. Feed solution of concentration 0.018 mg/ml was prepared by diluting 50 ml of lactic acid broth with distilled water.

At higher air flow rates the contact time of air bubbles with the liquid pool and thus protein molecules is decreased. This leads to a higher liquid entrainment, less coalescence and liquid drainage as a result of a more stable foam phase. All of these reduce the protein concentration in the foam phase (Brown *et al*., 1999).

In the experiments, as the air flow rate was increased above 38.5 cm^3/min, protein concentration in foam phase decreased. Higher foam heights were obtained and visual inspection indicated that liquid drainage and bubble coalescence had decreased.

The effect of air flow rate on enrichment ratio is shown in Figure 3.6. Enrichment ratio first increased and when it reached a maximum value decreased again as the air flow rate was increased. Under the conditions studied, minimum and maximum values of enrichment ratio were 34.8 and 172.2 and were obtained at air flow rate values of 24 cm^3/min and 38.5 cm^3/min, respectively. Low enrichment ratio at 24 cm^3/min was obtained, since the air flow rate was not high enough to produce sufficient foam for separation. Foam height was very low and liquid drainage was insufficient. Therefore, foam phase contained high amount of bulk liquid and as a result, protein concentration in the foam phase was low when compared to the values obtained for other air flow rates.

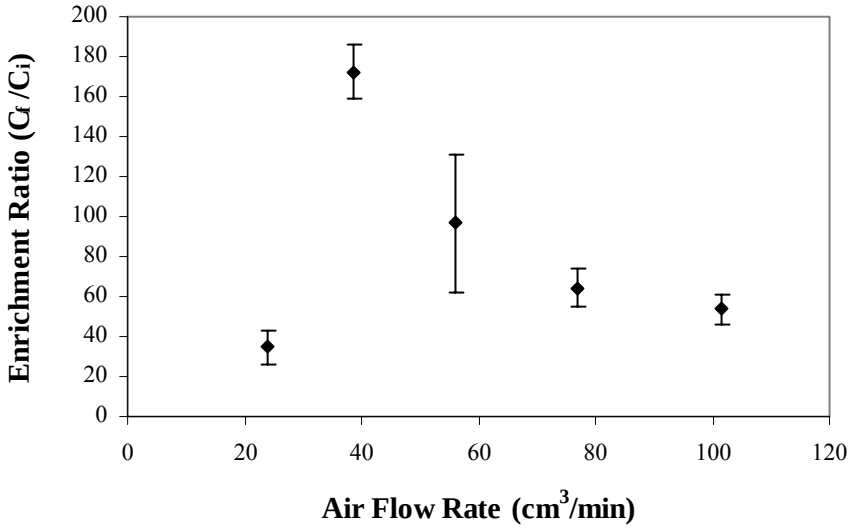


Figure 3.6 Effect of air flow rate on enrichment ratio (at room temperature: 20-25 ºC, air velocity: 24-101.5 cm^3/min, feed solution concentration: 0.018 mg/ml, feed volume: 200 ml). The points are average of triplicate experiments.

High levels of air flow rate causes (1) greater amounts of liquid to be taken into the foam, and (2) the residence time of foam in the column to be reduced limiting liquid drainage and internal reflux. Both these factors result in an increase in foam-flow rate, which leads to a reduction in foam protein concentration since a greater volume of bulk liquid is carried up into the foam. As a consequence, lower enrichment ratios are obtained. Conversely, at lower gas-flow rates, enrichment is greater because (1) less liquid is taken into the foam, and (2) the residence time of foam in the column is greater, allowing more time for drainage, and resulting in an increase in coalescence, especially at the top of the foam, where it is driest and more prone to rupture (Brown *et al*., 1999a).

Air flow rate affects separation ratio in a similar way as enrichment ratio. As mentioned before, low air flow rates promote higher protein concentrations in the

foam phase. Higher air flow rates increased the rate of protein removal from the liquid pool, however, due to greater liquid hold-up in the foam, the protein concentration in the foam was limited, resulting in lower separation ratios (Figure 3.7). The lowest value obtained for the separation ratio was 47.8 for an air flow rate of 24 cm^3/min and 314 for an air flow rate of 38.5 cm^3/min.

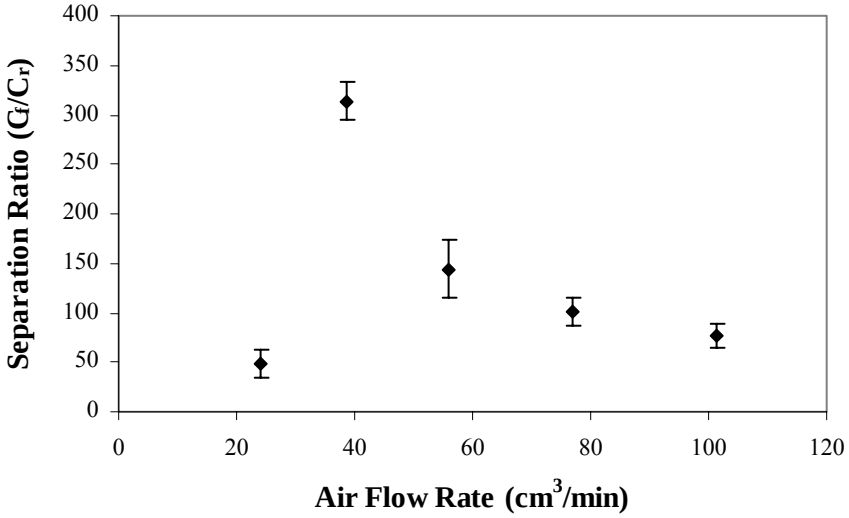


Figure 3.7 Effect of air flow rate on separation ratio (at room temperature: 20-25 ºC, air velocity: 24-101.5 cm^3/min, feed solution concentration: 0.018 mg/ml, feed volume: 200 ml). The points are average of triplicate experiments.

Effect of air velocity on percentage of total protein recovered in foam is shown in Figure 3.8. Both protein concentrations of the foam phase and volume of foam collected influence the protein recovery. As for enrichment ratio and separation ratio, lowest recovery (28%) was also obtained at an air flow rate of 24cm^3/min. At this flow rate, a small amount of foam was formed, and thus foam height obtained was very low. Liquid from the pool was carried up into the foam phase

continuously but, liquid drainage was slow. As a result a small amount of foam with low protein concentration was collected. Total effect of these resulted in a low value for protein recovery.

