This is to certify that the

dissertation entitled

The Use of Column Flotation in the Downstream Processing of Fermentation Products

presented by

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has been accepted towards fulfillment of the requirements for

Ph.D. degree in Chem. Eng.

Major professor

Date 3/9/93

DATE DUE	DATE DUE	DATE DUE
SEP 26 1994		
DEC 10 1995		

THE USE OF COLUMN FLOTATION IN THE DOWNSTREAM PROCESSING OF FERMENTATION PRODUCTS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemical Engineering

1993

ABSTRACT

THE USE OF COLUMN FLOTATION IN THE DOWNSTREAM PROCESSING OF FERMENTATION PRODUCTS

By

Everson Alves Miranda

Flotation has been considered a unit operation with a largely unexplored potential in biotechnology. There is a general lack of studies concerning its application in the recovery of fermentation products. This work is the study of column flotation for the recovery of products of fermentation.

Two approaches for the application of column flotation in biotechnology were addressed in this work. First, a unique separation of a product was accomplished by recognizing the natural floatability of a particulate separation system previously determined. A process to recover *Bacillus stearothermophilus* α-amylase expressed in *Escherichia coli* from fermentation broth and periplasmic extract had been developed (Summerfelt, 1988). The solid-liquid separation of the salted-out particles of (hydroxypropyl)methylcellulose by filtration and centrifugation was inefficient. Column flotation was able to recover virtually 100 percent of the enzyme partitioned to the salted-out particles with no loss of enzymatic activity. The recovery of the extracellular enzyme directly from the fermentation broth required the elimination of

small molecular weight compounds by ultrafiltration for improved recovery.

The second approach for application of flotation in biotechnology, the design and preparation of a flotation system to perform separation of proteins based on specific type of interaction between a product and a ligand, was also developed in this work. Hydrophobic interaction between albumin and hydrophobic groups was selected for the system developed. First, derivatives of methylcellulose with fatty acids were prepared by esterification. After characterization, the derivatives were tested in the precipitation of bovine serum albumin. One of the derivatives was shown to be efficient in the precipitation of the protein. The predictability of the recovery of the protein by flotation was good despite low floatability due to the large concentration of salt and protein.

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Gravity? Why fight it?

Fighting gravity is a losing proposition, and yet that is precisely what conventional flotation does. It fights gravity.

The column does not.

D. A. Wheeler

Acknowledgements

I would like to thank my advisor, Dr. Kris Berglund for the friendship and freedom he provided to develop a research project of my choice. Thanks also to the members of my committee, Dr. Oriel, Dr. Steffe, and Dr. Worden, for their encouragement and participation in fruitful discussions.

I am in great debt to my family for their patience and support during all these years.

I would also like to thank all my friends who made it possible to overcome the difficult times. I will always remember them.

Finally, I would like to express my deep appreciation for the financial support provided by the Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, Brazil.

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Introduction and Objective

1.1 - Scope: Biotechnology and Downstream Processing

Biotechnology, a science as old as history, deals with the use of animal cells, plant cells and microorganisms to produce products useful to humans. It was at the beginning, thousands of years ago, an unconscious use in the production of ethanol in the form of beer or wine, curdled milk products, cheese, and East Asian and Oriental foods. The pioneer work of Pasteur and other scientists showed that microorganisms were capable of metabolic reactions of practical utilization. What followed these findings was the development of manufacturing processes for primary metabolites, such as glycerol, citric acid, and butanol/acetone. A characteristic of such processes was their ability to be run without "absolute" prohibition of foreign infections. The next phase of biotechnology was the development of processes that were run with the exclusion of unwanted microorganisms. This opened the era for the production of compounds formed only in very small amount by cells. Microbiological transformations could be done and many secondary metabolites such as vitamin B_{12} , cortisone/cortisol, and estrogenic ovulation inhibitors could be manufactured. The most recent step in the evolution of biotechnology was the birth of the genetic engineering that provided a degree of control over microorganisms never deemed possible. Products can now be

"tailored-made" and microorganism growth and action on substrate optimized (Präve *et al.*, 1987).

Biotechnology is the result of the interweaving of many different areas of knowledge. Among them the three more relevant are microbiology, including microbial genetics, biochemistry, and process engineering including equipment construction.

One characteristic of biotechnology industries is the variety of the products involved. While a typical petrochemical company produces around 10 products, a typical drug company produces more than 200 which are, at least in part, made biochemically (Belter *et al.*, 1988). Regardless if a substance is produced by fermentation or extraction (from animal tissues or plants), it is often dilute and contaminated with other bioproducts. Concentration and purification of the desired product (or products) to a practical, economical, and useful level is accomplished by a series of process steps named downstream processing.

Downstream processing is carried out by the use of well-known unit operations of chemical engineering such as liquid-liquid extraction as well as by methods traditionally used in biochemical laboratories such as chromatography and electrophoresis. The scale of these processes vary from hundreds of milliliters to hundreds of cubic meters. The operations can be continuous or batch; steady- or unsteady-state; cocurrent or counter-current contacting. They may involve physical separations, rate controlled separations, and equilibrium controlled separations. Figure 1.1 shows a general flow sheet for a typical production process in biotechnology.

The viscous, heat unstable, and highly non-Newtonian feedstock, the low product concentration, and the chemical and physical similarities

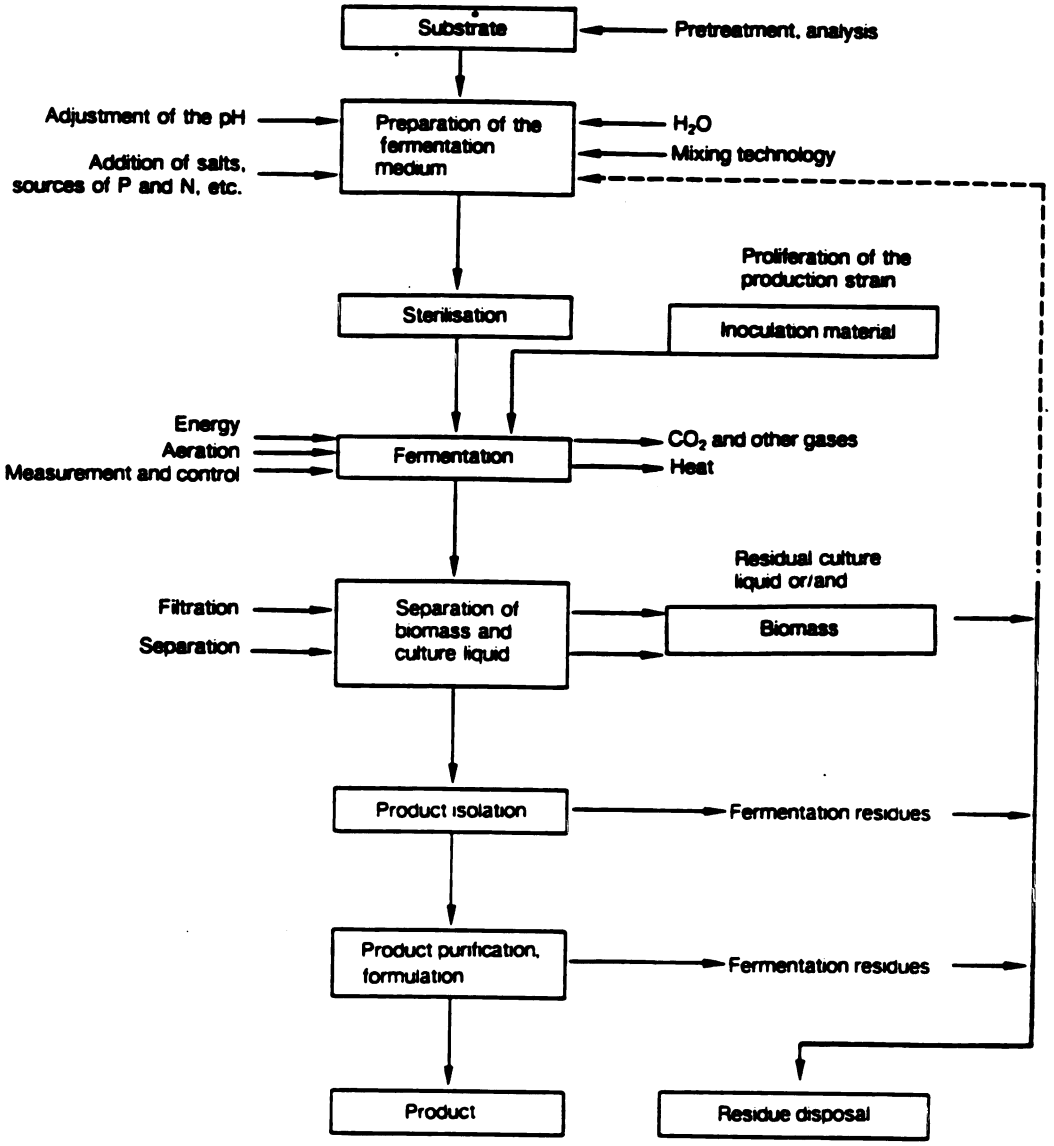

Figure 1.1 - General flow sheet of a typical production process in biotechnology (from Präve *et al.*, 1987).

among product and contaminants make downstream processing a difficult task. Typically, product specifications are attained only after different separation and/or concentration methods are applied, sometimes more than once. Product loss, space, and capital and operating costs often make downstream processing the major component in the economics of biotechnological processes. In fact, a company's competitiveness may depend on how well it accomplishes downstream processing. (Belter *et al.*, 1988; Dechow, 1989; Parker *et al.*, 1990; Wheelwright, 1987; Zlokarnik, 1990).

The search for simple, efficient and low cost downstream processes is a present challenge for the biochemical engineer (Kelly, 1987). The proposed research is one potential answer to this challenge.

1.2 - Flotation and its Potential in Biotechnology

1.2.1 - Flotation as a Unit Operation

Flotation is a technique mostly used in the selective separation and concentration of particles, mainly minerals. It is based on the ability of particles in a aqueous suspension to attach themselves to gas bubbles which carry them up in the liquid phase due to buoyancy. The removal of these particles as they reach the top of the liquid phase along with only a small amount of liquid results in a concentration effect (solid-liquid separation). If particle attachment is selective towards a specific class of particle, enrichment takes place.

Bubble generation is done by air dispersion (the most common procedure), by reducing air solubility with pressure reduction, or by electrolysis. These bubble generation methods characterize what is

referred as dispersed-air flotation, dissolved-air flotation, and electrolytic flotation, respectively (Matis and Mavros, 1991).

There are chemical agents that can change the surface properties of particles making possible the design and control of the specificity and yield of the separation. By the use of appropriate surfactants, particles can have their hydrophobicity increased. These surfactants are called collectors. Agents used to enhance or prevent interaction of the particles with collectors are called activators or depressors, respectively. Those agents which make the liquid foam are called frothers.

Flotation is conducted in special equipment called flotation cells. Leja reported the existence of more than 40 different designs of such cells which are variations of three basic types: mechanical cell, pneumatic cell, and cyclone cell (Leja, 1982). Figure 1.2 is a scheme of a mechanical cell. The impeller is a bubble generation device and keep particles in suspension in order to make particle-bubble contact and attachment possible. There is also a skimming device of some sort at the top of the cell to remove the froth which is rich in hydrophobic particles. The other two types of cells differ from the mechanical one mainly by the absence of an impeller. In pneumatic cells, feed is introduced over the top of the froth bed which is generated by air injection through a array of tubes located under this bed. In cyclone cells, highly pressurized feed together with air is introduced tangentially in a cyclone feeder located inside the cell that otherwise look similar to a mechanical cell.

The importance of flotation to the economy of the industrialized world can be illustrated by the amount of mineral materials floated in the United States: about 1.1 millions tones of ores and raw coal in 1985

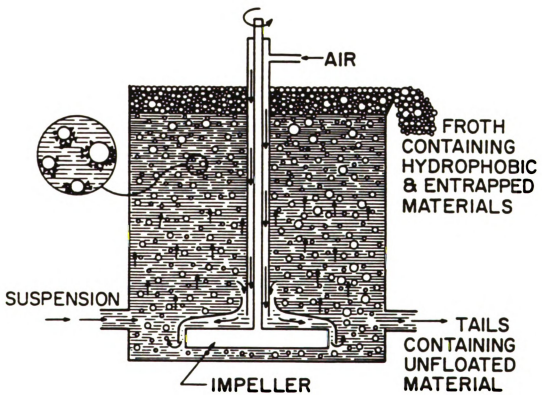

Figure 1.2 - Schematic diagram of a flotation cell (from Somasundaran and Ananthapadmanabhan, 1987).

(United States Bureau of Mines, 1985). This primary use of flotation to treat commodities of very low-value, ores, is confirmation of one the most important characteristics of flotation, its low cost. Other advantages are selectivity and versatility.

The first mention of a separation technique we could call flotation, a copper carbonate enrichment, dates back to the 15th century (Kitchener, 1984). The first patent related to mineral flotation, which was concerned with differences in wettability of minerals by water and oil, was issued in 1860 (Anonymous, 1982). The golden age of the invention of flotation was the period between 1910-1915. It was the result of small contributions of many people. T. J. Hoover, in 1912, named 57 people who had made significant contributions to the development of flotation up to that date (Kitchener, 1984). In 1921, a Norwegian engineer by the name Nils Padersen patented a flotation machine in the United States (Parker, 1958).

1.2.2 - Flotation in Biotechnology

The typical aqueous environment and the surface activity of many bioproducts certainly made the extension of the use of flotation to biotechnology a logical step [1]. Besides that, there has been a significant concern and knowledge about properties of biological media and products such as foaming and denaturation due their undesired effects like loss in activity and fermentation operational problems. However, natural properties such as hydrophobicity and foaming made the application of flotation not only possible but also viable, simple, and inexpensive. These

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[1] When looking through the biotechnology literature we must be careful in identifying what authors called flotation. Some authors called flotation some other adsorptive bubble separation techniques like bubble and foam separation. Description of these techniques follows.

characteristics are not only desirable in flotation but also a must since the addition of chemicals to promote hydrophobicity and foaming would be restricted in the case of dealing with food-grade or pharmaceutical products (Evans *et al.*, 1970; Kalischewski *et al.*, 1979; Kolsaridu *et al.*, 1983; Vardar-Sukan, 1988).

The first patents on the use of flotation in biotechnology may have been the processes for production of algin from seaweed by LeGloahec and Herter in 1938 and the separation of germinating from non-germinating seeds by Thomas Earle in 1939. The next decade saw a wide application of flotation in the processing of biological materials. Rubber, oils, and resins were recovered from plants, fat from wool and animal tissues, and proteins from dairy effluents. Sugar solutions were better clarified, starch and gluten were separated from each other, and the first flotation of microorganisms (Koch bacillus, *Escherichia coli, Staphylococcus albus, and Schirosaccharomyces sp.*) was done by Dognon and Dumontet in 1941 (Dognon and Dumontet, 1941; Gaudin, 1957; LeGloahec and Herter, 1938; Thomas and Winkler, 1977; Turrall *et al.*, 1943).

After the initial burst of broad application, flotation in biotechnology was restricted to waste treatment, probably due to the fear of loss of biological activity of fermentation products. Waste treatment targeted the removal of proteins, lipids, antibiotics, adsorbing agents as activated charcoal, and especially microorganisms. An advantage of microorganisms over minerals with respect to flotation is their homogeneity in shape and size. The disadvantage is their microscopic size that hinders attachment to the gas bubbles. Many different bacteria were concentrated, with or without the use of collectors (cell enrichment ratios up to 10,000,000); spores were separated from vegetative cells and cell

debris; non-foaming mutants were separated from foaming parent yeast cells; algae were recovered from cultures; and lead was removed from solution by flotation of microorganisms to which it had adsorbed and provided increase in hydrophobicity (Bishop, 1978; Gehle and Schürgel, 1984a; Golab and Smith, 1990; Grieves and Wang, 1966; Iibuchi *et al.*, 1974; Rubin, 1968; Rubin *et al.*, 1966; Thomas and Winkler, 1977).

1.2.3 - Potential of Flotation in Biotechnology

Limited interest of flotation lasted until recently when some investigators realized that biotechnology research has concentrated on the developing new bioreactors, the need for which was not evident, with disregard to the development of more efficient and less costly downstream process techniques. Among many techniques, flotation was cited as one with no doubt of successful application into biotechnology once process engineers recognize its potential (Gehle *et al.*, 1991; Hernáinz *et al.*, 1987; Zlokarnik, 1990). Parallel to that, the collection of data through the years made the fear of generalized loss of biological activity unfounded. The eventual accumulation of research data on the separation and concentration of an extended list of proteins showed that there is a considerable variation in the susceptibility of different proteins to denaturation. The loss in activity can vary from 0 to 50 percent, depending on the specific system and conditions used (Thomas and Winkler, 1977; Uraizee and Narsimhan, 1990). Therefore, for the right system, flotation is a viable option.

The potentially extensive application of flotation in biotechnology is its use as a solid-liquid separation or recovery unit operation in downstream processing, especially of fermentation products such as

proteins, amino-acids, antibiotics, and polysaccharides. Two approaches to accomplish these separations can be considered. First, some products are naturally insoluble or can be made insoluble in the media and then floated. Microorganisms, proteins, and polysaccharides are good examples. Second, most of the separation methods in biotechnology based on chemical or physical interactions can be adapted for flotation by the engineering of an appropriate carrying system. Details of these two approaches follow.

It was said above that flotation has been proven to be an efficient method to recover microorganisms in the treatment of waste water. Therefore, it would be logical to attempt to recover or concentrate cells from batch or continuous cultures. Miyazu and Yano determined that *Mycotorula japonica* can be effectively recovered from a aqueous suspension by flotation when the cells are grown with n-parafin instead of glucose (Miyazu and Yano, 1974). Continuous recovery of *Hansenula polymorpha* and *Saccharomyces cerevisiae* from a culture medium at the pilot plant level produced results better than laboratory tests at the Institut für Technische Chemie, Germany, one of the leading institutions in the development of flotation applications in biotechnology (Gehle *et al.*, 1991).

Proteinaceous materials and protein precipitates also can be sufficiently hydrophobic to attach and be lifted by air bubbles. Iibuchi and co-workers reported a much better recovery of hemoglobin and casein by particle flotation than by bubble separation when the proteins were in solution (Iibuchi *et al.*, 1974). In Northern Ireland, flotation was studied as a way to recover protein from fresh sea mussels. Recoveries up to 90 percent were reported (Holland and McComiskey, 1986; Holland and

Shahbaz, 1986). The advantage of flotation in this case is the possibility of simultaneous recover of insoluble and soluble protein (40 and 60 percent of the total protein, respectively) with the use of flocculants.

Polysaccharides with food and nonfood applications like xanthan, curdlan, levan, and cellulose derivatives are bioproducts that have at some point of their purification protocol an insolubilization step by the use of specific ions, pH, alcohol, increase in ionic strength, etc. (Cadmus *et al.*, 1976; Godet, 1973; Han, 1990; Harada *et al.*, 1968). Therefore, there are opportunities for the substitution of centrifugation, and especially filtration, by flotation in these processes since these products have a tendency to form gels that drastically clog filter media. Many of these compounds are non-ionic, a desirable characteristic when the final objective is to have a hydrophobic particle. One example of flotation of such a polysaccharide is the flotation of algin cited above (LeGloahec and Herter, 1938).

The second approach to recover bioproducts by flotation is the use of a carrying system. Soluble products can be floated by engineering of a carrying system which is insoluble (or can be insolubilized) and floatable. The motivation to design such a system is based on the fact that the majority of separations in biotechnology are based on the interaction between the molecule or molecules being separated with another molecule or group added to the system, as a solid, liquid or a species in solution, the agent of separation. Such interactions can be non-specific as in the case of adsorption on activated charcoal or specific such as electrostatic, hydrophobic, or affinity interactions with substrate analogs, inhibitors, triazine dyes, antigens, antibodies, etc. If the agent of separation is located on the surface of a particle, we have the case of adsorption, which

is usually carried out in packed beds. If it is in solution and two immiscible phases are formed, we have the case of aqueous two-phase extraction. The formation of an insoluble complex characterizes common precipitation or affinity precipitation. If the agent of separation is a high molecular weight compound and physical separation is accomplished by ultrafiltration, we have the case of affinity ultrafiltration. In general we must have a basic interaction between the molecule being separate and the agent of separation. The other details would dictate kinetics and the mechanics of the operation according the physical state of the phase in which the agent of separation is located. Therefore, if we engineered a system in which the agent of separation is associated with a carrying moiety that is or can be made insoluble and hydrophobic, the final complex "product-agent of separation-carrying moiety" can also be floated. With the exception of few techniques like reverse osmosis, electrodyalisis, and electrophoresis, almost all other separation methods in biotechnology have the potential to be practiced as flotation.

The use of these separation principles in the form of flotation would have many advantages. First, the immobilization of the product in a particle may contribute to a protection against denaturation. Second, when the agent of separation is in a liquid phase, flotation would combine advantages of adsorption on a solid (large selection of adsorbent groups to choose from) with advantages found in aqueous two-phase extraction (fast kinetics of liquid systems).

This new focus on flotation is occurring in parallel with the acceptance of a more efficient concept of a flotation device, the flotation column.

1.3 - Column Flotation: the New Concept of Flotation

Column flotation is the newest successful development in flotation equipment (Figure 1.3). Its mechanism of operation is very simple. Feed is introduced at an intermediate height in the column (approximately at the top one third) and bubbles are generated at the bottom. Hydrophobic particles from the feed are carried up to the froth at the end of the column. Wash water added at the top washes down hydrophylic particles and liquid from the feed. The overflown froth (called concentrate) contains the hydrophobic particles and liquid composed of wash water and a small portion of feed liquid, if any. Hydrophilic particles are eluted with the bottom stream (called tailings), which is the largest flow in the column.

Column flotation is the present trend in the mineral industry where it is replacing the traditional flotation cells for many reasons. The columns are highly efficient providing higher grade and recovery than conventional mechanical cells. They have high capacity despite their small size and low fixed and operating costs. The equipment can be constructed on the site and have no moving parts saving energy and maintenance. They can be fully automated and laboratory results are easily scaled-up (Foot *et al.*, 1986; Hernáinz *et al.*, 1987; Hyde and Stojsic, 1987; Sastry, 1988; Schubert, 1988).

1.4 - Objective

The objective of this proposed research is to study the application of the modern column flotation technique to biotechnology processes for the recovery of biomolecules produced by fermentation.

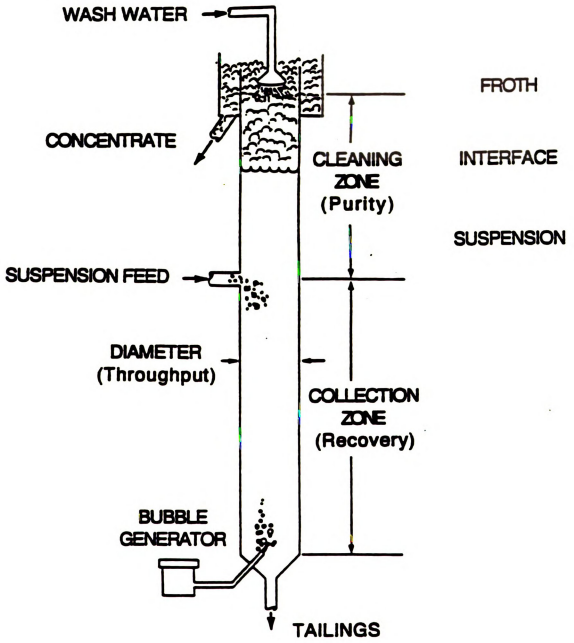


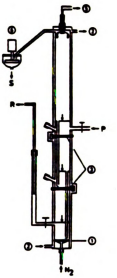
Figure 1.3 - General scheme of a flotation column (from McKay *et al.*, 1988).

The recognition of the potential of flotation has not yet been translated into a large number of processes developed and published research. In an extensive literature search, only a few papers dealing with application of flotation as a recovery method of species from culture media were found (Gehle and Schügerl, 1984a; Gehle and Schügerl, 1984b; Gehle *et al.*, 1991; Hernáinz *et al.*, 1987). However, these investigations dealt with the recovery of microorganisms or molecules from fermentation waste. No work on the recovery of a biomolecule as the major fermentation product was found. The development of new flotation applications is necessary to validate flotation as a viable and versatile downstream operation, especially at a commercial scale. Also, some aspects of how flotation have been practiced and developed in the field of biotechnology need to be addressed. Some of these issues are cited below.

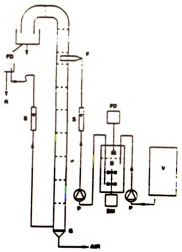
Virtually no published work uses the modern concept of column flotation. The major omission in the investigations is the use of equipment with the washing water injection, the simple, but most innovative characteristic of column flotation. It is the washing water that gives this type of column the capability to promote high purification, one of the main objectives in downstream processing.

The omission of this important characteristic, washing water, did not lead to simpler equipment. Instead, the different equipment reported in the literature either have the basic configuration of the old flotation cells or are rather complicated cylindrical devices, as it can be seen by the examples in Figure 1.4.

Another point of concern is that the majority of the flotation processes cited above made use of the addition of collectors, frothers, and modifiers. Those additives commonly used in the mineral industry like



from Gehle and
Schürgel, 1984b



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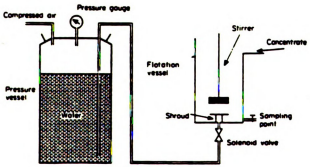
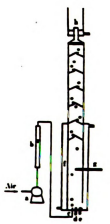
et al., 1991



from Holland and
McComiskey, 1986



from Miyazu and
Yano, 1974

Figure 1.4 - Some different types of flotation columns found in the biotechnology literature

kerosene, fuel oil, cyanide, and xanthates would not be approved for food or pharmaceutical applications. Flotation systems that naturally float or use food or pharmaceutical grade additives need to be developed.

This research addressed the two potential aproaches for flotation in biotechnology discussed in section 1.2.3. First, in the work described in Chapter 3 the natural floatability of a separation system to recover a product from fermentation broth was exploited: partition of a α-amylase to a salted-out (hydroxypropyl)methylcellulose phase from fermentation broth. Chapter 4 is the demonstration of the viability of development of a carrying system to perform flotation based on a specific product-ligant interaction. The example system consisted in modified methylcellulose as the carrying moiety, alkyl chains as ligands, and bovine serum albumin as the product. Chapter 2 is a background in flotation, especially column flotation, and related techniques classified as adsorptive bubble separation.

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Chapter 2

Flotation, an Adsorptive Bubble Separation

In order to better understand flotation in biotechnology, a literature search was conducted on this subject with special interest in its previous applications in downstream processing. Only a small number of publications about this use of flotation was found with the exception of reports concerning waste treatment. However, a large number of publications about techniques similar to flotation applied to or developed for downstream processing were found. These techniques, together with flotation, comprise what is called adsorptive bubble separations. Therefore, this literature review was not restricted only to column flotation since flotation and the related adsorption bubble separation techniques were developed simultaneously and findings in one area were used in the development of others.

Due to the difficulty of quantifying individual proteins in a mixture and the simplicity of some enzymatic assays in the early days of adsorptive bubble separations, enzymes were the compounds most used in the development of these techniques (Thomas and Winkler, 1977).

2.1 - Adsorptive Bubble Separation Techniques

2.1.1 - Concept and Classification

Adsorptive bubble separation is the generic name given to separation/concentration techniques based on surface activity (Lemlich,

1972). Species (compounds, particles, cells, precipitates, etc.) can be adsorbed or attached to rising bubbles in a liquid phase according to their surface activity. Transport of the attached species by the bubbles and foam causes their concentration at the top of the liquid phase or foam. If the adsorption/attachment is selective, enrichment takes place.

The different types of adsorptive bubble separation techniques are shown in Figure 2.1. The first distinction to be made is on the phase which will receive the species carried up by the bubbles. If there is no foam involved in this reception, the technique is classified non-foaming separation; otherwise, it is called foam separation.

Non-foaming separation is classified in two types: bubble fractionation and solvent sublation. In bubble fractionation, the separated species is left just at the liquid surface after bubble collapse. In solvent sublation, the separated species are transfered either to an immiscible liquid phase atop the lower liquid phase or to the interface between these two phases.

Foam separation is divided in foam fractionation and flotation. In the former, the species of interest, part of an homogeneous solution, is separated and/or concentrated by adsorption on bubble surface which are foamed out of the system. In the latter, the species is carried up by the gas bubbles as a particle or associated with one.

Lemlich (1972) subdivided flotation in many categories. However, for simplicity, these groups will be regrouped in only two groups: flotation for particle separation, wherein particles are the species to be separated (e.g., ore flotation), and separation by particle flotation, wherein specific particles, formed in the system or not, are the vehicle by which the

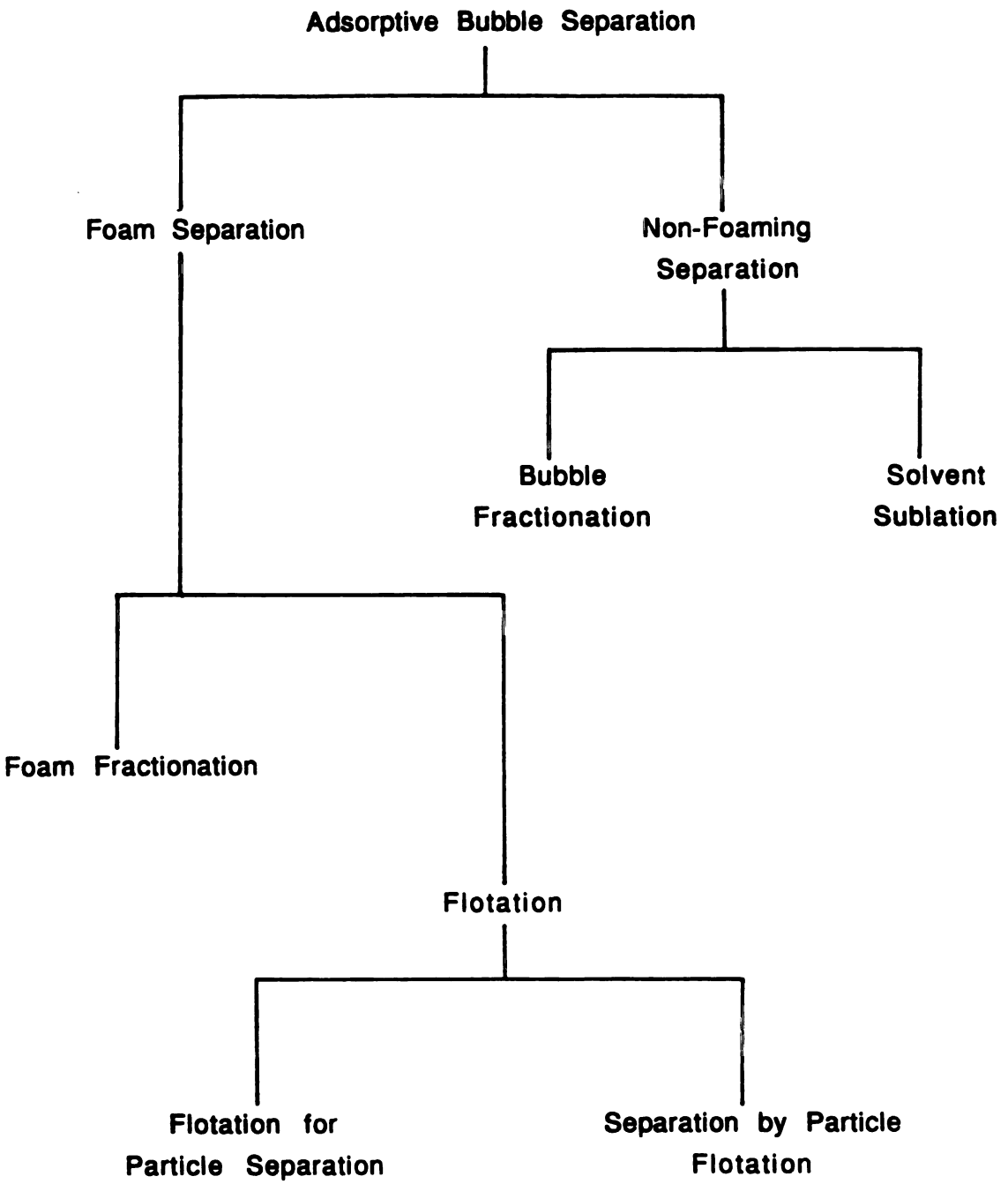


Figure 2.1 - Classification of the adsorptive bubble separation techniques (modified from Lemlich, 1972).

species of interest are separated. In this second group, a precipitate can be formed by addition of either any suitable chemical (precipitation flotation) or a collector (ion and molecular flotation). Another case of separation by particle flotation is the addition or formation of colloid particles which adsorb the species of interest (adsorbing colloid flotation).

2.1.2 - Non-Foaming Techniques: Bubble Fractionation

Dorman and Lemlich (1965) developed a foamless separation technique called bubble fractionation. The objective was to devise an adsorptive bubble separation technique applicable to liquid systems that do not foam. The separator was a bubble column: a vertical tube with a gas sparger at the bottom. In the case of continuous operation, inlet for the feed at intermediate height and outlets for rich and lean streams at the top and bottom, respectively, were installed. Monobutyl diphenyl sodium monosulphonate was concentrated more than four times by batch runs after several hours of bubbling.

Miyazu and Yano (1974) used a Dorman-Barker's apparatus as a bubble fractionation separator to study cell removal from aqueous suspension. The separator was a bubble column divided in two stages by inverted funnels to reduce back-mixing (Figure 1.4). They compared adsorption per unit area of bubbles and affinity of hydrocarbon-grown and glucose-grown cells to bubbles. High recovery was achieved for hydrocarbon-grown cells (up to 95 percent). Based on the results authors suggested that flotation could be an effective way to recovery hydrophobic cells.

Chiang and co-workers (1981) studied the separation of isoelectric precipitated hemoglobin and oil-hemoglobin with an apparatus similar to the one used by Miyazu and Yano (1974) cited above. The fundamental difference was the division of the column in stages by perforated disks instead of inverted funnels. The authors claimed that an unexpected reverse enrichment took place under certain conditions where the precipitate concentrated on the bottom of the separator. It seemed that a "not-so thick layer of foam" beneath the plates retained the particles from the upward stream of back-mixing.

Potter and co-workers (1990) developed a mathematical steady-state model for *in situ* separation by bubble fractionation. They studied the separation of proteins in the baker's yeast fermentation process using carbon monoxide either generated by fermentation or supplied externally as the gas phase.

2.1.3 - Foam Separation

2.1.3.1 - Foam Fractionation

According Charm and co-workers (1966) and Thomas and Winkler (1977), foam fractionation was developed by W. Ostwald in 1937. Ostwald first used this technique when he separated albumin from potato and beet juices. This work on the separation of protein from plant juices was continued later by other investigators (Davis *et al.*, 1949a; Davis *et al.*, 1949b; Weijenberg *et al.*, 1978).

