AQUEOUS TWO PHASE SYSTEMS FOR DOWN STREAM PROCESSING OF PROTEINS

A

THESIS

SUBMITTED TO DEVI AHILYA UNIVERSITY
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY

BY

KUMAR BABU KAUTHARAPU

BIOCHEMICAL ENGINEERING GROUP
CHEMICAL ENGINEERING & PROCESS DEVELOPMENT DIVISION
NATIONAL CHEMICAL LABORATORY, PUNE
&
SCHOOL OF BIOCHEMISTRY, DEVI AHILYA UNIVERSITY
INDORE

JUNE 2008

Aqueous two phase systems for down stream processing of Proteins

Thesis

Submitted for the Degree of

DOCTOR OF PHILOSOPHY

In

Biochemistry

By

Kumar Babu Kautharapu

National Chemical Laboratory, Pune
&
School Of Biochemistry, Devi Ahilya University, Indore.

JUNE 2008

Dedicated to loved ones…..

DECLARATION BY THE CANDIDATE

I declare that the thesis entitled **'Aqueous two phase systems for down stream processing of proteins'** is my own work conducted under the supervision of **Dr. Deepak Bhatnagar** (Supervisor) at School of Biochemistry, Devi Ahilya University, Indore, India approved by Research Degree Committee. I have put in more than 200 days of attendance with the supervisor at the center.

I further declare that to the best of my knowledge the thesis does not contain any part of any work, which has been submitted for the award of any degree either in this University or in any other University/Deemed University without proper citation.

(Dr. Deepak Bhatnagar) **(Kumar Babu Kautharapu)**

Signature of the Supervisor Signature of the Candidate.

Forwarded

(Dr. R. Gadre)

Signature of Head U.T.D

CERTIFICATE OF THE SUPERVISOR

CERTIFICATE

This is to certify that the work entitled **"Aqueous two phase systems for down stream processing of proteins"** is a piece of research work done by **Shri. Kumar Babu Kautharapu** under my (our) guidance and supervision for the degree of Doctor of Philosophy of Devi Ahilya University, Indore (M.P.) India. That the candidate has put-in an attendance of more than 200 days with me.

To the best of my knowledge and belief the thesis:

(i) embodies the work of the candidate himself;

(ii) has duly been completed;

(iii) fulfills the requirements of the Ordinance relating to the Ph.D. degree of the University; and

(iv) is upto the standard both in respect of contents and language for being referred to the examiner.

Forwarded

(Dr. Deepak Bhatnagar)
Signature of the Supervisor.

(Dr. R. Gadre)
Signature of Head U.T.D.

DECLARATION

I certify that the work incorporated in the thesis entitled **"Aqueous two phase systems for down stream processing of proteins**" submitted by **Kumar Babu Kautharapu** was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

(Co-Supervisor)

Date

Place. Pune

Dr. Surendra Ponrathnam
(Scientist-G)
Polymer Science and Engineering Division
National Chemical Laboratory, Pune,
India

Acknowledgements

It is my pleasure to acknowledge people who have given their help, moral support, valuable suggestions, learning experiences and peaceful ambience to finish my research work during Ph.D. and making it a memorable experience.

Firstly I would like to express my hearty thanks to Dr. B.D. Kulkarni for his valuable suggestions, which has contributed greatly in leading shape to my work and life. I express my gratitude and heartfelt feelings to Mr. Sanjay N. Nene who has shared his enthusiasm, time and knowledge to pursue a challenging research work in "Aqueous two phase partitioning" and gave valuable, timely suggestions during my Ph.D. and improved the quality of my research to establish my scientific career. I respect his personnel training, encouragement, thought provoking suggestions, positive criticism, scientific help, inspiration to do research, pleasant personality. My association with Dr. Surendra Ponrathnam has been multi-dimensional. Apart from his immense help in making me registered under him and stay in his laboratory, a pleasant and comfortable one. I admire the co-operation and keen interest shown by him.

I would like to express my heartfelt gratitude for my research guide, Prof. Deepak Bhatnagar for providing me with an opportunity to register and work in this esteemed institution. His principles and values have always have been a source of inspiration for me. His constant encouragement has greatly motivated me in all endeavors. I sincerely thank Prof. R. Gadre, Head, School Of Biochemistry for her kind cooperation during my master's degree at the department and for her help in completing this research work. I sincerely thank Dr. S.M. Gokhale and Dr. Meeta Jain Gupta for their timely suggestions and help during my research.

I would like to thank Mr. V.V Jogdand and Dr. R.V. Gadre for their valuable suggestions; critical discussions in getting senior research fellowship; Proof reading the thesis and research work. I thank Mr. Thakar. D.M for his kind co-operation, respect and maintaining a cheering atmosphere in lab and I shall also remember joyful moments shared with me. My sincere thanks are due to Ms. Varma madam, Dr. Gaikward and Dr. H.V. Adikane for their timely suggestions and kind patience shown by them helped me a lot in completing this work.

I deeply appreciate the help and support rendered by Dr. K.S.M.S. Raghavarao, Head, Food engineering, CFTRI, Mysore. I would like to convey my sincere thanks to his students Madhusudhan and Chatana for their help in measuring the interfacial-tensions.

No words can suffice encouragement and moral support rendered by Mr. Sandeep. G and Mr. Prabhakar. S, without their scarification and persistent motivation, this work would not have been possible.

I feel words are not enough to express my feelings to Ms. Geetanjali Lale and Dr. Sangeeta kasture madam who always helped me morally during my Ph.D. I am happy to receive personal and scientific advices for completing research work and future career. I express my hearty feelings to them for their personnel support and to create happy atmosphere during my stay.