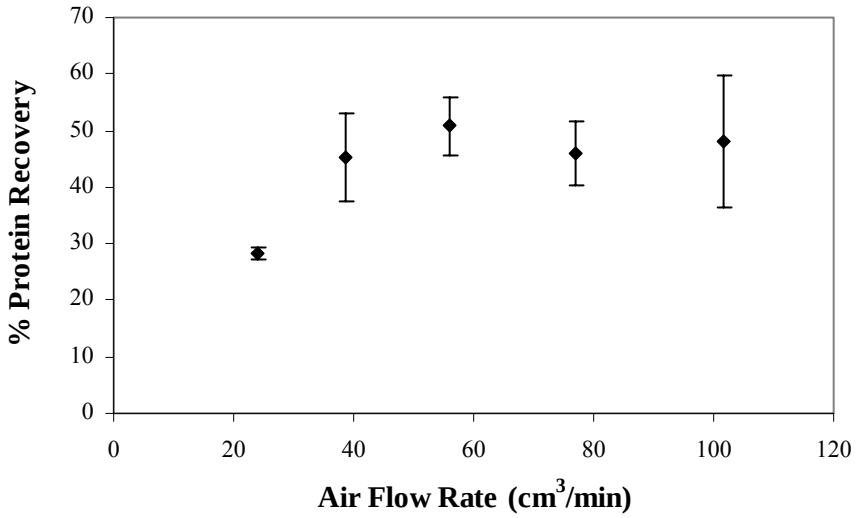


Figure 3.8 Effect of air flow rate on percentage of total protein recovered in foam (at room temperature: 20-25ºC, air velocity: 24-101.5 cm^3/min, feed solution concentration: 0.018 mg/ml, feed volume: 200 ml). The points are average of triplicate experiments.

When air flow rate was increased above 24 cm^3/min, protein recovery did not change significantly. As the air flow rate increased, high foam heights were obtained, and this allowed more time for liquid drainage and increased bubble coalescence. Since liquid drainage was high, foam obtained was dry for the most part of the foam height. As a consequence of these, the foam protein concentration decreased as the air flow rate increased, but foam volume increased. Low foam

concentration together with high foam volume resulted in similar protein recovery values (45-50%) under the conditions studied.

3.1.4 Effect of Liquid Pool Height on Performance of Foam Fractionation

Liquid pool depth above the air sparger is a significant parameter in foam fractionation process. The effect of liquid pool height on separation efficiency was studied by keeping the air flow rate at 38.5±1 cm^3/min, initial feed protein concentration at 0.031 mg/ml, and temperature at 20-25 ºC while changing the feed volume between 100 ml and 500 ml.

At constant air flow rate and initial feed concentration, an increase in liquid pool height results in an increase in residence time of air bubbles in the pool. As the bubbles more towards the liquid-foam interface, more proteins can adsorb on the surface of the bubbles if the contact time of the bubbles in the pool is high (Kumar *et al*., 1997).

In this work, when the liquid pool height was increased, enrichment ratio first increased and after attaining a maximum value, it decreased again when the pool height was increased further. Maximum enrichment ratio of 34.1 was achieved at a pool height of 33.5 cm (liquid volume/air flow rate ratio of 7.8) (Figure 3.9).

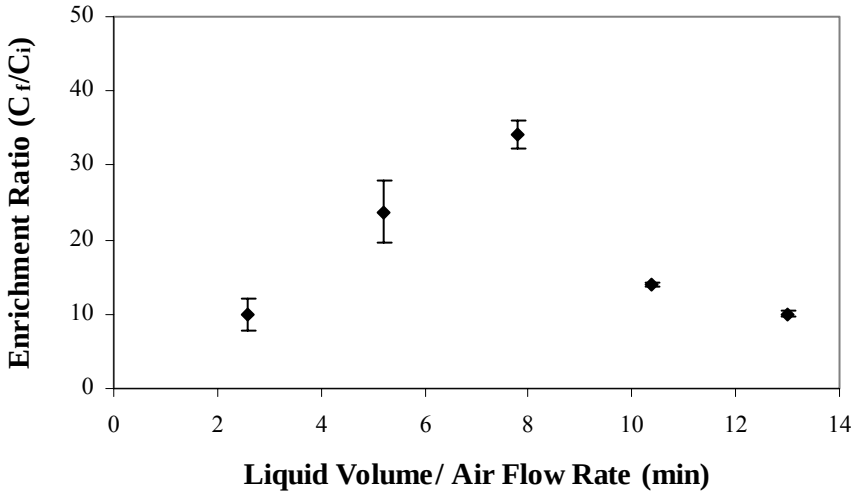


Figure 3.9 Effect of liquid pool height on enrichment ratio (at room temperature: 20-25ºC, air flow rate: 38.5±1 cm^3/min, initial feed concentration: 0.031 mg/ml, feed volume: 100-500 ml with liquid pool height of 13-53.5 cm, foaming time: 1 h). The points are average of triplicate experiments.

The increase in enrichment ratio with liquid pool height was expected since higher liquid heights provide more time for air bubbles to contact with protein molecules in the solution. Furthermore, similar results were found in published research. Ahmad (1975a) showed that enrichment of BSA increased with increasing liquid pool height from 10 cm to 40 cm. Gehle and Shügerl (1984) obtained a similar trend for BSA with a liquid height range of 11 - 44.2 cm. Both of these were results of continuous foam fractionation.

In the experiments conducted in this study, it was observed that, bubbles did not directly moved toward the liquid-foam interface, but instead formed eddies inside the liquid pool. Some of the air bubbles formed returned to the liquid pool when they reached the liquid-foam interface, and did not move into the foam phase

forming more foam cells. This effect was increased at higher liquid pool heights, and this might have caused the reduction in enrichment ratio with further increase in liquid pool height above 33.5 cm. A similar trend was obtained for the separation ratio which is shown in Figure 3.10.