However, in the same year, Schütz (1937) claimed foam fractionation development with the separation of cholic acid from its sodium salt and hemoglobin along with antibiotics from rabbit serum. A more complete description of the technique, that he called fractionation by

adsorption in foam, came later (Bader and Schütz, 1946; Schütz, 1946). These subsequent authors reported the purification of choline-estearase, sodium cholate, bile salts-saponin, lecithin, and horse serum albumin. Concentrations up to 16-fold were observed but foaming times were high, up to 3 days (Bader *et al.*, 1944). London and co-workers (1954) also had the goal "to start the development of a simple technique to aid in in the purification of enzymes" when they reported the purification of urease and catalase by foam fractionation.

Five different approaches to foam fractionation operation have been developed (Figure 2.2). One of the most significant changes in the mode of operation was the introduction of reflux to increase the overall separation analogous to distillation and extraction (Lemlich and Lavi, 1961). Concentration of the foamate increased 10-fold; however, foaming times were still long, around 48 hours. These large processing times may be one of the reasons why the potential applications of foam fractionation in the large-scale concentration/purification of enzymes suggested by Charm and co-workers (1966) did not materialize into actual commercial processes.

2.2 - Flotation and Column Flotation

2.2.1 - Basics of Flotation

Flotation is a technique mostly used in the selective separation and concentration of particles, mainly minerals. It had its inception and development in the area of mineral processing. Through the years and presently, mineral concentration and enrichment is by far the most important application of flotation. The vast literature in flotation is, as a consequence, concerned about ore flotation. The best treatments of

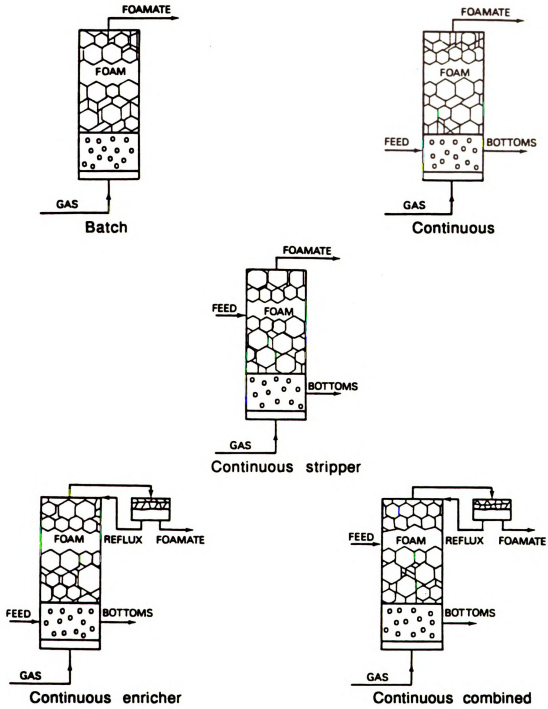


Figure 2.2 - Operational modes of foam fractionation (from Thomas and Winkler, 1977).

flotation and its basic principles are the works of Gaudin (1957), Fuerstenau (1976) and Leja (1982). Svarovsky (1981) has a less common treatment of flotation since it approaches flotation only as a unit operation for solid-liquid separation.

To better understand the importance of the different parameters related to flotation, an understanding of the most basic phenomenon in flotation, particle collection by bubbles, must come first. Figure 2.3 is a representation of this phenomenon. It is assumed that a bubble is a a rigid sphere travelling in the liquid were hydrophobic and hydrophilic particles are in suspension moving towards it at a low Reynolds number. As the particles approach the bubble following the stream lines, they begin to slide around it. The hydrophilic particle, despite the close positioning, slides all the way around the bubble and both, particle and bubble, continue their trajectories. They do not interact with each other. It is said that a collision happened and failed in producing attachment. However, the hydrophobic particle and the bubble, are pulled close towards each other by hydrophobic forces. The hydrophobic particle travels around the bubble moving to the inner streamlines. Therefore, the liquid film between them thins to a point where rupture occurs. This rupture means the formation of a solid-gas interface. What occurs next is a rapid expansion of the gas meniscus over the particle. If this expansion is fast enough the interaction between the hydrophobic solid and gas bubble is strong enough to support mechanical disturbances in form of future collisions. This way, a stable attachment takes place. We can say that the particle was collected by the bubble. The time the particle travels on the surface of the bubble is known as the sliding or contact time. The time necessary for the liquid film to

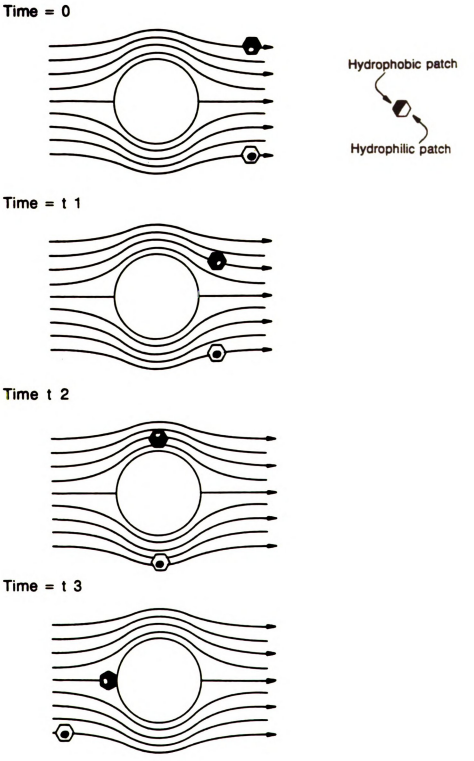


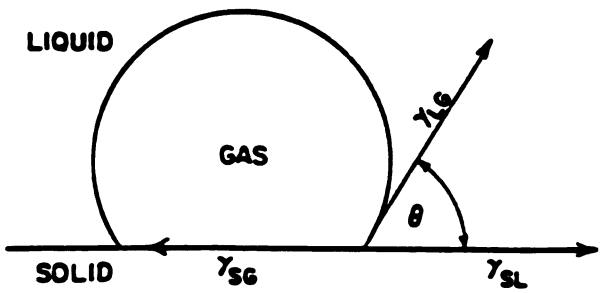
Figure 2.3 - Illustration of particle capture mechanism where $0 < t1 < t2 < t3$.

thin and rupture is known as the induction time (Dobby and Finch, 1986a; Dobby and Finch, 1987; Svarovsky, 1981).

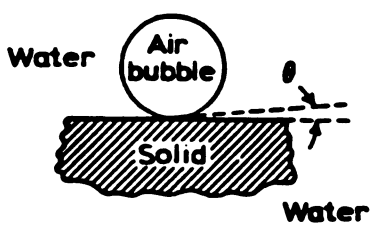
The key factor in the thinning and rupture of the liquid film between particle and bubble is the hydrophobicity of the particle surface (even though bubble surface charge may play a important part, according to Okada *et al.*, 1990). It is the degree of hydrophobicity of the particles that at the end determine which particles, after colliding with the bubble, will attach to it, and consequently, float.

A measurement of hydrophobicity is given by the contact angle ø measured according the scheme in Figure 2.4. Despite its popularity and widespread use, contact angle measurement is not always a viable technique. Problems include changes in the contact angle measured with gas adsorption on the surface of the particle, the need of a polished smooth surface, and the inapplicability for cases were the angle to be measured is smaller than 15°. Recently, a bubble pick-up method was improved as a alternative way to evaluate particle hydrophobicity. The method measures the extent of particle-to-bubble adhesion by the angle by which the gas bubble surface is covered with adhering particles under static conditions (Vinke *et al.*, 1991). The advantages of this method are the absence of particle pretreatment, its suitability for the determination of small angles, its applicability to porous materials, and that it is more closely related to the conditions occuring during flotation than other methods.

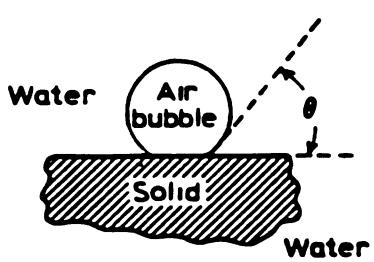
Despite its drawbacks, the thermodynamic interpretation of contact angle has a great significance. The thermodynamic condition for the existence of three phase contact is represented by Young's equation



Contact equilibrium



Contact angle for a solid with low hydrophobicity



Contact angle for a solid with high hydrophobicity

Figure 2.4 - Contact angle and hydrophobicity (from Lemlich, 1972; Svarovsky, 1981).

$$\gamma_{sg} = \gamma_{sl} + \gamma_{lg} \cos \phi \qquad (2.1)$$

where γ represents interfacial tension of solid-gas, solid-liquid, and liquid-gas phases according the subscripts indication (l, g, and s stand for liquid, gas, and solid, respectively). The free energy change due to attachment ΔG is given by

$$\Delta G = \gamma_{sg} - (\gamma_{sl} + \gamma_{lg}) \qquad (2.2)$$

This is an account for the energy lost due to the disappearance of solid-liquid and liquid-gas interfaces and the energy gained with the creation of the new one, the solid-gas interface.

Applying Young's equation results in

$$\Delta G = \gamma_{gl} (\cos \phi - 1) \qquad (2.3)$$

This equation, known as the Young-Dupré equation, shows that for a finite contact angle there will be a decrease in the free energy of the system. Contact angles over 30 degrees are usually necessary for a good flotation (Lemlich, 1972; Svarovsky, 1981).

A more practical indicator of particle hydrophobicity is the electrokinetic potential also known as zeta potential. A charged surface particle generates a electrical potential gradient through the liquid phase. The potential at the slip plane of a particle when it is moving it is known as zeta potential. The exact position of this slip plane is not known but the thickness of the liquid layer adhering to the solid is believed not to be more than few angstroms (Leja, 1982). Therefore, the zeta potential is a

close indication of the charge at the particle surface as well as of a bubble (Yoon and Yordan, 1986). Figure 2.5 depicts the striking relation between the zeta potential and the floatability for some materials.

The paramount importance of bubble capture, for which the mechanism was discussed above, relies on the fact that it determines how operating parameters affect flotation performance. Specifically, it determines the rate by which particle concentration changes with time in the flotation equipment (Yoon *et al.*, 1988). This rate of collection is given by

$$dC/dt = -K[C(t) - C_\infty]^n \qquad (2.4)$$

where t is time, C is particle concentration, C_∞ is the terminal concentration of particles (concentration of apparently floatable material that would remain in the flotation cell after long times), n is the order of the rate equation, and K is the rate constant (Lemlich, 1972).

The constant K, a lumped parameter involving physical, chemical, and surface properties of the system, is where the collection effect is accounted by a parameter called collection efficiency, E_k, defined as the fraction of all particles swept out by the projected area of the bubble that collide with, attach to and remain attached to the bubble until reaching the froth. This efficiency can be understood as the product of two other efficiencies related to the two subprocess in the collection phenomenon: collision and attachment. Efficiency of collision, E_c, is defined as the rate at which particles collide with the bubble divided by the rate at which particles flow across the projected area of the bubble. Efficiency of attachment, E_a, is defined as the fraction of all colliding particles that reside on the bubble for a time greater than the induction time, i.e. have a

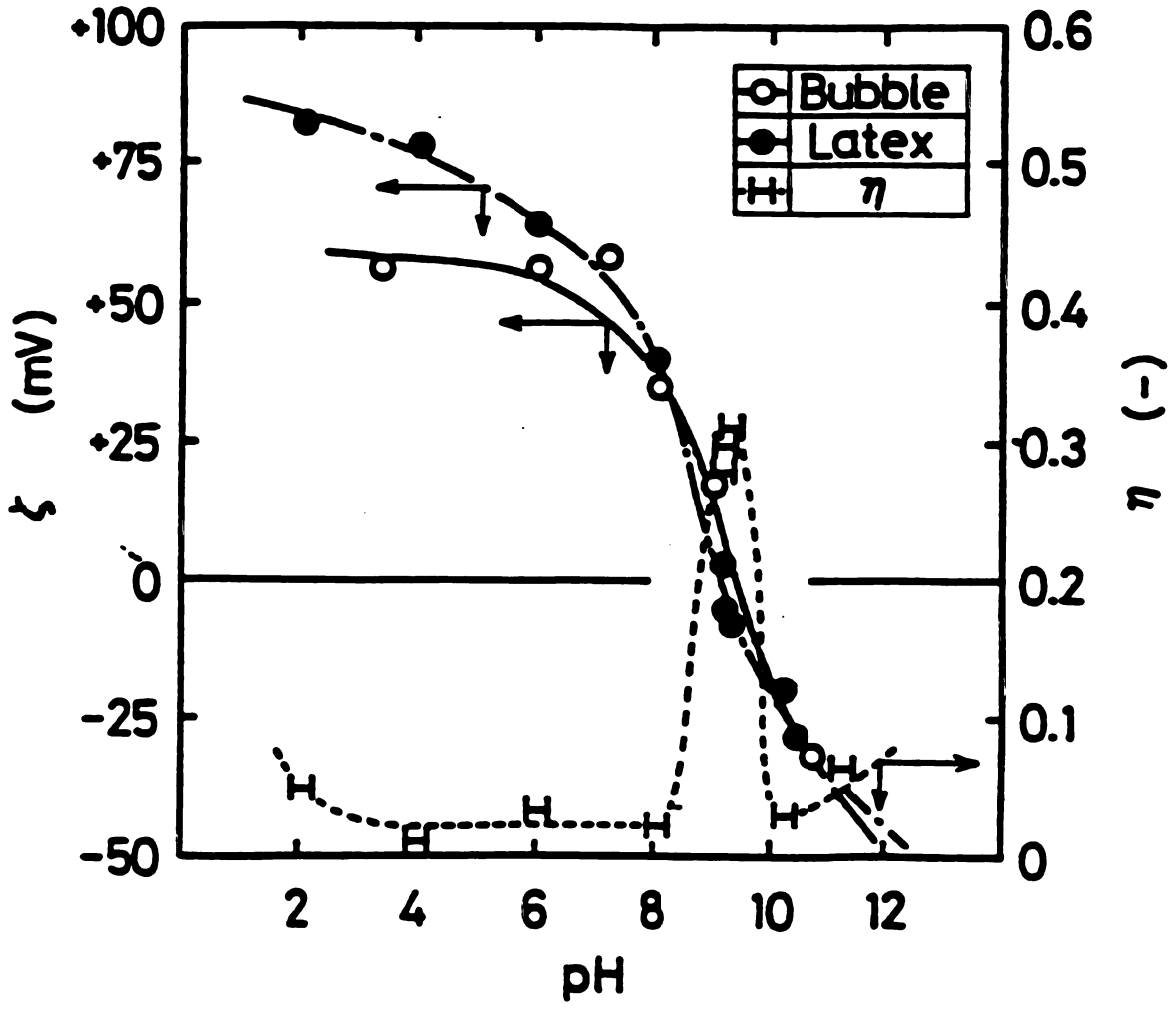


Figure 2.5 - Effect of zeta potential of the bubbles and latex particles (ζ) on flotation efficiency (η) in a cationic surfactant solution, with change of pH (from Okada *et al.*, 1990).

sliding time or contact time larger than the induction time (Dobby and Finch, 1986a; Dobby and Finch, 1987). Therefore,

$$E_k = E_c \cdot E_a \qquad (2.5)$$

Dobby and Finch (1987) developed a fundamental model for the capture process which includes dependence on the particle size and density, bubble size, and liquid viscosity. Particle size affects the collision and attachment efficiencies differently. As the diameter of the particle increases the efficiency of collision increases due to the higher inertia of the particle. Simultaneously, efficiency of attachment decreases due to higher sliding velocity around the bubble. The net result is the existence of am optimum particle size with respect to collection efficiency which is in accord with recovery-size data reported for many mineral systems.

Particle density markedly affects the collision efficiency through effect on inertia in the same manner that particle size does. However, the net result is the prevalence of the increase in the collision efficiency in the range of 1.3 to 7.0 g/mL.

Both collision and attachment efficiencies vary inversely with bubble size. The increase of collision efficiency with bubble size decrease can be explained in part by the higher rigidity of small bubbles (Jameson *et al.*, 1977). Since the bubble interface is deformable, a particle with high inertia may cause a deformation in the bubble surface and even bounce off it which means no collision. In the case of the more rigid small bubbles, a particle would have a lesser chance to slide around it and not experience this bouncy effect (Schulze *et al.*, 1989). The increase in attachment

efficiency is due to a smaller sliding velocity of smaller bubbles (bubble rising velocity decrease with decrease in bubble size) which exceeds a decrease in the sliding distance.

Attachment efficiency was insensitive to particle hydrophobicity (represented by induction time) for very small particles (less than 5 μm) due to the long sliding time of small particles. This time would be enough for a highly hydrophobic particle as well as for a not so hydrophobic particle to adhere to the bubble by the thinning of the liquid layer that separates bubble from liquid. The importance of this phenomenon is the decrease in selectivity. An example is given where separation between particles with 40 and 15 ms of induction time changes from excellent to virtually no separation for particle size change from more than 20μm to less than 5 μm.

Dobby and Finch (1987) also predicted the negative effect of the increase in viscosity on the collection efficiency due to decreasing bubble rise and particle settling velocities and increase in induction time.

Besides the physical aspects of bubbles and particles, their surface chemistry, especially of the particles, is of large significance in flotation. Few materials are naturally hydrophobic enough to be floated, particularly minerals. The solution is the use of collectors that incorporate on the solid surface imparting hydrophobicity to the particles. Collectors are amphipatic surfactants, R-Z type of molecules, where R and Z represent a non-polar and a polar group, respectively. The association of the collectors on the particle surface is the result of many different interactions operating individually or in combination with each other. Electrostatic interaction between charged areas on the solid and ionic species are common. Some collectors are covalently bound to the surface

by chemisorption while others interact by hydrogen bonds. Species may undergo chain to chain interaction upon adsorption that reinforces the individual adsorption. A collector chain to particle surface interaction may also occur. The driving force for adsorption, the free energy of adsorption, is therefore, a summation of all the free energies related to these interactions plus the free energy due to solvation or desolvation attributed to these species during adsorption (Somasundaran and Ananthapadmannabhan, 1987).

These particle-collector interactions depend on pH, ionic strength, collector concentration, type of solid surface, temperature, presence of other chemical species, etc. Therefore, each system has a different mechanism playing the central role in the adsorption of the collector. The many possible combinations of chemical process variables give flotation a large degree of flexibility. As an example, Lemlich (1972) discusses the concentration of iron ores where hematite is to be separated from quartz. Six different processes have been developed, the first four in the list below have been put in commercial operation:

1 – Flotation of hematite using a sulfonate as the collector at pH 2 to 4.

2.– Flotation of hematite with a fatty acid as the collector at pH 6 to 8.

3 – Flotation of quartz with an amine as collector at pH 6 to 7.

4 – Flotation of quartz activated with calcium ions at pH 11 to 12, using a soap as a collector together with starch to depress the hematite.

5 – Flotation af hematite with an amine as collector at pH 1.5 in the presence of hydrochloric or sulfuric acid.

6 – Flotation of hematite with hydroxamate as a collector at pH 8.5 and methylisobutylcarbinol as a frother.

The first process is based on the electrostatic interaction between the negatively charged sulfonate with the positively charged hematite. The quartz under these conditions is negatively charged. In the second process, the fatty acids chemisorb only on the hematite. Flotation of quartz in the third process is due to its electrostatic interaction with the cationic amine (the hematite is essentially uncharged in the process conditions). In the fourth process, starch chemisorbs on the surface of the hematite preventing its flotation.

Collector adsorption on the surface of particles driven by electrostatic interactions are dictated by the particle surface charge. This charge is originated by the breaking of bonds during particle size reduction, ions dissolution, etc. A practical measurement of this charge is given by the electrokinetic potential also known as zeta potential. Its measurement is based on the electrical potential gradient generated by the charged particle surface. This potential causes a specific arrangement of ions throughout the interface as a way of neutralizing the electrical charge. First, there is a layer of ions, termed counter ions, that adsorb on the interface generating a double layer of charged ions. Due to thermal agitation the next layer is not fixed in a plane by it diffuses throughout the liquid over a finite distance from the particle surface. The potential at the slip plane of a particle when it is moving relatively to the water is known as zeta potential. The exact position of this slip plane is not

known, but the thickness of the liquid layer adhering to the solid is believed not to be more than few angstroms (Leja, 1982). Therefore, the zeta potential zeta is a close indication of the charge at the particle surface. Charge of bubbles can also be evaluated by the same principle (Yoon and Yordan, 1986). Figure 2.6 is a schematic representation of this electric double layer.

2.2.2 - Column Flotation

2.2.2.1 - Operational Principles and Advantages

Bubble columns, commonly used in chemical engineering practice as chemical reactors, were first used as flotation devices for the treatment of fine particle systems. The first flotation column were patented in Canada in the early 60's. The mineral industry seemed to be reluctant in using the new devices, choosing to deslime the ores prior to flotation despite the loses of valuable product in the finer materials. The main reason for this was probably the marked difference in design and operating philosophy between flotation column and conventional mechanical flotation equipment. The breakthrough in North America happened only in the beginning of the 80's. The necessity of treating ores of increasing difficulty of separation and the need for minimization of losses led industries to adopt the flotation column concept. However, since the early 60's the Russians also have been working in this new concept and the Chinese already have columns in operation (Dobby and Finch, 1986a; Hu and Liu, 1988; Mavros *et al.*, 1990; Schubert, 1988).

A schematic of a flotation column showing the delineation of the two distinct sections in the equipment, the collection or recovery zone and the cleaning or washing zone, was presented in Figure 1.3. Also, the

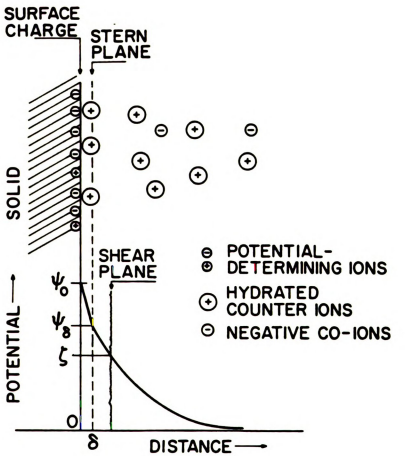


Figure 2.6 - Schematic representation of the double layer according to Stern's model (from Somasundaran, 1975).

location of feed, washing water, and gas inlets, and tailings and concentrate outlets are indicated. The tailings volumetric flow rate is slightly higher than the feed volumetric flow rate due to the introduction of the washing water at the top. A small part of this water goes to the concentrate (overflow) and the rest of it goes to the tailings (underflow). This imbalance between feed and tailings volumetric flow rate is called bias. The collection and cleaning sections work under countercurrent flow, but with distinct characteristics and functions. Ideally, the column should work in a plug flow regime (Wheeler, 1986).

In the recovery section, there is a countercurrent flow of suspension and air bubbles generated in the bottom of the column. There is no need of moving parts to agitate and keep particles in suspension so they can contact the air bubbles. In conventional machines, impellers are used for this purpose and, at the same time, to generate and/or introduce the air bubbles in the system. However, the turbulence necessary for particle-bubble collision is, at the same time, deleterious to the flotation phenomenon because it causes detachment between particles and bubbles attached in previous contact.

The descending particles in the recovery section meet a upstream of moving bubbles during all their residence time. Therefore, particles have a chance to collide and possibly attach to bubbles during all their way down in the column. This means that they spend their whole time productively in the collection zone. In conventional cells, however, after particles and bubbles leave an active zone their velocities are lowered which reduces the probability of collision and attachment between them. This may be why flotation columns use up to eight times less air per volume than mechanical cells. Moreover, the recovery zone is one hundred percent

available for a particle coming from the cleaning section due to undesired accidental detachment. In contrast, the horizontal configuration of the mechanical cells would offer a detached particle only a fraction of the total retention time (the fraction corresponding to the last cells of the bank). Due to the bottom location of the tailings exit and to the top to bottom liquid flow, the column never sands up.

The most unique characteristic of the flotation column, the washing water, is located in the cleaning section. In the conventional flotation devices, there is a draining froth bed where non-flotable particles that were pulled up in the wake of the rising bubbles are separated from the bubble-attached particles. The bubbles are surrounded by large amounts of suspension at their arrival at the liquid-froth bed interface. As the bubbles move up in the bed, the suspension containing the non-flotable particles drains back to the liquid phase. However, this makes the bubbles closer to each other due to the thinning of the liquid wall between them up to a point that they work as a filter trapping non-flotable particles among them. In flotation columns, the washing water flowing down the cleaning section sweeps away the tail of non-flotable particles following the bubbles. Moreover, it thickens the liquid wall between the bubbles preventing any mechanical entrainment, just the opposite of what happens in the draining froth bed. This avoids even non-flotable slimes from reaching the concentrate by washing down feed water. Consequently, the water in the overflow is wash water.

In columns there is no single draining froth bed, but a froth structure composed of two sections due to the injection of washing water at a internal point of the column (Figure 2.7). The section above the injection point is just froth that characterizes it as a conventional

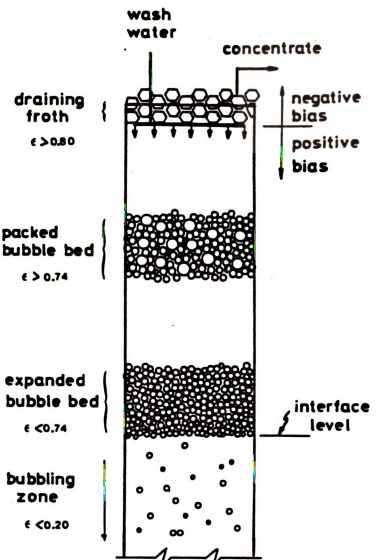


Figure 2.7 - Froth structure. ε is the fractional gas holdup (from Yanatos, *et al.*, 1986).

draining froth bed. However, the portion under the injection point is a more loose configuration of bubbles due to the washing water flowing downwards, termed bubble bed. Due to the existence of a water content gradient in this bubble bed, it can be further subdivided in two subsections. At the bottom, right at the interface with the suspension, the bubbling zone, is the expanded bubble bed; above it is a less loose bed termed packed bubble bed (Yanatos *et al.*, 1986).

The positioning of the washing water is a valuable parameter of operation since it affects the amount of liquid that overflows the column. Therefore, it partially controls how much liquid is recovered with the floated particles. This is measured by the volumetric concentration ratio, i.e. the ratio between the feed and the concentrate volumetric flow rates. A high volumetric concentration ratio is a desirable characteristic of an unit operation in downstream processing and it is almost a must if the unit operation is applied as one of the first steps in a multiple step purification/recovery scheme.

The most important practical consequence of these fundamental differences between columns and conventional mechanical cells is the higher separation/recovery performance. Wheeler (1986) reported increase in grades for a cleaning operation of a complex copper ore in Peru from 22.5 to 30.1 percent and, for a molybdenum circuit at Gaspé Copper Mines, Canada, increase in recovery of 100 percent (from 40 to 80 percent). Other advantages of flotation columns over conventional devices are ease of control, higher capacity (up to ten times faster), lower energy consumption, less space for installation, less maintenance, and lower operating costs.

2.2.2.2 - Operational Parameters

Schubert (1988) pointed out the following essential column design and operational parameters:

- Column height L;
- Column diameter D;
- Column height to column diameter ratio L/D;
- Position of feed inlet;
- Type of aerator;
- Bubble size;
- Superficial air velocity;
- Superficial pulp velocity;
- Volume percent of solids in the feed; and
- Superficial velocity of wash water.

The five first parameters are determined by design. The rest can be adjusted within given operational limits. Typical industrial columns are 13 m high by 0.3 to 2.0 m in diameter, either circular or square in cross-section. The collection section is typically about 10 m. Typical operating conditions are: superficial gas velocity, from 1 to 3 cm/s; superficial suspension velocity, from 0.5 to 2 cm/s; bubble diameter, from 0.05 to 0.2 cm; and gas hold-up, from 10 to 25 percent (Dobby *et al.*, 1988).

Yianatos and co-workers (1988) studied the effect of height and diameter on column performance. They addressed the question as to why industrial columns have about recovery section height (H) and diameter (D) of about 10 and 1 m, respectively. Simulation studies showed the existence of a compromise among degree of mixing and volumetric bias rate versus volumetric gas rate. The longer the column, the smaller the degree of mixing which leads to better performance (grade and recovery).

However, the gain in recovery levels off as the the ratio H/D increases: due to the fixed superficial gas velocity, the gas flow rate decreases which causes the column to approach overloading. Their final conclusion was that for typical operating conditions, a height to diameter ratio of 10 is a reasonable compromise.

The feed inlet position indirectly affects the cleaning action of the wash water. Because the froth bed can not be longer than the cleaning section, the feed position determines its limit. However, froth bed height is not the only factor upon which cleaning is controlled. Yianatos and co-workers (1987), used laboratory and large scale experiments to determine hydraulic entrainment of fine particles in industrial columns (12 m in height). Minimum entrainment was found for froth height of at least 1 m as long as superficial gas velocity and bias rate were less than 1.5 cm/s and in the range of 0.2 to 0.4 cm/s, respectively. A remarkable finding was that the main cleaning action occurred close to the interface, approximately 10 cm at superficial gas rates lower than 1 cm/s.

Superficial pulp velocity and pulp density (a combination of particle density, size, and population density) determine the column capacity which is limited by the rate of concentrate removal. The rate at which particles can be removed depends on the individual bubble loading (mass of solids per area of bubble surface) and bubble surface production rate (area of bubble per time). Espinosa-Gomez and co-workers (1988a and 1988b) studied carrying capacity in full scale columns. Based on data from six different plants, an empirical relationship was found that relates the carrying capacity to particle size and particle density:

$$C_a = 0.0682\ (d_{80})\ (r_p) \qquad (2.6)$$

where C_a is the carrying capacity expressed as mass of solids to overflow per time per unit of column crossectional area in grams/minutes/cm^2, d_{80} is particle size as the 80 percent passing size of the concentrate in μm, and r_p is particle density in g/mL. The authors noted that there is evidence that C_a is independent of column diameter and it has potential as a scale-up parameter.

Superficial gas rate is, therefore, responsible for the recovery as the determinant of bubble surface production rate. However, an upper limit for this parameter exists above which flooding will occur (Dobby and Finch, 1986b). Smaller bubbles and higher downward liquid velocity act to lower this limit. For a specific bubble size the maximum collection rate constant is obtained while operating at a superficial gas velocity V_g at (or close to) the maximum V_g. Since the bubble size increases with increasing gas rate, design of gas spargers has a twofold converse objective: producing small gas bubbles at high gas velocity.

Since bubble size is the most important physical parameter in column flotation, the sparger is the heart of the equipment (Wheeler, 1986). As the bubbles get smaller, the surface area per unit volume increases. Therefore, there is more surface available for bubble-particle contact. Moreover, the larger the bubbles, the larger the wake they cause drawing more material (liquid and hydrophilic particles) up as the bubbles rise. This contributes to particle entrainment and, consequently, lower grades.

Spargers for industrial columns are made of flexible materials such as perforated rubber or filter cloth. One singular characteristic of the flexible spargers is a self-regulating property, with hole size increasing in direct proportion to pressure drop across sparger. Laboratory and pilot

scale columns usually have rigid spargers made of ceramic or porous metal for convenience. The choice of flexible spargers for industrial columns is based on the decreased chance that these spargers will get plugged with particles or salt deposits. Additionally, Rice and co-workers (1981) and Weber and co-workers (1990) found that flexible spargers are more efficient bubbling devices than rigid ones producing more uniform emulsions, smaller bubbles, larger voids, and reduced dispersion coefficients. Saxena and co-workers (1990) verified that this emulsion uniformity is relative since they detected a segregation of bubbles through the cross sectional area of the column, with small bubbles concentrating towards the wall.

A study by Xu and Finch (1989) showed a modest effect of the sparger material on the bubble diameter. Moreover, this paper brings three other conclusions that deserve mention:

- Bubble size decreases as the column cross-section area to sparger surface area ratio decreases. This is in accordance with results showed by Reddy and co-workers (1987).

- Bubble diameter correlates with gas flow rate per unit area of sparger by

$$d_b \propto (R_s \cdot V_g)^{0.25} \qquad (2.7)$$

  where d_b is the bubble diameter, V_g is the superficial gas rate, R_s is the ratio of column crossectional area to sparger surface area, and, consequently, $R_s \cdot V_g$ is the gas flow rate per unit area of sparger.

- As a scale-up criterion, R_s should be preserved to ensure

the same d_b and V_g in an up-scaled column.

Methods for bubble size distribution measurement include photography, high speed cinephotography, fiber optics technique, and drift flux analysis (Dobby *et al.*, 1988; Saxena, *et al.*, 1990).

2.2.2.3 - Modeling and Scale-Up

Ease of modeling and scaling-up based upon fundamental physico-chemical principals are other advantages of flotation columns over the conventional cells. Dobby and Finch (1986a) pointed out four reasons for this. First, there is a well defined flow regime of particles and bubbles in the columns. Second, there is no turbulence to be matched in laboratory machines as is the case of mechanical cells. Third, there is a minimum particle entrainment in the froth contrary to which happens in the draining froth employed in the conventional cells. Fourth, the existence of a single three-dimensional froth structure in a single device makes froth modeling for the column less complicated than the conventional case where there is a bank of cells each one with different froth characteristics.

Therefore, many have centered their research efforts in understanding the basic fundamentals necessary to be incorporated in models (Dobby and Finch, 1985; Jameson *et al.*, 1977; Laplante *et al.*, 1988; Pal and Masliyah, 1989; Schulze *et al.*, 1989; Xu, 1985) and different models have been developed.

Dobby and Finch (1986a) developed a model based on the kinetics of flotation. Their work was based on the fact that for a first-order rate process the conversion of a reactor can be predicted using the values of the rate constant and two reactor mixing parameters: mean residence time and dispersion number. The approach for the modeling was to develop an

understanding of the mixing and the kinetics of the two column sections, collection and cleaning zones, separately. The overall model was obtained by combining the model for these two regimes (Castillo *et al.*, 1988; Dobby and Finch, 1985).

Another model based on kinetics, simpler than the one above, was used to study oil separation from aqueous emulsion. The model adequately described the oil recovery and the order of the flotation rate was determined to be 0.6, away from the generally accepted value of 1.0 for mineral systems.

Sastry and Lofftus (1988) developed a general dynamic mathematical model for column flotation based on mechanistic representation. The model equations were normalized and dimensionless groups directly responsible in determining the column behavior were identified. These parameters were the normalized particle-bubble attachment rate, the normalized particle-bubble detachment rate, the Peclet number representing the dispersion of solid particles in the froth phase, and the Peclet number representing the dispersion of solid particles in the liquid phase. The resulting model equations, with no analytical solution, were incorporated in a computer simulation.

Luttrel and co-workers (Luttrel *et al.*, 1987; Luttrel *et al.*, 1988; Yoon *et al.*, 1988) developed a basic model where transport and rate terms were considered. The model was developed by describing the column as divided in sections according the different flow conditions. Each section was further divided in one or more well mixed zones. For each zone a mass or volume balance was applied for each particulate class present. The classes that were considered were air, unattached solids, and solids attached to air bubbles. Particulate solids were further classified in two

categories, coal or mineral matter (at first, the model was developed for column flotation of coal). The model was considered an excellent tool to independently asses the effects of various operating parameters.

2.2.2.4 - New Concepts and Design Variations

Many variations of flotation column have been studied and conventional equipment, some not used as flotation machines before, has been modified to incorporate the principles of column flotation.