I thank my colleagues Abijit. K, Balachandra. V, Banin. R, Chandra shekar. G, Chetan. B, Deepali. P, Gayatri. I, Hemangi. J, Hithesh. S, Jahnavi. S, Kiran. K, Mayura. D, Mukesh. P, Neha, Priyanka. S, Rahul upadhyay, Rashmi. R.M, Renuka. J, Santosh. D, Trupti. J, Tushar. D and Ummi. K for their help and creating a cooperative and pleasant environment in lab. A special thanks to Vaishali. S, Asiya. K and Laxman. S for their help in preparation of Ph.D. thesis. I thank Mahendra. J, Nimhan. S and Pingale. B.G for their continuous support to the lab and technical help. I Thank Amol. D for patient listening to me and waiting late nights in the lab along with me while writing the thesis.

I thank Ganesh. I, Giri, Hari. R, Poojari. N, Praveen. S, Sarika, Sata, Dr. Shini, Dr. Smita madam, Sunny, Supriya, Tim and Vasif for their timely help when necessary.

I thank Council of Scientific and Industrial Research, New Delhi, India for providing fellowship during my Ph.D. and also Department of Biotechnology, New Delhi, India for financial support.

I express my heart-felt feelings to Rajendra Prasad. G, Dr. Ravi. D.S, Mahesh. R.E, Siva shankar, K. Sreenath, P. L. Raghukumar, N. Kiran, Mauli, Pavan and P. Sudheer kumar for their continuous support to personnel life, making my stay a happy, memorable experience and also to be at my side in happy and difficult situations.

I thank my family members' mother: Krishna veni; father: Sathyanarayana; brother: Madhu Babu; sister-in-law: Laxmi and nephew: lucky for their continuous support.

I love to express deep, hearty feelings for the one who have always supported me in entire my life with necessary encouragement, provided a pleasant environment in home, gave inspiration to take my own decisions in life and encouragement to enter into business of research.

Kumar Babu Kautharapu

Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ρ	Density of given phase
ρ_B	Density of bottom phase
ρ_o	Density of the initial solution
ρ_T	Density of top phase
ATPE	Aqueous Two Phase Extraction
ATPS	Aqueous Two Phase System
BSA	Bovine Serum Albumin
C_B	Concentration of solute in bottom phase
CM-GG	Carboxymethyl guar gum
CM-TKP	Carboxymethyl Tamarind kernel powder
C_T	Concentration of solute in top phase
DNA	Deoxyribonucleic acid
DS	Degree of Substitution
DSP	Down Stream Process
D_{Vf}	Difference in free volume between two phases
FDA	Food and Drug Administration
GG	Guar Gum
HLB	Hydrophilic Lypophilic Balance
HP-GG	Hydroxypropyl guar gum
HP-TKP	Hydroxypropyl Tamarind Kernel Powder
IL	Ionic Liquid
k	Boltzmann Constant
K	Partition coefficient of the solute
LLE	Liquid-Liquid Extraction
MW	Molecular Weight
PDI	Polydispersity Index
PEG	Polyethylene Glycol
pH	Potential of hydrogen
pI	Isoelectic point
PPO	Polyphenol oxidase
PVA	Polyvinyl Alcohol
PVP	Polyvinyl Pyrrolidone
RNA	Ribonucleic acid
rpm	Rotations per min
s	Interfacial Tension
T-500	Dextran (molecular weight 5 million)
TKP	Tamarind kernel powder
TLL	Tie Line Length
V_B	Volume in bottom phase
V_f	Free volume of a given phase to other phase
V-GG	Vinyl pyrrolidone guar gum
V_I	Initial volume of the solute added to the phase system
V_R	Phase volume ratio
V_T	Volume in top phase

ΔK	Difference in partition coefficient
Δr	Density difference between two phases

Summary

Introduction

In recent years progress in biotechnology and cell biology depends to a great extent on the production and development of efficient separation methods. This holds for both soluble substances such as proteins and nucleic acids and for suspended particles such as cell organelles and whole cells of human interest from various sources like plants, microbes, tissue culture (plant and animal). However, technology for down stream processing of biological products, has not kept pace with the advances in upstream operations. The reason for the failure of biological process at commercial scale can be traced to non-viable unit operations for down stream processing of biological molecules.

Advances in biotechnology have simplified production of a large number of new proteins that are important to the food, pharmaceutical, medical and chemical industries. The application of traditional separation processes suggest that 50-80 % of the total cost of therapeutic proteins is incurred at the purification stage which is why the replacement of multi-step processes by a single step can be vastly beneficial (Jones, 1991). Isolation and purification of biomolecules on a large scale is of increasing importance in industry for example, as the production of pure enzymes, cell organelles and viruses for vaccines etc. Normally liquid-liquid extraction involves the use of organic solvents that are not suitable for protein recovery, as proteins are either insoluble in organic solvents or are denatured due to exposure to solvents (Kula *et al.,* 1982). The cost of recovery is a major factor influencing the recovery process to be used (Cramer *et al*., 1996).