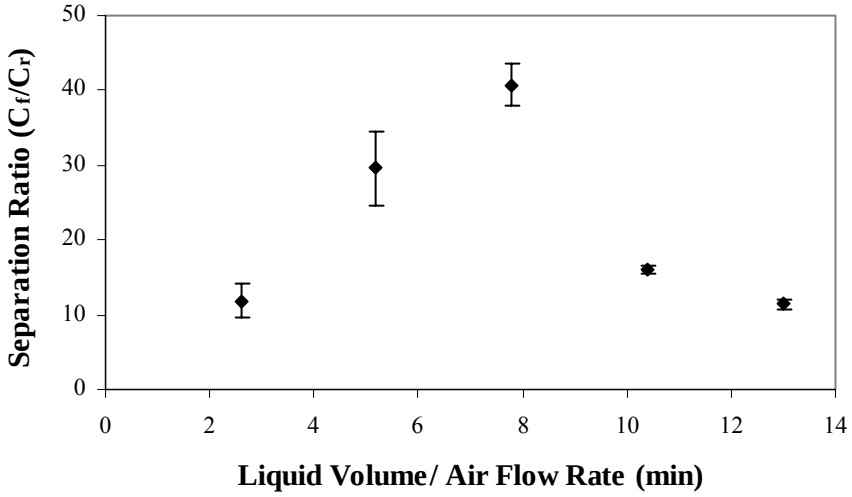


Figure 3.10 Effect of liquid pool height on separation ratio (at room temperature: 20-25 ºC, air flow rate: 38.5±1 cm^3/min, initial feed concentration: 0.031 mg/ml, feed volume: 100-500 ml with liquid pool height of 13-53.5 cm, foaming time: 1 h). The points are average of triplicate experiments.

The effect of liquid pool height on % protein recovery is shown in Figure 3.11. Total protein recovery remained constant at about 32% except the foaming experiment at liquid pool height of 13 cm (100ml feed volume). Since the liquid height above the air sparger was very low, the residence time of air bubbles in the liquid was low; therefore protein molecules had little time to adsorb onto the surface of the bubbles. As a result, foam with a low protein concentration was

obtained at this condition and this lead to the lowest value for protein recovery (23.7%).

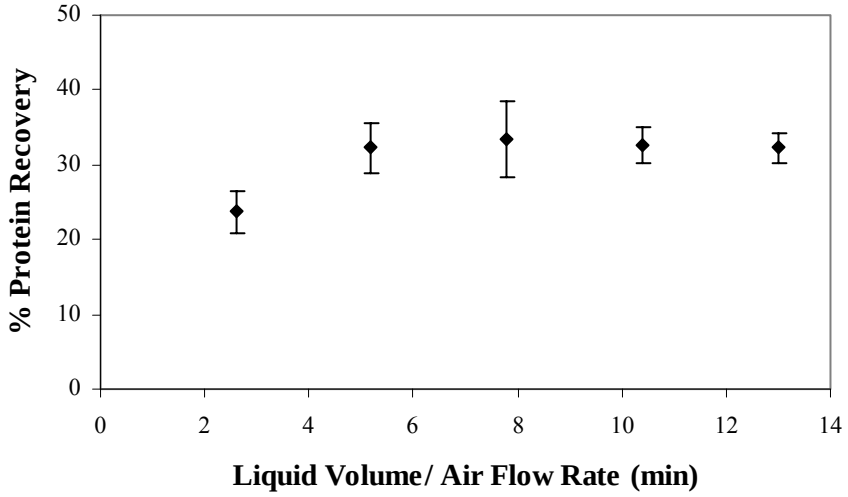


Figure 3.11 Effect of liquid pool height on % protein recovery (at room temperature: 20-25 ºC, air flow rate: 38.5±1 cm^3/min, initial feed concentration: 0.031 mg/ml, feed volume: 100-500 ml with liquid pool height of 13-53.5 cm, foaming time: 1 h). The points are average of triplicate experiments.

3.1.5 Effect of Foaming Time on Performance of Foam Fractionation

In order to determine the effect of foaming time on separation efficiency, 250 ml of lactic acid broths with an initial feed protein concentration of 0.064 mg/ml (prepared by diluting 225 ml lactic acid broth to 1.5 L) were foamed at an air flow rate of 38.5±1 cm^3/min for different foaming times (10-60 min).

Both enrichment ratio and separation ratio increased as the foaming time increased (Figure 3.12 and Figure 3.13). At higher foaming times, greater foam heights were obtained and this increased the time for liquid drainage. Therefore, drier foam with higher foam protein concentration was obtained when the solutions were foamed for longer times.

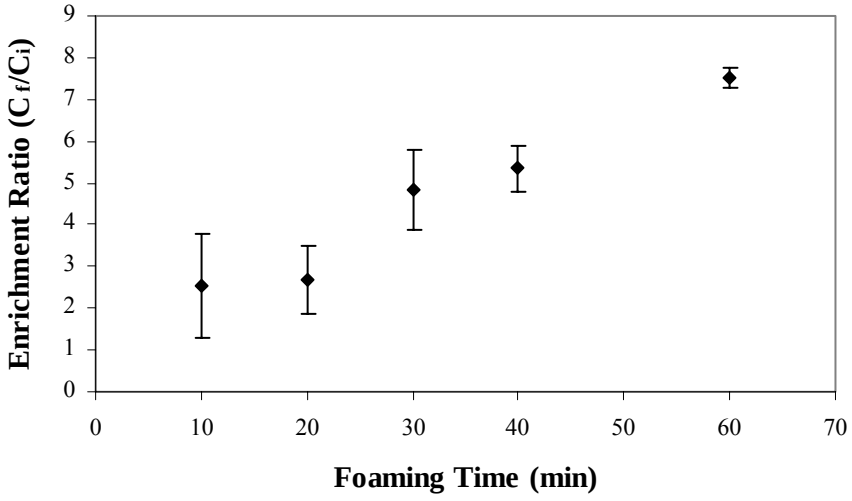


Figure 3.12 Effect of foaming time on enrichment ratio (at room temperature: 20-25 ºC, air velocity: 38.5±1 cm^3/min, feed solution concentration: 0.064 mg/ml, feed volume: 250 ml, foaming time: 10-60 min). The points are average of triplicate experiments.