Rice and co-workers (1974) used funnellike baffles inside the column to reduce axial dispersion. The main objective was achieved and the baffles did not present any problems regarding solids build up or clogging. The use of baffles in bubble columns was also reported by Miyazu and Yano (1974) and later on by Iibuchi and Yano (1980) and Gehle and Schügerl (1984).

In China, a type of column takes advantage of the two common column geometries, round and square (Hu and Liu, 1988). These columns have a square upper part with a round lower body. The authors listed the following merits of the combined geometry column:

- It is easy to remove the froth by scraper;
- There is more uniform pulp loading;
- A square cross-section with a side length equal to a diameter of the round cross-section gives a larger area, thus allowing the height to be decrease; and
- Several square columns can use a common wall, thus saving in construction and floor space.

Yang (1988) developed a packed column with packing similar to plates of a heat-exchanger. The equipment is said to be ideal for cleaning of fine particles that can not be efficiently treated in conventional flotation columns. The key mechanism seems to be in the small passages provided by the packing that causes an intimate particle-bubble contact. As air moves upwards in the column it is broken in small bubbles of relative uniform size. The author claimed that no fine bubble generator is necessary.

A flotation column that physically is more similar to a conventional cell due to the existence of a impeller and the feed inlet at the bottom was reported by Degner and co-workers (1988). The Leeds flotation column, as it is known, consists of a series of horizontal baffles designed to strip gangue particles from loaded bubbles as it rises to the top of the column. This baffle system consists basically in set of two rods of different densities. The more dense is positioned over the less dense rod. The difference in buoyance creates a hydrostatic pinch that act on the bubbles removing entrained hydrophilic particles from their surface.

The disadvantages of a tall column such as high ceiling requirement, widespread concentrate and feed stream, high volume of pulp inside the column, and cost of pumping feed up and providing air at sufficient pressure to overcome the hydrostatic head was addressed by Jameson (1988) with a new column design. The height of the column could be reduced to one fifth of the height of conventional columns by pre-mixing air and feed in a short tube called a downcomer that is positioned through the cleaning section. The air-feed mixture is discharged into a shallow pool of pulp where non-attached particles disengage from bubbles and flow downwards as tailings. Bubbles and the hydrophobic particles attached to

them rise to the froth bed. In other words, the collection section was substituted by the downcomer which has approximately one tenth of the height of the collection zone of a conventional column. Wash water inlet, concentrate and tailings withdrawal are similar to the conventional columns.

Schneider and Weert (1988) reported the development and full scale operation of a new flotation column called a Hydrochem column. The column is basically a conventional column with radial centered mechanical agitators through all its height similar to the one used by Parker (1958). The main difference between them is that in this new concept the mixers are designed to give to different sections of the column different degrees of mixing. The full scale prototype column is divided in five sections: a suspension zone for suspension of tailings to be discharged; a gas dispersion zone where gas bubbles are generated by suspension agitation; a collection zone where agitation is optimized to increase particle collection; a froth zone which is the zone with the minimum agitation; and the washing zone.

Raju and co-workers (1988) incorporated the concept of electroflotation in column flotation that is the generation of fine and uniform bubbles electrolytically. Three different ores were used and control experiments with conventional columns were ran. At least for one type of ore, chalcopyrite, the electro-column was more effective.

Entrainment of magnetic gangue particles was hindered by the use of magnetic field around part of the collection section in a new design by Sonokilar and co-workers (1988). The column design differs from the conventional by the absence of a froth bed and the lateral outlet for concentrate withdrawal.

Ye and co-workers (1988) developed a new device by the adaptation of a well known solid-liquid separator, the hydrocyclone. The new flotation column, or the swirl-flow flotation column, was said ideal for the treatment of ores with low grades due to its high capacity: 100 to 600 tons per day per cubic foot of cell volume for a nominal 5 cm x 50 cm system compared to 1 to 2 tons per day per cubic foot of cell volume for conventional columns and mechanical machines.

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Chapter 3

Recovery of a Genetically Engineered α-Amylase

Abstract

A novel technique for recovery of amylases from fermentation broth was developed by combining a unique separation of amylases with flotation. The partition uses (hydroxypropyl)methylcellulose (HPMC), a food grade polymer, and ammonium sulfate. First, the enzyme is removed from the liquid phase by partition to a salted-out HPMC phase. The enzyme containing polymer flocs are then floated from the liquid in a flotation column by exploiting the natural floatability of these flocs. *Bacillus stearothermophilus* α-amylase expressed in *Escherichia coli* was recovered from fermentation broth by such a technique. Recovery of active enzyme was as high as 90 percent with throughput as high as 94 $m^3/(h.m^2)$. Floatability of the enzyme from a periplasmic extract was higher than extracellular enzyme in the broth due to the presence of depressors of molecular weight lower than 10,000 in the broth.

3.1 - Introduction

3.1.1 - Expression of Heterologous Protein

Until the beginning of the 80's, research and commercial needs for plant or animal proteins could not be matched in cases where relatively large amounts were required. While some products like hemoglobin, proteases, and insulin were naturally available in large quantities, others

were not. As an example, production of 80 µg of pure and biologically active secretin required 3,000 Kg of bovine intestine for its production. No general method for the production of these proteins was available (Josephson and Bishop, 1988; Wilcox and Studnicka, 1988).

The development of molecular biology, including the techniques collectively called recombinant DNA technology, made possible the production of virtually any protein or peptide in large amounts. This is due to the ability to identify and isolate the gene responsible for the protein or peptide production in the original animal or plant and then transfer it to a selected organism. This transfer is done such that the host organism is able to use the foreign gene to produce the desired product (heterologous protein) in a relatively controlled and predictable way. The overall process is often termed as genetic engineering.

Despite the application of general basic concepts, the overall process to develop a final expression system has a large empirical component. Each gene or cDNA expression represents a peculiar case with specific problems to be overcome (Goeddel, 1990).

There are many host organisms or expression systems in use. The most common are the prokaryotes *Escherichia coli, Bacillus subtilis, Staphilococcus aureus, Streptomyces lividans*, and the eukaryotes *Saccharomyces cerevisae* and mammalian cells.

The most basic choice among these different expression systems must be made between the type of microorganism (prokaryote or eukaryote host) due to significant difference in their genetic machinery. Besides the fact that eukaryotes are more complex and difficult to handle, they are able to perform sophisticated post-translational modifications and intron splicing that prokaryotes do not do. However, the most used expression

system is *E. coli* which is linked to the development of the recombinant DNA technology itself. *E. coli* physiology and genetics are much more well understood than those of other organisms which makes for easier cloning and high levels of the expression of heterologous proteins. *E. coli* can also be grown to a high cell density with possible yields of one gram of heterologous protein per liter of culture (Abrahmsén *et al.*, 1986; Georgiou *et al.*, 1988; Gold, 1990; McGregor, 1982).

The most common locus of heterologous proteins expressed in *E. coli* is in the cytoplasm. There are cases where they are transported to the inner membrane or to the periplasmic space. Rarely are they excreted to the medium. Secretion to the periplasm (which contains only 4 percent of *E. coli* protein) or excretion to the medium would be highly desirable to facilitate the downstream processing. In addition, extracellular release would allow microorganisms to be grown chemostatically instead of batch by batch (Uhlén and Moks, 1990; Wilcox and Studnicka, 1988).

When the expressed protein is confined to the cytoplasmic space, four deleterious phenomena may occur. First, proper folding based on disulfide bridges may not occur due to the reducing environment of the cytoplasm resulting in an inactive protein. Second, the expressed product may be degraded by proteases (*E. coli* has at least eight soluble proteolytic activities). Third, the expressed protein may accumulate as an aggregate of denaturated chains termed inclusion bodies. After solubilization and purification the proteins are often in a non-active form. Complex renaturation procedures are required to obtain an active product. And fourth, the heterologous protein may be extremely toxic to *E. coli* causing cessation of protein synthesis shortly after induction of the heterologous gene. This toxicity may be represented by ribosome inactivation due to

enzymatic activity or association with membrane potential (Abrahmsén *et al.*, 1986; Davies, 1988; Gold, 1990; McGregor, 1982; Mitraki and King, 1989).

As in the case of traditional biotechnology, downstream processing is a key component in the overall process of production of genetically engineered proteins. Specific problems in separation/purification are the frequent intracellular location, usually as inclusion bodies of non-active protein, and hydrolysis by host proteases cited before.

It is interesting to note that one solution for the problems of downstream processing of genetic engineered proteins lies in the application of the same technique which made possible their expression in the first place, recombinant DNA technology. This solution is the expression of fusion proteins.

3.1.2 - Fusion Proteins and Purification Fusion

Fusion proteins are the result of expression of *in vitro* fusion of genes or gene fragments. One of the genes or gene fragments codes for the desired product while the other codes for a carefully chosen protein or peptide. This protein or a peptide, a carrier, is selected for being expressed extracellular, having a cheap, simple, and efficient purification protocol, masking product toxicity towards the host, and/or protecting the it from hydrolysis by the host proteases. If the fusion strategy is successful, the carrier imparts some of its properties to the hybrid protein. If the hybrid can be recovered or purified by exploiting the advantageous properties previously recognized on the carrier, we have the case of purification fusion. The product is termed the "wagon" moiety while the other part, the carrier, is termed "mule" moiety, purification

tail, purification carrier, affinity tail, affinity tag, or affinity handle. If it is necessary, the carrier can be removed by site-specific cleavage to generate the native gene product. The idea of such constructions came from the existing natural gene fusion events such as gene duplication and exon shuffling. Proteins seem to be tolerant to changes involving whole domains (Uhlén and Moks, 1990).

Several fusion schemes have been devised (Figure 3.1) and a large number of carriers identified some of which are listed in Table 3.1. Most of these carriers were designed for purification purposes. Chromatography (affinity, chelation, ion-exchange, etc) was generally the method of choice but aqueous two-phase extraction has also been used (Veide *et al.*, 1987). However, in an extensive literature search only one example of extracellular fusion release was found. This was the human insulin-like growth factor fused to IgG-binding domain from staphyloccocal protein A (Moks *et al.*, 1987). It was also the only example found of a fusion protein processed in large scale.

3.1.3 - α–Amylase/Alkaline Phosphatase Fusion Proteins

The study of a thermostable α-amylase from *Bacillus stearothermophilus* by Oriel and Schwacha led to its recognition as a potential purification carrier. When expressed in *E. coli* this enzyme was surprisingly excreted to the medium (up to 28 percentage) in both logarithmic and stationary phase (Oriel and Schwacha, 1988; Sen and Oriel, 1989). The study of continuous culture of the transformed *E. coli* showed a extracellular activity 100 percent higher than the extracellular activity of *B. stearothermophilus* batch culture and 10-fold more activity release to the medium. The compartmentalization of the α-amylase was unaffected

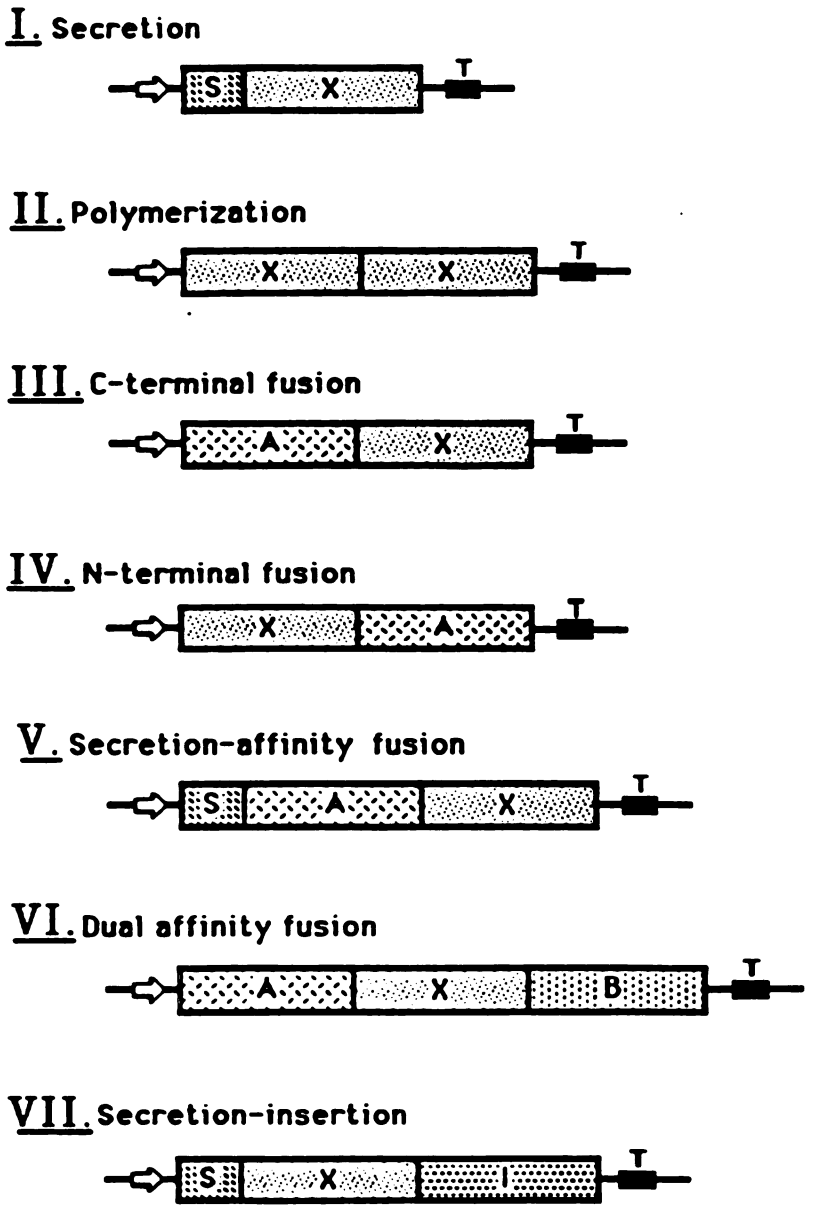

Figure 3.1 - Examples of gene fusions used for the expression of a recombinant protein (X). Promoters are symbolized by arrows and transcription sequences by rectangles. The boxes represent signal sequence (S), different affinity "handles" (A and B), and a domain for insertion into membranes or cell wall (I) (according Uhlén and Moks, 1990).

Table 3.1 - Carrier moieties for protein fusion

CARRIER	REFERENCE
Poly-arginine peptide	Brewer and Sassenfeld, 1985
E. coli outer membrane OmpF	Nagahari *et al.*, 1985
	Seo *et al.*, 1988
ß-galactosidase	Veide *et al.*, 1987
IgG-binding domain	Moks *et al.*, 1987
Maltose binding protein	Guan *et al.*, 1988
	Bedouelle and Duplay, 1988
	Maina *et al.*, 1988
Poly-histidine peptide	Hochuli *et al.*, 1988
Cellulose-binding domains	Ong *et al.*, 1989a
	Ong *et al.*, 1989b
Flag™ peptide	Sassenfeld, 1990
Protein A	Sassenfeld, 1990
Chloramphenicol acetyltransferase	Sassenfeld, 1990
Streptavidin	Sassenfeld, 1990
Poly-glutamic acid peptide	Sassenfeld, 1990
Phosphatase-binding protein	Sassenfeld, 1990
Cystein	Sassenfeld, 1990
Protein G	Sassenfeld, 1990
Glutathione S-transferase	Uhlén and Moks, 1990

by dilution rate and the specific productivity increased with dilution rate (Alexander *et al.*, 1989).

In searching for the cause of the aforementioned release, two 5' (N terminus) fragments of the amylase gene containing the putative translocation sequence were fused with truncate *E. coli* alkaline phosphatase. These fusions contained the amylase control and signal sequences and the desired N-terminal fragment, followed by the alkaline phosphatase coding region lacking its own control regions, signal sequence, and the first four residues of the mature phosphatase. The resulting hybrid protein was also excreted to the medium. Alkaline phosphatase activity in the medium was as high as 18 percent of the total phosphatase activity produced for the hybrid protein with the longer α-amylase sequence (Oriel, 1989).

Recognizing the potential use of this α-amylase as a carrier of fusion systems for expression, excretion, and purification of other proteins and peptides and the importance of α-amylase itself (in volume, the second largest group of enzymes produced for biotechnological purpose according Vihinen and Mäntsälä, 1990), a process to recover this α-amylase from fermentation broth was developed (Summerfelt, 1988; Miranda and Berglund, 1990). Description of this process follows.

3.1.4 - The Recovery Process by HPMC/Salt Partition

The process for amylase recovery from fermentation broth applicable to the amylase/phosphatase fusion above is based on partition of the enzyme to an ammonium sulfate salted-out cellulose ether phase previously added to the broth (Figure 3.2). These cellulose ethers can be (hydroxypropyl)methylcellulose or methylcellulose, termed HPMC or MC,

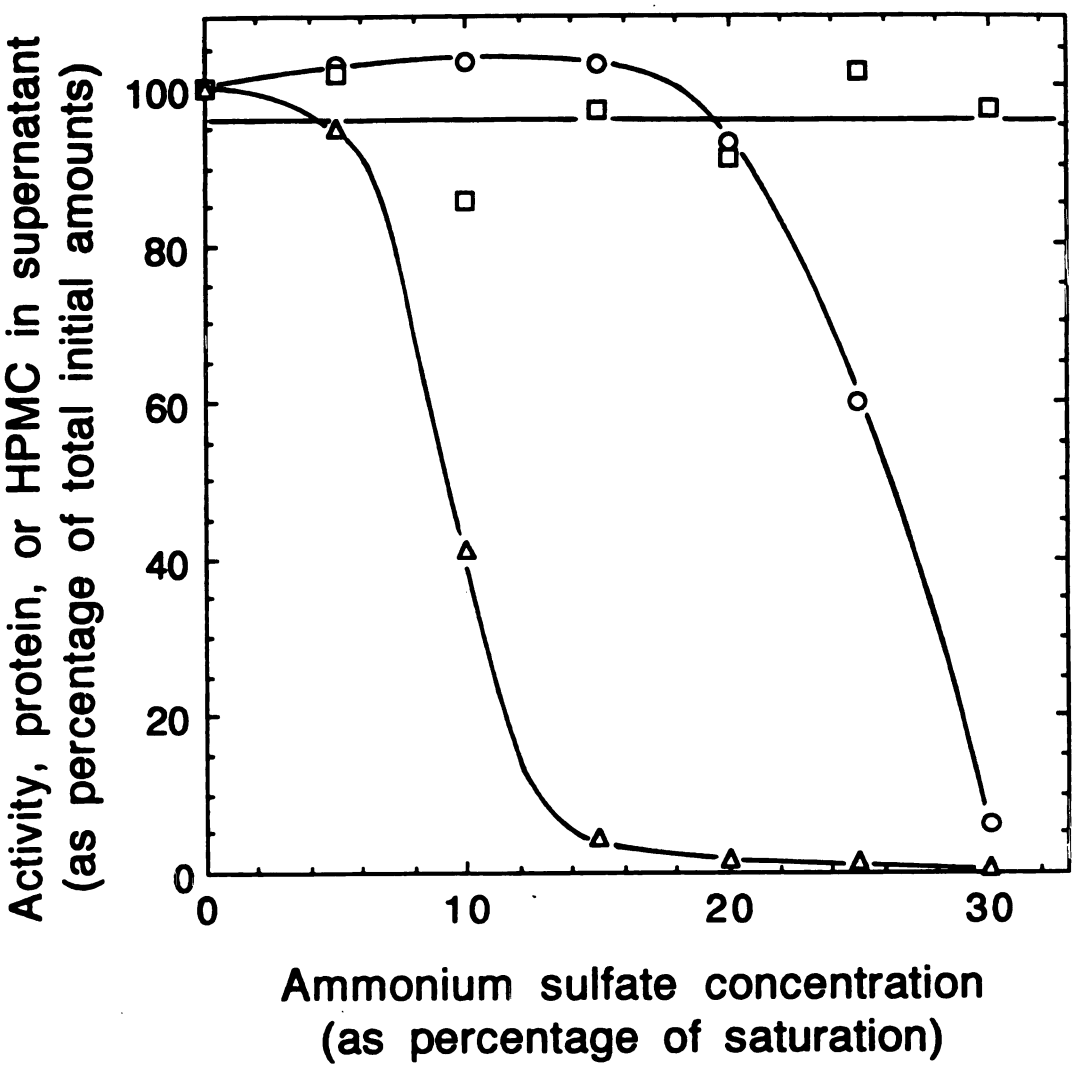

Figure 3.2 - Effect of ammonium sulfate in the partition of α-amylase (0), partition of total protein (□), and salting-out of HPMC (Δ). Data from Miranda (1988) and Summerfelt (1988).

respectively. The enzyme containing particles are then pelleted by centrifugation or filtration. The HPMC or MC polymer can be separated from the enzyme after dissolution of the pellet and addition of ammonium sulfate to salt-out the polymer at a lower salt concentration than at partition. Therefore, the supernatant contains the enzyme.

The nature of the interaction between amylases and the salted-out polymer is believed to be specific since the process works poorly for amylase grown in medium containing starch as the carbon source. The backbone of the polymer, cellulose, may mimic the structure of the linear part of the amylases substrate, starch.

The process has potential applicability at large scale for many reasons. Recovery is as high as 96 percent and requires less salt than regular salting-out. Salt savings are 67 and 57 percent for the α-amylase and α-amylase/phosphatase fusion, respectively. Purification for the extracellular α-amylase was as high as 59-fold. Up to 95 percent of the polymer can be separated from the enzyme and recycled. Polymer loading is low: 1.1 and 2.6 mg of polymer per mg of total protein for α-amylase and fusion, respectively. The polymer used has many advantages over polymers used in aqueous two-phase partition like food-grade status, low cost, and ready availability. The salt used, ammonium sulfate, does not have negative environmental impacts when compared with the salt usually required in these extractions, phosphates (Kula *et al.*, 1982).

The difference between results obtained for the α-amylase and for the fusion α-amylase/phosphatase is probably due to the use of fusion containing only 10 percent of the whole amylase. The use of fusions with a more complete amylase moiety may drive these results close to the ones for α-amylase.

This partition process is potentially applicable not only to other amylases and amylase fusions discussed above, but also to the purification of other fusions whose affinity tag may bind to the HPMC. Some candidates are the fusions of cellulose-binding domains and fusions of maltose-binding proteins cited before (Bedouelle and Duplay,1988; Guan *et al.*, 1988; Maina *et al.*, 1988; Ong *et al.*, 1989a; Ong *et al.*, 1989b). The former has a specific affinity for cellulose, the back-bone of the HPMC structure. The latter binds to maltose which is the substrate of an enzyme also purified by the HPMC partition process, ß-amylase (Miranda and Berglund, 1990).

3.1.5 - The Problem

During development of the partition process for amylases described above, pelleting the enzyme containing polymer phase for solid-liquid separation was difficult. The salted-out polymer particles are better described as light flocs made by the agglomeration of small elongated fiberlike particles. Filtration was not efficient due to gel characteristics of the cake formed. Filters were rapidly clogged. Centrifugation failed in producing a compact pellet containing all flocs and a clear supernatant. A significant number of small flocs were always present either floating in the supernatant or attached to air bubbles at the surface of the liquid. These phase separation problems would translate into in a combination of larger equipment, higher processing times, loss of product, loss of activity, and product contamination. It was concluded that an alternative method for phase separation had to be developed in order to make the partition process feasible for commercial scale application.

3.1.6 - The Proposed Solution

The unit operation chosen for the separation of α-amylase containing HPMC or MC pellets from the liquid phase was flotation. Since centrifugation and filtration, separation methods based on size and specific gravity, were not suitable for the polymer particle recovery, we looked at the surface properties of the polymer particles and solution. The choice of flotation, an adsorptive bubble separation technique, was based on the hydrophobic character of the HPMC or MC molecules and on the low surface tension of their aqueous solutions. These polymers are the result of hydroxypropyl and/or methyl substitutions on the cellulose that give them hydrophobicity while preserving the original non-ionic character of cellulose (Dow Chemical, 1988; Sarkar, 1979). Therefore, the polymer particles could be preferentially attached to air bubbles rising in a liquid and be lifted. In fact, this behavior was observed in some partition experiments. At the same time, the HPMC or MC remaining in solution lowers the surface tension of the water making the polymer a frother (Greminger and Krumel, 1980). As a consequence, no addition of chemicals as collectors or frothers is necessary which is important in the case of food related or pharmaceutical products. Moreover, the amylases seemed to be stabilized by the interaction with the polymer in a way similar to the same effect given by immobilization of enzymes on solid matrices (Miranda, 1988). Therefore, no loss in activity due to bubbling is expected.

The overall α-amylase purification process can be classified as a adsorbing colloid flotation according Wilson (1971) since the enzyme is scavenged on a floc which is then floated out of the suspension.

3.2 - Materials and Methods

3.2.1 - Cellulose Ether and Other Chemicals

The cellulose ether chosen for this study was (hydroxypropyl)methylcellulose (HPMC), type K4M supplied by Dow Chemical, Midland. This is a product with 85,000 nominal molecular weight and hydroxypropyl molar substitution in the range of 0.1-0.3. Stock solutions of approximately 1 percent in concentration were prepared according to the manufacturer's instructions: wetting and dispersing the particles in hot water and then adding cold water to dissolve the wetted particles (Dow Chemical, 1988).

Soluble starch (media preparation type) was obtained from Difco, Detroit, and ammonium sulfate (ACS grade) from Columbus Chemical Industries, Columbus. All other chemicals were reagent grade.

3.2.2 - Organism, Cultivation, and Broth and Periplasmic Extract Preparation

The microorganism for the production of the α-amylase was the *E. coli* DH1 transformed with the α-amylase gene from *Bacillus stearothermophilus* (EC 217) provided by Dr. Patrick Oriel, Michigan State University. The microorganism was grown overnight at 37 °C in LB medium (Lennox L broth base, Gibco Laboratories) containing 50 μg/mL of ampicillin. The cells were separated by centrifugation in a Sorvall RC5B centrifuge at 6000 rpm for 10 min. Calcium chloride was added to the supernatant (15 mmoles/L) for the subsequent heat treatment at 80 °C for 45 min to denature and precipitate contaminants. After cooling to 4 °C, the precipitate was removed by centrifugation at 6000 rpm for 40 min and sodium azide was added to the broth as a preservative (100 mg/L).

Periplasmic extracts were prepared by osmotic shock according to methodology developed by Nossal and Heppel (1966) with the exception that six times more mass of cells per volume of solution were used throughout the procedure. Calcium addition and heat treatment were performed as for the case of the broth. Cell and cell debris removal and heat treatment were used to obtain a stable source of enzyme. Calcium was added for enzyme stability and activity.

3.2.3 - Diafiltration

An Amicon hollow fiber cartridge system composed of a membrane cartridge H1P10-43 with a 10,000 nominal molecular weight cut-off, 20.3 cm in length, and 0.03 m^2 of surface area mounted in a DH2 holder was used for diafiltration. Batch diafiltration of approximately 1 L of broth at recirculation rate around 100 mL/min gave a filtration rate of 8 mL/min. The buffer used for these purifications was 0.010 M sodium acetate with 0.005 M calcium chloride pH 5.25.

3.2.4 - Enzyme Partition and HPMC Salting-Out

The aqueous solution of HPMC used for enzyme partition or partition-flotation was termed synthetic mixture while the HPMC containing broth was termed broth mixture. Periplasmic extracts were diluted six times with 0.020 M sodium acetate buffer pH 5.25 containing 0.005 M calcium chloride (termed herein acetate buffer) prior use in partition experiments. Average broth activity was 8.5 units/mL (standard deviation=1.6, n=14) and activities of the two batches of periplasmic extract used were 596 and 237 units/mL, respectively.

Enzyme partitions for continuous flotation experiments were carried out in a batch fashion at a HPMC concentration of 0.1 percent (before salt addition) and final ammonium sulfate concentration of 30 percent saturation by addition of a 90 percent saturation salt solution with a peristaltic pump. Mixing was provided by a magnetic stirrer bar at a speed that provided enough mixing without excessive bubble generation. The reactors were cylindrical in shape and had different capacities according to the requirement of the particular experiment (from 1.5 to 10 L).

Enzyme partitions for batch experiments were similar to the ones described above. However, some conditions were fixed: reactor volume was 150 mL, volume of the HPMC containing solution was 100 mL, and volume of salt solution was 50 mL added by a syringe pump at rate of 3.3 mL/min.

Control experiments for the level of recovery of α-amylase activity by partition at 0.1 percent HPMC and 30 percent ammonium sulfate saturation were also carried out as batch experiments. Nine milliliters of broth or periplasmic extract were added to 1.0 mL of 1 percent HPMC stock solution in a test tube. During mixing in a vortex, 4.3 mL of a saturated solution of ammonium sulfate were added drop by drop. The particles formed were separated by centrifugation in a Sorvall centrifuge RC5B for 10 min at 15,000 rpm. The pellet formed was dissolved in 9.0 mL of acetate buffer. The dissolved pellet solution and supernatant were analyzed for activity.

Experiments to determine the effect of pH on the salting-out of HPMC at 30 percent saturation were conducted in batch experiments similarly to the determination of level of α-amylase recovery by partition described above. Nine milliliters of water were added to 1.0 mL of 1

percent HPMC stock solution. During mixing in a vortex, 5.0 mL of 90 percent saturated solution of ammonium sulfate were added drop by drop. The pH of the suspension formed was then adjusted to the desired values with ammonium hydroxide and sulfuric acid. The particles formed were separated by centrifugation in a Sorvall centrifuge RC5B for 10 min at 15,000 rpm. Pellets were dissolved in 35 mL of water and both pellet solution and supernatant were analyzed for total carbohydrates.

3.2.5 - Continuous Flotation: System and Runs

All columns used were made of pyrex glass. Spargers were rigid cylinders made of sintered glass with pore sizes from 40 to 60 μm, diameter of 1 cm, and length of 2 cm fixed at the tip of a glass tube. Two similar columns were used for continuous flotation experiments. The basic design of column #2 is shown in Figure 3.3. The enlargement at the top of the column is to avoid particle accumulation on the internal surface of the column (Oliveira, 1990). The enlargement at the bottom compensates for the reduction of the crossectional area due to the presence of the sparger to avoid clogging. The cuplike device around the top of the column collects the foam that overflows the column (the concentrate fraction). Column #1 differs from #2 by the absence of the top enlargement and internal part of the feed injection port and by differences in the recovery and cleaning zone lengths, 31 and 13 cm, respectively. The feed line consisted of a glass tip immersed into the feed suspension, plastic tubing, and a peristaltic pump. The tailings line was a siphon made of plastic tubing connected to the bottom of the column with a glass tip at the other end. This glass tip end was raised up to a few centimeters above the top of the column where it was turned downwards.

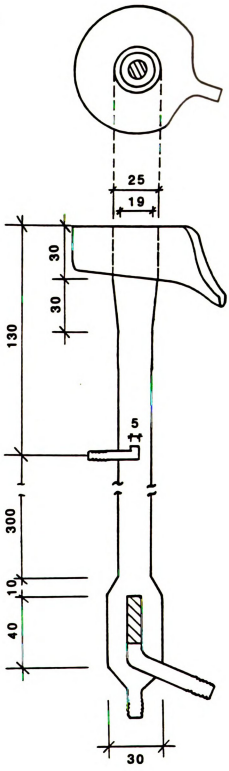

Figure 3.3 - Basic design of column #2. Measurements in millimeters.

By lowering and lifting this glass tip, the tailings flow rate and, consequently, the level of the froth inside the column, could be adjusted to a desired level. A funnel positioned under the glass tip collected the tailings into a graduated cylinder. The gas line was a plastic tube connecting the air reservoir with the fritted sparger. The wash line consisted of a wash solution reservoir, a peristaltic pump, plastic tubing, and the injection device (a syringe needle size 18 G $1^1/2$ radially centered and immersed approximately 0.5 cm into the column).

Continuous experiments were started by flowing air (27.5 mL/min) and pumping suspension and wash solution into the column at desired rates until it overflowed. The tip of the siphon for tailings withdrawal was lowered stepwise to set the froth-suspension interface to the desired level. Once the air flow rate and froth-suspension interface level were set to the desired values, the column was operated in a pre-run mode for a time equivalent to the pumping of at least three recovery zone volumes of suspension-washing solution at the current feed flow rates. The corresponding concentrate and tailings collected were discarded. The actual run was initiated with time recording and concentrate and tailings collection. Concentrate was collected over water or acetate buffer for dissolution of the HPMC particles. A graduated cylinder was used to collect the tailings. In flotation experiments with synthetic mixtures, the tailings (particles and liquid) were dissolved in water. However, in the case of broth flotation the particles from tailings were separated by filtration on a sintered glass filter and dissolved in acetate buffer. Synthetic and broth mixtures, concentrate, tailings liquid, and tailings particles were analyzed for α-amylase activity and total carbohydrates.

3.2.6 - Batch Flotation: System and Runs

Due to the absence in the literature of description of batch flotation columns, a column was developed for this purpose along with a procedure for its use. The batch column was a straight vertical tube with 66 cm in height, 1.7 cm in diameter, internal volume of 140 mL with a bottom outlet and overflow collector similar to the continuous columns. Design and construction precautions were taken to minimize the "dead volume" below the porous part of the sparger (the effective sparger area).

Batch flotation was initiated with the air flow through the column. The suspension to be tested was then poured into the column until it overflowed. Flotation time was recorded with the start of bubbling and, simultaneously, the air flow rate was set at 4.0 mL/min. To avoid a large drop in liquid level inside the column due to loss of liquid with the overflown froth, a solution was continuously added at the top of the column. This solution had the same salt concentration and pH of the suspension under test. Its addition started at 1 min. of flotation at a rate of 1.6 mL/min. A needle was used as the injection device and the solution was pumped by a peristaltic pump. The concentrate overflow was collected over water. The test was terminated with a fast drainage of the column by opening the bottom outlet (this drained suspension was termed tailings). After HPMC particle dissolution, both concentrate and tailings solutions were used for total carbohydrate determination. In the case of broth flotation, the volume of feed liquid that overflowed was estimated by the ratio between absorbance at 257 nm of concentrate solution and feed liquid (this was an empirical method using the maximum of the absorbance spectrum of broth between 200 and 400 nm). Knowing this volume and its total carbohydrate concentration, the amount of total

carbohydrate in the concentrate due to liquid overflow could be calculated. This amount was deducted from the amount of total carbohydrate determined in the concentrate to give the amount of HPMC particles floated. Due to high floatability of the HPMC particles, especially in the synthetic systems, batch flotation conditions were set to avoid total recovery. In this way, any possible effect of variables under study on the HPMC particle recovery could be detected.

The hydrodynamics of this column are certainly different from the continuous column. However, the objective was to have a batch equipment that needed small amounts of sample and time for operation rather than a device that exactly represented column flotation. The conventional batch devices like the Hallimond tube, bench mechanical cell, and those developed by Fuerstenau (1964) and Partridge and Smith (1971) were inappropriate due to complex design and requirements like particle-bubble disengagement at the suspension-froth interface and either too low or too high sample volume. Moreover, they are further away from duplicating the hydrodynamics of a flotation column in continuous operation than the column developed here.