Aqueous two-phase extraction (ATPE) has been widely used for protein recovery and purification. ATPE has been recognized as an economical and efficient downstream

processing method and offers many advantages, such as low process time, low energy consumption and a biocompatible environment (Srinivas *et al*., 1999). Liquid-liquid binary phases can also be formed using two polymers/polymers or polymer/salt solutions. Albertsson partitioned microorganisms, cell walls, chloroplasts and vesicles selectively between the phases of an aqueous two-phase system composed of Polyethylene glycol (PEG)/dextran and PEG and potassium phosphate. Two recent reports on biphasic systems containing polysaccharides, i.e guar/amylopectin polyethyleneoxide (PEO)/maltodextrin (Closs *et al*., 1998; 1999; Silva *et al*., 2000a; 2000b), cashew-nut tree gum/polyethylene glycol and aqueous guar/dextran suggest that aqueous two-phase systems using, cheap eco-friendly polymers is an attractive development. The basis of separation is the selective distribution of substances between the two-phases.Whereas, small molecules are evenly distributed between the phases and macromolecules tend to partitioning between the two phases to variable extent (Albertsson, 1986).

Biological molecules such as proteins are generally obtained in very dilute solutions. Hence, the first step in their recovery is augmenting the concentration. An aqueous two-phase system can be used to concentrate samples by choosing the specific phase composition. A single or multi-step procedure may be used depending on the partition characteristics of the desired and undesired substances (Kaul *et al*., 2000). Traditionally, purification is done in steps involving clarification - using solid/liquid separation techniques (centrifugation, filtration), followed by concentration. The application of ATPS that has attracted interest as isolation and recovery of proteins can be achieved from crude feedstock. Pilot-scale studies to assess the feasibility of PEG/salt systems for

extraction of enzymes (superoxide dismutase) from macerated bovine liver tissue have been carried out (Boland *et al*., 1991). The subject matter of the thesis is presented in six chapters.

Chapter 1: General Introduction and the scope of present work

This chapter comprises of literature survey with reference to the principles properties, different aqueous two phase systems and applications of ATPs. The important parameters that have been considered in the development of new ATPs like pH, salt concentration, protein properties, polymer concentration, temperature, viscosities and phase properties are discussed. The production of α-galactosidases with reference to plant, animal and microbial α-galactosidases, their occurrence, properties and applications. has been reviewed in detail. As introduction of natural gums, their classification, their environmentally safe to economically feasible and their uses are discussed. Further, the uses of carbohydrates, commercially available derivatized carbohydrates and derivatizaition of natural gums for different aqueous two phase systems have been discussed. The main scope and applications of the ATPs are explained in this chapter.

Chapter 2: Partition of proteins in aqueous two phase system based on Hydroxypropyl-guar gum derivative - Dextran: Effect of salt and pH

The partitioning of two proteins, BSA and Lysozyme was studied in two different aqueous two phase systems based on a guar gum derivative and dextran. Phase diagrams were formulated provided for a) Guar gum-Dextran 2000 and b) Hydroxyl propyl guar gum (HPGG)-Dextran 2000 systems. Protein partition data was compared for both the

systems. The HPGG rich phase supported a high concentration of proteins in solution as compared to GG rich phase. Effects of pH and NaCl concentration were studied on the partition coefficient of BSA and lysozyme. Partitioning of both proteins were significantly affected by addition of NaCl in the HPGG-Dx system. The data on partitioning of various proteins in this system indicates that it can be used for selective separation of a particular protein from a protein mixture.

Chapter 3: α-Galactosidase hydrolyzed guar based aqueous two phase system

Response surface methodology (RSM) analysis was used for medium optimization to enhance the production of enzyme α-Galactosidase (α-D-galactoside galactohydrolase E.C.3.2.1.22) using *Aspergillus awamori* (NCIM -1225). The production of α-Galactosidase is growth associated. The concentration of carbon and nitrogen sources was optimized by RSM. Guar gum is the main source for the production of the α-Galactosidase but organic nitrogen sources such as soya bean meal, wheat bran and kurma (seed coat of guar seeds) and inorganic nitrogen sources such as ammonium nitrate and ammonium sulphate were found to affect the production of the α-Galactosidase. The RSM method was able to predict higher production rate with a minimum amount of carbon and nitrogen sources. The best combination of guar (4.41 g/l), soybean meal (1.38 g/l), yeast extract (0.12 g/l) and ammonium nitrate (0.52 g/l) was optimized to give maximum production of the alpha-galactosidase (14.917 μmole/ml) after 72 hours. The hydrolysis of guar and hydroxylpropyl-guar gum was investigated. The enzyme hydrolyzed guar gum was investigated for the formation of aqueous two phase system.

Chapter 4: Protein partition on Phospho-Guar gum aqueous two phase system

A new aqueous two phase system based on a Phospho-guar gum (PGG) and Polyvinyl pyrrolidone (PVP) was studied. The Phase Diagram for the systems of PGG and PVP were determined at 25^{o}C. Partitioning of BSA and Polyphenol oxidase (PPO) was studied with two tie-line compositions of PGG/PVP aqueous two phase system. The protein partitioning of BSA and PPO were determined by partition coefficient (K_a). The K_a >1 indicates that BSA was partitioned in to the PVP enriched top phase, while PPO values <1 represents that PPO was partitioned in to PGG enriched bottom phase, in both the tie-line compositions. The influence of pH and concentrations of polymers on the partitioning of both BSA and PPO proteins were investigated. The results demonstrated the importance of the polymer characteristics, for partitioning in aqueous two phase system.