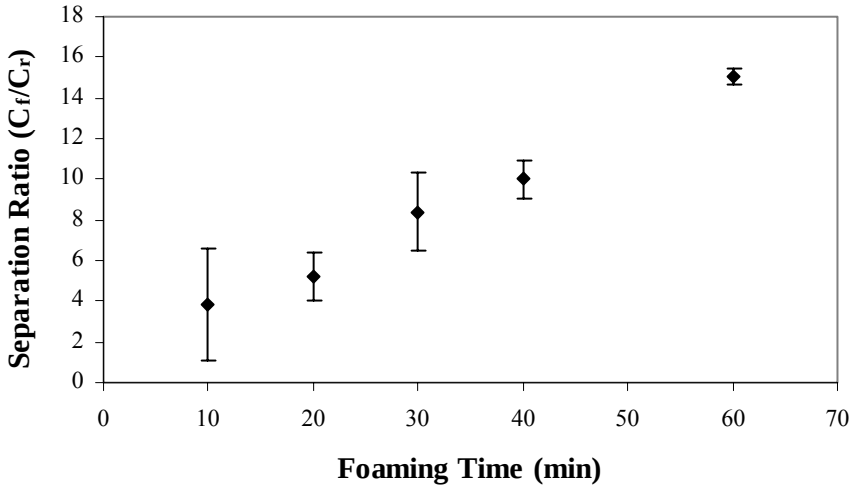


Figure 3.13 Effect of foaming time on separation ratio (at room temperature: 20-25ºC, air velocity: 38.5±1 cm^3/min, feed solution concentration: 0.064 mg/ml, feed volume: 250 ml, foaming time: 10-60 min). The points are average of triplicate experiments.

As shown in Figure 3.14, protein recovery first increases to a maximum of 43.9 % and then levels of to 32 % as the foaming time was increased and remained approximately constant at this value with further increases in foaming time. A low protein recovery was obtained by foaming the broth for 10 min, because a small volume of foam was formed and both the foam height and drainage time was insufficient to achieve high protein concentration in foam phase. As a result, a small volume of foam with low protein concentration caused the total protein recovered in the foam phase to be low. When the solution was foamed for 20 min, a high foam column was obtained, but the foaming time was insufficient for adequate liquid drainage, and low protein concentration was found for the foam phase. Although the foam protein concentration was low, high foam volume ensured the maximum protein recovery at this condition. Further increases in

foaming time produced greater foam heights, and hence higher liquid drainage. High foam protein concentrations together with low foam volumes obtained at these foaming times resulted in similar protein recoveries.

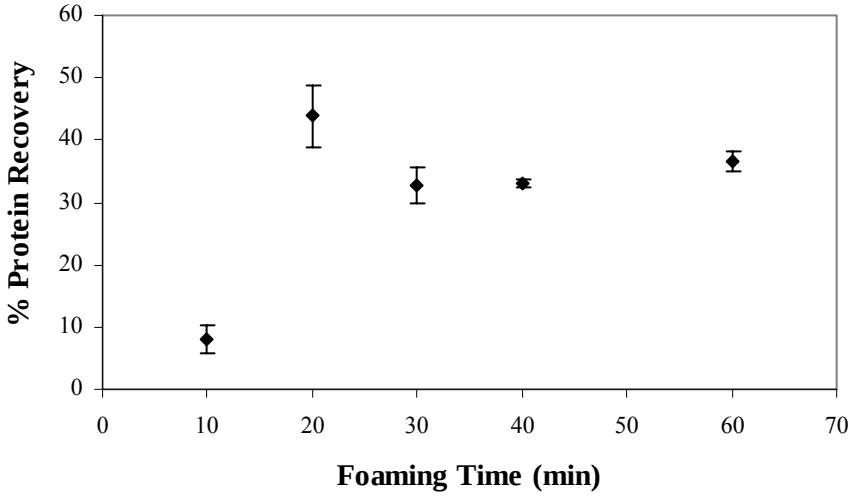


Figure 3.14 Effect of foaming time on % protein recovery (at room temperature: 20-25 ºC, air velocity: 38.5±1 cm^3/min, feed solution concentration: 0.064 mg/ml, feed volume: 250 ml, foaming time: 10-60 min). The points are average of triplicate experiments.

3.2 Recovery of Proteins from Spent Brewer's Yeast Extract Solution

Efficiency of foam fractionation technique in recovery of proteins from industrial wastes was investigated using spent brewery yeast extract.

Feed solution was prepared by dissolving 10g of spent brewer's yeast extract powder in 1 L distilled water. The solution was centrifuged at 11000 rpm for 5 min and filtered through coarse filter paper before use.

Effect of pH on fractionation performance was studied over a pH range from 3 to 11. pH of the feed solutions were adjusted by adding either 1.0M HCl or 1.0M NaCl. Experiments were carried out in semi-batch mode in a glass column of inside diameter 2.8 cm and total length 62 cm. Air was forced through a wooden sparger at a flow rate of 18.5 cm^3/min, which was determined as the minimum air flow rate for onset of foaming in yeast extract solution. Water loss in the effluent air stream was minimized by humidifying the air prior to column entry. Air was allowed to flow into the column for 30 minutes, and the total volume of foam formed was collected into a vessel using a vacuum pump. Foam samples were allowed to collapse at 4ºC for 24 hours, before analyzed for protein concentration using the Bradford Coomassie Blue method at 595 nm.

Surface tensions of the solutions were measured at 20ºC with a Kruss surface tensiometer using the ring method.

The effect of pH on the surface tension of spent yeast extract solution is shown in Figure 3.15.

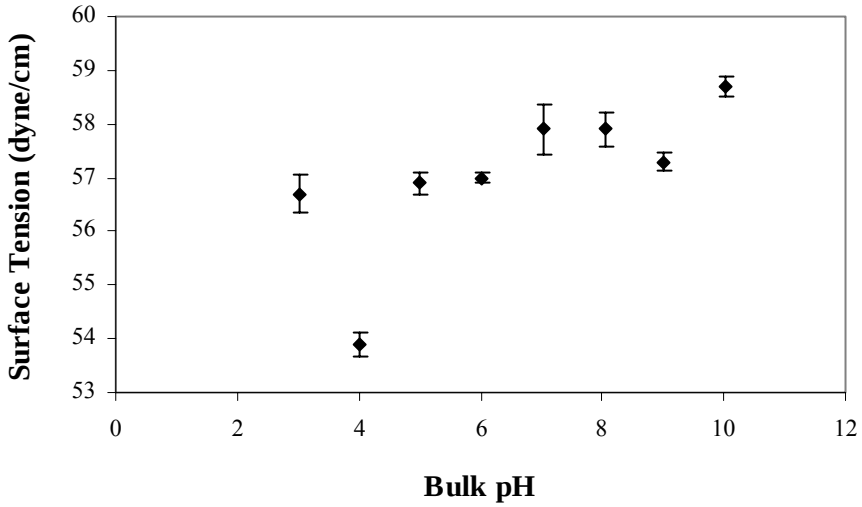


Figure 3.15 Effect of pH on the surface tension of spent yeast extract solution. (initial protein concentration: 0.504 mg/ml, changed within a range of 0.399-0.570 mg/ml after pH adjustment. The points are average of duplicate experiments.