3.2.7 - Enzyme Activity Assay

α-Amylase activity was measured using a modified iodine method described by Yoo and co-workers (1987). One unit of activity was defined as the of amount of substrate hydrolyzed, calculated as micromoles of maltose, per minute under the assay conditions. Substrate solution was 1% starch in 0.1 M sodium acetate with 0.025 M calcium pH 5.25. The hydrolysis was carried out at 70 °C by incubating 24 µL of the enzyme solution with 238 µL of substrate solution. Incubation was terminated by

addition of 238 µL of 0.5 N hydrochloric acid. Five milliliters of iodine solution (5 percent potassium iodide with 0.5 percent iodine) freshly diluted 50 times was added to develop color. Absorbance at 555 nm was taken with water as blank (readings termed R). The reference for color reduction was done by adding the enzyme aliquot only after acid addition (readings termed Ro). Control for background color of iodine solution was prepared by substituting buffer for starch solution in the incubation mixture (readings termed B). Activity was determined based on the fractional reduction of absorbance f calculated as

$$f = (Ro - R) / (Ro - B) \qquad (3.1)$$

As suggested by Yoo and co-workers (1987) and confirmed by the results, f values between 0.20 and 0.70 were linear with respect to enzyme concentration.

3.2.8 - Total Carbohydrates Assay

Total carbohydrates (TCH) were determined by a method based on the one described by Dubois and co-workers (1956). HPMC can be determined by this method because polysaccharides and their derivatives, including the methyl ethers with free or potentially free reducing groups, give a orange-yellow color when treated with phenol and sulfuric acid. The procedure consists in preparing a reaction mixture with 0.2 mL of sample and 0.2 mL of 5 percent phenol solution in 13x100 mm glass test tubes. The addition of 1.0 mL of concentrated sulfuric acid to the surface of the reaction mixture is performed as fast as possible to generate high temperature during the color development period (at least 20 min). The tubes were manually shaken and, after 10 min. the contents were mixed

made with water in place of sample was used as reference. Glucose was used as the standard.

3.2.9 - Other Experimental Methods

Different particle sizes were obtained by changing the rate of addition of the ammonium sulfate solution and the mixing during partition. Particle size was analyzed in a Horiba Capa-700 particle analyzer. This equipment operates based on the sedimentation speed of particles (gravitational or centrifugal sedimentation) which is measured by absorbance of the particle suspension. Basic data to be fed to the analyzer was dispersant density and viscosity and particle density. The first two were measured using the filtrate of a suspension, the actual dispersant of HPMC particles (1.08 g/mL and 1.18 cP, respectively). Viscosity was measured using a capillary viscometer. However, due to the gel characteristics of the HPMC particles, which prevent them from being dried without destructing them, particle density was not determined. Particle density was assumed to be the density of cellulose, 1.3 g/mL. Bubble sizes were estimated to be in the range of 0.1 to 0.2 mm by photographic analysis.

3.3 - Results

3.3.1 - Experiments with Synthetic Mixtures

Small variations in the salt solution flow rate and/or mixing during partition resulted in the change of the size and morphology of salted-out particles. At high mixing conditions and slow salt addition individual particles of a fraction of a millimeter were formed. For a slightly faster salt addition or lower mixing we would get elongated fibers of a few

millimeters which formed flocs. These irregular shapes made their size determination by traditional methods inappropriate. The effect of particle size on flotation of HPMC was demonstrated at two sizes where particles were mostly individual, approximately spherical to rodlike particles, with nominal sizes of 10 and 16 μm (Table 3.2 and Figure 3.4). The differences in conditions for production of these particles were relatively large (salt solution flow rate of 5 and 20 mL/min, respectively). Although the particle sizes obtained were less than one order of magnitude different from each other, the presence of some agglomerates of filaments was noticed in the suspension. A remarkable effect of particle size on recovery was noticed by the different air flow rates required to maintain a certain recovery level.

Knowing this significant effect of particle size on flotation recovery and due to the difficulty in control and analysis of their size distribution, care was taken to assure a constant particle size distribution within an experiment. Each experiment was done from a single partition or when treatments of experiments were carried out from different batches, these batches were processed with the same conditions (volumes, mixing and rate of salt addition). Conditions were set such that small flocs were formed without excessive agglomeration into large flocs or elongated strings.

Figure 3.5 shows how the recovery of HPMC is affected by the ammonium sulfate concentration of the supernatant. Salt concentration lower than 45 percent saturation allowed recovery of virtually 100 percent of the polymer. A sharp decrease in recovery takes place as ammonium sulfate concentration increases. We qualitatively noticed a

Table 3.2 - Particle size distribution of HPMC particles suspension used for experiments illustrated in Figure 3.4.

Nominal size (μm)	Mean x	Mean sd	Standard Deviation x	Standard Deviation sd	n
10	9.96	0.55	12.55	1.12	4
16	16.15	0.75	9.74	0.75	4

Particle size analyzer: Horiba Capa-700.

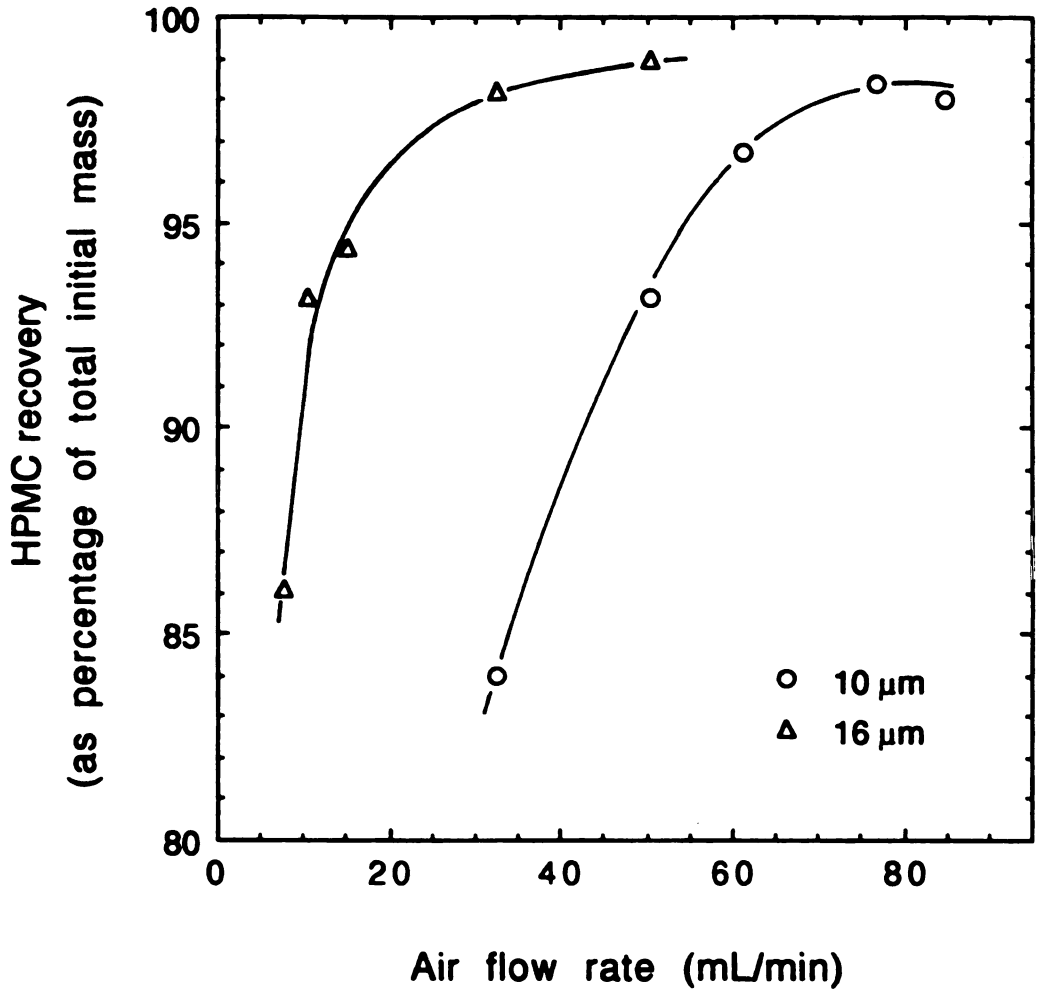

Figure 3.4 - Response of HPMC flotation to particle size. Partition of synthetic mixture: batch volume, 4 L; ammonium sulfate addition flow rate, 5 and 20 mL/min for 10 and 16 µm, respectively. Continuous flotation with no washing: column used, #1; feed flow rate, 27. mL/min; froth height, 0.5 to 3 cm.

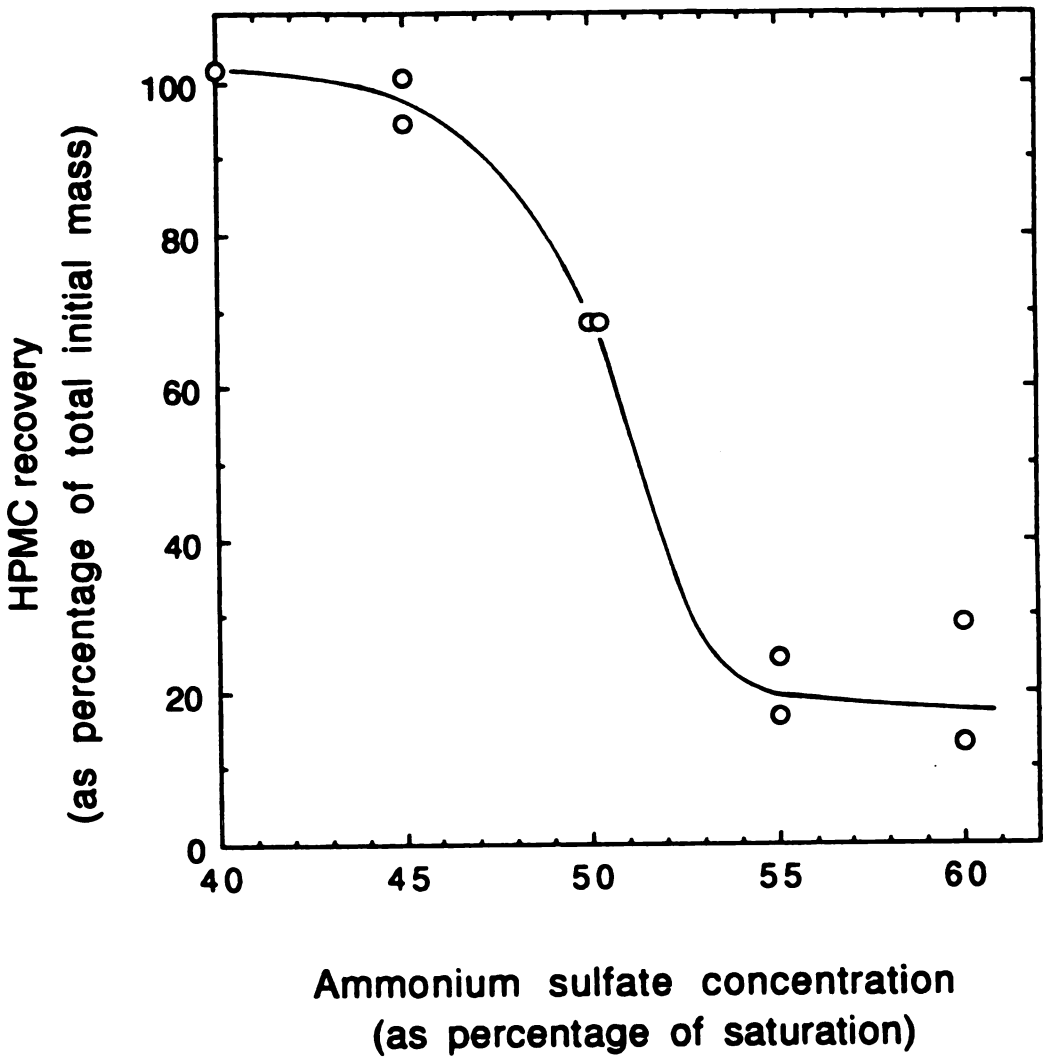

Figure 3.5 - Effect of ammonium sulfate concentration on the recovery of HPMC by flotation. Salting-out of synthetic mixture: batch volume, 1.5 L; HPMC concentration before salt addition, 0.07 percent; ammonium sulfate addition flow rate, 14 mL/min. Continuous flotation with no washing: column used, #1; feed flow rate, 36 mL/min; froth height, 1 cm.

decrease in the foamability of the suspension at higher salt concentrations.

An apparent column capacity for synthetic mixtures was determined by feeding the column at increasing flow rate (Figure 3.6). The breakthrough point (a sharp decrease in HPMC recovery) as the feed flow rate reached a certain value is defined as apparent column capacity. Its nominal value was determined by fitting separate straight lines to the lowest 7 and highest 9 feed flow rate points and finding their intercept. The apparent capacity found was 156 mL/min which corresponds to 94 cubic meters per hour per square meter of column crossectional area. A bubble size increase was observed with the increase of the feed flow rate.

Concentrate purification by washing of liquid from the feed out of the froth was studied with the help of the tracer alkaline copper sulfate, which could be detected by absorbance at 700 nm. The purification quantified in Figure 3.7 could also be visually detected due to the strong color of the feed liquid. Not only the change in the blue color of the froth from treatment to treatment was noticeable, but a gradient of color was clearly present throughout the froth.

Changes in the pH of the suspension by addition of sulfuric acid or ammonium hydroxide did not significantly affect HPMC recovery (Figure 3.8). pH changes were limited to the range of 3.0 to 9.0 due to the buffering capacity of the system. A control experiment showed that pH changes do not affect solubility of HPMC in this range.

3.3.2 - Experiments with Broth Mixture and Periplasmic Extract

Continuous experiments with broth mixtures at different levels of diafiltration showed increased recovery of HPMC with increasing

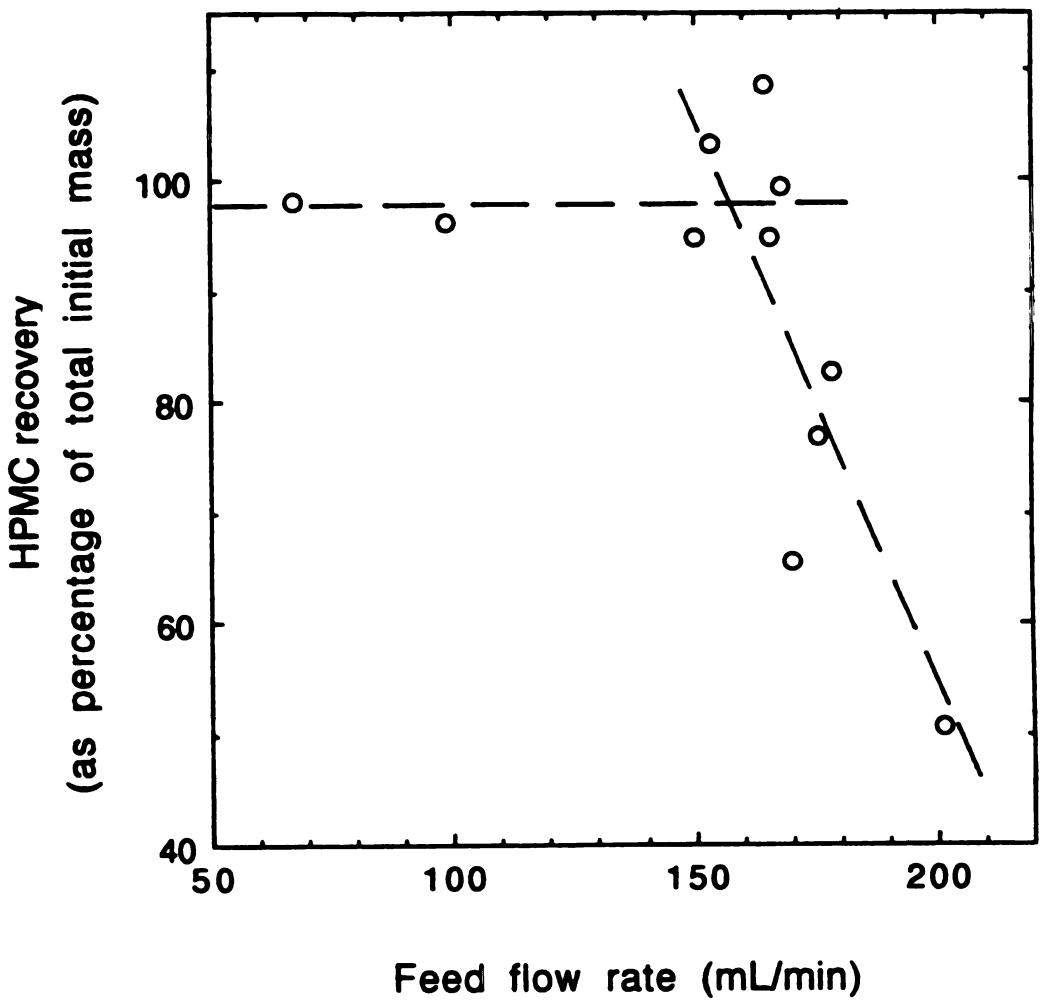

Figure 3.6 - Determination of apparent column capacity. Partition of synthetic mixture: batch volume, 10 L; ammonium sulfate addition flow rate, 60 mL/min. Continuous flotation with no washing: column used, #1; froth height, 3 cm.

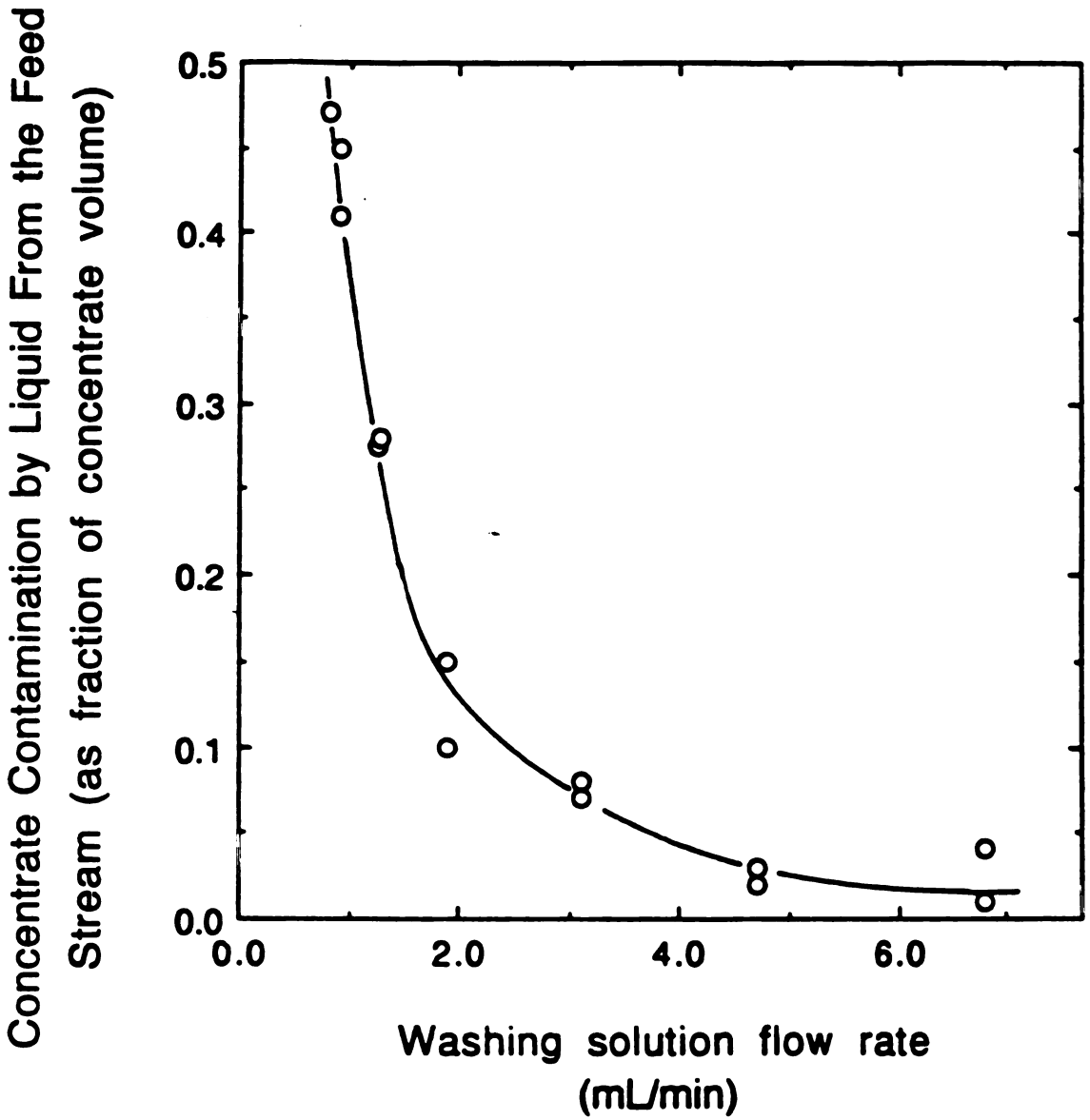

Figure 3.7 - Effect of washing on concentrate purity. Partition of synthetic mixture: batch volume, 10 L; ammonium sulfate addition flow rate, 60 mL/min; tracer, 2.0 percent copper sulfate ($CuSO_4.5H_2O$), 0.4 percent sodium hydroxide, and 2.0 percent sodium potassium tartrate. Continuous flotation: column used, #1; feed flow rate, 40 mL/min; froth height, 4 cm; washing solution, 2.0 percent sodium potassium tartrate in 30 percent saturation ammonium sulfate.

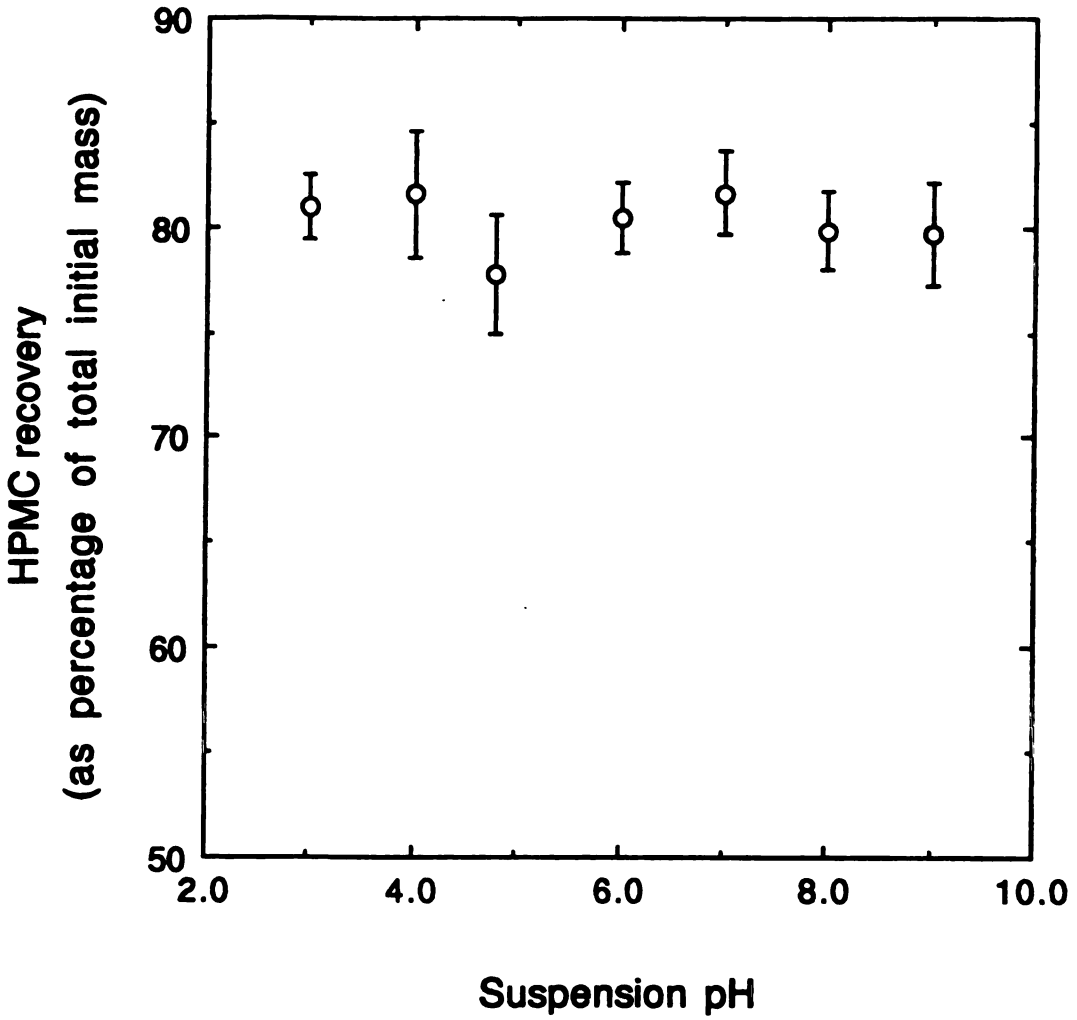

Figure 3.8 - Effect of pH on flotation recovery of HPMC from synthetic mixture by batch flotation. Partition: batch volume, 150 mL; HPMC concentration, 0.1 percent; ammonium sulfate concentration and addition rate, 30 percent saturation and 3.3 mL/min, respectively. Flotation: air flow rate, 4.0 mL/min; make-up water flow rate, 1.6 mL/min; flotation time, 5 minutes.

purification of the broth up to the point of total recovery (Figure 3.9). Activity recovery followed the same pattern increasing up to the total recovery possible by the partition (91 percent, determined by control partition experiments). Both maximum HPMC and activity recoveries took place when broth was diafiltrated with at least two volumes of buffer per volume of broth. A linear relationship was observed between the recovery of HPMC and recovery of activity with HPMC particles. Figure 3.10 is a plot of the actual recovery of activity versus the theoretical recovery of activity based on the recovery of HPMC particles (slope of 0.88 and regression coefficient of 0.992). The slope deviated slightly from the expected value of one indicating that the flotation operation recovered all the enzyme that was associated with the particles.

Periplasmic extracts were much more efficiently treated than broth with virtually 100 percent recovery of particles and total possible activity recovery (90 percent, determined by the control partition experiment). An attempt to reduce the salt used in the flotation was made by lowering the salt concentration in the washing solution (Table 3.3). However, the slight reduction from 30 to 25 percent saturation resulted in about one third reduction in the activity recovered (from 90 to 62 percent of total initial activity).

pH dependence of HPMC recovery from a broth mixture was demonstrated by batch experiments (Figure 3.11); the lower the pH, the higher the HPMC recovery. Continuous experiments were done to evaluate the influence of pH on the recovery of activity (Figure 3.12). HPMC recovery had the same behavior as in the batch flotation experiments for the pH range from 3.0 to 7.0: HPMC recovery increased as the pH was lowered. The lowering of the pH to 2.5 decreased the HPMC recovery

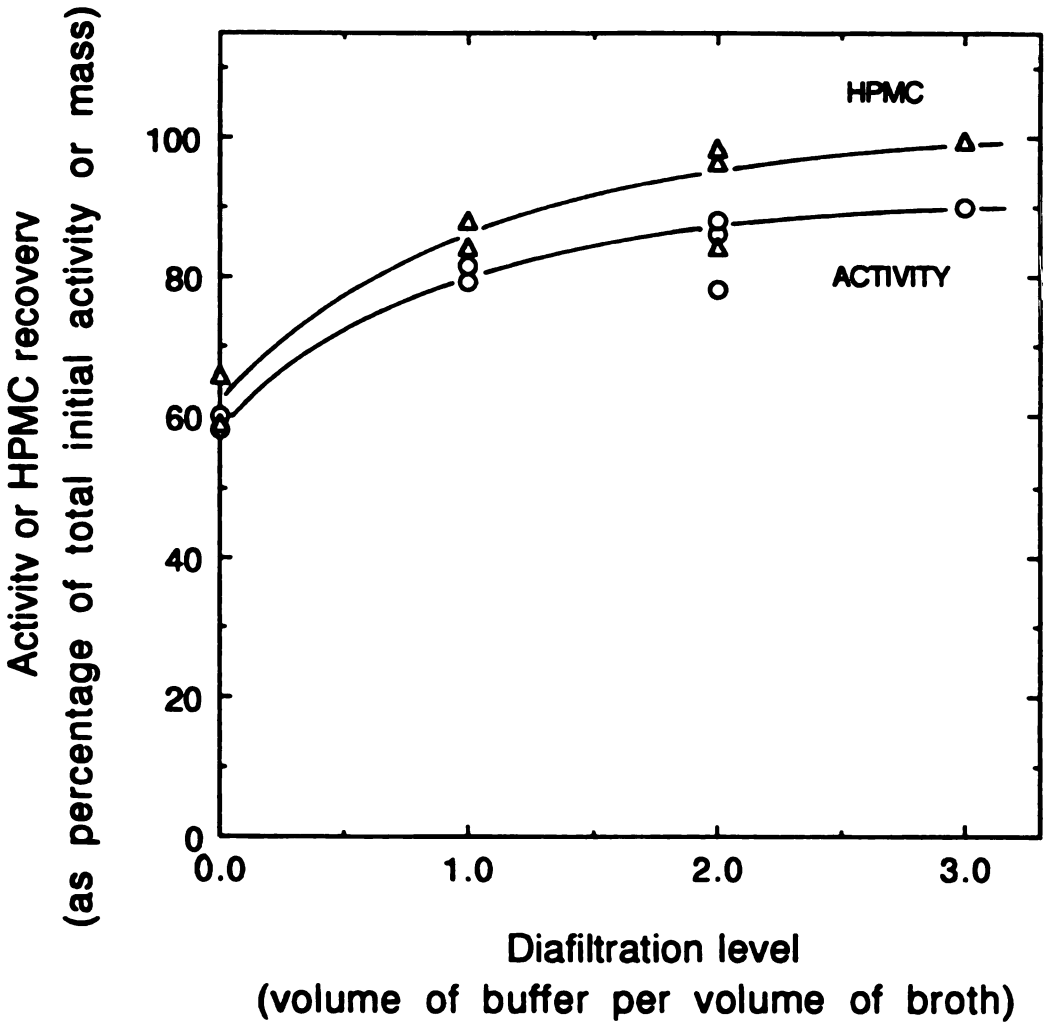

Figure 3.9 - Flotation response to broth pre-purification by diafiltration. Partition of broth mixture: batch volume, 1.5 L; ammonium sulfate addition flow rate, 31 mL/min. Continuous flotation without washing: column used, #2; feed flow rate, 20 mL/min; froth height, 5 cm.

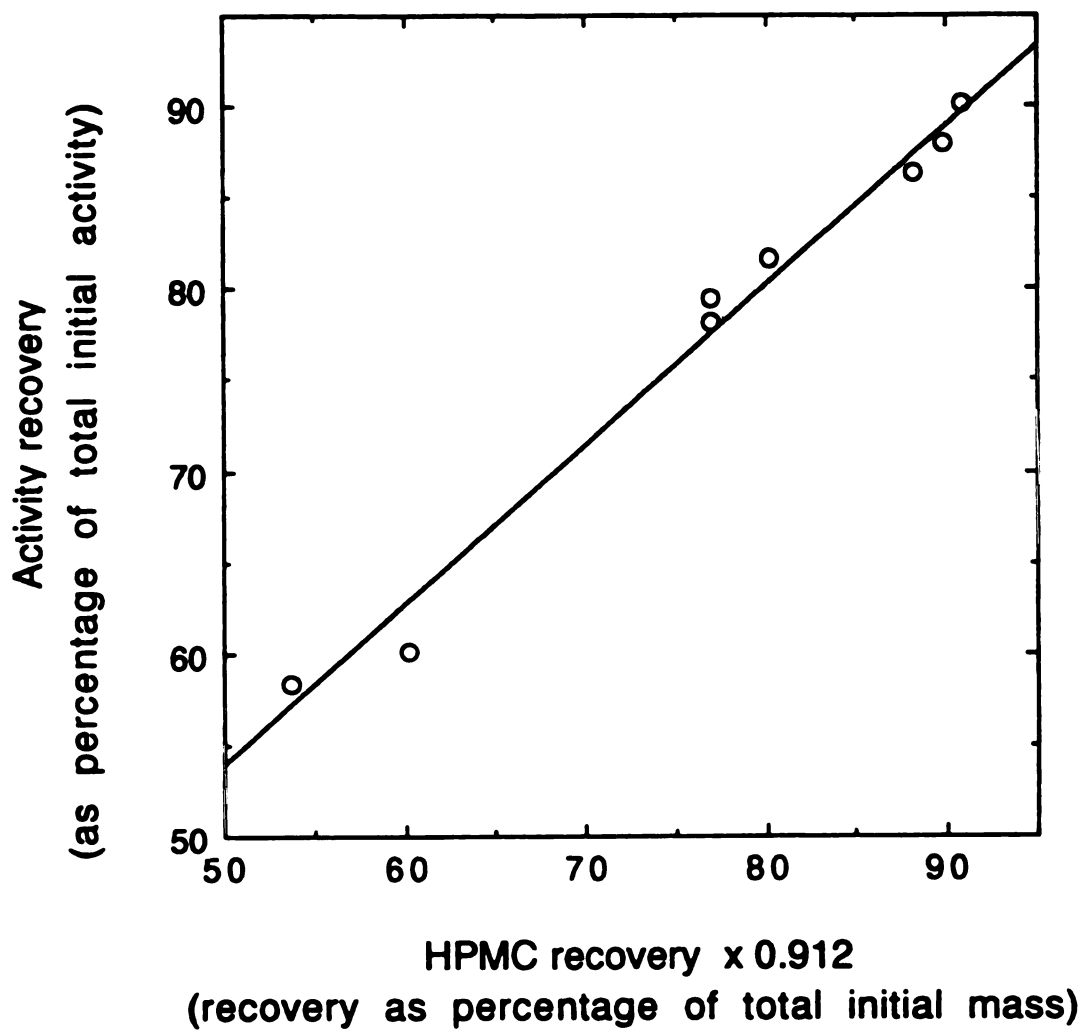

Figure 3.10 - Correlation between actual activity recovery and theoretical activity recovery calculated as 0.912 x HPMC recovery. Data from experiment depicted on Figure 3.9.

Table 3.3 - Flotation of periplasmic extract and the effect of lowering the ammonium sulfate concentration in the washing solution.

Concentration of ammonium sulfate in the washing solution (as percentage of saturation)	Activity recovery (as percentage of total activity)		
	Run#1	Run#2	Average
30	91	88	90
25	62	62	62
20	45	41	43

Conditions: Partition of periplasmic extract: batch volume, 600 mL; ammonium sulfate addition flow rate, 10 mL/min. Continuous flotation: column used, #2; feed flow rate, 20.0 ml/min; washing flow rate, 10 mL/min; froth height, 7 cm.