Chapter 5: Vinyl-2-pyrrolidone derivatized guar gum based aqueous two phase system

Aqueous two phase systems (ATPS) are formed when certain combinations of two polymers or polymer and salt are added to water. Phase separation occurs at certain specific concentrations that have similar densities and low interfacial tensions. The present study involves synthesis and application of a novel derivatized guar in aqueous two phase system. The derivation of guar was performed by a grafting of 1-vinyl-2-pyrrolidone by a redox reaction in aqueous medium. Effect of concentration of redox pair, ceric ammonium nitrate (CAN) and potassium peroxydisulfate ($K_2S_2O_8$) was investigated on the two critical parameters of grafting viz. grafting efficiency (%GE) and

ratio of grafting (%G) 56% and 70.1 respectively. The grafted copolymers were characterized for percent yield and absorbance.

A new aqueous two-phase extraction protocol was established using a mixture of derivatized guar and dextran. A phase diagram was constructed based on the phase separation exhibited by the system at 25^{o}C. The protein partitioning studies with standard bovine serum albumin (BSA), lactate oxidase (LO) and phytase were 0.479, 1.981, and 2.586 respectively in the 1-Vinyl-2-pyrrolidone guar gum (V-GG)/Dextran (T500)/H_2O system. The influence of an ionic salt sodium chloride, (NaCl) was investigated on the partitioning of BSA.

Chapter 6: A new aqueous two phase system based on Tamarind kernel powder

This chapter deals with the formation of a new aqueous two phase system comprising of tamarind kernel powder (TKP), Hydroxypropyl-TKP (HP-TKP) and Carboxymethyl-TKP (CM-TKP). The physical characteristic of the top and bottom phases were characterized. The main advantage and novelty of employing ATPs is that tamarind gum forms one of the phase forming components along with the other counter polymer. This new tamarind system was also employed for the partitioning studies of the model protein, Bovine Serum Albumin (BSA), which showed that most of the proteins tested could be partitioned by this system. The effect of salts and pH in the enhancement of protein partition was studied.

Chapter 1

General introduction and the scope of present work

1.1. General Introduction

Biochemicals are those chemical substances that are made by living organisms like microorganism, plants and animals. Depending upon their nature and function, they may be synthesized intracellularly or secreted extracellularly by the organism. Biochemicals derived from microorganisms, plants and animals often command a high price. They find use in the pharmaceutical and health care industry in the preparation of diagnostic reagents and in the food and cosmetic industries. The purity of the biochemical is a very important consideration which also decides its price. Biochemicals are normally sold on the basis of their chemical or bioactivity and purity.

Isolation and purification of a particular biochemical from a complex mixture decides the cost of the product. This makes the choice of separation method(s) very important in deciding its purity and cost. In most cases a number of different purification steps e.g. cell disruption, filtration/centrifugation, precipitation and chromatography have to be employed in combination to reach the desired purity of final product. These purification steps are generic in nature and can be used on a laboratory scale or industrial scale. Both economic and environmental factors are decisive in making a suitable choice of separation steps.

The increasing demand for proteins, vaccines and bio-pharmaceuticals etc have lead to considerable research in devising new methods for their production on a large scale. In recent years progress in molecular biology and biotechnology has led to a dramatic increase in diverse proteins from cloned microorganisms. Thus developing an efficient

separation and purification method has become a challenging task. This holds true for both soluble substances such as proteins and nucleic acids and insoluble materials such as cell organelles and whole cells of human interest from microorganism, plant and animal sources.

Use of traditional separation media has demonstrated that a 50-80% increase in cost of the purification of therapeutic proteins is due to multiple steps involved for obtaining pure protein. Therefore more emphasis is given on replacing these purification stages by a single step which has impact on price of therapeutic proteins (Jones *et al*., 1991). However, technology for down stream processing of biological products, has not kept pace with the advances in upstream operations. The reason for the failure of biological process at commercial scale can often be traced to non-viable unit operations for recovery of product. The separation of many biomolecules is still performed in a batch mode using column chromatography, salt or solvent induced precipitation and electrophoresis. These operations have problems associated with scale up. Therefore current research in the area of down stream processing (DSP) is gaining momentum towards the development of less expensive, more efficient and scalable methods of separation preferably in a continuous mode of operation.

Aqueous two phase extraction (ATPE) is well recognized for DSP of biomolecules and has been used as a primary step for product recovery. The process is amenable to scale-up and there can be several fold concentration of product with a high degree of selectivity. The major advantages of ATPE include higher efficiency of extraction, more solid handling capacity, biocompatible environment, low interfacial tension (leading to energy intensive operation), high recovery, less process time and good selectivity in extraction at

the initial stage of purification. Further, it offers ease of scale up, continuous operation and allows easy adaptation of the equipment as well as the methods of conventional organic aqueous extractions. The technique is effective in removing not only solids but also contaminating materials such as polysaccharides and nucleic acids. Affinity, ion-exchange and hydrophobic binding can also be carried out in aqueous two phase systems (ATPS) by employing polymers to which suitable ligands are attached. One of the shortcomings of ATPE has been the relatively high cost of polymers used for phase formation.

The main scope of the thesis has been to study and develop new polymers for aqueous two phase systems usful for the primary recovery of proteins produced by fermentation (both intracellular and extra cellular). Less expensive, natural, derivatized polymers and recyclable polymers were selected to design systems that can be applied on a laboratory, as well as large scale in an industry. The aqueous two phase systems used were composed of two incompatible polymers in water. The aqueous two phase systems are characterized for polymers and water content in both the phases. The extraction procedures are comparatively fast and no sofisticated equipment is needed. This thesis shows that it is possible to purify wide range of intra and extra cellular proteins under a wide range of conditions of temperature, pH and ionic strength.