The surface tension of the yeast extract solution at concentration of 0.399-0.570 mg/ml, showed high decreases relative to that of pure water, (approximately 72.6 dyne/cm at 20ºC) at all pH levels, indicating significant protein adsorption at the air-water interface.

At pH 4, a high decrease in surface tension was found relative to that at other pHs. This decrease might be due to increase in hydrophobicity of the protein molecules at this acidic condition. There was slight decrease at pH 6 and 9. These minimum values in surface tension might correspond to isoelectric point values of proteins that were present in high amounts in yeast extract solution.

In the foaming experiments, highest protein concentration in the foam phase were obtained at pH 4, 8 and 9, the values being 2.04, 1.78 and 1.84 mg/ml respectively

(Figure 3.16). These pH values also correspond to the conditions at which highest enrichment and separation values were obtained.

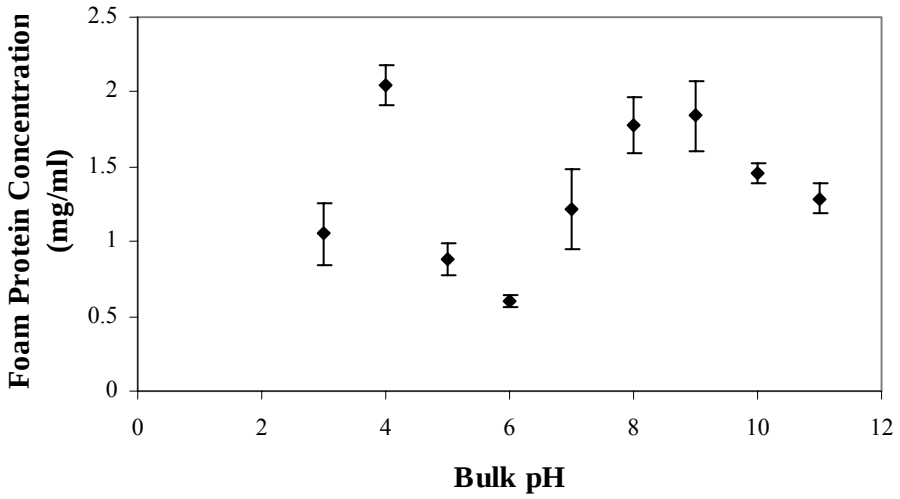


Figure 3.16 Change of foam protein concentration with respect to bulk solution pH (initial protein concentration: 0.504 mg/ml, changed within a range of 0.399-0.570 mg/ml after pH adjustment; total foaming time: 30 min, temperature: 20-25ºC). The points are average of duplicate experiments.

As shown in Figure 3.17 and 3.18, in the conditions studied, highest enrichment and separation ratios were obtained at pH 4, the values being 5.1 and 21.7, respectively.

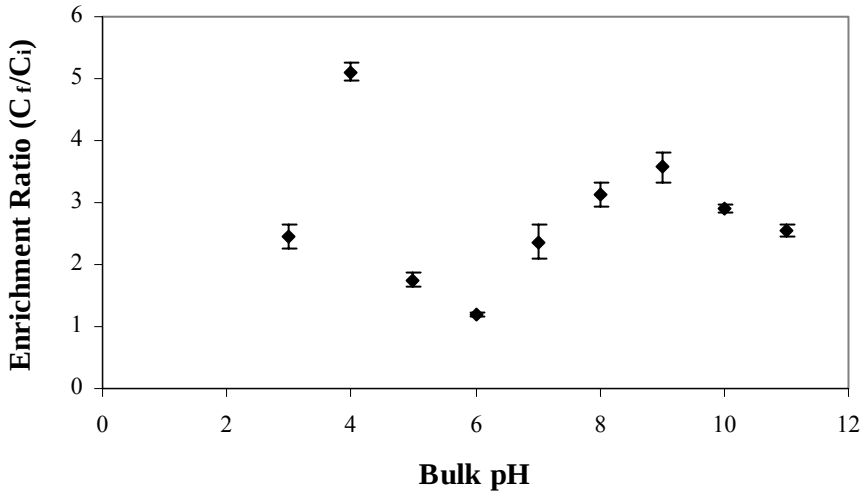


Figure 3.17 Effect of bulk solution pH on enrichment ratio (initial protein concentration: 0.504 mg/ml, changed within a range of 0.399-0.570 mg/ml after pH adjustment; total foaming time: 30 min, temperature: 20-25ºC). The points are average of duplicate experiments.

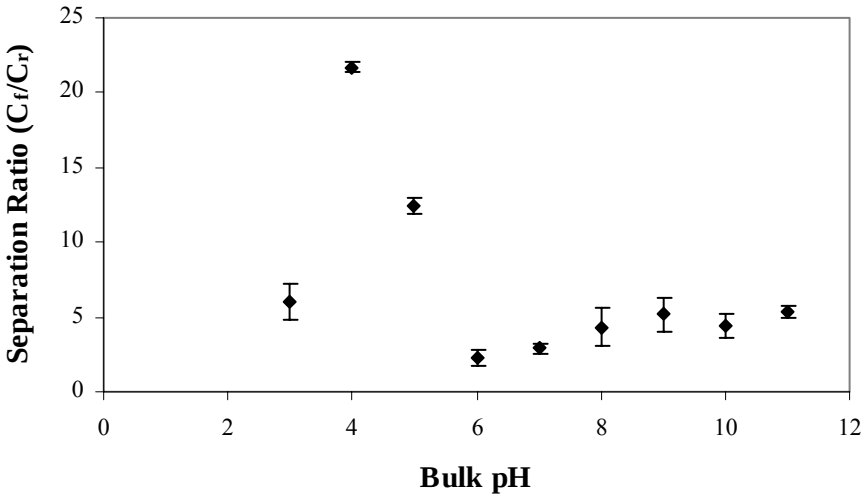


Figure 3.18 Effect of bulk solution pH on separation ratio (initial protein concentration: 0.504 mg/ml, changed within a range of 0.399-0.570 mg/ml after pH adjustment; total foaming time: 30 min, temperature: 20-25ºC). The points are average of duplicate experiments.

3.3 Selectivity of Foam Fractionation in Recovery of Proteins

Selectivity of foam fractionation technique in recovery of proteins was investigated using spent brewery yeast extract.