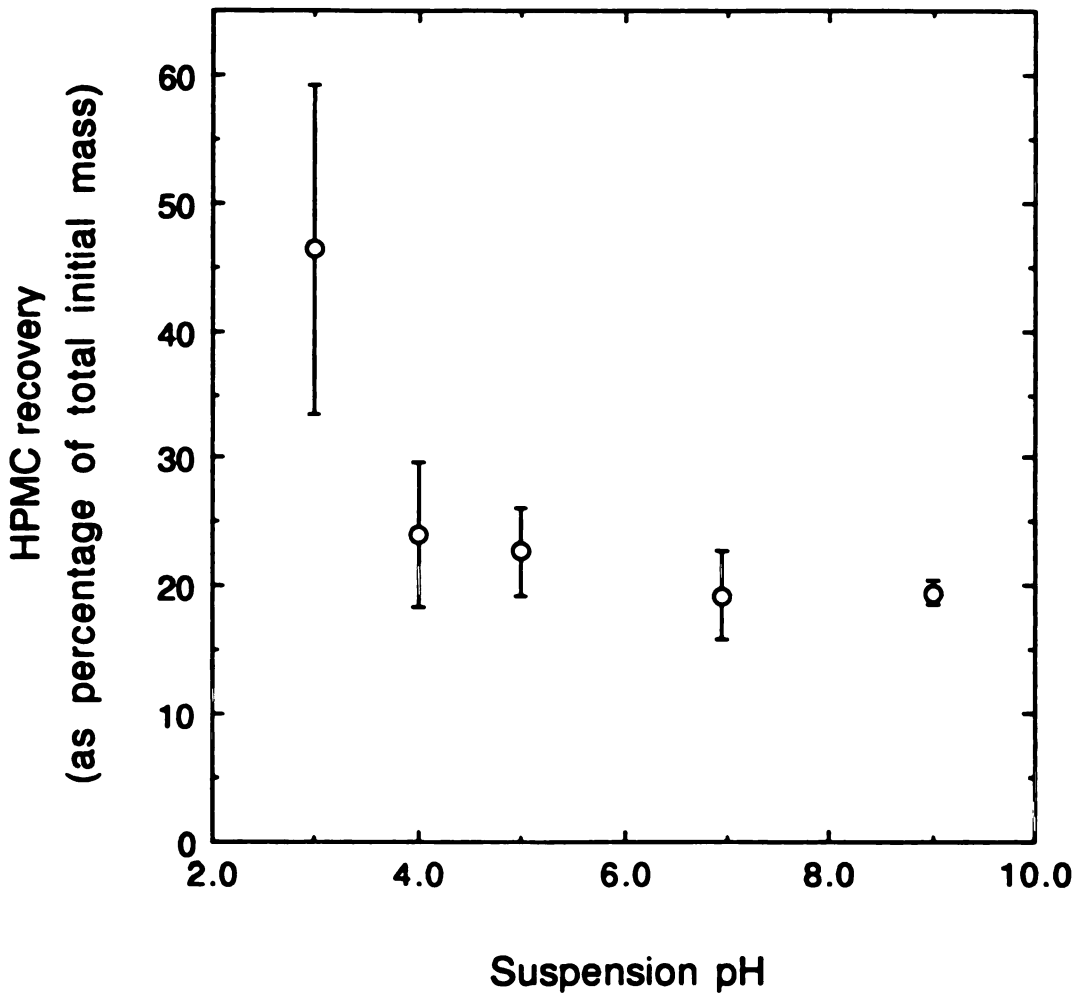

Figure 3.11 - Effect of pH on flotation recovery of HPMC from broth mixture by batch flotation. Partition: batch volume, 150 mL; HPMC concentration, 0.1 percent; ammonium sulfate concentration and addition rate, 30 percent saturation and 3.3 mL/min, respectively. Flotation: air flow rate, 4.0 mL/min; make-up water flow rate, 1.6 mL/min; flotation time, 5 minutes.

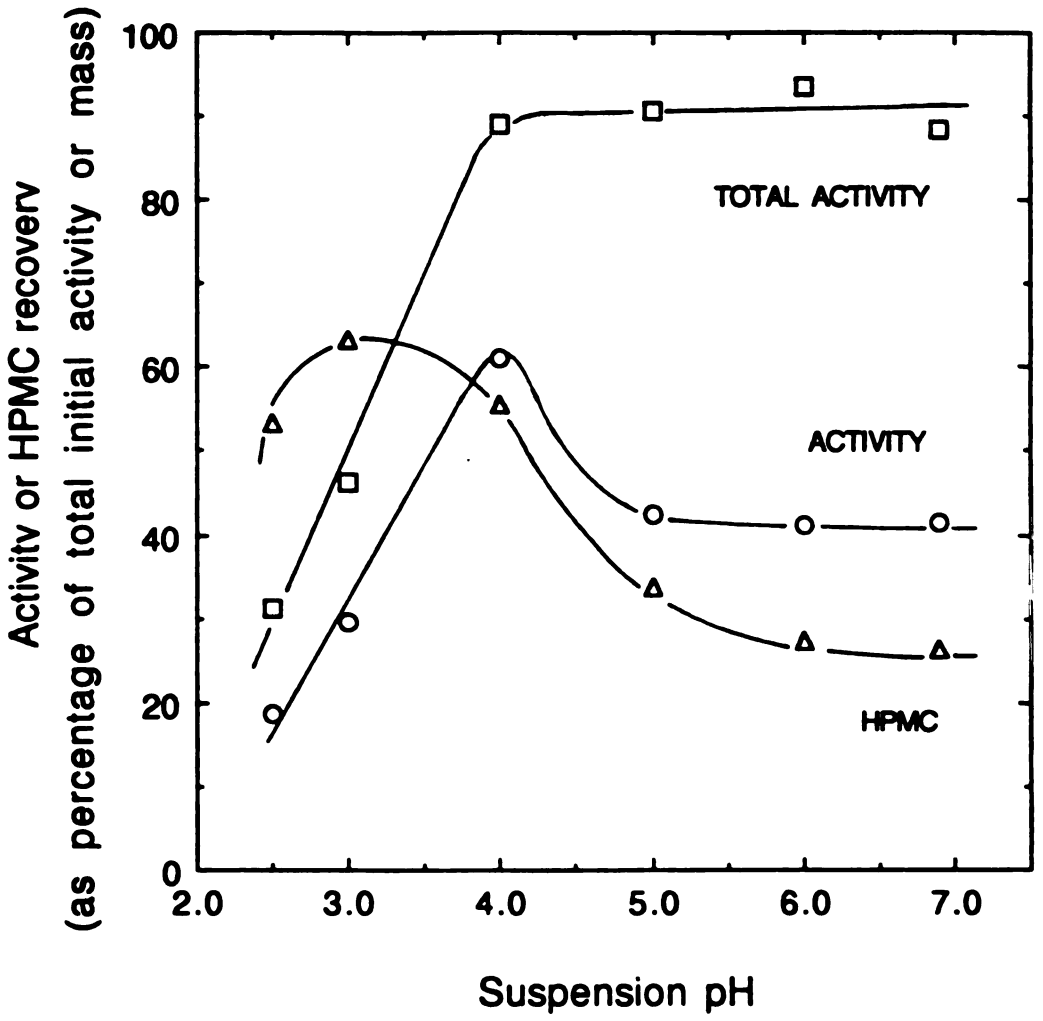

Figure 3.12 - pH effect on recovery of HPMC particles, activity associated with HPMC particles, and total activity. Partition of broth mixture: batch volume, 4 L; ammonium sulfate addition flow rate, 42 mL/min. Continuous flotation : column used, #2; feed flow rate , 20 mL/min; washing solution flow rate, 7 mL/min; froth height, 7 cm.

making the pH 3.0 a maximum. Activity recovered with the particles also increased with pH decrease to a value at which a maximum was reached. However, the maximum for activity recovery took place at a higher pH than that for HPMC recovery (pH 4.0 instead of pH 3.0). Total activity recovery was as high as 90 percent for pH equal to or higher than 4.0 with a continuing decrease for lower values.

Continuous experiments produced a concentration ratio from 3 to 4 (ratio of volume of feed to volume of concentrate) and concentrate contamination by the feed liquid around 30 percent.

Temperature increase resulted in a decrease in the recovery of particles (Table 3.4). An increase in bubble size could be seen as the temperature was raised.

3.4 - Discussion

Nearly total recovery of HPMC particles from both the synthetic mixture and the periplasmic extract was possible. The broth mixture had a lower, but significant floatability. However, pre-purification of the broth made it possible to achieve total recovery of activity and HPMC particles. Moreover, the recovery can be optimized with the increase of the air flow rate. Typical superficial air velocity in mineral processing is in the range of 1 to 3 cm/s while we used a value of 0.18 cm/s in these continuous experiments (Dobby *et al.*, 1988). The linear relationship between recovery of HPMC particles and recovery of enzyme activity indicates that no significant enzyme denaturation occurred due to flotation (the activity recovered in the concentrate was proportional to the recovery of the particles). The recovery of active enzyme/HPMC for the pH experiments was lower than the recovery for the diafiltration experiments. This

Table 3.4 - Temperature effect on the flotation recovery of HPMC from broth

Temperature (°C)	HPMC recovery (as percentage)		
	Run#1	Run#2	Average
25±0.5	19	25	22
40±2.0	15	11	13
56±4.0	6	5	6

Conditions: Partition of broth mixture: batch volume, 600 mL; ammonium sulfate addition flow rate, 10 mL/min. Continuous flotation: column used, #2; feed flow rate, 20.0 ml/min; washing flow rate, 10 mL/min; froth height, 7 cm.

decrease in efficiency is probably caused by hydrodynamic disengagement due to the introduction of washing at this experiment.

The low activity recovery when the salt concentration in the washing solution was decreased is the result of the large ratio of volume of washing solution to volume of suspension in the froth (approximately 2 due to wash solution flow rate of 10 mL/min and concentrate overflow around 5 mL/min). The salt concentration is close to the value for the washing solution and partition strongly depends on the salt concentration of the system (Figure 3).

The low floatability for the broth mixture is not surprising. The electrokinetic properties of the solid/water interface of hydrophobic solids are primarily or completely determined by the aqueous phase. The electric double layer is entirely on the water side of the interface. Also, the behavior of the solid/water interface is similar to the behavior of the air/ water interface (Arbiter *et al*, 1975). Due to the complex composition of the broth, interactions of its components at the solid/liquid and gas/liquid interfaces were expected. However, not all are expected to be neutral toward floatability. The components may act as depressors, activators, or collectors. In this specific case, a depressant effect prevailed. The improved floatability of the broth pre-purified by diafiltration indicates that these depressors have, or are activated by, low molecular weight molecules (less than 10,000). These molecules are probably amphipatic entities of which nonpolar ends interact with the nonpolar structure of the HPMC exposing the charged groups to the liquid. These charged groups may impart to the particle a net electric charge responsible for the decrease in hydrophobicity and, as a consequence, decrease in floatability.

Some insight into this depressor phenomenon was provided by the pH experiments. No alteration in floatability was noticed when the pH of suspension of synthetic mixture was modified. However, in the case of broth mixtures, pH changes seemed to interfere with intermolecular interactions. The higher recovery at low pH suggested a negatively charged molecule as the depressor which is neutralized as pH is lowered. This depressor could be an amphiphilic molecule having its hydrophobic moiety positioned towards the hydrophobic phases (particles and air bubbles) and its charged moiety towrds the water phase. Therefore, this negative charge increases both the particle affinity to water and the repulsion from an also negatively charged air bubble. An excessive decrease in pH would cause its protonation to a positive net charge with decrease in floatability.

The media used in the fermentation, LB medium, is rich in peptides and polypeptides since it contains peptone and yeast extract. Due to the diversity of chemical character that polypeptides can have, some may act as depressors of the HPMC particles. Polypeptides that are relatively rich in glutamic, aspartic, and hydrophobic residues like leucine, isoleucine, phenylalanine, tryptophan, and tyrosine are consistent with the depressor pictured above, i.e., a negative charged molecule at neutral to moderate low pH with hydrophobic patches.

The effect of particle size on flotation can be explained by the limited carrying capacity of bubbles and different specific area of particles of different sizes. A bubble has a limited area to which particles can attach (only the bottom part is effective on carrying particles). The smaller the particles, the larger their specific surface area which means that small particles have more surface area than large

particles for the same mass. Consequently, small particles require more bubble area to achieve the same level of flotation; or small particles have lower recovery for the same bubble area available (gas flow rate).

The column capacity measured is termed apparent capacity because it is lower than the true column capacity. In the determination of apparent capacity, the increase in the feed flow rate at a fixed particle concentration causes an increase in the amount of HPMC fed to the column. Simultaneously, it also decreases the residence time of particles in the equipment which reduces their chance to be picked up by the bubbles. The net result is lower recovery. However, this apparent capacity is a good indicator for capacity and consequently column size for a specific separation. Despite of the high column throughput for this system, 94 $m^3/(h.m^2)$, this is not a optimized value. The dependence of capacity on the bubble area available for flotation makes it possible to increase the capacity by operating at higher air flow rates as long as flooding does not occur.

The bubble size increase observed with the increase of the feed flow rate may be due to the heavy bubble load conditions reached. As two bubbles get close to each other, the particles attached to them seem to be in a thin liquid film. Bubble coalescence caused by hydrophobic particles in thin films is a known phenomenon and there are two mechanisms by which it takes place. The hydrophobicity of the particle induces the film thinning by the hydrophobic interaction between the solid and the gas phase to a point that ruptures occur. Also, the particle may partially dissolve in this film spreading its surfactant molecules over the air/water interface. Water in the film flows along with these molecules

(the Marangoni effect) away from the particle causing the film to thin around it up to the rupture point (Walstra, 1989).

Extensive washing accomplished with the synthetic mixture is the characteristic that enables column flotation to combine the two goals of solid recovery from liquid suspension (particle concentration and liquid removal) in one step. Washing efficiency will vary from system to system due to different liquid viscosity and density, bubble size, and how the froth is packed. However, the result obtained here (Figure 3) illustrates the potential of this flotation column feature in contributing to the overall purification of the partition process.

The level of concentrate contamination (30 percent) obtained for the broth mixture during the pH experiments is high compared with the results obtained for the synthetic mixture. Also, the volumetric concentration ratio from 3 to 4 is not overly impressive. This high contamination and low volumetric concentration ratio are the result of a finer froth structure composed of small bubbles. As a consequence, the liquid content of the froth is higher than the values for synthetic mixture. Therefore, there is a higher overflow rate out of the column. This results in a higher rate of feed liquid in the froth to be pushed down the column by the washing solution. The final result is a reduced concentration ratio and purification efficiency.

This froth characteristic may be due to an increased stability conferred to the bubbles by surfactants from the fermentation broth. It is known that proteins or polypeptides at the air/water interface may promote a concentration of polysaccharide frothers in this interface by interacting with them. The result is a more stable foam due to the increase of the thickness and mechanical strength of the film between

bubbles (Dickinson, 1989). Polypeptides and proteins in the broth may be enhancing the action of the soluble HPMC as a frother beyond a desired level. The use of food-grade antifoams may set foamability to a optimum level.

The washing effect is expected to be improved in a scaled-up column with a larger cross-sectional area due to a better distribution of the washing solution traveling down the column. At low shear rates, foam flowing inside a column exhibits a non-Newtonian behavior following a Casson model. Due to a yield stress, the velocity profile is flat at the center of the column. At the wall, there is a thin layer of liquid devoid of gas bubbles which is characteristically Newtonian, called a skimming layer. Therefore, the velocity profile close to the wall is linear and goes through a transition towards the central region where it reaches the plateau (Mahalingam *et al.*, 1975). As a consequence, there may be a uneven distribution of the washing solution throughout the cross-sectional area of the column. The high inertia of the foam at the central part of the column may divert the washing solution towards the wall. With most of the washing solution flowing away from the center of the column, excessive washing may take place at the region close to the wall at the expense of poor washing at the center. This can result in chanelling resulting in poor utilization of the wash solution. This phenomenon can be called a wall effect. A column with a larger cross-sectional area will experience a smaller wall effect due to a larger cross-sectional area-to-perimeter ratio.

More complete insolubilization of the HPMC, the frother of the system, at high salt concentration and temperature would contribute to the froth instability and to the low recovery at these conditions. Larger

bubbles in the recovery zone and coalescence to even larger bubbles at the froth bed decrease the bubble surface area available for particle pick-up in the suspension and their transport through the froth. We should note that HPMC has a "inverse" solubility property, i.e., it is more soluble in water at low temperatures than at high temperatures (Sarkar, 1979). Another way to improve volumetric concentration ratio is by lowering the injection point of wash water. This increses the length of the draining froth bed. More liquid drains down the column and, consequently, a drier foam overflows.

3.5 - Conclusions

In summary, the recovery of α-amylase by the combination of partition to a HPMC/salt phase with subsequent solid-liquid separation in a flotation column was shown to be feasible. Processing of synthetic systems showed that high polymer recovery, and consequently high enzyme activity recovery, and equipment capacity are possible with good purification even before optimization of operational variables. The processing of fermentation broth and periplasmic extract confirmed the feasibility of the process, despite results not as good as for the synthetic systems.

Scaling-up of the process seems to be straightfoward and suitable for large scale operations due to its simplicity, efficiency, high throughput, and low cost.

Overall, the flotation of this biological system behaved similarly to flotation of mineral systems. The same basic principles and mechanisms were valid. Specifics due to the biological nature of the feedstock exist and, consequently, solution of some problems required approaches more

restricted to biotechnological processing (e.g., diafiltration). Other problems were addressed by procedures common to mineral processing (e.g., pH adjustment). As it was shown for this partition process, it is possible that other particulated systems of difficult solid/liquid separation used in separations in biotechnology can be processed in as flotation.

The important conclusions from the experimental results are:

a) The potential capacity of flotation columns in the treatment of (hydroxypropyl)methylcellulose particles salted-out at 30 percent saturation of ammonium sulfate is on the order of 100 $m^3/(h.m^2)$. It is independent of pH and dependent on the particle size and salt concentration.

b) The washing capability of the column can allow the produciton of concentrates with low contamination of feed liquid (as low as 2 to 3 percent).

c) The recovery of α-amylase directly from the fermentation broth by flotation is feasible at reasonable capacity. The capacity can be increased by the elimination of small molecular weight compounds (less than 10,000) from the enzyme preparation that act depressants of the flotation. Also, these depressant effects can be counterbalanced by pH lowering down to pH 3.

d) The flotation column demonstrated higher capacity for the flotation of periplasmic α-amylase than for the extracellular enzyme.

e) There is no detectable enzyme denaturation due to the partition or the flotation steps.

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Chapter 4

Affinity Flotation Based on Ligand-Ligate Hydrophobic Interaction

Abstract

A flotation system was developed to perform protein separation based on hydrophobic interaction. It consisted of a ligand covalently attached to the carrier molecule methylcellulose, a polymer that when salted-out by ammonium sulfate can be floated. The ligands were saturated fatty acids of linear chains attached to the methylcellulose by esterification. These methylcellulose derivatives were partially soluble in water and their salting-out properties and floatability were close to the unsubstituted methylcellulose. When the derivatives were tested for the precipitation of bovine serum albumin, the butyric acid ligand showed a higher efficiency than others tested. However, the floatability of the protein-derivative particles was low due to the high salt concentration required for binding and the high protein concentration. There is evidence that the major driving force for the precipitation is hydrophobic interaction.

4.1 - Introduction and Objectives

One of the most successful strategies for product recovery in downstream processing of fermentation products, especially proteins, is

the use of affinity separations. These are separation techniques based on affinity interactions; i.e., the result of a selective and reversible interaction between two entities called the ligate and the ligand. The main advantage of these techniques is the high purification attained by selective binding of ligates to ligands. Ligands can recognize either an entire class of ligates (general ligands) or only one or two (specific ligands).

Ligate-ligand interactions occur in accordance with their biological functions. For example, coenzymes, antibodies, inhibitors, and substrates are commonly used as ligands for protein purification (Scouten, 1981). Some ligands such as hydrophobic groups are not classified as affinity ligands when ligate-ligand bonding is not related to the biological activity.

The unit operation by which the separation is actually carried out depends on the physical characteristics of the ligand or ligand-ligate complex under the conditions of separation. Therefore, affinity precipitation, affinity ultrafiltration, affinity aqueous two-phase extraction, and affinity adsorption have been developed (Flygare *et al.*, 1982; Janson, 1984; Male *et al.*, 1987; Mattiasson *et al.*, 1986; Scopes, 1987). The most common affinity unit operation is the adsorption of the ligate onto a solid matrix where the ligand isimmobilized. Despite the use of membranes, fibers, and sponges the most popular solid matrix used, in both laboratory and industrial scale, is small agarose beads.

In the vast majority of the adsorption processes described in the literature, the adsorption and desorption steps are carried-out in batch fashion with the beads contained in chromatographic columns due to the convenience of handling beaded shaped adsorbents in such devices. Therefore, such separation techniques are called affinity chromatography.

However, among the many cases of affinity chromatography reported in the literature, only few cases of true chromatography (the simultaneous but differential migration of different molecules along a packed bed) are cited (Janson, 1984).

The drawbacks of affinity chromatographic processes are similar to the problems found in other processes that utilize packed beads. The difficulty in operating on a continuous basis, the need to find the optimum flow rate (balance between high capacity and adsorption efficiency), the consolidation of the bed at high flow rates due to mechanical weakness of beads, and the need of a feed free of particulate material are a few of them. The significance of these problems are magnified by the fact that affinity chromatography is a four-stage operation including adsorption, washing, desorption, and washing.

To circumvent these problems, systems like CARE (continuous affinity-recycle extraction), a combination of a batch adsorption with packed bed desorption, partition of adsorbents to aqueous two-phase system, fluidized beds, and magnetized adsorption beads have been suggested (Gordon *et al.*, 1990; Janson, 1984; Pungor *et al.*, 1987; Yang *et al.*, 1989; Yang and Goto 1992). To date, it seems that these techniques have only been used on a laboratory scale.

Flotation is a potential unit operation for affinity separations without the negatives associated with packed bed operation. The basic requirement for affinity flotation is that the ligand-ligate complex must be insoluble and floatable under the binding conditions. The literature is abundant with reports of successful affinity precipitations that may suit these requirements (Scopes, 1987). However, the potential of affinity flotation is not restricted to known affinity precipitation systems. We

can design and prepare molecules for this purpose. If the complex ligate-ligand is not insoluble or floatable, the attachment of the ligand to a carefully chosen carrier may produce a ligate-ligand-carrier complex that is insoluble and floatable. Its important to understand the properties of the carrier to a considerable depth since, ideally, its properties would markedly be expressed by the ligand-ligate-carrier complex. Therefore, the knowledge of carrier precipitation and flotation could be used to predict ligand-ligate-carrier precipitation and flotation.

The advantages of flotation over adsorption on solid matrices are many. First, flotation is a typical and a simple continuous unit operation well know for its high throughput. By designing a system where ligate-ligand binding precedes insolubilization, we can take advantage of the fast kinetics typical of liquid systems. The presence of particles other than ligate containing particles is of little concern due to the ability of the flotation process to reject non-floatable particles in the tailings stream by the action of the wash solution. This feature also reduces the four steps needed when packed beds are used (adsorption, washing, desorption, and washing) to two steps (adsorption-washing and desorption-washing) since, ideally, the washing rejects all feed liquid from the concentrate.

This chapter describes the development of an example of the affinity flotation system discussed above composed of flotation of bovine serum albumin (BSA) bound by hydrophobic interaction to a linear alkyl chain attached to a floatable methylcellulose. The carrier, methylcellulose, and type of interaction for ligand-ligate binding (hydrophobic interaction) were selected based on the common characteristic of high salt concentration requirement. Hydrophobic interaction, as discussed above, is usually driven by high salt concentration. Flotation of cellulose ethers

was discussed in Chapter 3. A high salt concentration is also required for insolubilization and flotation of the cellulose ether. Thus, high salt concentration favors both the chemical and physical requirements of flotation. Also, there is a vast literature in this field describing the purification of a large number of different proteins, including enzymes, by hydrophobic chromatography. Particularly, bovine serum albumin has been extensively studied as a model system for hydrophobic chromatography. One of serum albumin's biological functions is the transport of fatty acids in the blood stream (Peters *et al.*, 1973; Wichman and Anderson, 1974); therefore, fatty acids are a widely used ligand for serum albumin.

Derivatization using linear aliphatic chains was used to impart different degrees of hydrophobicity to methylcellulose by varying the size and the degree of attachment. Acid catalyzed esterification by fatty acids.was used since it is simpler than the other methods for derivatization of agarose. The common procedure for hydrophobic chromatography is to activate the matrix, attach a linear spacer, and then attach the ligand to this spacer. However, a spacer may not be as important in the case of soluble ligand-carriers as in the case of solid matrices since the flexibility of a soluble carrier of high molecular weight may make the ligand accessible to the ligate. The esterification of the hydroxyl groups of the methylcellulose is not only a simpler procedure, but also may subject the methylcellulose to fewer side-reactions. An extensive literature search,did not reveal any examples of agarose derivatization by esterification. It is possible that the acid conditions of such a reaction causes degradation of the agarose beads altering their porosity, shape, and their physical strength. As mentioned before, the

physical strength of the beads is an important characteristic for adsorbents used in packed beds.

4.2 - Hydrophobic Interaction

The term hydrophobic was first used by J. Perrin in 1905 to refer to colloid sols that readily coagulate by addition of electrolytes (Melander and Horváth, 1977). Hydrophobic substances are defined by Tanford (1980) as those readily soluble in nonpolar solvents and only sparingly soluble in water. These substances are nonpolar or have a structure primarily nonpolar. The apparent repulsion from water is a common property and explains the name. However, some disagree with this name claiming that there is a significant attraction between these substances and water molecules (Bull and Breese, 1980). Their association in aqueous media is of enormous importance, especially in biological systems, where they are responsible for the organization of living matter as a coherent entity. In 1981 about 2000 titles in the Science Citation Permuterm Index contained the word hydrophobic (Franks, 1982).

At first, hydrophobic interaction was believed to be due to attraction between the nonpolar moieties of the interacting species. More recently, it has been accepted that the attraction between hydrophobic molecules or moieties is only marginal in the overall hydrophobic effect. The basis for the effect is believed to be the strong preference of water molecules in interacting with other water molecules through hydrogen bonding rather than with hydrophobic sites. This causes a rearrangement of the water molecules around hydrophobic sites to maximize hydrogen bonding among themselves. This high energy structure of water is lowered by the reduction of the hydrophobic area exposed to the water as the

hydrophobic sites interact. In parallel, other forces like Van der Waals forces and charge transfer bonds contribute to maintain the interaction.

The noncooperative migration of molecules of amphiphiles (molecules bearing both polar and nonpolar moieties) containing long hydrophobic chains (e.g., fatty acids, alcohols, and amides) to the surface of aqueous solution at low bulk concentrations demonstrated this preferential interaction of the water with itself (Tanford, 1980). Under this condition, the solution surface is sparsely populated and contact between hydrocarbon chains is negligible. The surface concentration was found to be proportional to the bulk concentration and this would not be expected if the amphiphile molecules had a strong attraction for themselves. Moreover, the ratio between amphiphile surface concentration and bulk concentration increased three fold for each CH_2 group added to the alkyl chain. Since interaction among the hydrophobic chains are negligible, this effect of the additional CH_2 must reside on the lack of affinity of water to the hydrocarbon chain. This dominant effect of water self-attraction in hydrophobic interaction was also demonstrated by measurement of the free energy of attraction of two liquids (water and hydrocarbons) that were previously exposed to air and then put in contact with each other. This free energy of attraction is reported as the surface tension of pure liquids or interfacial tension of hydrocarbon-water mixtures. The free energy of attraction for water-hexane and water-octane systems are about -40 erg/cm^2. The same value was found for attraction of the hydrocarbons to themselves. However, the free energy of attraction of water for itself is about -144 erg/cm^2. The hydrophobic effect of dilute solutions of salt is essentially the same as that of pure water.

Thermodynamically, hydrophobic interaction is seen as an entropically driven phenomenon because of the more loose structure attained by the water molecules that were surrounding the hydrophobic species before interaction. The introduction of a hydrophobic species to water causes the breaking of hydrogen bonds between water molecules so these species can be accommodated in the bulk of the liquid. It is as if a cavity were created. The water, which originally had a dynamic, relatively loose structure provided by hydrogen bonds, acquires a more rigid structure around the hydrophobic species to minimize the loss of hydrogen bonding. This increase in the organization of the system translates into a decrease in its entropy. Therefore, association of hydrophobic species is expected to take place along with an increase in the entropy of the system since it releases these more structured water molecules to their initially more loose configuration (Figure 4.1). However, this release is accomplished at the expense of hydrogen bonds that gives a positive enthalpy contribution for the change (Creigthon, 1984).

The illustration of the solvation of a hydrophobic species through the the idea of cavity formation is supported by the linear correlation between the free energy of transfer of hydrocarbon or amphiphile molecules of different surface areas from water to pure hydrocarbon (Creighton, 1984; Tanford, 1980). The larger the area of the molecule transferred, the larger is the decrease in the free energy of transfer due to a larger restoration of original water structure for the transfer from water of the larger molecules.

The cavity formation model with the surface area effect on the solvation energy led to the investigation of the role of the surface tension of the aqueous solution on the hydrophobic effect. The increase of surface

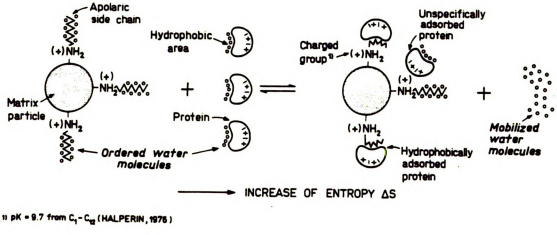


Figure 4.1 - Schematic representation of hydrophobic interaction. The situation refers to binding of a protein to a solid matrix as in hydrophobic chromatography (from Mohr, 1985).

tension of water by salts was found to correlate directly with the strength of the hydrophobic effect. The molal surface tension increment of a salt was proposed as a measurement of its ability to affect hydrophobic interaction and the basis for a natural lyotropic series (Melander and Horváth, 1977).

4.3 - Hydrophobic Interaction Chromatography of Proteins

Proteins can experience intramolecular and intermolecular hydrophobic and hydrophilic interactions since they are amphiphilic molecules. The side chains of the amino acids that make up the backbone of proteins can have character ranging from the highly hydrophobic to the highly ionic. Tryptophan and aspartic acid are good examples of these extremes. In water soluble proteins, 25 to 30 percent of the amino acid residues are strongly hydrophobic while 45 to 50 percent are typically hydrophilic. The hydrophobic residues tend to bury themselves in the interior of the protein to minimize the energy state of the molecule. In doing this, they bring with them some hydrophilic groups due to the primary structure of the protein: the highly hydrophilic peptide bond and hydrophilic amino acid residues. To compensate for the lost interaction with water, the internal hydrophilic groups must undergo hydrogen bonding among themselves. In the case of the peptide bond, this compensation is especially important because the hydrogen bonding between the hydrogen of the amino group and the oxygen of the carbonyl group are responsible for the protein secondary structure (α-helix and the β-sheet). In contrast to this burial of hydrophilic residues into the interior of the protein molecule, there are always some hydrophobic amino acid residues that are present at the surface of the molecule or in crevices. Forming patches or

found in crevices, they are sites for hydrophobic interaction with other protein molecules or hydrophobic ligands (Creighton, 1984; Tanford, 1980).

Hydrophobic characteristics of proteins have been extensively exploited as a way to purify these molecules by hydrophobic interaction chromatography. Besides versatility, this technique is able to produce high levels of purification, up to 200-fold (Bigelis and Umbarger, 1975). Relevant literature is abundant. Review articles are available and books about affinity chromatography always contain a chapter about it.

The basic procedure for the separation is shown in Figure 4.1. First, agarose matrix is derivatized with a hydrophobic chain. The matrix is packed in a column and after equilibrating with a buffer, usually with a high salt concentration, the protein is introduced into the column. Adsorption takes place and after washing the column, conditions (e.g., salt concentration, aditives, pH) are changed to promote protein desorption. The high salt concentration found in hydrophobic chromatography systems offers important advantages to the separation in addition to increased binding. First, the proteins are often stabilized in the solution of the salts employed and second, bacterial growth is inhibited (Porath, 1987).

The matrices and the procedures for activation and derivatization are in general the same as used in other affinity chromatographic technique discussed above. Agarose is generally the matrix of choice. Activation with cyanogen bromide is the most common procedure to prepare the matrix for coupling with the spacer or ligand. The cyanogen bromide reaction with the hydroxyl groups of agarose forms cyclic imidocarbonate that can react with nucleophiles. Spacers (e.g., NH_2-$(CH_2)_n$-COOH, NH_2-$(CH_2)_n$-NH_2) and ligands (e.g.,R-NH_2) have amino groups that react with activated agarose forming N-substituted carbamate, N-

substituted imidocarbonate, and isourea derivatives. These isourea functions give an ion-exchange property to the matrix which is deleterious for some separations (Scouten, 1981).

The attachment of ligands to the spacers, besides the direct attachment to the activated matrix, can be done by a variety of reactions as shown in Figure 4.2. There is also a large variety of hydrophobic ligands available as depicted in Figure 4.3. Additional series of ligands can be obtained by using other functional groups, placing the functional group at various position along the chain, or having more than one functional group on the chain (Shaltiel *et al.*, 1978). The use of linear aliphatic chains is very common due to the possibility of changing the hydrophobicity by changing the length of the chain and also due to the benefits of having a flexible ligand (Figure 4.4). Shaltiel (1978) verified the importance of length of linear chains during purification of glycogen phosphorylase b (Figure 4.5). Total retention of the protein was possible only for ligands with more than 3 carbons in the chain. Moreover, desorption was possible only from the ligand with 4 atoms of carbon despite the use of drastic desorption conditions. This "irreversible" binding has been exploited as a way to prepare immobilized enzymes for bioconversion in fixed bed reactors (Caldwell *et al.*, 1975; Hofstee 1976).