1.2. Down stream processing of biomolecules

1.2.1. General partitioning theory

Many traditional chemical industries have been undergoing a change in their orientation from conventional chemicals to life-science products. Recent developments in biochemistry, along with recognition for the need of renewable resources, have accelerated biotech research and development, in academia, industry and government-funded laboratories. Bioseparation entails the recovery and purification of biochemicals from various biological feed streams. Bioseparation using chromatography entails separation of components from an aqueous mixture by loading a sample of the mixture onto an absorbent column. Due to the difference in binding potential, each component binds to the matrix to different extents. The product is recovered by an elution process that gives the desired product as a concentrated band. This process is used for purification of proteins, chemicals and biochemicals used in the manufacture of pharmaceutical, food products, agriculture, purification of water and for other biochemical processes [Figure 1.1]. To optimize output from this process, it is important to know precisely when and at what concentration a particular product can be obtained. Bioseparation processes for life sciences require different approaches from those used in traditional chemical industries (Karsten, 2001). For example, in the pharmaceutical industry, only a few kilograms of a protein might be produced per year priced in millions of dollars. In industrial biotechnology, thousands of tons of bio-based polymers, such as dextran, xanthan gum, enzymes could be priced at less than a hundredth of this price per unit weight. Separation of biological products often needs entirely new processes to handle unusual material properties, such as low bulk density. The desired product might be a single component present at low concentration that must be separated from bulk water and other soluble components.

Separation technologies can be categorized according to their underlying principle. Often the effort involved in thermal and chemical separation techniques is higher than that in mechanical techniques. Although thermal and chemical separation tasks will not change greatly, when applied to the field of bioseparation; mechanical separation will involve new engineering challenges, for example, in bioseparation, the handling of soft bio-particles is particularly crucial (Schurr *et al.,* 2000; Karsten, 2001). A broader analysis indicates that there will be three major challenges in the engineering of downstream processes:

1. Managing variables
2. Product behavior and
3. Selection of the proper separation technology [Figure 1.2].

The challenge for the future will be to develop and adapt improved technologies or to create innovative separation technologies to meet the bioprocess requirements (Karsten, 2001). Therefore, separation methods are often combined and/or modified to suit different types of solutions. Depending on the use of the target protein the demand of purity vary, e.g. purity of a protein to be used in a washing powder application would be far less than that to be used as a drug. It is rare that a single purification step is enough to reach the required degree of purity. Hence it is important to have a different purification method readily available to design fast, powerful and cost efficient purification strategies. There is always a loss of material in every purification step. Thus, the number of purification steps in a process should be kept to a minimum.

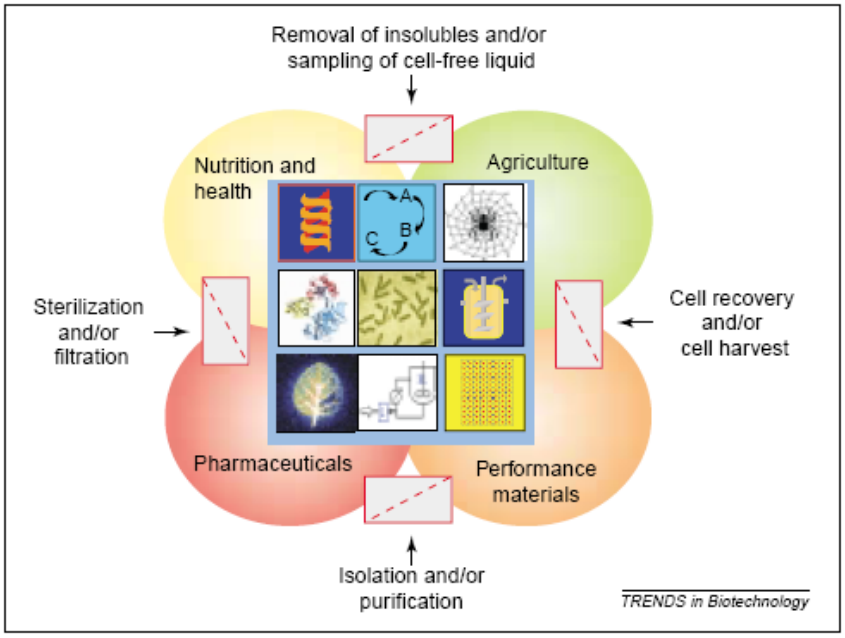


Figure 1.1: In the future, various bioseparation technologies are needed for a broad range of business areas (Courtesy by Karsten, 2001).

Due to loss of material, the purification costs increase dramatically with increase in number of purification steps. On the other hand, manufacturing a therapeutic protein often requires many purification steps to remove important undesired such as endotoxins, deoxiribose nucleic acid (DNA) and virus particles in order to meet the demands from regulatory agencies such as the US Food and Drug Administration (FDA).