Feed solution was prepared by dissolving 2.8 g of spent brewer's yeast extract powder in 1 L distilled water. The solution was centrifuged at 11000 rpm for 5 min and filtered through coarse filter paper before use.

200 ml of yeast extract solution was foamed at room temperature at an air flow rate of 200 cm^3/min for 15 min using a glass foam fractionation column of inner diameter 2.8 cm and total height 62 cm.

SDS-PAGE was used to determine differences in protein content of the feed solution, residual solution and foam sample by comparing the intensity of protein bands of these solutions.

In SDS-PAGE, proteins are roughly separated according to their size (molecular weight). Depending on their size, proteins differentially migrate through the gel matrix: smaller proteins move easily through the pores in the gel and travel farther down the gel, while the mobility of larger ones are retarded by the sieving effect of the gel and they remain closer to the point of origin.

SDS-PAGE analysis of samples from foam fractionation of spent yeast extract solution is shown in Figure 3.19.

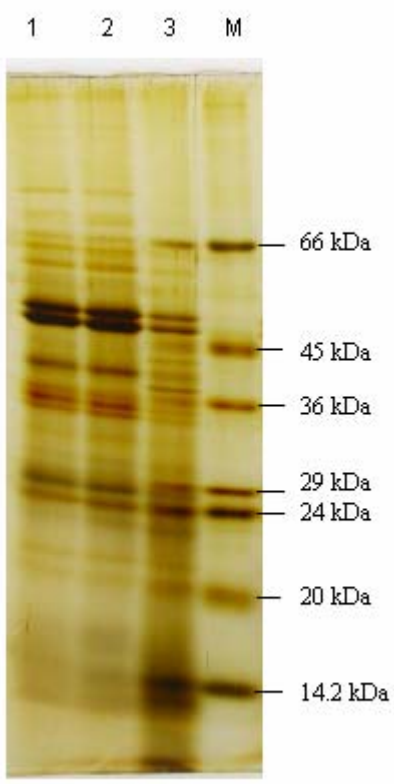


Figure 3.19 SDS-PAGE analyses of spent yeast extract foam fractionation samples. The proteins on the gel were silver-stained. Molecular weight standard proteins are marked as "M" above the lane, lane 1: feed solution, lane 2: residual solution, lane 3: foam sample. Feed protein concentration: 0.147 mg/ml; residual solution protein concentration: 0.106 mg/ml; foam sample protein concentration: 0.328 mg/ml.

The amount of protein in samples loaded to each well was 10 g. Feed solution contained a large number of proteins having molecular weights within a range of 85kDa to 14 kDa. As can be seen from the figure (Figure 3.19), some bands were thicker in the foam sample (lane 3), while the others in the feed and residual sample. This resulted since the proteins were present in larger amounts in that

sample when compared to the others. From the SDS-PAGE analysis, it can be said that some proteins were concentrated in the foam samples while others mainly remain in the residual solution, and selective separation/concentration of proteins is possible with foam fractionation technique.

3.4 Effect of Foam Fractionation on Protein Structure

An attempt has been made to determine the effect of foam fractionation on protein structure using nondenaturing polyacrylamide gel electrophoresis (native-PAGE). For this purpose, 150 ml of waste beer was foamed at an air velocity of 24 cm^3/min at room temperature.

In native-PAGE, proteins are separated according to both their size and charge. Migration through gel occurs because most proteins carry a net negative charge at slightly basic pH. Proteins with higher negative charge density (more charges per molecule mass), migrates faster. At he same time, frictional force of the gel matrix retards movement of the proteins according to their size and smaller proteins move faster than the larger ones.

In this study, native-PAGE is employed to determine differences in feed solution, residual solution and foam sample by comparing the differences in protein bands in the lanes of these solutions.

Native-PAGE analysis of foam fractionation of waste beer is shown in Figure 3.20. As can be seen from the figure, the bands that are present in the feed solution lane (lane 1) were not recognizable in the lane for foam sample. This might be caused by breaking of the proteins into smaller molecules.

From the Native-PAGE analysis, it can be said that, foam fractionation might cause changes in protein structure. Based on the studies of Clarkson *et al*. (1999)

and Phillips *et al*. (1995), these changes can be attributed to surface denaturation caused during adsorption of proteins and shear stress which might be caused during foam collection due to the use of vacuum pump for collection.

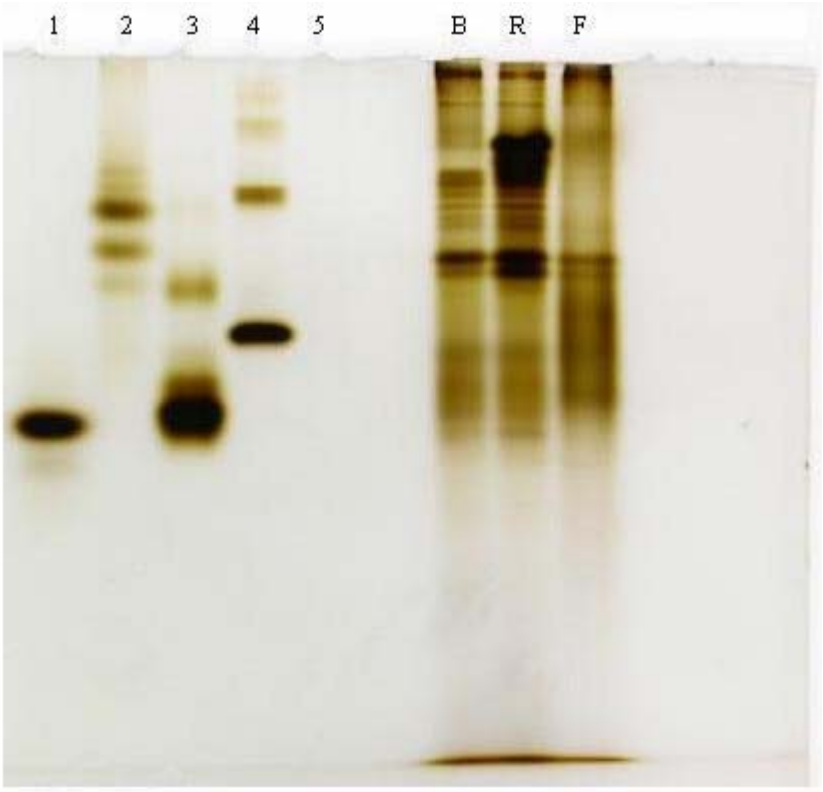


Figure 3.20 Native-PAGE analysis of residual beer foam fractionation. Lanes 1-4 are molecular weight markers (1. α-lactalbumin, 2. carbonic anhydrase, 3. chicken egg albumin, 4. BSA), B: residual beer feed solution, R: residual solution, F: Foam sample. Each sample contains 10 μg of protein.