Another factor concerning the degree of hydrophobicity of the matrix is the the extent of derivatization or density of ligands on the matrix. Jennissen (1976; 1978) demonstrated that the binding of glycogen phosphorylase b on three different alkylamine-sepharoses (methylamine-, ethylamine-, and butylamine-sepharose) is a function of the density of matrix substitution. However, he found that there was a minimal density,

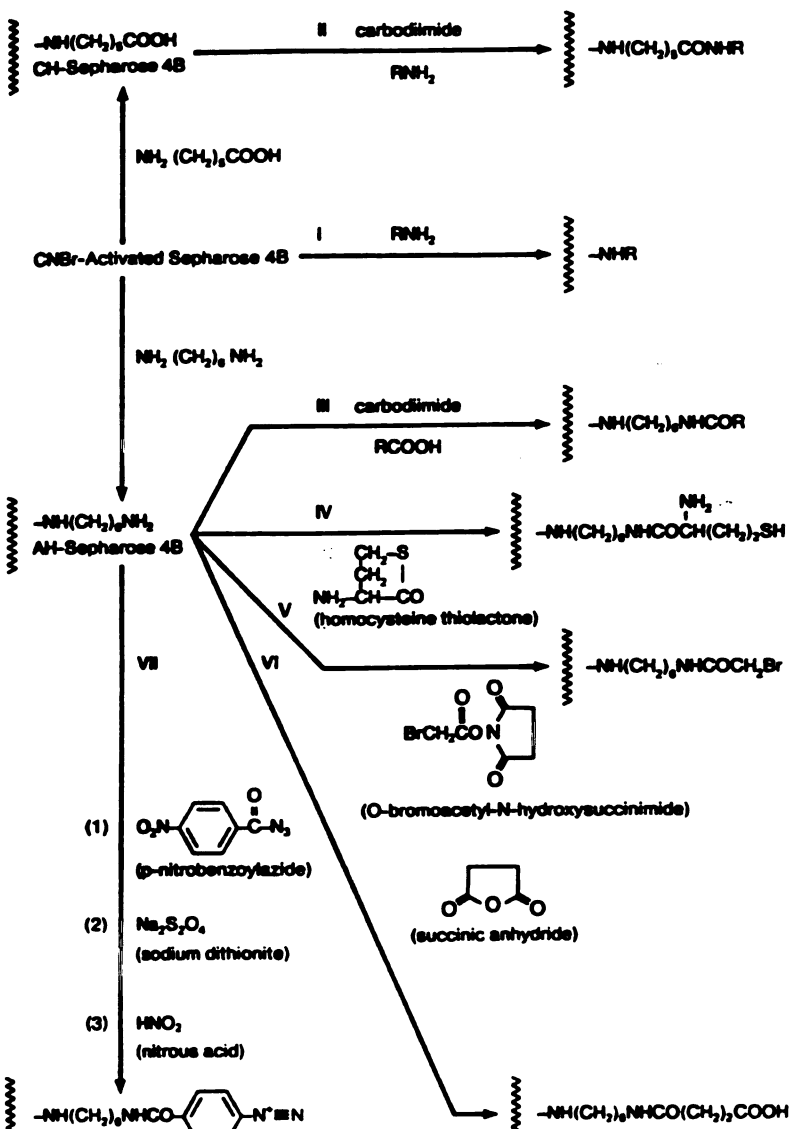


Figure 4.2 - Reactions used to couple ligands to agarose (from Pharmacia Fine Chemicals, 1983).

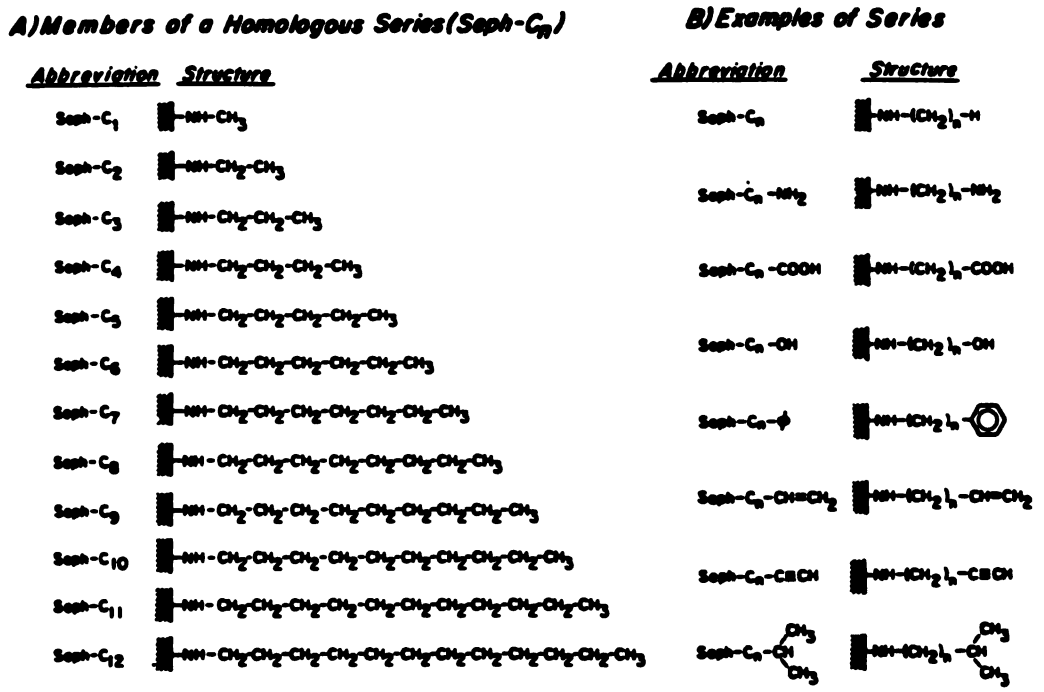


Figure 4.3 - Homologous series of alkyl agaroses which can be prepared for use in hydrophobic chromatography (from Shaltiel *et al.*, 1978).

Figure 4.4 - Schematic illustration of how a flexible linear ligand can accommodate itself in different hydrophobic sites on a protein molecule (from Shaltiel *et al.*, 1978).

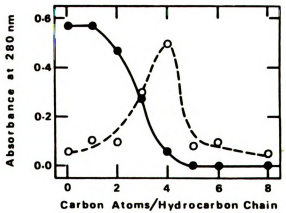


Figure 4.5 - Effect of length of a linear ligand on the adsorption (●) and desorption (○) of glycogen phosphorylase b on a hydrophobic agarose (from Shaltiel, 1978).

called critical density, under which no binding took place. A study of the binding of phosphorylase kinase led the author to conclude that the binding of this enzymes does not follow a simple key-lock mechanism. Instead, it was found that positive cooperative interaction of the substituted alkyl ligands is responsible for the adsorption in a multipoint interaction between the ligand and the proteins.

The role of the spacers is to provide the right steric conditions for the binding between ligand and ligate. Therefore, some optimization of the length of the spacer is required because the improper length of spacers can reduce the binding due to inaccessibility of the binding site to the ligand or vice-versa (Figure 4.6).

Protein elution, as well as adsorption, depends on the additives to the solvent in use. As discussed above, the salts usually used for adsorption are those strongly excluded from the protein and have large molal surface tension increments. The salts that show little preferential exclusion from or bind to proteins and have a low or negative molal surface tension increment are used for desorption. However, the most common elution strategy is the decrease of the concentration of salt which promoted binding in first place. Compounds like ethylene glycol, polyethylene glycol, dimethyl sulfoxide, urea, glycerol, guanidine hydrochloride, and chaotropic salts are employed when desorption is difficult (Arakawa and Narhi, 1991; Raymond *et al.*, 1981). pH changes have also been used as a part of elution strategy (Porath *et al.*, 1973).

Despite the many applications of hydrophobic chromatography, its detailed understanding is incomplete. In practice, the binding of hydrophobic species is never the result of only hydrophobic interaction. There is always the overlapping of a medley of interactions such as

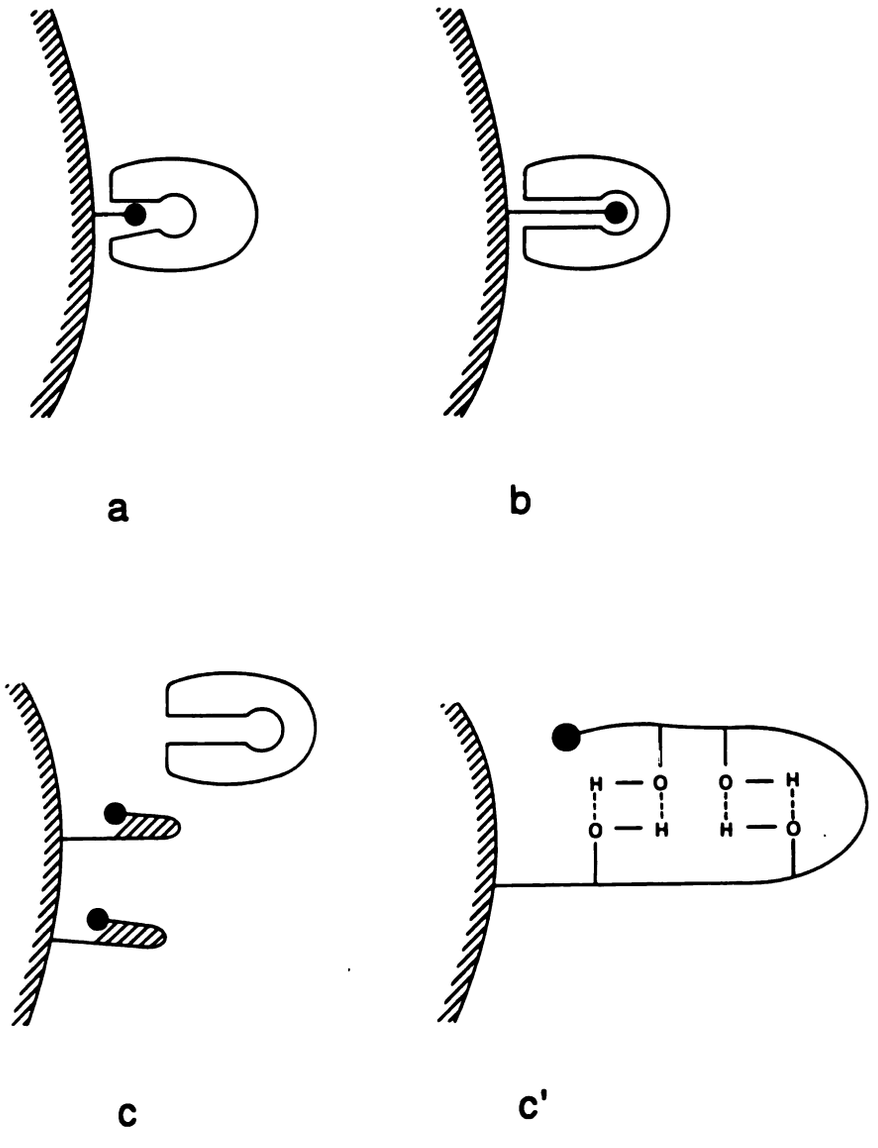


Figure 4.6 - Illustration of the role of the length of the spacer in the binding efficiency of a ligand to a protein. a) spacer is too short; b) spacer is of right size; c) and c') spacer is too long (from Scouten, 1981).

electrostatic interactions, hydrogen bonds, Van der Waals forces, and charge transfer bonds. Also, the conformation of the protein, and possibly of some ligands, can be altered due to binding or changes in the solvent composition. Therefore, the determination of the character of the binding is not a simple task (Raibaud *et al.*,1975). While some authors claim the achievement of "true" hydrophobic chromatography (Halperin *et al.*, 1981; Mevarech *et al.*, 1976), others found that their separation was the result of combination of hydrophobic and electrostatic interactions (Hofstee, 1973).

The changes in adsorption temperature and elution with ethylene glycol are examples of how elusive the pursuit of the mechanism of binding can be (Hjertén, 1973; Hjertén *et al.*, 1974). Since hydrophobic interaction is a entropy driven phenomenon, a decrease in protein binding as the temperature is lowered is expected. Breitenbach (1976) found the opposite when studying the separation of hemoglobins and myoglobins in alkyl-Sheparoses. When alkyl-Sepharose was replaced by aminohexyl-Sepharose, binding was stronger at higher temperature. However, the author attributes the increase in binding to a temperature dependence of the isoelectric point of the proteins. Ethylene glycol has been used to determine the contribution of hydrophobic interaction in hydrophobic chromatography due to its capacity for disrupting native conformation of proteins and nucleic acids (Nemat-Gorgani and Karimian, 1982). However, Hofstee and Otillio (1978) desorbed γ-globulin from butylamine-Sepharose and control Sepharose (unsubstituted, inactivated, cyanogen bromide-treated agarose). The first desorption could have led one to interpret the adsorption as a hydrophobic based phenomenon; however, the desorption from the control indicates that this is not the case.

4.4 - Materials and Methods

4.4.1 - Chemicals

Methylcellulose was obtained from Dow Chemical, Midland, MI., with a molecular weight of 85,000 and a degree of substitution in the range of 1.6 to 1.8. Fatty acids were obtained from Aldrich, Milwaukee. IR grade KBr was purchased from Sigma, St. Louis. Bovine serum albumin (fraction V, heat shock) was obtained from Boehringer Mannheim Biochemicals, Indianapolis. ACS grade ammonium sulfate was obtained from Columbus Chemicals, Columbus. All other chemicals were reagent grade.

4.4.2 - Methods

4.4.2.1 - Esterification of Methylcellulose

A linear series of alkyl carboxylic acids were used to prepare different derivatives of methycellulose. Propanoic (propionic), butanoic (butyric), pentanoic (valeric), hexanoic (caproic), octanoic (caprylic), decanoic (capric), dodecanoic (lauric), tetradecanoic (myristic), and hexadecanoic (palmitic) acids which contain 3, 4, 5, 6, 8,10, 12, 14 and 16 atoms of carbon, respectively, were used. In this work, derivatives of the acids were labeled as Cn where n represents the number of atoms of carbon of the acid used in their preparation. Unsubstituted methylcellulose was labelled C1.

Esterification of methylcellulose with fatty acids was performed in batches of 1 and 20 g of the polymer in glass vials. Each batch consisted of 8 mL of fatty acids containing 5 to 80 µL of sulfuric acid per gram of methylcellulose. The fatty acid concentration is at least five times the stoichiometric requirement to esterify the average number of hydroxyl groups available in the methylcellulose. The objective was to have an

excess of acid to shift the reaction equilibrium towards the esterification. The reaction was carried out in an oven at temperatures from 25 to 100 °C. After reacting from 3 to 216 hours, the mixtures were filtered in sintered glass filters. The cake was transferred to a beaker and it was washed with mixing with ethanol (40 mL of ethanol per 1 g of methylcellulose fed) for 10 minutes. The ethanol washing was repeated twice and, after partial drying on the filter, the cake was washed three times with ethyl ether (same proportion as used for ethanol). After suction drying on the filter the powder obtained was spread in a tray, dried at room temperature for approximately 12 hours, and weighed.

Large batches of C4, C8, and C12 derivatives were prepared for the flotation experiments and others that followed. Esterification times were 4 hours for the C4 and C8 derivatives and 24 hours for the C12 derivative. The extent of reaction was 0.43, 0.32, and 0.22 for the C4, the C8, and the C12 derivative, respectively.

4.4.2.2 - Apparent Solubility of the Derivatives

Apparent solubility of derivatives was determined by studying 1 mg/mL solutions. These solutions were prepared according to the general procedure for dissolving methylcellulose and other cellulose ethers: hydrating the particles at high temperature and dissolving them at low temperatures. One hundred milligrams of derivatives was added to 20 mL of water at 70 to 90 °C under mixing. Twenty milliliters of cold water was added and after mixing in a ice-water bath for one hour more water was added to make a final volume of 100 mL. A 1.4 mL sample of the suspension was taken and the soluble and the insoluble fraction were

separated by centrifugation (14,000 rpm, 20 minutes in a Eppendorf centrifuge model 5415C).

4.4.2.3 - Solutions of Derivatives for BSA Precipitation

Individual solutions of the derivatives for BSA precipitation were prepared by dispersing the derivatives (10 g for C4 and C8 and 20 g for C12 derivatives) in approximatelly 500 mL of hot water (from 70 to 90 °C) under strong mixing. The volume was doubled with cold water and mixing was continued for 48 hours in a ice-water bath. After this period, centrifugation at 8,000 rpm for 20 min in a Sorvall RC-5B centrifuge separated a gel cake and a slightly milky solution free of particles that were used for the precipitation experiments. Concentration of solutions of the C4 and C8 derivatives was around 8 mg/mL while solutions of C12 was around 15 mg/mL.

4.4.2.4 - Salting-out of the Derivatives

Salting-out of the soluble fraction of the derivatives with ammonium sulfate was done by adding different volumes of saturated salt solution to 1 mL of the soluble fraction. After mixing and 10 minutes of aging, the supernatant was separated and analyzed for total carbohydrates.

4.4.2.5 - Flotation: System and Runs

The flotation column was made of pyrex glass. Spargers were rigid cylinders made of sintered glass with pore sizes ranging from 40 to 60 μm, diameter of 1 cm, and length of 2 cm fixed at the tip of a glass tube. The basic design of the column is shown on Figure 4.7. The enlargement at the top of the column is to avoid particle accumulation on the internal

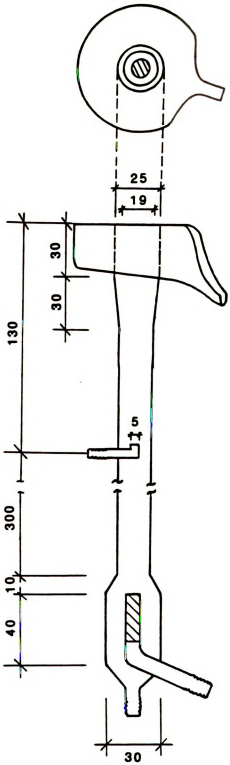


Figure 4.7 - Basic design of flotation column. Measurements in millimeters.

surface of the column (Oliveira, 1990). To avoid clogging, the enlargement at the bottom compensates for the reduction of the cross-sectional area due to the presence of the sparger. The cup-like device around the top of the column collects the foam that overflows the column (the concentrate fraction). The feed line consisted of a glass tip immersed into the feed suspension, plastic tubing, and a peristaltic pump. The tailings line was a siphon made of plastic tubing connected to the bottom of the column with a glass tip at the other end. This glass tip end was raised up to a few centimeters above the top of the column where it was turned downwards. By lowering and lifting this glass tip, the tailings flow rate and, consequently, the height of the froth inside the column, could be adjusted to a desired level. A funnel positioned under the glass tip collected the tailings into a graduated cylinder. The gas line was a plastic tube connecting the air reservoir with the fritted sparger.

Salted-out derivatives were prepared for flotation experiments using 20 percent saturation of ammonium sulfate, salt addition of 40 mL/min, and derivative concentration of 0.3 mg/mL (before salt addition). Flotation conditions were feed flow rate of 100 mL/min, air flow rate of 5 mL/min and froth heigth of 2 cm.

Batches of BSA precipitates were prepared for flotation experiments by using 600 mL final volume, pH 6.0, 45 percent saturation of ammonium sulfate, salt solution added at the rates of 10 and 50 mL/min (for C1 and C4, respectively), Cn/BSA (mass of derivative per mass of protein) equal to 2 for a mass of protein of 397.2 mg, and aging of 1 hour. Flotation conditions were feed flow rate of 20 mL/min, air flow rate of 5 and 16 mL/min (for C1 and C4, respectively)

Flotation experiments, done in triplicates, were started by flowing air and pumping suspension into the column at desired rates until it overflowed. The tip of the siphon for tailings withdrawal was lowered stepwise to set the froth-suspension interface to the desired level. Once air flow rate and froth-suspension interface level were set to the desired values, the column was operated in a pre-run mode for a time equivalent to the pumping of at least three recovery zone volumes of suspension-washing solution at the current feed flow rates. The corresponding concentrate and tailings collected were discarded. The actual run was initiated with time recording and concentrate and tailings collection. Concentrate was collected in a beaker and a graduated cylinder was used to collect the tailings. Tailings and concentrate had particles and liquid separated by filtration in a sintered filter of 30 mL ASTM 40-60 C. The particles were dissolved in 0.01 M sodium acetate buffer pH 6.0 and together with the liquid from concentrate and tailings they were analyzed for protein or total carbohydrates.

4.4.2.6 - De-fatting of BSA

To assure that the BSA was free from fatty acids, the protein was de-fatted. De-fatting of the BSA was done according to the procedure developed by Chen (1967). Five grams of albumin were dissolved in 50 mL of water and 2.5 g of activated charcoal were added. The pH of the suspension was adjusted to 3.0 with 4 N HCl. The suspension was kept in ice-water for 1 hour under mixing. The particles were separated by centrifugation (12,000 rpm for 20 minutes in a Sorvall RC-5B centrifuge). The supernatant pH was adjusted to 6.0 with sodium hydroxide and sodium acetate was added to a final concentration of 0.01 M.

4.4.2.7 - Precipitation of BSA with Salt

Precipitation of bovine serum albumin at 60 percent saturation of ammonium sulfate was done at different pH values. A solution of bovine serum albumin was prepared by diluting 402 µl of a stock solution of the protein (82.75 mg/mL) in 20 mL of 0.01M sodium acetate buffer. Thirty milliliters of saturated solution of ammonium sulfate was added to the protein solution at the rate of 1 mL/min through a syringe pump (Sage Instruments model 355). The pH of the mixture was set around 9 (Orion pH meter model 501) with sodium hydroxide and, under mixing, a sample of approximately 1 mL was taken. The pH was lowered approximately 0.2 pH units with sulfuric acid and the sampling was repeated. This procedure of pH lowering and sampling was repeated until pH 3 was reached. After 90 minutes of aging, the samples were centrifuged in a Eppendorf centrifuge at 14,000 rpm for 10 minutes and the supernatant collected. Absorbance at 280 nm was used to evaluate the amount of protein precipitated. In a similar way, a control was done with no ammonium sulfate.

4.4.2.8 - BSA Precipitation with Derivatives at Different pH

The study of BSA precipitation in the presence of derivatives was done at 60 percent saturation of ammonium sulfate and different pH values. Final batch volumes were 50 mL due to the addition of 30 mL of saturated ammonium sulfate solution at pH 9 to 20 mL of the derivative-protein solution. The salt addition was done by a syringe pump at the rate of 1 mL/min. The Cn/BSA ratio was kept constant at a value of 2 for a mass of protein of 33.1 mg. Therefore, due to the different concentrations of the derivative solutions, different volumes were used. The derivative-

protein mixture volume was increased to 20 mL by addition of 0.01 M sodium acetate at pH 9.0. A 5 mL sample of the suspension was taken. The pH was lowered approximately 1 pH unit and another sample was taken. The procedure of pH lowering and sampling was repeated 3 more times until a pH around 5.5 was reached. After two hours of aging, the samples were filtrated in 30 mL sintered glass filter (ASTM 10-15 C) and filtrate absorbances at 280 nm were taken.

4.4.2.9 - Time Course of Precipitation of BSA with C4 Derivative

BSA batch precipitation with a C4 derivative at a Cn/BSA ratio of 1.5 was achieved as described in section 4.4.2.8 above with the exception of addition of the salt solution at a much faster rate (30 mL in 25 seconds or 72 ml/min). Samples of 5 ml were taken at different times after the addition of the salt solution, filtered in a sintered glass filter (ASTM 10-15 C), and the absorbance at 280 nm was recorded. The Cn/BSA ratio was changed from 2 to 1.5 to avoid extensive precipitation of the protein. The objective was to avoid low absorbance of the filtrate at extensive precipitation which can be significantly affected by background.

4.4.2.10 - BSA Precipitation at Different Salt Concentration

Test tubes batches of 2 mL of C4 derivative-BSA mixtures were prepared using the same proportions as before (section 4.4.2.8), but with a Cn/BSA ratio equal to 2. During mixing in a vortex, a saturated solution of ammonium sulfate was added to achieve different salt concentrations. After one hour of aging, the suspensions were filtered in a sintered glass filter (ASTM 10-15 C) and filtrate absorbances at 280 nm were recorded.

4.4.2.11 - Total Carbohydrates Assay

Total carbohydrates (TCH) were determined by a method described by Dubois and co-workers (1956). Methylcellulose can be determined by this method because polysaccharides and their derivatives, including the methyl ethers with free or potentially free reducing groups, give a orange-yellow color when treated with phenol and sulfuric acid. The procedure consists in preparing a reaction mixture with 0.2 mL of sample and 0.2 mL of 5 percent phenol solution in 13x100 mm glass test tubes. One milliliter of concentrated sulfuric acid is added to the surface of the reaction mixture as fast as possible to generate a high temperature during the color development period (at least 20 min). The tubes were manually shaken and, after 10 min the contents mixed with a vortex. Absorbances at 488 were taken after at least 10 minutes. A blank made with water in place of the sample was used as reference. Glucose was used as the standard.

4.4.2.12 - Protein Assay

Protein concentration was determined by two methods: by absorbance at 280 nm and by a dye-binding assay from Bio-Rad, Richmond, CA. Bovine serum albumin was used as the standard. Absorbance readings were recorded with a Perkin-Elmer absorption spectrometer, model Lambda 3A.

4.4.2.13 - Infra-red Spectra

Infra-red spectra were taken using the KBr technique. A few milligrams of the sample were ground with approximately 100 mg of KBr in a mortar. Pellets were made by pressing the mixture with a hand-press. Liquid samples were dried directly in the mortar (100 to 200 µL) at

approximately 90 °C by a heat gun. Spectra were taken using a Perkin-Elmer Infra-Red Fourier Transform spectrometer, model 1750 interfaced to a Perkin-Elmer 7700 Professional Computer.

4.5 - Results

4.5.1 - Esterification of Methylcellulose and Characterization of the Derivatives

The infra-red spectra of both carboxilic acids and esters have a characteristic band due to the carbonyl stretch. The carbonyl stretch of acids appears between 1700 to 1725 cm^{-1} while that of esters occurs around 1740 cm^{-1}. Esterification of a given carboxilic acid can be verified by the appearance of the shifted carbonyl stretch of the ester. Likewise, spectral phenomena can be utilized to detect the esterification of methylcellulose (Morrison and Boyd, 1973).

The first verification of methylcellulose esterification was accomplished with octanoic acid derivatives. The spectrum of octanoic acid showed a sharp peak at 1710 cm^{-1}. The methylcellulose spectrum did not show a peak in this region. However, the derivative of this acid showed a sharp peak at 1740 cm^{-1}. Spectra obtained immediately after mixing methylcellulose with this acid at room temperature and no catalyst present showed a doublet with peak maxima ocurring at 1710 and 1740 cm^{-1}. The peak at 1710 cm^{-1} corresponds to the free acid. The other peak, at 1740 cm^{-1}, corresponds to some esterification during the preparation of the pellet, probably because of high pressure used in the process.

An arbitrary quantity to follow the progress of the esterification of methylcellulose was developed. It was termed "Extent of Reaction" and it

was defined as the ratio between the transmittance measurements at the characteristic maxima of the ester carbonyl group and the methylene group, at 1740 and 2930 cm^{-1}, respectively (Pradeau *et al.*, 1982). Figure 4.8 shows a typical spectrum of the derivatives.

A characteristic of esterification is the effect of inorganic acids (e.g., sulfuric or hydrochloric acids) as catalyst. The effect of sulfuric acid as a catalyst on the esterification of methylcellulose was checked by running two series of reaction mixtures, one containing the catalyst and other without it (Figure 4.9). The catalytic effect of the sulfuric acid is easely recognized by the higher extent of reaction of the reaction mixtures which contained it.

A short optimization of the esterification was done with regard to two variables: temperature and amount of sulfuric acid (Figure 4.10). The use of 80 µL of sulfuric acid per gram of methylcellulose resulted in total degradation of the methylcellulose at all temperatures studied. Also the use of 100 °C in a reaction system with 20 µL of acid per gram of methylcellulose caused the total degradation of the polymer. As it is showed in Figure 4.10, a higher volume of sulfuric acid resulted in a faster reaction. High temperatures increased the reaction rate; however, the combination of high temperature and high amount of sulfuric acid led to degradation of the methylcellulose.

In preparation for the production of derivatives of the different fatty acids at different degrees of esterification, an evaluation of their reactivities regarding the esterification was done. This evaluation consisted in determining the extent of reaction of derivatives produced

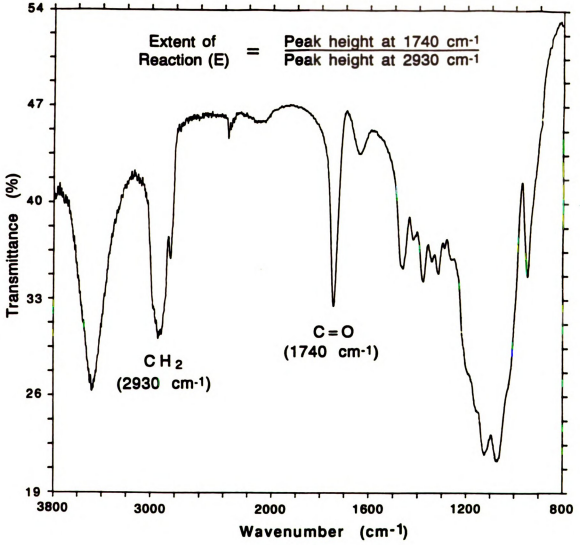


Figure 4.8 - Typical spectrum of methylcellulose derivative. Fatty acid, butyric acid; volume of sulfuric acid, 5 μL/g of methylcellulose; temperature, 65 °C; reaction time, 15 hours.

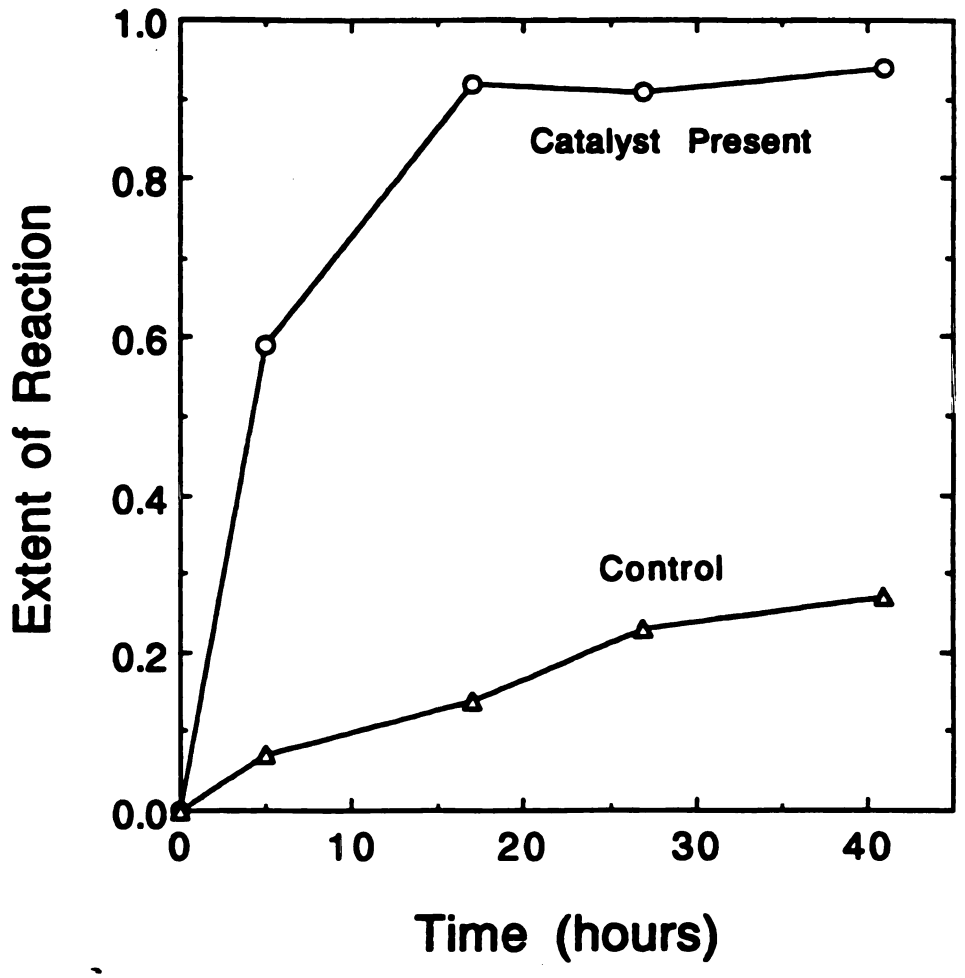
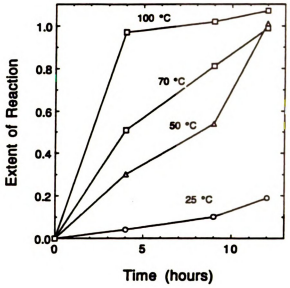
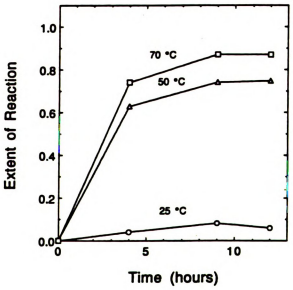


Figure 4.9 - Effect of sulfuric acid as a catalyst on the esterification of methylcellulose using octanoic acid; catalyst 20 µL/g of methylcellulose; temperature, 65 °C.



5 μL of sulfuric acid / 1 g of methylcellulose



20 μL of sulfuric acid / 1 g of methylcellulose

Figure 4.10 - Effect of temperature and amount of sulfuric acid on the esterification of methylcellulose using octanoic acid.

under the same conditions (Figure 4.11). The longer the linear chain, the lower was the esterification of the methylcellulose. Based on these results, an experiment was designed to produce derivatives of the different fatty acids at different extents of reaction (Figure 4.12). The faster reaction rate of the smaller acids was again clearly evident in these experimental results. Evaluation of degradation of the methylcellulose by the reaction conditions was done by determining the yield (mass of derivative after washing and drying). Instead of a weight gain, expected if esterification was the only reaction to take place, a weight loss took place in almost all preparations. The smaller fatty acids showed a much larger weight loss than the longer ones (Figure 4.13).

Characterization of the derivatives was based on their solubility in water, salting-out with ammonium sulfate, and floatability. The importance in determining derivative solubility is the desire to use a carrier-ligand system soluble in water. Faster kinetics are expected when liquid phases are used for ligand-ligate binding. Note that methylcellulose is very soluble in water with concentrations as high as 100 mg/mL having been reported (Dow Chemical, 1988). However, the attachment of a hydrophobic chain should decrease this solubility. This was confirmed by results of the measurement of the apparent solubility of the derivatives previously made (Figure 4.14). Since the maximum concentration achievable was 1 mg/mL, the apparent solubility is also a measurement of the fractional soluble mass. An inverse dependence of the apparent solubility on the extent of reaction was noted.

Figure 4.14 was divided into two plots as shown in Figure 4.15. Three groups of derivatives with close apparent solubility dependencies on the extent of reaction are evident. Derivatives of C3 and C4 form one

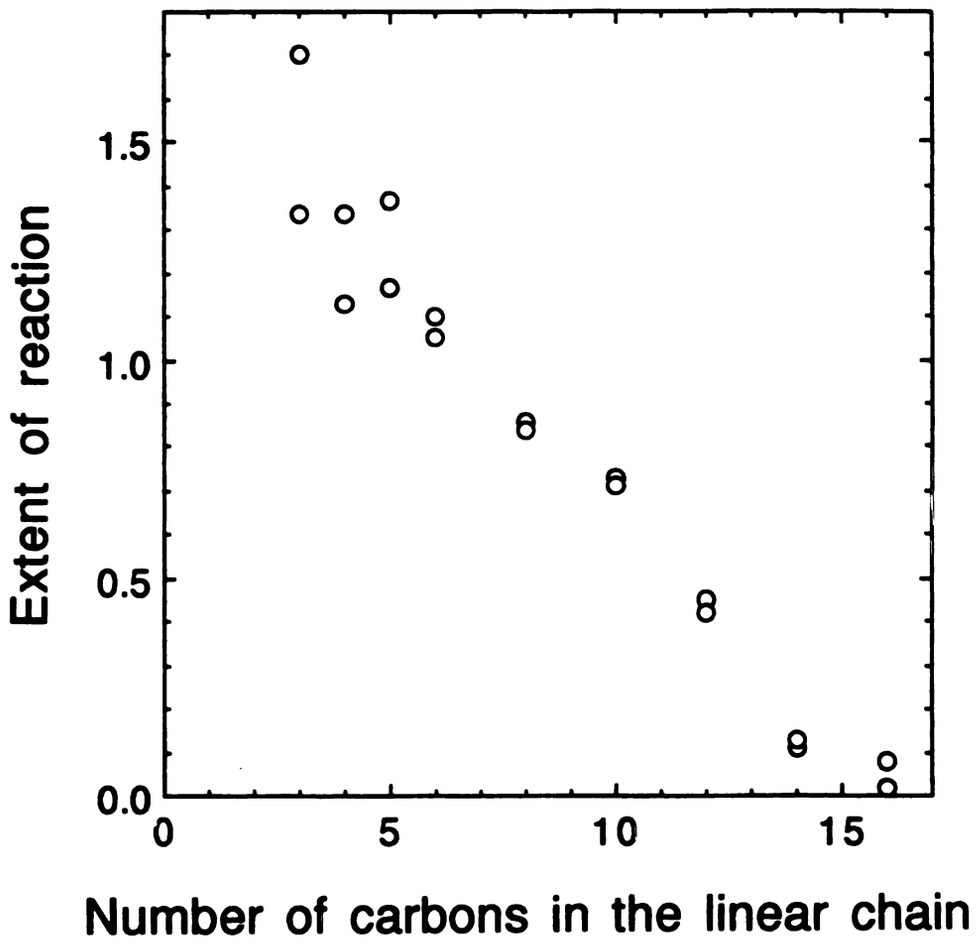


Figure 4.11 - Reactivity of different fatty acids with respect to the esterification of methylcellulose. Volume of sulfuric acid, 5 µL/g of methylcellulose; temperature, 65 °C; reaction time 24 hours.