The purification procedure for any biomolecules (protein) can be strategically programmed in to four groups:

1. Cell harvesting *(Filtration, Centrifugation and Adsorption)*
2. Extraction *(Extraction, Ultra filtration, Aqueous Two Phase Systems and precipitation)*

3. Purification (*Chromatography, Crystallization and Precipitation)*

4. Polishing Step *(drying and crystallization).*

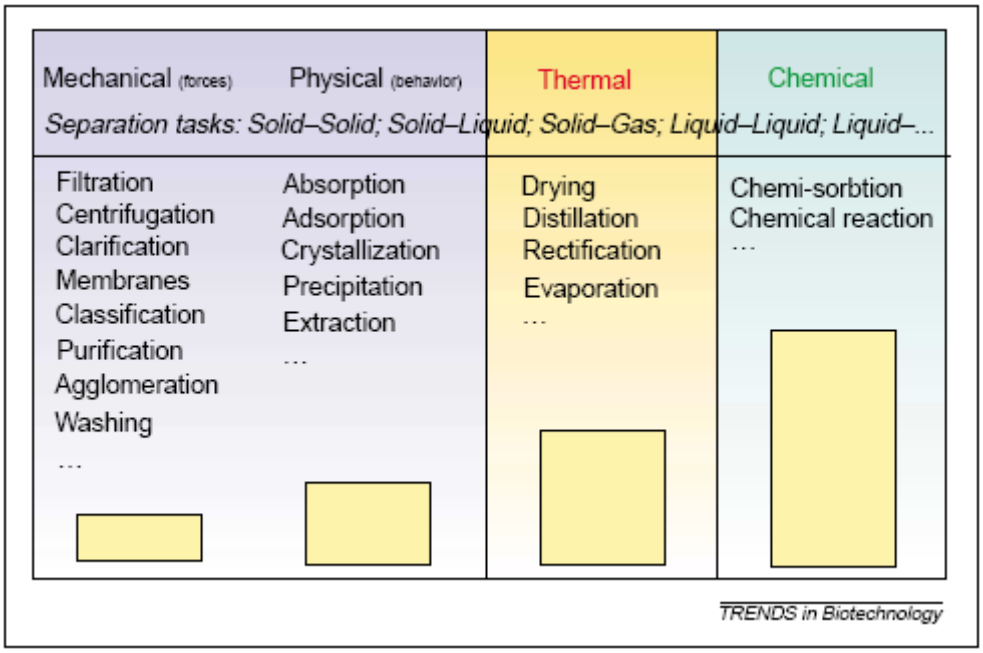


Figure 1.2: Separation technologies can be categorized according to their underlying principles. The bars represent the relative cost of each process (Courtesy by Karsten *et al*., 2001).

1.2.2. Fermentation

The ultimate destination of the target biomolecules determines the emphasis on various groups. For instance design of further purification steps after the fermentation depends on whether the target molecules are extracellular or intracellular in the fermentation fluid e.g. if the target molecule is intracellular, importance is given to the cells and if the target molecule is extracellular, the supernatant fluid is of importance.

1.2.3. Cell harvesting

Proteins produced by microorganisms can be intracellular or extracellular. In either case, the first step is to separate the cells from the culture broth. The most common techniques used for this purpose are centrifugation and filtration (Enfores *et al*., 2000).

1.2.4. Filtration

Filtration is a technique used to separate solid from the suspending liquid by a porous barrier. The principle of filtration is based on the passage of fluid through a permeable barrier based on a differential pressure across the barrier. It is governed by Darcy's law of filtration equation.

$$\text{Rate of filtration} = \frac{1}{A}\frac{dv}{dt} = \frac{\Delta p}{\mu R} \qquad [1]$$

The rate of filtration is defined as the volume of filtrate V (in m^3) collected per unit time, t (in minutes) per unit area A (in m^2) of the filtration medium with a medium of viscosity μ, resistance R by a porous bed and a pressure drop caused by filter cake and medium Δp.

The filtration flow rate at constant driving pressure become a function of time because the liquid is presented with two resistance R in series, one of which, the medium resistance R_m may be assumed constant and the other, the cake resistance R_c increases with time.

It is convienient to write the cake resistance R_c in terms of a specific cake resistance α as follows:

$$Rc = \alpha\rho_c\left(\frac{V}{A}\right) \qquad [2]$$

Where, ρ_c is the mass of dry cake solids per volume of filtrate. Thus resistance increases with volume filtered.

$$\frac{1}{A}\frac{dv}{dt} = \frac{\Delta p}{\mu_0[\alpha\rho_c(V/A) + R_m]} \qquad [3]$$

The variable constituting the slope in equation [3], the only one that could change appreciably with Δp is α, the specific cake resistance. The slopes are increasing with volume filtered, indicating that α is increasing. However, α can increase if the cake is compressed. Cakes are compressed when cells and other biological materials are being filtered.

Parameters that will determine efficiency of the filtration derive are surface properties of the membrane, flow rate of the feed stream and surface area of the membrane. The advantage of membrane filter press are higher yield, drier cake, easy cake removal. Dead end filtration is most widely used on smaller scale while cross flow filtration is utilized on large scale. The main disadvantage of crossflow filtration is higher capital investment.

1.2.5. Centrifugation

A centrifugation is a device that is used for separating material of different densities when gravitational force is insufficient for separation. The basic principle involved in centrifugal separation of solids is the density difference between the solids and the surrounding fluid. Normally in a solution, particles whose density is higher than that of the solvent sink (sediment) and particles that are lighter than it float to the top. The greater the difference in density, the faster is the rate of settling. This process is known as sedimentation. If there is no difference in density (isopyknic conditions), the particles do not settle. To take advantage of even small density differences between various particles in solution, gravity can be replaced with a more powerful "centrifugal force" provided by a centrifuge. Rate of sedimentation of particle by centrifugation is dependent on the density of the particle size of the particle and viscosity of the medium.