CHAPTER 4

CONCLUSION

In this work, application foam fractionation technique on partial removal of proteins from fermentation broth and recovery of proteins from industrial wastes was investigated. An attempt was made to determine the optimum operating conditions for removal of proteins from lactic acid broth and efficiency of protein recovery from brewery wastes.

A study of different parameters affecting process efficiency revealed that high enrichments together with high recovery can be obtained at the optimum values of operating variables. It was found that low initial feed concentration, low air flow rate, and high foaming time enhanced separation efficiency. Larger liquid pool heights were also found to assist separation. Conditions which tend to promote high enrichments, due to high protein concentrations in the foam, were also found to promote high separation ratios. pH was found to have a significant effect on the foam fractionation efficiency of brewer's yeast solutions.

Based on the experimental results, it can be said that foam fractionation is an efficient method of separation that can be used industrially to concentrate dilute solutions fermentation broth and to recovery proteins from industrial waste streams.

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APPENDIX A

BRADFORD'S DYE BINDING PROTEIN ASSAY

Preparation of Solutions:

A) Bradford's Reagent (1X):

100 mg Coomassie Brilliant Blue G 250 (Eastman)
50 ml 95 % Ethanol
100 ml phosphoric acid (85 % pure)
After the dye is completely dissolved, dilute the solution to 1 L with d H_2O. Filter through ordinary filter paper into a dark bottle (reagent is sensitive to light).
This solution can be kept at room temperature in tightly closed dark bottle up to 2 weeks.

B) BSA Protein Standard (1 mg/ml):

Weigh 104.17 mg of BSA (Sigma A2153) 96% and dissolve in 0.85 % NaCl and 0.1 sodium azide as preservative. This solution can be stored at +2 to +8 °C.

APPENDIX B

STANDARD CURVE PREPARATION TABLE AND STANDARD CURVE FOR BRADFORD METHOD

Table A.1 Standard Curve Preparation Table for Bradford Method

Tube #	**BSA Standard (μL)**	**Distilled Water (μL)**	**Bradford Solution (ml)**	**Protein Concentration (μL)**
1	-	500	5	0
2	5	495	5	0,01
3	10	490	5	0,02
4	15	485	5	0,03
5	20	480	5	0,04
6	25	475	5	0,05

Bradford solution must be at room temperature before addition.

After addition of the Bradford's solution, samples were vortexed and mixed well, waited for 10 minutes and optical density were measured at 595 nm within 1 hour.

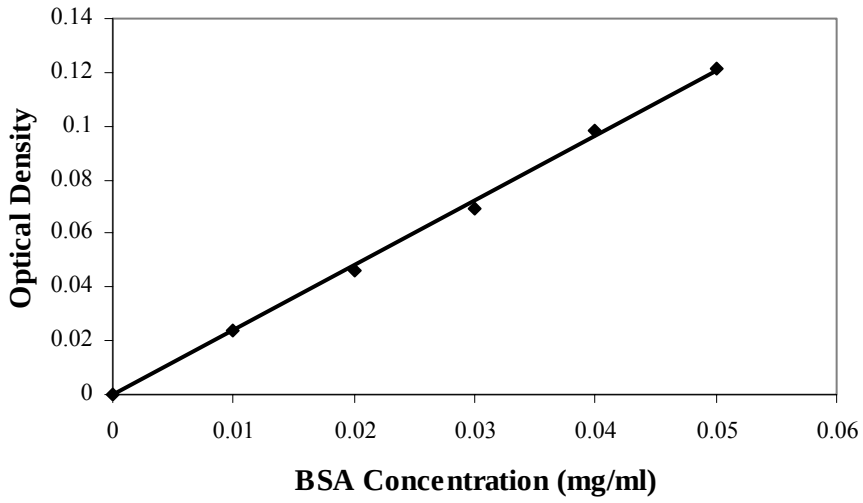

Figure A.1 Standard Curve for Bradford Method

APPENDIX C

SDS- POLYACRYLAMIDE GEL ELECTROPHORESIS

Reagents

A) 30% Acrylamide/bis Solution

Acrylamide is a nerve toxin. Therefore, precautions should be taken by wearing gloves and mask during preparation of this solution.

- Weigh 58.4 g acrylamide in a 500 ml beaker.
- Add 1.6 g NN-bis-methylene-acrylamide
- Pour 100 ml dH_2O into the beaker, cover the beaker with aluminum foil due to light sensitivity, and mix on a magnetic stirrer.
- When it is completely dissolved, complete the volume to 200 ml with dH_2O.
- Store the solution in dark bottle at 4ºC.
- After 30 days of storage, discard the remaining solution after polymerizing.

B) 10% SDS Solution

- Dissolve 10 g of SDS (Sodium Dodecyl Sulfate) in 100 ml dH_2O.

- Gently stir to prevent foaming of SDS.

C) 1.5 M Tris-HCl, pH 8.8 Buffer

- Weigh 54.45 g Tris base and dissolve in 150 ml dH_2O by stirring on a magnetic stirrer.
- Adjust the pH to 8.8 with concentrated HCl while continuously stirring.
- Complete the volume to 300 ml with dH_2O and store at 4ºC.
- Discard the remaining solution after 30 days.

D) 0.5 M Tris-HCl, pH 6.8 Buffer

- Weigh 6 g Tris base and dissolve in 60 ml dH_2O.
- Adjust the pH to 6.8 with concentrated HCl while mixing on a magnetic stirrer.
- Complete the volume to 150 ml with dH2O and store at 4ºC.
- Discard the remaining solution after 30 days.

E) 10% Ammonium Persulfate (APS)

- Dissolve 100 mg APS in 1ml dH_2O in an Eppendorf by vortexing.
- Prepare freshly on the day of usage.

F) Sample Buffer

Mix together

- 1 ml of 0.5 M Tris-HCl, pH 6.8 buffer
- 0.8 ml glycerol
- 1.6 ml of 10% SDS solution

- 0.4 ml β-mercaptoethanol
- 0.4 ml of 0.05% (w/v) bromophenol blue (in water)
- 3.8 ml dH_2O

Do not add 3.8 ml distilled water in this solution to prepare concentrated sample buffer.