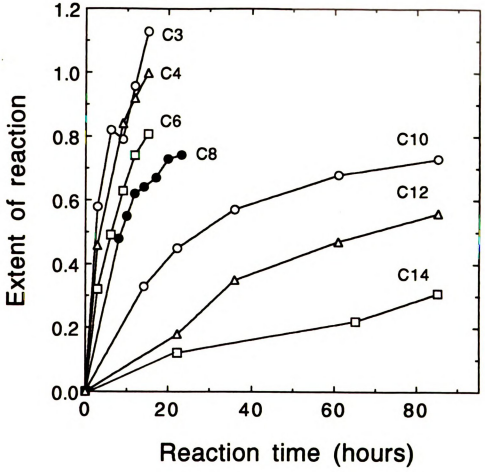
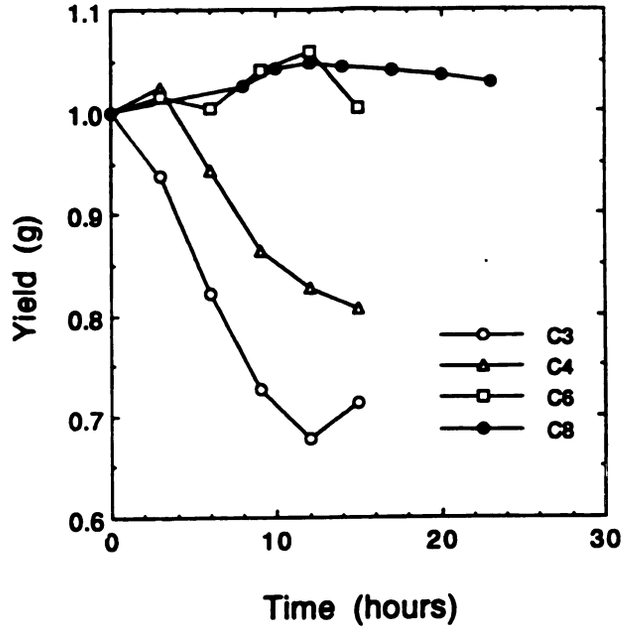


Figure 4.12 - Kinetics of esterification of methylcellulose by fatty acids. Volume of sulfuric acid, 5 μL/g of methylcellulose; temperature, 65 °C.



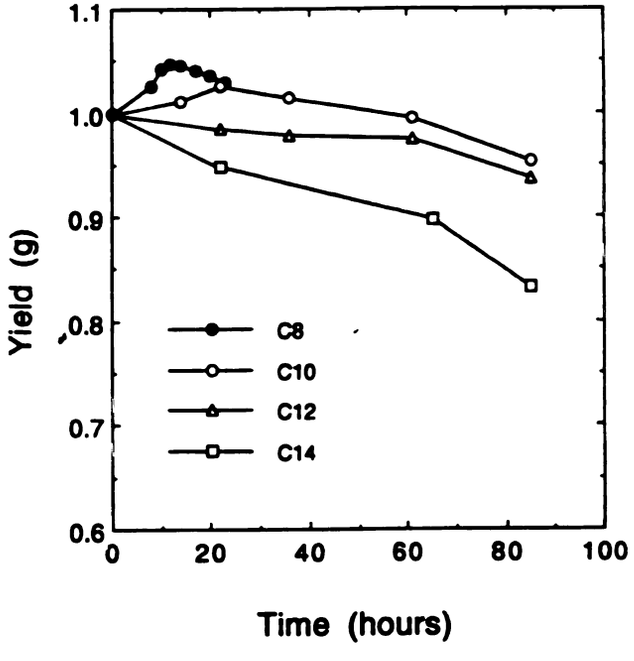


Figure 4.13 - Hydrolysis of methylcellulose during esterification measured as decrease in yield. Volume of sulfuric acid, 5 μL/g of methylcellulose; temperature, 65 °C.

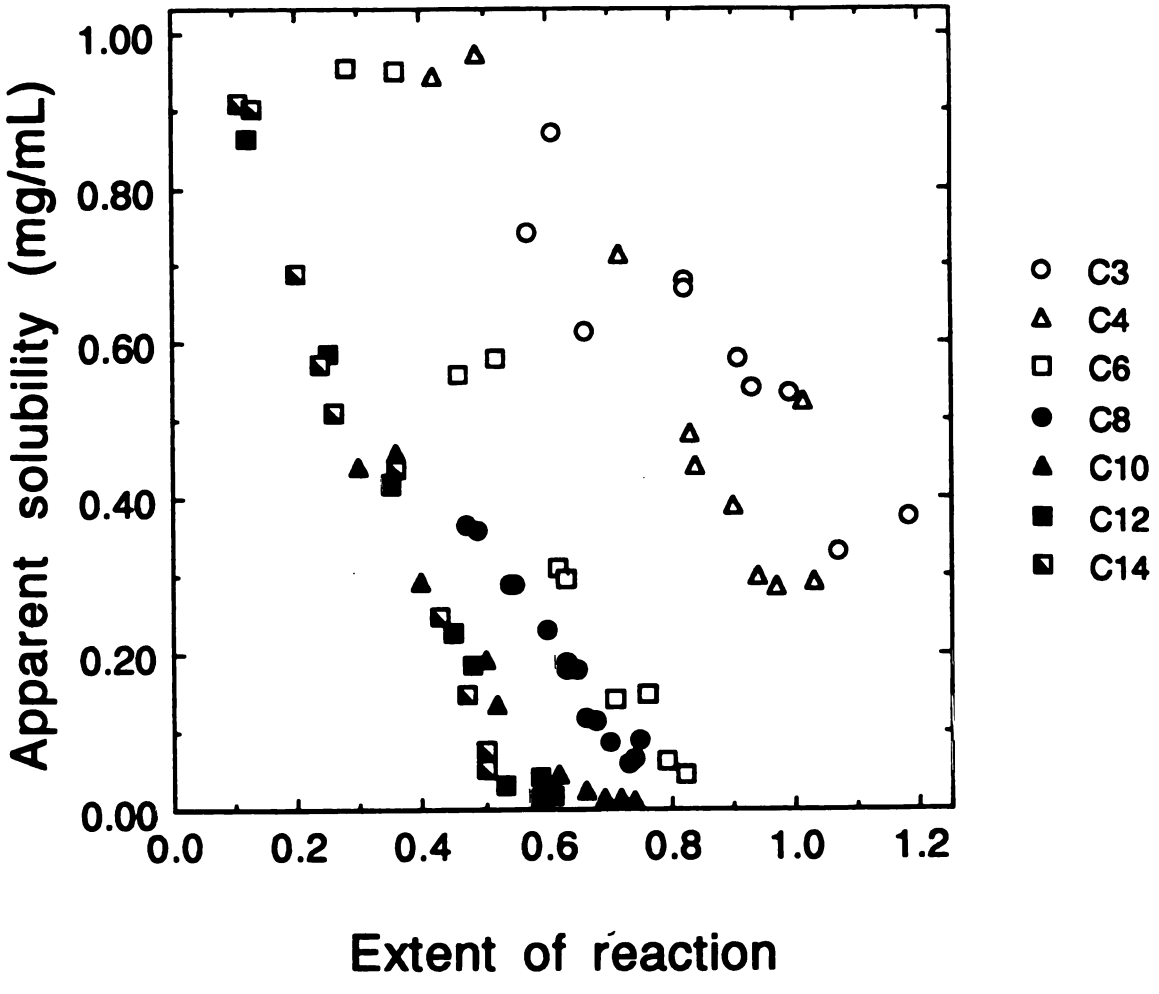


Figure 4.14 - Apparent solubility of the derivatives (C3 to C14). Derivatives were treated according to the procedure to dissolve methylcellulose as for the preparation of a 1 mg/mL solution. The concentration of derivative in the liquid phase was determined and was called apparent solubility.

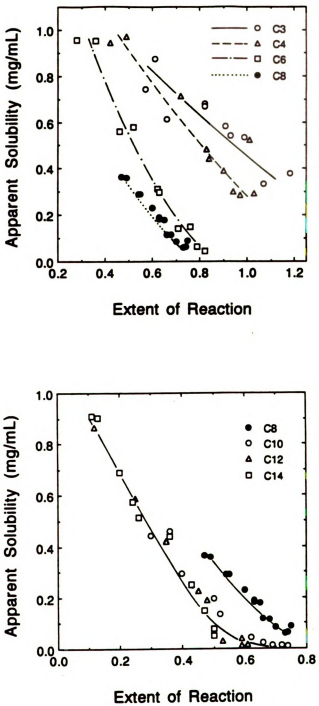


Figure 4.15 - Apparent solubility of the derivatives (C3 to C8 and C8 to C14). Derivatives were treated according to the procedure to dissolve methylcellulose as for the preparation of a 1 mg/mL solution. The concentration of derivative in the liquid phase was determined and was called apparent solubility.

group, C6 and C8 form a second one, and C10, C12, and C14 form a third one. Instead of studying the derivatives of the seven fatty acids, one member from each one of the three groups was chosen for the subsequent experiments. The objective was to reduce the number of experiments by studying derivatives that are representatives of the entire range of derivatives. C4, C8, and C12 derivatives were used in the subsequent experiments.

Due to the objective of using a soluble derivative for the protein binding applications, the extent of reaction of the bulk derivatives and soluble and insoluble fractions were compared (Figure 4.16). The existence of a limiting extent of reaction for solubility was evident. The longer the fatty acid chain, the lower is this limiting extent of reaction.

Besides the knowledge on the solubility of the derivatives and their degree of esterification, another important solubility related behavior is their insolubilization with salts. Therefore, the soluble fraction of the C4, C8, and C12 derivatives were salted-out with ammonium sulfate parallel to salting-out of unsubstituted methylcellulose (Figure 4.17). The three derivatives have salting-out properties very close to each other. However, salting-out started at lower salt concentration. than the unsubstituted methylcellulose although the minimum concentration of ammonium sulfate necessary to salt-out virtually all material is approximately the same (around 20 percent saturation).

As the last characterization of the derivatives, their floatability was compared to the floatability of the unsubstituted methylcellulose under similar conditions (Table 4.1). C4 and C8 derivatives showed the same level of recoveries by flotation as the unsubstituted methylcellulose while C12 derivative showed a much lower recovery.

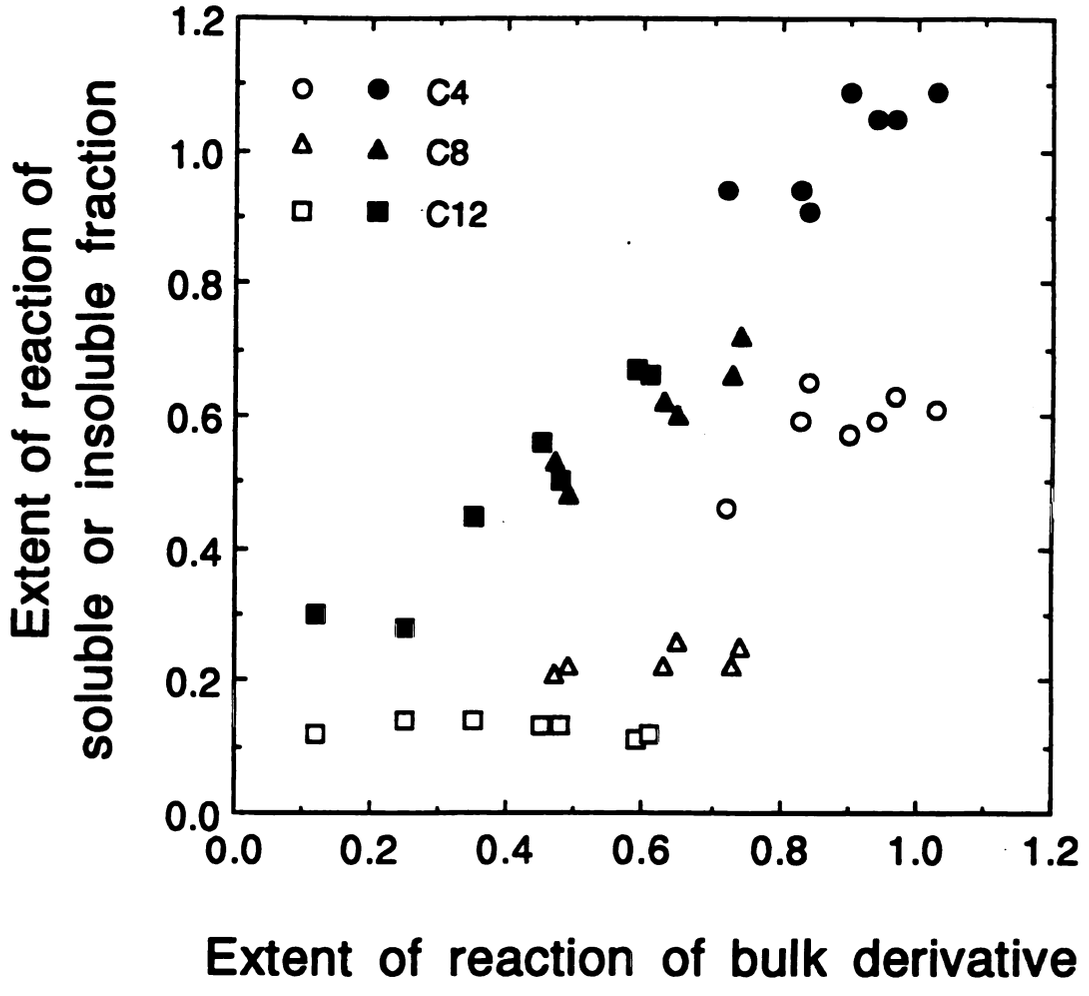


Figure 4.16 - Differences in the extent of reaction of soluble and insoluble fractions of different derivatives. Soluble and insoluble fractions of the derivatives resulted from experiment showed in Figures 4.14 and 4.15 were dried and their extent of reaction measured. Open symbols, soluble fraction; closed symbols, insoluble fraction.

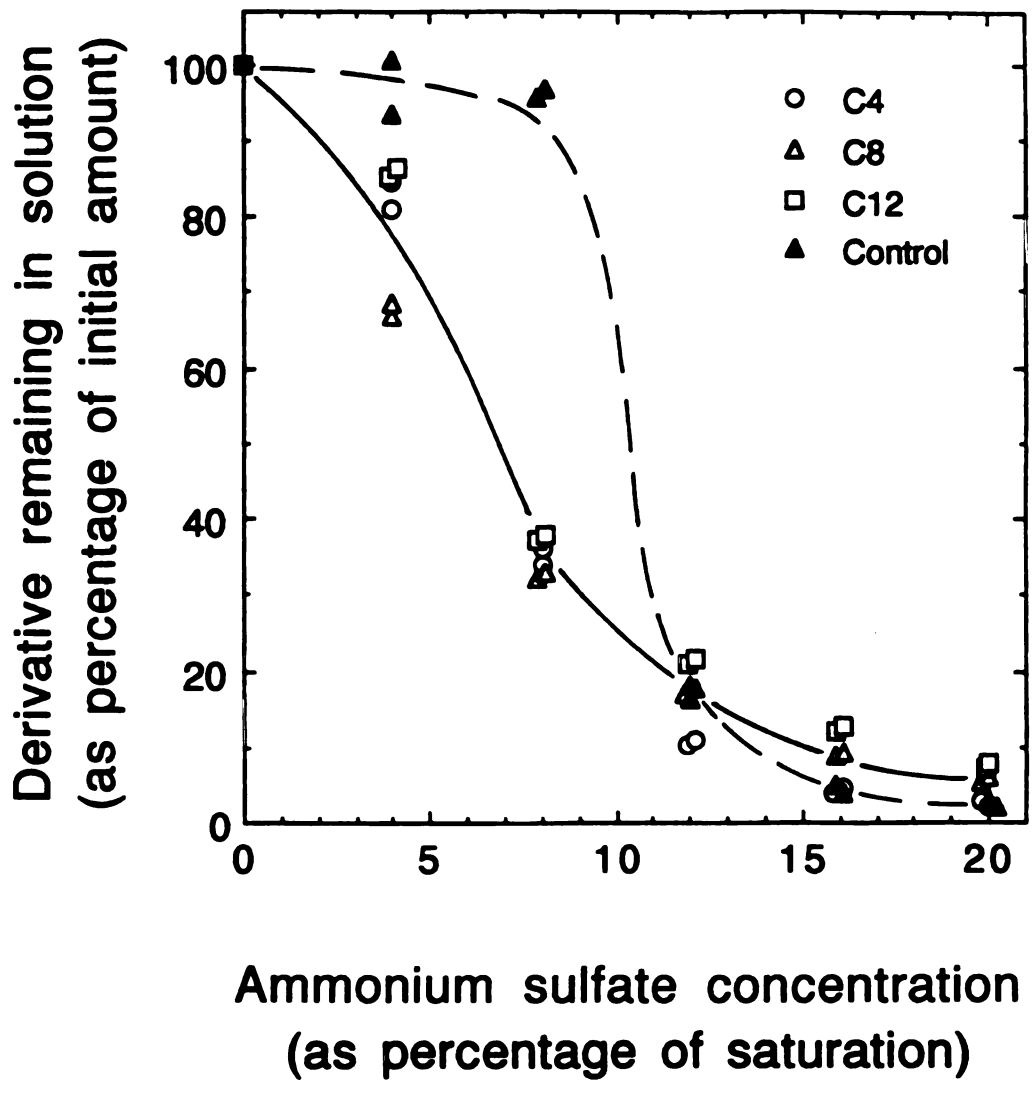


Figure 4.17 - Salting-out of derivatives and unsubstituted methylcellulose. Soluble fractions of the derivatives resulting from experiment showed in Figures 4.14 and 4. 15 were salted-out with ammonium sulfate. The concentration given by the remaining amount of derivatives in the solution was measured and compared with the original concentration

Table 4.1 - Comparison among floatability of the derivatives and unsubstituted methylcellulose (C1).

DERIVATIVE	X	s	n
C1	66.6	1.3	3
C4	70.0	3.6	3
C8	62.0	4.5	3
C12	18.1	1.2	3

X = average recovery; s = standard deviation; n = number of treatments

Conditions - Batch partition: derivative concentration before salt addition, 0.3 mg/mL; salt concentration, 20 percent saturation. Continuous flotation: feed flow rate, 100 mL/min; air flow rate, 5 mL/min

4.5.2 - Precipitation of BSA with Methylcellulose Derivatives

C4, C8, and C12 derivatives were tested for the precipitation of BSA at 60 percent saturation of ammonium sulfate. First, due to the high salt concentration, control of precipitation of BSA at different pH with and without salt was necessary (Figure 4.18). BSA showed an extensive precipitation at pH values lower than 4.7 . Therefore a lower limit for the precipitation in the presence of the derivatives was set at pH 5.5. A higher limit was set at pH 9 due to the strong buffer capacity of the suspension at high pH values.

A modest enhancement of BSA precipitation was realized for all derivatives except great enhancements of the C4 derivative (Figure 4.19). Virtually all BSA was precipitated when C4 derivative was present. For all three derivatives and unsubstituted methylcellulose, little protein precipitation was noted as the pH was lowered from around 9 to around 5.5. Due to the superior capability of the C4 derivative in the precipitation of BSA, it was the only one selected to be used for the experiments in which aging time, salt and protein concentration, temperature, and floatability were studied.

The kinetics of the precipitation were estimated by following the amount of protein precipitated out of solution with time (Figure 4.20). The precipitation seemed to take place in a short time period relative to the experimental time. Based on this finding, a one hour aging time was used for the subsequent experiments.

Changes in salt concentration strongly affected the precipitation of the BSA with the C4 derivative (Figures 4.21 and 4.22). The higher the salt concentration, the more complete was the protein precipitation. The concentration of the C4 derivative had a similar effect on the BSA

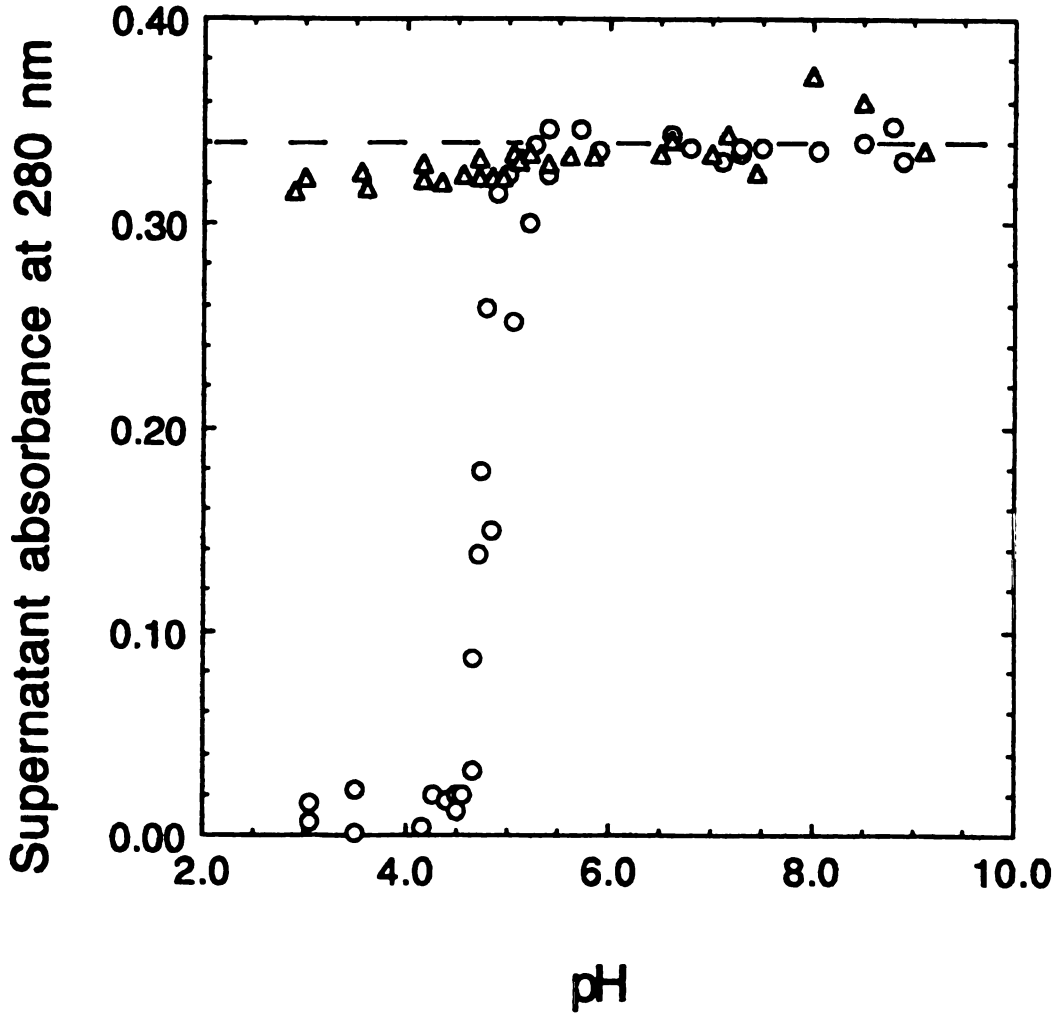


Figure 4.18 - pH precipitation curves for BSA. O - 60 percent saturation of ammonium sulfate; Δ - no ammonium sulfate. Protein concentration, 0.66 mg/mL; aging time, 2 hours. The dashed line is the expected absorbance in the case of no precipitation.

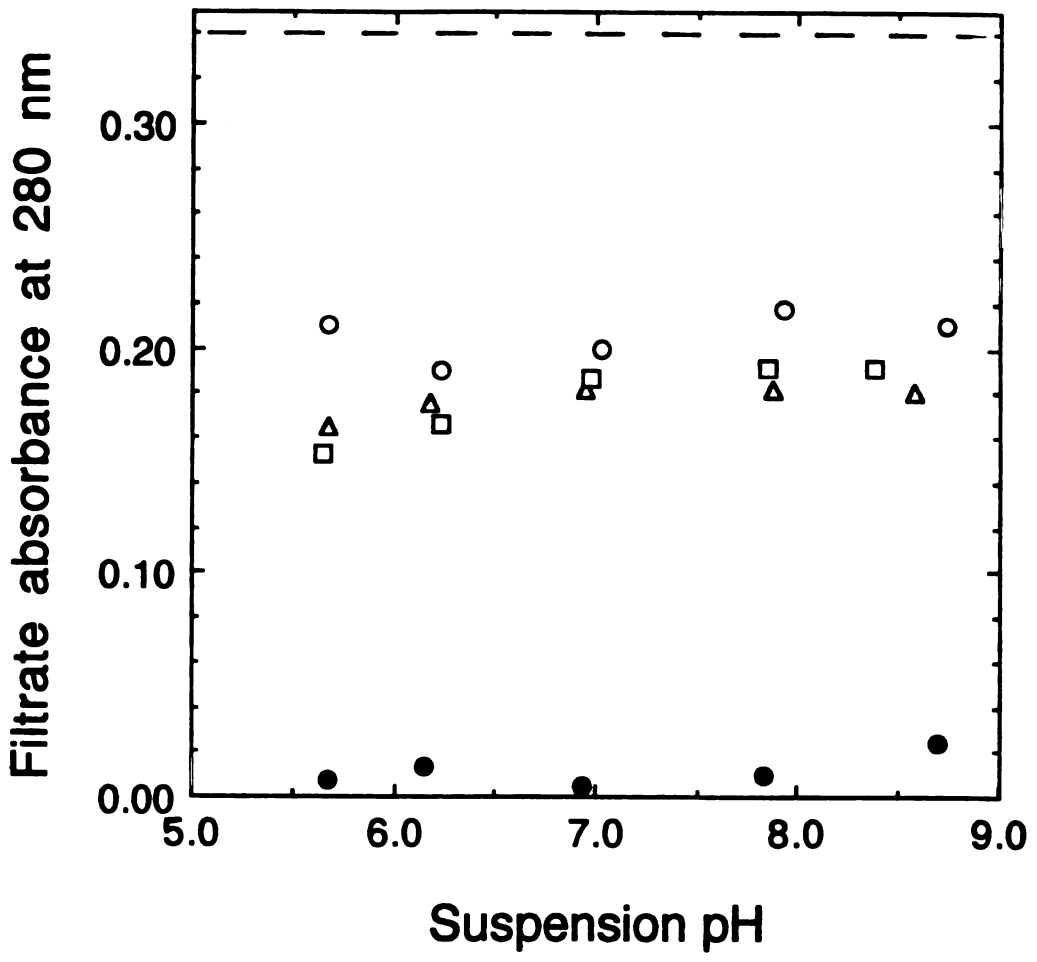


Figure 4.19 - Precipitation curves for BSA in the presence of derivatives (●, C4; Δ, C8;□, C12) and unsubstituted methylcellulose (O). Cn/BSA = 2; protein concentration, 0.66 mg/mL; salt concentration, 60 percent saturation; aging time, 2 hours. The dashed line is the expected absorbance in the case of no precipitation.

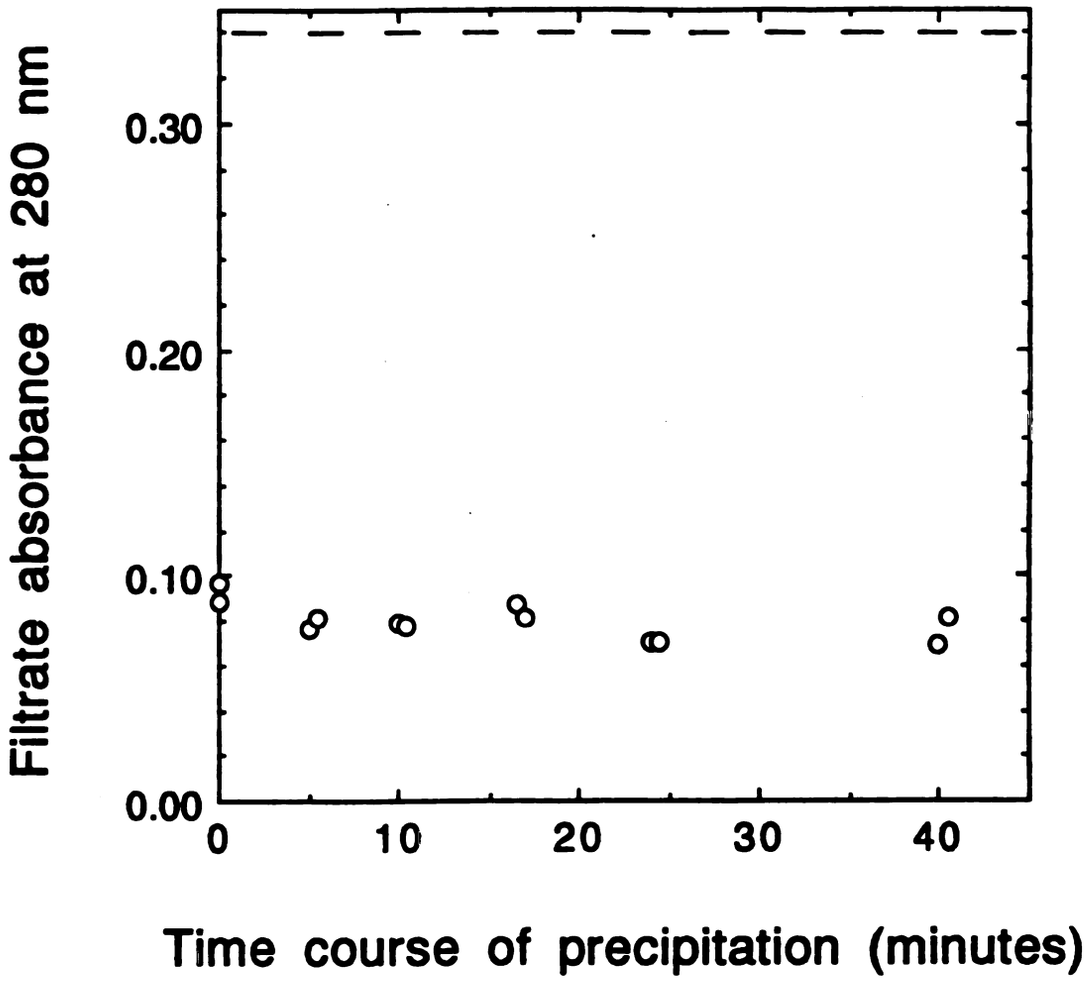


Figure 4.20 - Time course for precipitation of BSA with C4 derivative. Time recording started after addition of the salt solution. Cn/BSA = 1.5 (decreased from 2 to avoid supernatants with low absorbance at 280 nm); protein concentration, 0.66 mg/mL; salt concentration, 60 percent saturation; pH, 6.0. The dashed line is the expected absorbance in the case of no precipitation.

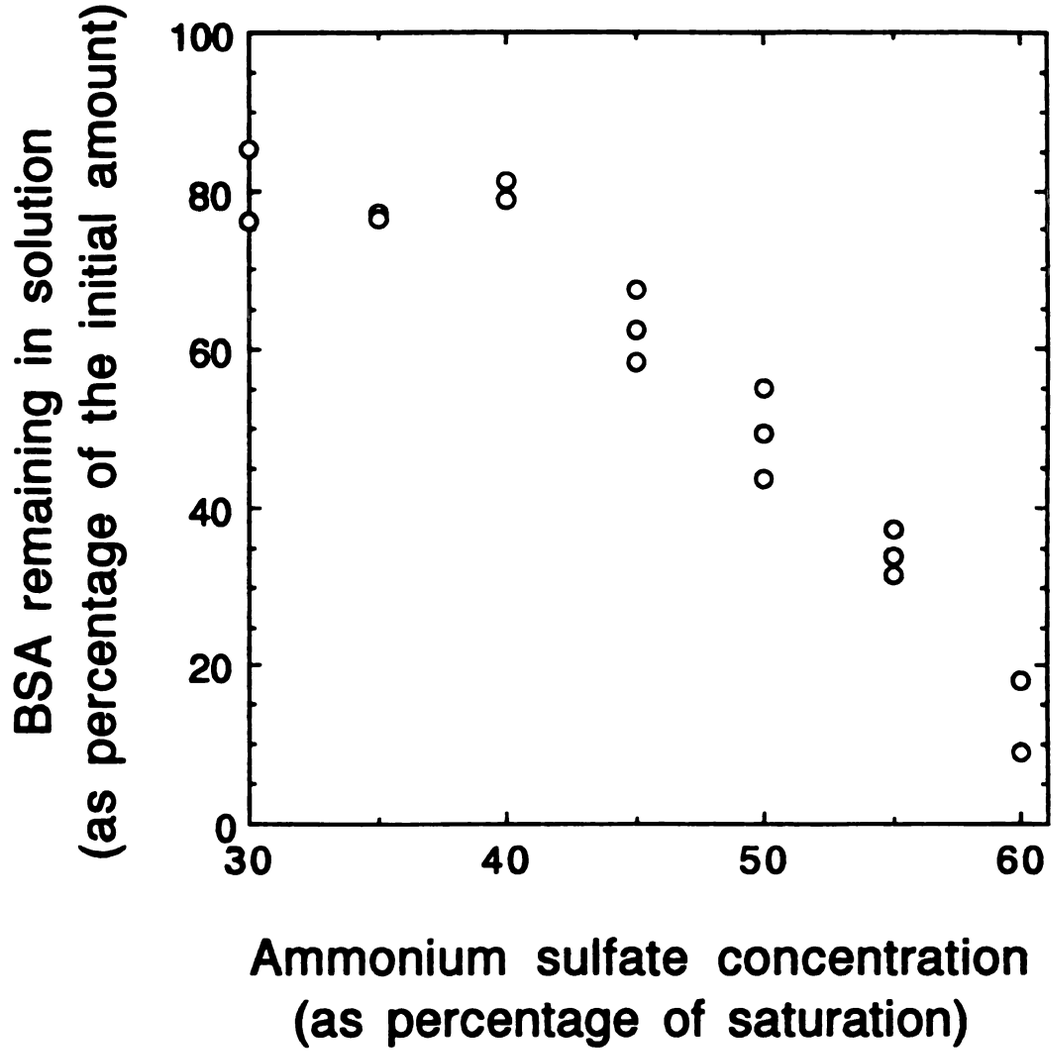


Figure 4.21 - Effect of ammonium sulfate concentration on the precipitation of BSA with C4 derivative. Cn/BSA = 2; protein concentration, 0.66 mg/mL; pH, 6.0; aging time, 1 hour.