The separation process is performed by applying a high gravitational force to the sample. During centrifugation, the circular motion of the rotor provides a centrifugal force F_c (in Newton, N) acting on a particle which is related to the angular velocity W (radian/s) and the radial distance, r (m) and is representated as:

$$F_c = mW^2 r \qquad [4]$$

Where W^2 = acceleration due to centrifugal force. The tangential velocity of the particle, v (m/s) is given by the relationship, $v = Wr$. Equation may be written as

$$F_c = mr\left(\frac{v}{r}\right)^2 = \frac{mv^2}{r} \qquad [5]$$

The rotational speed of the centrifuge (n) is generally expressed in terms of number of revolutions per minute (rpm). The centrifugal force may be expressed by substituting the angular velocity in Eq [4] by he relationship $W = 2\pi n/60$.

$$F_c = mr\left(\frac{2\pi n}{60}\right)^2 = 0.01097 mrn^2 \qquad [6]$$

The centrifugal force may be expressed in terms of gravitational force F_g=mg where g is the acceleration due to gravity, 9.80665 m/s^2) as

$$\frac{F_c}{F_g} = \frac{r}{g}\left(\frac{2\pi n}{60}\right)^2 = 0.001118 rn^2 \quad \text{(since n = } 60v/2nr\text{)} \qquad [7]$$

Thus force developed in a centrifuge is W^2r/g or v^2/rg times as large as the gravitional force and is expressed as equivalents of *g* force (from equation 4 and 5).

On a large scale, particles are best separated from soluble components using a centrifugal separator (i.e. Separation of cells from soluble materials). The problem of disposing solid material from the separator may be encountered if the heavy phase is too viscous to flow out of the separator. But there are examples where this problem has been overcome by appropriate design of the separator i.e. by discontinuous emptying of the bowl.

1.2.6. Extraction

Liquid-liquid extraction or solvent extraction as is commonly known is a classical and versatile method for recovery as well as concentration of a variety of products. The method has been a workhorse in chemical, pharmaceutical and hydrometallurgical industries. It is used in biotechnology industries for the recovery of antibiotics and organic acids from fermentation broths. The advantage of solvent extraction includes:

- Selectivity of extraction directly from fermentation broths or from reaction medium in the case of biotransformations
- Reduction in product loss due to hydrolytic or metabolic/microbial degradation as the product is transferred from one phase to another
- Ease of scale up over a wide range of operation.

Extraction of whole broths is possible by combining solid/liquid separation and product enrichment in one step. Here two phases come in to contact whereby a solute is transferred from one phase to another, at the same time the suspended material (cells and organelles etc) are separated in the other phase. An example of such a method is the whole broth extraction of penicillin in a decanter centrifuge. Here penicillin can be enriched in a solvent such as butyl acetate. The partitioning of solute between the two phases is expressed quantitatively on the basis of the thermodynamic principle of partition coefficient or distribution coefficient. The partition coefficient *"K"* is the ratio of the solute concentration in the organic phase, *Y* (called the extract phase) to that in the aqueous phase, *X* (called the raffinate phase), is represented by Eq. [8].

$$K = \frac{Y}{X} \qquad [8]$$

The value of *"K"* is independent of the solute concentration for a given solvent pair and is a constant at a given temperature.

It is usually not feasible to extract proteins with organic solvent as proteins are either insoluble/denatured in organic solvents (Kula *et al*., 1982). Proteins are often extracted by means of two immiscible liquid phases that consists of solutions of two water soluble but incompatible polymers or one polymer and high concentration of certain salts (Albertson, 1986).

1.2.7. Purification

Purification is the final and in many cases, the essential step in the recovery chain. The degree of purification step depends on the targeted use of the protein or biological product. The cost of the product depends generally on the degree of purification step (i.e. generally high cost and relatively low yields). Selective distribution of the components of a mixture between two immiscible phases in intimate contact with each other forms the basis of separation in any chromatography technique. In chromatography, separation is caused by the difference in affinity or interaction of components with the solid phase. One of the phases, called the stationary phase is a solid or an immobilized fluid (i.e. a liquid coated on a finely divided inert solid). The other phase, called the mobile phase (eluent or carrier phase) percolates through the stationary phase. The sample is usually dissolved in the mobile phase applied for fractionation of biomolecules on a relatively

smaller scale. In many fermentation processes, chromatographic techniques are used to isolate and purify relatively low concentrations of metabolic products. In this context, liquid chromatography is concerned with the passage and separation of different solutes as liquid is passed through the column (Peter *et al.,* 1997). Both liquid chromatography and adsorption chromatography processes are based on the differential affinity of various soluble molecules for a specific matrix. In such processes, equilibrium is reached between a solid phase (a resin or matrix) and the soluble molecules in the liquid phase. The first chromatography resin (matrix) for bioseparation was available in 1959 by Pharmacia. Since then a number of different matrices have been produced with varying particle, pore size and surface properties. The components in a solution can be separated according to the difference in size (Gel permeation chromatography or size exclusion chromatography), charge (anion or cation exchange chromatography) or hydrophobicity (hydrophobic interaction chromatography (HPC). There is also a more selective technique for separation called affinity chromatography. It is possible to use affinity chromatography for separation of most biomolecules on the basis of their function and chemical structure e.g. enzyme-substrate, enzyme-inhibitor, antigen-antibody etc. Purification of active material may be achieved with up to thousand fold purity. The method is however costly and time consuming. Alternative affinity methods such as affinity cross-flow filtration, affinity precipitation and affinity partitioning offer advantages over normal affinity purification (Janson, 1984; Luong *et al*., 1992). In the scale-up of affinity chromatographic process, the bed height limits the superficial velocity of the liquid, thus scale up requires an increase in bed diameter or adsorption capacity (Katoh, 1987).