G) Running Buffer

- Weigh 9 g Tris base and 43.2 g glycine and dissolve in 600 ml dH_2O
- Complete to volume to 1000 ml and transfer the solution into the electrophoresis tank.
- Dilute the solution to 3 ml with dH_2O.
- Add 3 g SDS and stir on a magnetic stirrer.
- Place the tank in cold room and stir continuously.

Procedure

A) Preliminary Preparation

- Clean the surface of the glasses with pure ethanol
- Align the spacers at the two edges of the big glass and place the small glass on it. Adjust the bottom edges very carefully in order not to have leakage.
- Install the clamps and fasten screws.
- Place the glass sandwich on the base and check that the bottoms of the glasses are properly sealed by pouring dH_2O inside the sandwich.
- Dry the glasses with filter paper.

B) Preparation of SDS-PAGE Gel Solution

Separating Gel (for two 10 % Slab Gel):

Add the followings into an Erlenmeyer flask and shake gently:

- 33.5 ml of 30 % acrylamide/bis solution
- 40 ml of distilled water
- 25 ml of 1.5 M Tris-HCl, pH 8.8 buffer
- 1.0 ml 10% SDS solution
- 500 μl of 10% APS
- 50 μl of TEMED

Add APS and TEMED when you are ready to pour the gel inside the glass sandwich.

Immediately collect the gel solution into a glass pipette using a pump and discharge it into the space between the glasses very gently not to cause formation of bubbles. Fill the space up to 5 cm below the upper edge of the small glass.

With a Pasteur pipette, pour distilled water onto the gel in order to avoid the contact of the gels with air that prevents polymerization of the gel.

Allow 1 hour for complete polymerization.

Stacking Gel (for one 4% gel):

Add the followings into an Erlenmeyer flask and shake gently:

- 1.3 ml of 30 % acrylamide/bis solution
- 6.1 ml of distilled water

- 2.5 ml of 0.5 M Tris-HCl, pH 6.8 buffer
- 100 μl 10% SDS solution
- 50 μl of 10% APS
- 10 μl of TEMED

Dry the upper part of the gel with a filter paper. Immediately pour the gel solution between the glasses and place the comb inside the glass sandwich carefully not to cause any air bubbles. Allow 30 minutes for polymerization.

After polymerization is completed, take off the comb and fill the wells with loading buffer.

Samples and molecular weight markers are loaded into the wells with the help of a Hamilton syringe. The syringe should be inserted to about 1-2 mm from the well bottom before delivery & rinsed between samples. The samples are prepared by heating them in the presence of SDS and β-mercaptoethanol, the aim of this step is to denature the protein chains.

Load the standard molecular weight markers and each sample into one well. Install the slab gel sandwiches to the cooling core. Fill the upper chamber of the core with loading buffer. Gently place the cooling core into the electrophoresis tank. Make sure that there are no bubbles trapped on the upper side of the glass sandwich. If bubbles are present, remove them with a glass rode, since these bubbles can act as an insulator.

Conduct the separation at 4°C by operating at 25 mA constant current in the region of stacking gel and then at 35 mA per gel for the separating gel.

When the separation is over, carefully extrude the gels and immerse them in fixing

solution containing 50 % methanol, 12% acetic acid and 0.5 ml of 37% formaldehyde/L. perform shaking for 5 minutes on a platform shaker and keep the gels in fixing solution for at least 1 hour.

Stain the gels with silver staining procedure of Blum *et al.* (1987).

Molecular weight markers used for SDS-PAGE are given in Table A.2.

Table A.2 Molecular weight marker proteins used in SDS-PAGE

Proteins	***Approx. MW (kDa)***
α-lactalbumin	14.2
Trypsin inhibitor	20
Trypsinogen	24
Carbonic anhydrase	29
Glyceraldehyde-3-phosphate dehydrogenase	36
Chicken egg ovalbumin	45
BSA	66

APPENDIX D

NATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS

All steps are the same as in the SDS-PAGE except for there is no SDS solution and β-mercaptoethanol in the sample buffer, loading buffer and gel solution of Native-PAGE, distilled water is added instead.

Molecular weight markers used are given in Table A.3.

Table A.3 Molecular weight marker proteins used in native-PAGE

Proteins	***Approx. MW (kDa)***
α-lactalbumin	14.2
Carbonic anhydrase	29
Chicken egg albumin	45
BSA	66

Each standard marker protein is loaded into a separate well.

For silver staining, samples are prepared in order that each sample contains approximately 2 μg of protein and same volume of sample buffer.

APPENDIX E

SILVER STAINING OF THE SLAB GELS

Reagents:

A) Fixer:

Mix 150 ml methanol with 36 ml acetic acid and add 150 microliter of 37 % formaldehyde and complete the volume to 300 ml with d H_2O

B) 50 % Ethanol:

Prepare 1 liter of 50 % ethanol

C) Pretreatment Solution (Sodium thiosulfate solution):

Dissolve 0.08 g sodium thiosulfate ($Na_2S_2O_3.5H_2O$) in 400 ml d H_2O, mix with a glass rod and take 8 ml for further use in developing solution preparation

D) Silver Nitrate Solution:

Dissolve 0.8 g silver nitrate in 400 ml d H_2O and add 300 microliter 37% formaldehyde.

E) Developing Solution:

In an Erlenmeyer flask measure 9 g potassium carbonate and ad 8 ml previously kept pretreatment solution and 300 microliter 37% formaldehyde. Complete the

volume to 400 ml with d H_2O.

F) Stop Solution:

In an Erlenmeyer flask mix 200 ml methanol and 48 ml acetic acid and complete the volume to 400 ml with d H_2O.

Procedure:

The procedure given in the table below was followed. All steps were performed by constant shaking on a platform shaker.

Table A.4 Procedure of silver staining

	Step	**Solution**	**Time**	**Comments**
1	**Fixing**	Fixer	> 1 hour	
2	**Washing**	50 % Ethanol	3 x 20 min	
3	**Pretreatment**	Sodium thiosulfate solution	1 min	Time exact
4	**Rinse**	d H_2O	3 x 20 sec	Time exact
5	**Impregnate**	Silver nitrate solution	20 min	
6	**Rinse**	d H_2O	2 x 20 sec	Time exact
7	**Developing**	Developing solution	App. 5 min*	
8	**Wash**	d H_2O	2 x 2 min	
9	**Stop**	Stop solution	> 10 min	

* Time was adjusted according to the color development.