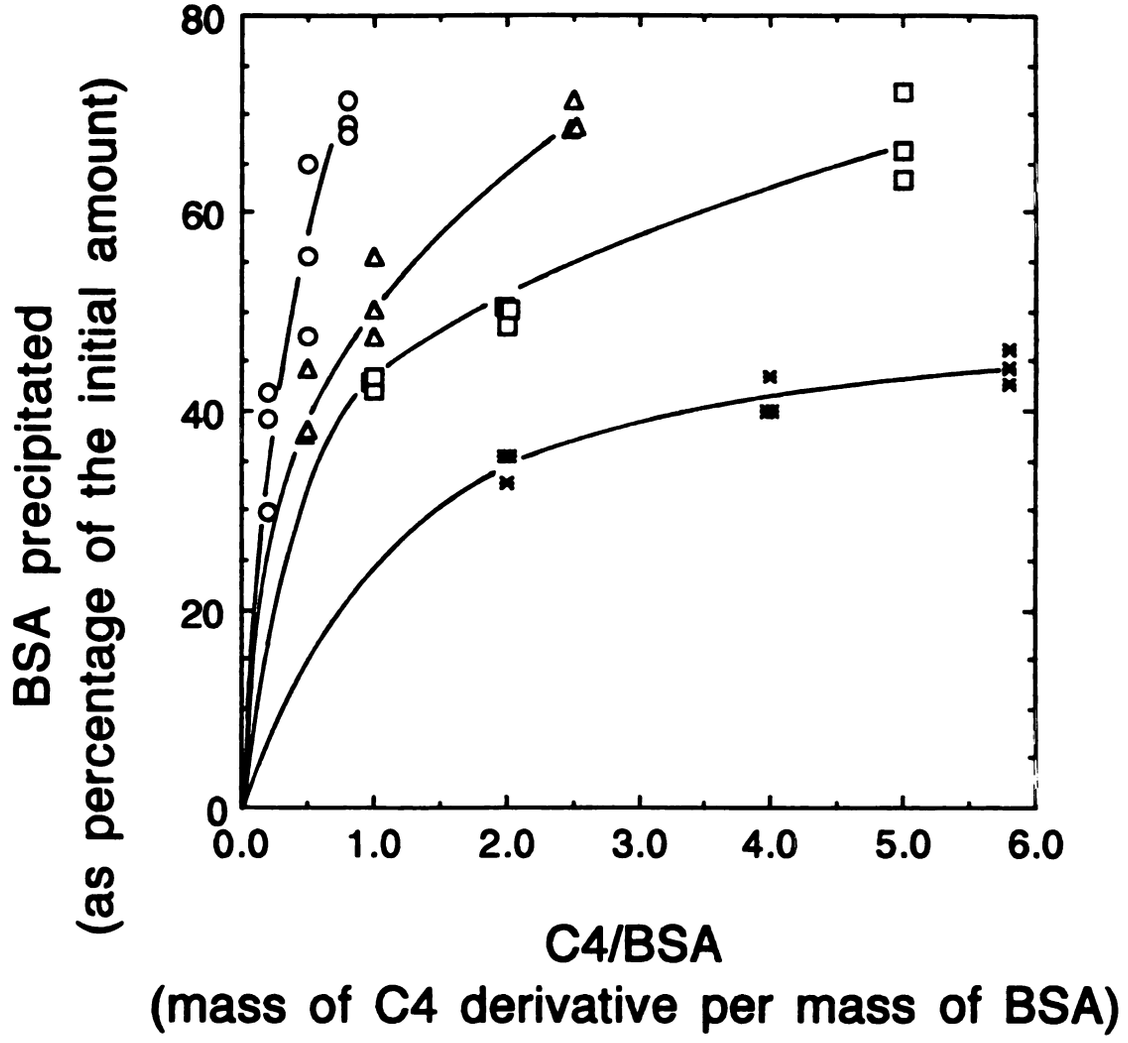


Figure 4.22 - Effect of the ratio C4/BSA on the precipitation of BSA with C4 derivative at different ammonium sulfate concentrations (O, 60 percent saturaton; Δ, 55 percent saturation; □ , 50 percent saturation; ⁎, 45 percent saturation). Protein concentration, 0.66 mg/mL; pH, 6.0; aging time, 1 hour.

precipitation. However, there was less dependence of the precipitation on the derivative concentration as the salt concentration was lowered from 60 to 45 percent saturation.

The last precipitation variable studied was aging temperature wherein precipitated batches were aged at three temperatures (Table 4.2). The results showed an increase in precipitation with an increase in temperature. At the same time, virtually no derivative was detected in the filtrate (data not shown).

Flotation experiments (Table 4.3) first targeted the effect of the BSA on the flotation recovery of unsubstituted methylcellulose. The results showed a reduction in the recovery from 99 to 25 percent when BSA was added to the system. Subsequently, it was estimated whether total protein recovery would correspond to the amount predicted by precipitation with the derivative and flotation recovery combined. BSA precipitation under this conditions is 35 percent according data from Figure 4.22. The flotation experiments showed a recovery of derivative particles in the concentrate of 27 percent (as percentage of total mass of derivative fed). The recovery of protein associated with these particles was 13 percent as percentage of total protein fed (analyzed by the dye method). This 27 percent recovery of derivative by flotation and the 35 percent recovery of protein by precipitation combine to predict a protein recovery by the combination of precipitation and flotation of 9 percent. The experimental value found was slightly higher, 13 percent.

4.6 - Discussion

Esterification as a route to derivatize methylcellulose with linear chains of carboxylic acids proved feasible. As typical in esterification,

Table 4.2 - Effect of temperature on the precipitation of BSA with C4 derivative

Temperature (°C)	Filtrate Absorbance at 280 nm*
0	0.292
24	0.244
55	0.169

*Average of three treatments
Cn/BSA, 1 (decreased from 2 to avoid filtrate with low absorbance at 280 nm); protein concentration, 0.66 mg/mL; pH, 6.0; salt concentration, 50 percent saturation; aging, 1 hour; expected absorbance at 280 nm for no precipitation, 0.338.

Table 4.3 - Flotation of the BSA-derivative precipitates*

Run	Precipitation Recovery(1)	Flotation Recovery Derivative(2)	Flotation Recovery BSA	(1) x(2)
C4 + BSA	35	27	13	9
C1	--	99	--	--
C1 + BSA	--	25	--	--

* Average of three treatments
Precipitation Recovery (1) is the expected percentage of protein precipitated at the experimental conditions given below and acccording to Figure 4.22.
Flotation Recovery Derivative(2) gives the derivative particles recovered by flotation as a percentage of total initial amount.
(1)x(2) is the predicted recovery of BSA as a result of a combination of precipitation and flotation.
Conditions:
Precipitation: Cn/BSA = 2; protein concentration, 0.66 mg/mL; 45 percent saturation of ammonium sulfate; pH = 6. Flotation: feed flow rate, 20 mL/min; air flow rate, 5 and 16 mL/min (C1 and C4 derivative, respectively).

the catalytic effect of an inorganic acid (sulfuric acid) was observed. The presence of degradation of the methylcellulose by parallel reactions was also evident. However, mild conditions provided sufficient kinetics and yield.

The dimensionless parameter extent of reaction proved to be a simple and useful tool in following the progress of the reaction. A weak point of the method is that the reference peak for the measurement of the carbonyl stretch peak (stretch of the methylene groups) also increases as the reaction progresses: the methylene groups are the majority of the components of the added chain to the methylcellulose. However, the effect on the extent of reaction seemed to be minimal probably due to the originally relatively high degree of substitution of the methylcellulose (1.6 to 1.8 where the possible maximum is 3.0) and, consequently, the low esterification levels achieved. Therefore, the methylene groups introduced may have been significantly less in number than the ones present in the structure of the unsubstituted methylcellulose.

The different reactivity of the acids (the shorter chain acids being more reactive in terms of producing derivatives with higher extent of reaction and giving lower yield due to degradation) was expected since the shorter chain acids tend to react more vigorously than the longer bulkier ones. Large groups attached to the carboxylic groups are known to create steric problems and lower the reactivity of organic acids in esterification (Morrison and Boyd, 1973).

The low apparent solubility of the derivatives was the result of increasing of the hydrophobicity of the methylcellulose by attaching hydrophobic groups to it. The spreading of the apparent solubility curves (Figure 4.15) should be analyzed in terms of increased hydrophobicity of

the derivative molecule. The reaction of fatty acids of different sizes with methylcellulose produce different derivatives. These derivatives differ in the hydrophobicity due their different number of carbons in the chain added. This is noted by the position of the curves which is, from top to bottom, C3, C4, C6, and C8. However, as the ligand increases in size, hydrophobicity added by a few more carbons in the chain is not as significant as when the derivative was much shorter. As a consequence, the difference in hydrophobicity between the derivatives of same extent of reaction is negligible and the apparent solubility curves overlap as in the case of C10, C12, and C14 derivatives. The limiting extent of reaction of the soluble fractions of the different derivatives is another example of the lower hydrophobicity of the shorter chains. The shorter the fatty acid used, the higher the extent of reaction of the soluble fraction. The values of these extent of reaction limits (approximately 0.60, 0.25, 0.10 for C4, C8, and C8 derivative, respectively) are spaced in the same pattern of the apparent precipitation curves.

Another possible factor in the differing behavior of the derivatives is the modification of the methylcellulose caused by reactions other than esterification. One of these reactions is the acid hydrolysis of the methylcellulose. The different yields of derivatives obtained with the different fatty acids indicates different degrees of hydrolysis. This results in derivatives of different molecular weight distributions that may influence their solubility and binding characteristics.

The precipitation of BSA with the derivatives, especially the C4 derivative, does not seem to be a simple phenomenon. The BSA removal from solution only occurs at salt concentrations higher than the minimum concentration for the salting-out of the derivatives. It seems that the

protein adsorbs to the salted-out derivative. However, the Cn/BSA ratios used are high enough to discard the concept of adsorption in this case.

The better effectiveness of the C4 derivative may not be due only to the right size of ligand, but it may also be due to the large extent of reaction achieved with butyric acid. This would provide not only the conditions for the right solvation environment, but also a density of substitution larger than the critical density necessary for the binding to occur as previously discussed (Jennissen, 1976 and 1978).

The separation required a high salt concentration to significantly precipitate BSA contrary to most reports in the literature where binding to chromatographic matrices is achieved at low ionic strength (Hofstee and Otillio, 1978; Nemati-Gogani and Karimian, 1982; Wichman and Anderson, 1974; Peters *et al.*, 1973). However, Påhlman and co-workers (1977) reported the need of high salt concentration for the binding of the protein to pentyl-Sepharose gel (ionic strength as high as 3 M). These authors correlated the decrease in the binding with changes in the conformation of the BSA when different salts were used in the protein solution. Therefore, it is possible that the BSA binds to the C4 derivative at low ionic strength, but the increase in salt concentration to precipitate the derivative disrupts the binding due to conformational changes in the protein molecule. Parallel to that, we must consider also changes in the conformation of the derivative. At low ionic strength, the hydrophobic chains may be available for binding. As the salt concentration increases, not only does the protein conformation change, but as the derivative is precipitated, its conformation may also change. The hydrophobic n-propyl chain of the reacted butyric acid may preferentially interact among

themselves. Only at high salt concentration can the conditions be reversed enough for binding to occur.

Despite the murky picture of the overall phenomenon, there is evidence that hydrophobic interaction plays a role in the separation. First, the precipitation is not very sensitive to pH in the range studied indicating that electrostatic forces do not play a major part in the precipitation. Second, the dependence of the precipitation on the ammonium sulfate concentration is typical of hydrophobic based phenomena. Third, the temperature experiments showed an increased precipitation as the temperature was raised, a behavior related to the principle that hydrophobic interactions are entropically driven. The complete insolubilization of the C4 derivative at 60 percent saturation of ammonium sulfate ruled out the possibility that the increase in BSA precipitation is due to a higher insolubilization of the derivative at higher temperatures.

The flotation results showed that the derivatization does not affect floatability significantly, expect for the C12 derivative. The large reaction time for the production of this derivative (24 hours compared to 4 hours for the others) and, consequently, the long exposure to sulfuric acid may be the reason for this different behavior.

The floatability of the BSA precipitated with C4 derivative was not high. The presence of the BSA in a high concentration, as it was seen in the flotation of the unsubstituted methylcellulose, certainly contributed to the expected low floatability due to the high salt concentration. As it was seen in Chapter 3, the floatability of a very similar polymer, (hydroxypropyl)methylcellulose, is strongly affected by the increase of ammonium sulfate from more than 40 percent saturation.

However, the recovery of BSA with the particles floated was, within a margin of error, the percentage predicted by the combined recoveries of precipitation and flotation. This result demonstrated the feasibility of the system developed. A known amount of protein was precipitated with a chemically engineered molecule. The flotation step did not affect the distribution of the BSA between solid and liquid phases. As long as the particle recovery by flotation is known, the combined recovery of precipitation and flotation can be predicted. The prediction of the flotation step is complicated in this case due to the high concentration of protein. However, its dependence on bold changes in the system is a characteristic of the flotation system.

In summary, the development of the proposed flotation system to perform protein separation by hydrophobic interaction between a ligand and a ligate was accomplished although the protein chosen required conditions that required the system to be run at suboptimal conditions.

4.7 - Conclusions

Besides the overall conclusion that the design and production of a flotation system to perform protein separation by flotation based on protein-ligand hydrophobic interaction is possible there are other conclusions from this study. They are as follows:

a) Direct esterification of methylcellulose by fatty acids can be performed at mild temperature (around 65 °C). Sulfuric acid is an efficient catalyst for this reaction.

b) The longer is the fatty acid, the slower is the reaction with the methylcellulose.

c) The longer the fatty acid, the smaller is the soluble fraction of its derivative for a fixed extent of reaction.

d) the longer the fatty acids, the lower is the extent of reaction of the soluble fraction of its derivative.

e) The derivatives are salted-out from aqueous solutions at lower ammonium sulfate concentration than the unsubstituted methylcellulose.

f) The floatability of derivatives of butyric and octanoic acids are similar to the unsubstituted methylcellulose while the dodecanoic acid has a much lower floatability.

g) The butyric acid derivative promotes extensive precipitation of the bovine serum albumin at 60 percent saturation of ammonium sulfate.

h) pH does not significantly affect the precipitation of bovine serum albumin by the derivatives used.

i) The precipitation with C4 derivative of bovine serum albumin takes place in less than one hour.

j) The precipitation of bovine serum albumin with the butyric acid derivative is dependent on the salt concentration and on the ratio mass of derivative to mass of protein.

k) The presence of bovine serum albumin is deleterious to the floatability of the unsubstituted methylcellulose.

l) The interaction that causes the precipitation of bovine serum albumin seems to be at least partially of a hydrophobic nature.

4.8 - List of References

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Chapter 5

General Conclusions and Recommendations

5.1 - General Conclusion

The general conclusion of this dissertation is that flotation can be applied to the recovery of fermentation products. This work explored and showed the feasibility of the two routes through which this can be accomplished. First, was the use of polymer-protein precipitates already in use in the separation of proteins. The natural floatability of these precipitates make the development of the flotation processes a relatively easy task. The partition of the protein to a particulate system seems to protect the protein from denaturation and loss of activity during flotation. The recovery of α-amylase from fermentation broth and periplasmic extract by partition to (hydroxypropyl)methylcellulose and subsequent flotation were the example of such a route.

Second, a flotation system was designed to recover products according to a specific interaction in which they participate. The chemical engineering of a floatable molecule containing a ligand that is also floatable with the product bound to it was the core of this work. The feasibility of such an approach to flotation in biotechnology was exemplified by the production of fatty acids derivatives of methylcellulose and their use in the precipitation and flotation of bovine serum albumin.

5.2 - Recommendations

As said in the beginning of this dissertation, little has been done in the field of flotation applied in the recovery of biomolecules from fermentation broth. Therefore, there are many areas were future work can contribute to the expansion of flotation in biotechnology.

In terms of the experimental work done in the recovery of α-amylase, the identification of the molecules that have the depressant effect on flotation would be helpful in improving the process as well in helping to understand the flotation of (hydroxypropyl)methylcellulose. Since this polymer showed good qualities as a flotation carrier, it is important to optimize its flotation regarding particle size distribution. Due to the better floatability of the flocs of twisted filaments of salted-out polymer, a better understanding of the filaments and floc formation is necessary. Followed by the study of how they are attached to air bubbles and lifted, an optimization of the flotation of these particles can be done.

The production of derivatives of methylcellulose needs optimization in few areas. According to the protocol used, large amounts of fatty acids are wasted and it is difficult to assure homogeneity in the reaction mixture. The use of an inert solvent for the fatty acid can improve both of these problems. The washing of the derivatives needs to be changed after the optimization of the esterification reaction. The lower load of fatty acids to be washed will result in lower solvent requirements. Also, one must investigate if the different derivatives have significantly different molecular weight distributions due to acid hydrolysis. If so, the effect of molecular weight on the properties of the derivatives should be investigated.

Looking now in broader terms, there is work needed in the two routes for the application of flotation in biotechnology. In the area of exploring the natural floatability of protein-polymer precipitates known to biotechnology, a screening of these precipitates regarding their floatability is lacking. Such a study would not only identify precipitates that are floatable but also develop basic knowledge of which conditions and which characteristics protein precipitates must have to be floatable. In the design of flotation systems, there is a need for development and characterization of carriers. The literature is abundant with descriptions of ligands and conditions for their binding with a variety of proteins. Therefore, these carriers must be developed with this in mind. They must be able to be insolubilized in a variety of conditions to match with the ligand-protein binding conditions. Parallel to this, the attachment of different ligands to these new carriers would open the door for the purification of a variety of different proteins by flotation.

APPENDIX

Raw data used in the preparation of tables and figures.

Table A.1 - Raw data of Figure 3.4.

Air flow rate (mL/min)	HPMC recovery (as percentage of initial amonut)	
	10 μm	16 μm
84.8	98.0	-
76.8	98.4	-
61.3	96.7	-
50.5	93.2	99.0
32.5	84.0	98.2
15.2	-	94.4
10.6	-	93.2
7.8	-	86.1

Partition of synthetic mixture: batch volume, 4 L; ammonium sulfate addition rate, 5 and 20 mL/min for 10 and 16 μm, respectively. Continuous flotation with no washing: column used, #1; feed flow rate, 27 mL/min; froth height, 0.5 to 3 cm.

Table A.2 - Raw data of Figure 3.5.

Ammonium sulfate concentration (percent of saturation	HPMC recovery (as percentage of initial amount)
40.0	101.5
45.0	94.8
45.0	100.6
50.0	68.4
50.0	68.5
55.0	16.6
55.0	24.6
60.0	13.3
60.0	29.1

Salting-out of synthetic mixture: batch volume, 1.5 L; HPMC concentration before salt addition, 0.07 percent; ammonium sulfate addition flow rate, 14 mL/min. Continuous flotation with no washing: column used, #1; feed flow rate, 36 mL/min; froth height, 1 cm.

Table A.3 - Raw data of Figure 3.6.

Feed flow rate (mL/min)	HPMC recovery (as percentage of initial amount)
67.3	98.0
99.0	96.2
150.1	94.8
153.5	103.2
164.9	108.5
165.5	94.8
168.0	99.4
170.0	65.5
175.6	76.9
178.2	82.7
201.3	50.7

Partition of synthetic mixture: batch volume, 10 L; ammonium sulfate addition flow rate, 60 mL/min. Continuous flotation with no washing: column used, #1; froth height, 3 cm.

Table A.4 - Raw data of Figure 3.7.

Wash water flow rate (mL/min)	Concentrate contamination by liquid from the feed stream (as fraction of concentrate volume)
0.90	0.410
1.25	0.275
1.90	0.100
3.10	0.070
4.70	0.020
6.80	0.010
0.80	0.470
0.90	0.450
1.30	0.280
1.90	0.150
3.10	0.080
4.70	0.030
6.80	0.040

Partition of synthetic mixture: batch volume, 10 L; ammonium sulfate addition flow rate, 60 mL/min; tracer, 2.0 percent copper sulfate ($CuSO_4.5H_2O$), 0.4 percent sodium hydroxide, and 2.0 percent sodium potassium tartrate. Continuous flotation: column used,#1; feed flow rate, 40 mL/min; froth height, 4 cm; washing solution, 2.0 percent sodium potassium tartrate in 30 percent saturation ammonium sulfate.

Table A.5 - Raw data of Figure 3.8.

pH	HPMC recovery* (as percentage of initial amount)	Standard deviation
3.0	81.0	1.6
4.0	81.6	3.0
4.8	77.8	2.8
6.0	80.5	1.7
7.0	81.7	2.0
8.0	79.9	1.9
9.0	79.7	2.4

*Average of three treatments.
Partition: batch volume, 150 mL; HPMC concentration, 0.1 percent; ammonium sulfate concentration and addition rate, 30 percent saturation and 3.3 mL/min, respectively. Flotation: air flow rate, 4.0 mL/min; make-up water flow rate, 1.6 mL/min; flotation time, 5 minutes.

Table A.6 - Raw data of Figure 3.9 and Figure 3.10.

Diafiltration level	Recovery (as percentage of initial amounts)		
	Activity	HPMC	HPMC x 0.912
0	58.4	58.9	53.7
0	60.1	66.0	60.2
1	81.6	88.0	80.3
1	79.4	84.3	76.9
2	78.1	84.4	77.0
2	86.4	96.7	88.2
2	88.0	98.5	89.8
3	90.2	99.7	90.9

Partition of broth mixture: batch volume, 1.5 L; ammonium sulfate addition flow rate, 31 mL/min. Continuous flotation without washing: column used, #2; feed flow rate, 20 mL/min; froth height, 5 cm.

Table A.7 - Raw data of Figure 3.11.

pH	HPMC recovery* (as percentageof initial amounts)	Standard deviation
3.0	46.3	12.9
4.0	24.0	5.6
5.0	22.7	3.5
6.9	19.3	3.4
9.0	19.5	0.9

*Average of three treatments.
Partition: batch volume, 150 mL; HPMC concentration, 0.1 percent; ammonium sulfate concentration and addition rate, 30 percent saturation and 3.3 mL/min, respectively. Flotation: air flow rate, 4.0 mL/min; make-up water flow rate, 1.6 mL/min; flotation time, 5 minutes.

Table A.8 - Raw data of Figure 3.12.

pH	HPMC recovered*	Activity recovered*	
		with particles	Total
7.0	26.4	41.4	88.5
6.0	27.2	41.3	93.5
5.0	33.7	42.3	90.8
4.0	55.6	61.1	89.0
3.0	63.4	29.5	46.3
2.5	53.4	18.8	31.3

* As percentage of initial amounts. Average of two treatments. Partition of broth mixture: batch volume, 4 L; ammonium sulfate addition flow rate, 42 mL/min. Continuous flotation: column used #2; feed flow rate, 20 mL/min; washing solution flow rate, 7 mL/min;froth height, 7 cm.

Table A.9 - Raw data of Figure 4.9.

Reaction time (hours)	Extent of reaction of mixtures	
	with catalyst	without catalyst
5	0.59	0.07
17	0.92	0.14
27	0.91	0.23
41	0.94	0.27

Fatty acid, octanoic acid; catalyst 20 µL/g of methylcellulose; temperature, 65 °C.

Table A.10 - Raw data of Figure 4.10.

Time	Extent of reaction						
	5 µL acid/g methylcellulose				20 µL acid/g methylcellulose		
	25 °C	50 °C	70 °C	100 °C	25 °C	50 °C	70 °C
4	0.04	0.30	0.51	0.97	0.04	0.63	0.74
9	0.10	0.54	0.81	1.02	0.08	0.74	0.87
12	0.19	1.01	0.99	1.07	0.06	0.75	0.87

Fatty acid used: octanoic acid.

Table A.11 - Raw data of Figure 4.11.

Number of carbons in the chain	Extent of reaction
16	0.02
16	0.08
14	0.13
14	0.11
12	0.42
12	0.45
10	0.71
10	0.73
8	0.84
8	0.86
6	1.10
6	1.05
5	1.37
5	1.17
4	1.13
4	1.34
3	1.70
3	1.34

Volume of sulfuric acid, 5 μL/g of methylcellulose; temperature, 65 °C; reaction time, 24 hours.

Table A.12 - Raw data of Figure 4.12.

Time (hours)	Extent of reaction of the different derivatives*						
	C3	C4	C6	C8	C10	C12	C14
3	0.58	0.46	0.32	-	-	-	-
6	0.82	-	0.49	-	-	-	-
9	0.79	0.84	0.63	-	-	-	-
12	0.96	0.92	0.74	0.62	-	-	-
15	1.13	1.00	0.81	-	-	-	-
8	-	-	-	0.48	-	-	-
10	-	-	-	0.55	-	-	-
14	-	-	-	0.64	0.33	-	-
17	-	-	-	0.67	-	-	-
20	-	-	-	0.73	-	-	-
23	-	-	-	0.74	-	-	-
22	-	-	-	-	0.45	0.18	0.12
36	-	-	-	-	0.57	0.35	-
61	-	-	-	-	0.68	0.47	-
85	-	-	-	-	0.73	0.56	0.31
65	-	-	-	-	-	-	0.22

*Average of two treatments.
Volume of sulfuric acid, 5 μL/g of methylcellulose; temperature, 65 °C.

Table A.13 - Raw data of Figure 4.13.

Time (hours)	Yield of the different derivatives (g)*						
	C3	C4	C6	C8	C10	C12	C14
3	0.938	1.024	1.016	-	-	-	-
6	0.82	0.944	1.003	-	-	-	-
9	0.727	0.864	1.041	-	-	-	-
12	0.678	0.826	1.059	1.048	-	-	-
15	0.714	0.807	1.003	-	-	-	-
8	-	-	-	1.026	-	-	-
10	-	-	-	1.042	-	-	-
14	-	-	-	1.045	1.011	-	-
17	-	-	-	1.041	-	-	-
20	-	-	-	1.035	-	-	-
23	-	-	-	1.029	-	-	-
22	-	-	-	-	1.026	0.984	0.948
36	-	-	-	-	1.015	0.978	-
61	-	-	-	-	0.994	0.974	-
65	-	-	-	-	-	-	0.897
85	-	-	-	-	0.953	0.936	0.833

*Average of two treatments.
Volume of sulfuric acid, 5 μL/g of methylcellulose; temperature, 65 °C.

Table A.14 - Raw data of Figure 4.14, Figure 4.15 and Figure 4.16

Derivative	E bulk	E soluble	E insoluble	Apparent solubility (mg/mL)
	0.57	-	-	0.744
	0.61	-	-	0.872
	0.82	-	-	0.670
	0.82	-	-	0.681
	0.66	-	-	0.614
C3	0.91	-	-	0.582
	0.99	-	-	0.536
	0.93	-	-	0.541
	1.07	-	-	0.333
	1.18	-	-	0.378
	0.42	-	-	0.943
	0.49	-	-	0.970
	0.72	0.46	0.94	0.715
	1.01	-	-	0.524
	0.84	0.65	0.91	0.441
C4	0.83	0.59	0.94	0.483
	0.94	0.59	1.05	0.301
	0.90	0.57	1.09	0.389
	0.97	0.63	1.05	0.288
	1.03	0.61	1.09	0.293
	0.28	-	-	0.954
	0.36	-	-	0.952
	0.46	-	-	0.560
	0.52	-	-	0.580
	0.63	-	-	0.297
C6	0.62	-	-	0.311
	0.76	-	-	0.149
	0.71	-	-	0.141
	0.79	-	-	0.062
	0.82	-	-	0.045

Table A.14 - cont'n

Derivative	E bulk	E soluble	E insoluble	Apparent solubility (mg/mL)
	0.47	0.21	0.53	0.365
	0.49	0.22	0.48	0.359
	0.55	-	-	0.290
	0.54	-	-	0.291
	0.60	-	-	0.231
	0.63	-	-	0.181
C8	0.65	0.26	0.60	0.179
	0.63	0.22	0.62	0.189
	0.68	-	-	0.114
	0.66	-	-	0.117
	0.70	-	-	0.085
	0.75	-	-	0.089
	0.74	0.25	0.72	0.064
	0.73	0.22	0.66	0.060
	0.36	-	-	0.461
	0.30	-	-	0.442
	0.50	-	-	0.195
	0.40	-	-	0.294
	0.52	-	-	0.135
C10	0.62	-	-	0.044
	0.66	-	-	0.024
	0.69	-	-	0.014
	0.74	-	-	0.011
	0.72	-	-	0.014
	0.25	0.14	0.28	0.589
	0.12	0.12	0.30	0.865
	0.35	0.14	0.45	0.423
C12	0.35	-	-	0.418
	0.45	0.13	0.56	0.227
	0.48	0.13	0.50	0.188
	0.59	0.11	0.67	0.040
	0.53	-	-	0.032
	0.59	-	-	0.013
	0.61	0.12	0.66	0.018

Table A.14 - cont'n

Derivative	E bulk	E soluble	E insoluble	Apparent solubility (mg/mL)
	0.13	-	-	0.902
	0.11	-	-	0.909
	0.24	-	-	0.573
	0.20	-	-	0.690
	0.36	-	-	0.438
C14	0.26	-	-	0.512
	0.47	-	-	0.149
	0.43	-	-	0.249
	0.50	-	-	0.077
	0.50	-	-	0.051

E soluble and E insoluble stand for extent of reaction of the soluble and the insoluble fractions of the derivatives, respectively.

Table A.15 - Raw data of Figure 4.17.

Salt concentration*	Derivative precipitated (as percentage of initial amounts)			
	C4	C8	C12	C1
4	81.3	67.0	85.5	93.4
4	84.7	68.7	86.3	100.6
8	36.0	32.2	37.3	95.5
8	34.1	32.8	38.0	96.6
12	10.2	17.0	21.0	16.2
12	10.9	17.7	21.6	17.9
16	3.8	8.8	11.9	4.8
16	4.5	9.3	12.6	3.8
20	2.0	5.4	7.0	2.2
20	2.9	6.1	7.8	2.9

Soluble fractions of the derivatives resulted from experiment showed in Figures 4.14 and 4.15 were salted-out with ammonium sulfate. The concentration given by the remaining amount of derivatives in the solution was measured and compared with the original concentration

Table A.16 - Raw data of Figure 4.18.

pH	Supernatant absorbance at 280 nm	
	Ammonium sulfate	Buffer
8.89	0.330	-
8.50	0.340	0.359
8.05	0.336	0.373
7.50	0.337	0.325
7.10	0.330	0.343
8.80	0.348	-
7.30	0.337	-
6.80	0.337	-
5.90	0.336	0.333
5.40	0.324	-
5.20	0.300	0.335
5.05	0.252	0.335
4.85	0.150	0.322
4.65	0.032	-
4.50	0.012	-
4.15	0.004	0.321
3.50	0.001	0.325
3.05	0.007	0.322
7.30	0.335	-
6.60	0.344	0.341
5.70	0.346	-
5.40	0.346	0.329
5.25	0.338	-
5.10	0.332	0.330
5.00	0.324	-
4.90	0.315	0.323
4.80	0.258	-
4.70	0.138	0.322
4.75	0.178	0.332
4.65	0.086	-
4.55	0.020	0.324
4.50	0.020	-
4.40	0.017	0.320
4.25	0.020	-
3.50	0.022	0.325
3.05	0.016	0.322
7.00	-	0.335
6.50	-	0.334

Table A.16 - Cont'n

pH	Supernatant absorbance at 280 nm	
	Ammonium sulfate	Buffer
3.60	-	0.318
2.90	-	0.316
9.10	-	0.336
5.60	-	0.333
4.15	-	0.329

Ammonium sulfate concentration, 60 percent saturation. Protein concentration, 0.66 mg/mL; aging time, 2 hours.

Table A.17 - Raw data of Figure 4.19.

pH	Filtrate absorbance at 280 nm			
	C1	C4	C8	C12
8.30	0.210			
7.93	0.218			
7.03	0.199			
6.23	0.190			
5.68	0.210			
8.70		0.024		
7.83		0.009		
6.93		0.005		
6.15		0.013		
5.68		0.007		
8.58			0.180	
7.88			0.182	
6.95			0.182	
6.18			0.176	
5.68			0.165	
8.38				0.191
7.85				0.191
6.98				0.186
6.23				0.166
5.65				0.153

Cn/BSA = 2; protein concentration, 0.66 mg/mL; salt concentration, 60 percent saturation; aging time, 2 hours. The dashed line is the expected absorbance in the case of no precipitation.

Table A.18 - Raw data of Figure 4.20.

Time (hours)	Filtrate absorbance at 280 nm
0	0.097
0	0.088
5	0.077
5	0.081
10	0.079
10	0.078
16	0.087
16	0.081
24	0.071
24	0.071
40	0.069
40	0.081

Cn/BSA = 1.5 (decreased form 2 to avoid supernatants with low absorbance at 280 nm); protein concentration, 0.66 mg/mL; salt concentration, 60 percent saturation; pH, 6.0.

Table A.19 - Raw data of Figure 4.21.

Ammonium sulfate concentration (as percentage of saturation)	Protein precipitated (as percentage of initial amount)
30	85.1
30	76.2
35	77.1
35	76.5
40	81.3
40	78.9
45	58.5
45	62.5
45	67.5
50	49.3
50	55.1
50	43.5
55	33.8
55	31.4
55	37.1
60	18.1
60	9.1

Cn/BSA = 2; protein concentration, 0.66 mg/mL; pH, 6.0; aging time, 1 hour.

Table A.20 - Raw data of Figure 4.22.

Ammonium sulfate concentration (as percentage of saturation)	C4/BSA	Protein precipitated (as percentage of initial amount)
60	0.8	68.0
	0.8	71.3
	0.8	68.9
	0.5	65.1
	0.5	47.6
	0.5	55.6
	0.2	29.9
	0.2	42.0
	0.2	39.3
55	2.5	71.4
	2.5	68.8
	2.5	68.5
	1.0	55.5
	1.0	50.3
	1.0	47.4
	0.5	37.5
	0.5	38.0
	0.5	44.3
50	5.0	72.1
	5.0	63.3
	5.0	66.4
	2.0	48.6
	2.0	50.5
	2.0	50.3
	1.0	43.0
	1.0	43.4
	1.0	42.2
45	5.8	44.4
	5.8	42.7
	5.8	46.2
	4.0	43.6
	4.0	40.1
	4.0	40.1
	2.0	35.4
	2.0	35.4
	2.0	32.8

Protein concentration, 0.66 mg/mL; pH, 6.0; aging time, 1 hour.