1.2.8. Polishing

The step of removing water or drying is the final step in bioseparation process. Removal of water or concentrating the product is very important mainly in relation to heat sensitive biochemicals (Cube *et al.,* 1984; Coulson and Richardon, 1991). Drying converts the product to a crystalline form. In a crystalline form of the product has considerable advantage such as reduction of transportation cost, ease of handling, packaging, no fear of contamination and storage (Peter *et al,* 1997). The method of crystallization or drying depends on the temperature stability of the product. For example a drum drier is used for heat stable products while a spray drier is more suited for heat stable biochemicals while lyophillization (freeze drying) finds use for many biological and pharmaceutical products such as hormones, enzymes etc.

Depending on the moisture content of the material and the relative humidity of the surrounding air, the material may either absorb or desorb moisture. The equilibrium moisture content is the minimum moisture to which a hygroscopic material can be dried. The basic mechanisms involved in the rates of drying of materials are yet to be understood clearly.

The drying cycle consists of a number of stages as shown by the variation of drying rate as a function of moisture content. The initial stage represents the settling stage period in which solid surface comes to equilibrium with the drying air. The second stage represents the constant drying rate period. At this stage the surface of the solid remains saturated with liquid water because of the movement of the water with in the solid to the surface

takes place at a rate greater than the rate of evaporation from the surface. Drying takes place by the movement of water vapor from the saturated surface through a stagnant air film in to the main stream of drying air. The rate of drying is dependent on the rate of heat transfer to the drying surface. The third stage represents the falling rate period because as drying proceeds, the rate of movement of moisture with in the material to the surface decreases and the surface begins to dry out. The fourth stage represents the falling rate period in which surface temperature begins to rise as drying proceeds.

1.3. Aqueous two phase systems

Beijerinck, first described aqueous two phase phenomenon in 1896. He observed the formation of two phases after mixing agar with soluble starch or gelatin (Abbot *et al*., 1990). These binary systems are called aqueous two phase system (Dobry *et al*., 1947). Albertsson in 1956 showed that microorganisms, cell wall, chloroplasts and other biological molecules partitioned selectively between the phases of an aqueous two phase system, composed of either two polymers (PEG & dextran) or a polymer and a salt (PEG and Potassium phosphate) (Albertsson, 1956). He further studied the effect of different polymers, their concentration, molecular weight and represented in the form of phase diagrams for several systems. Since then the attention for these systems had been growing rapidly in order to acquire more fundamental knowledge about ATPS, particularly for various biochemicals.

1.3.1. Theory/Principles of partitioning

Pre-Ake Albertsson at Sweden in the mid-1950 showed that aqueous two phase partitioning technique may be used for separation of biological materials. Later Walter G, Kula, M.R and Zaslavasky gave the constructive shape to this finding. Aqueous two phase provides an alternative and efficient approach for purification of biomolecules by partitioning between two liquid phases (Albertson, 1986). The basis for separation of a two phase extraction is the selective distribution of substances between the phases. For soluble substances, distribution takes place mainly between the two bulk phases and the partition is characterized by the partition co-efficient (*"K"*).

$$k = \frac{Ct}{Cb} \qquad [9]$$

Where C_t and C_b are the concentrations of the partitioned substance in moles per liter of top and bottom phase respectively. Ideally, the partition coefficient is independent of concentration and also independent of the volume ratio of the two phases. It is mainly a function of the properties of the two phases, the partitioned substance and temperature (Albertsson, 1986).

The interface between the phases should, however, also be considered. It has a certain capacity for adsorption of the partitioned substance. This does not usually play any significant role with respect to soluble substances but when suspended particles are present in the interface may adsorb relatively large quantities of the material. Therefore in the separation of cell particles there are in-fact three "phases" to consider; the upper

phase, the inter phase and the lower phase. It is the selective distribution between these phases which forms the basis for separation of particles by a two phase system.

The choice of a suitable phase system is the key step in aqueous two phase partitioning along with several factors like compatibility of the partitioned biomolecules in aqueous solutions, pH, ionic composition, osmotic pressure and denaturing effects.

1.3.2. Phases diagram

Aqueous two phase system is obtained when the constituents of the composition are mixed at certain range of proportions. The constituent compositions at which phase separation occurs may be represented in the form of phase diagram. Each two phase system has a unique phase diagram under a particular set of conditions such as pH and temperature (Albertsson, 1971).

The concentrations of the components are expressed in the form of percentage. The “P and Q” are two aqueous polymer solutions. The “P and Q”, the concentrations of the components are expressed in the form of percentage. The curved line separating two areas is called Binodial. The point on the binodial at which the composition and volume of both phases are almost equal is called critical point or cloud point (Kaul, 2000)**.**

The typical phase diagram for a polymer-polymer water phase system is shown [Figure 1.3]. It is based on the compositions of the top and bottom phases of different mixtures of Dextran T-500 (Pharmacia Biotechnology AB, Uppsala, Sweden) and polyethylene

glycol (PEG-3350, Union carbide, New York) in water. The curved line is the binodal curve; the lines connecting top and bottom phases are tie lines. All compositions represented by points (nodes) above the binodial curve give a two phase system. For example the phase system 5.7% Dextran and 5.7% PEG consists of an upper phase with 0.4% Dextran and 7.92% PEG and a lower phase with 14.38% dextran and 2.57% PEG. Points representing the top or bottom phase all lie on the binodial curve. The line connecting the composition of two phases in equilibrium is called tie-line. The tie-line has points which show the compositions of the total system, top and bottom phases. If the polymer concentrations are expressed in percent weight/weight (%w/w), the weight ratio of top/bottom phase is determined by calculating the ratio between lengths on the tie-line.

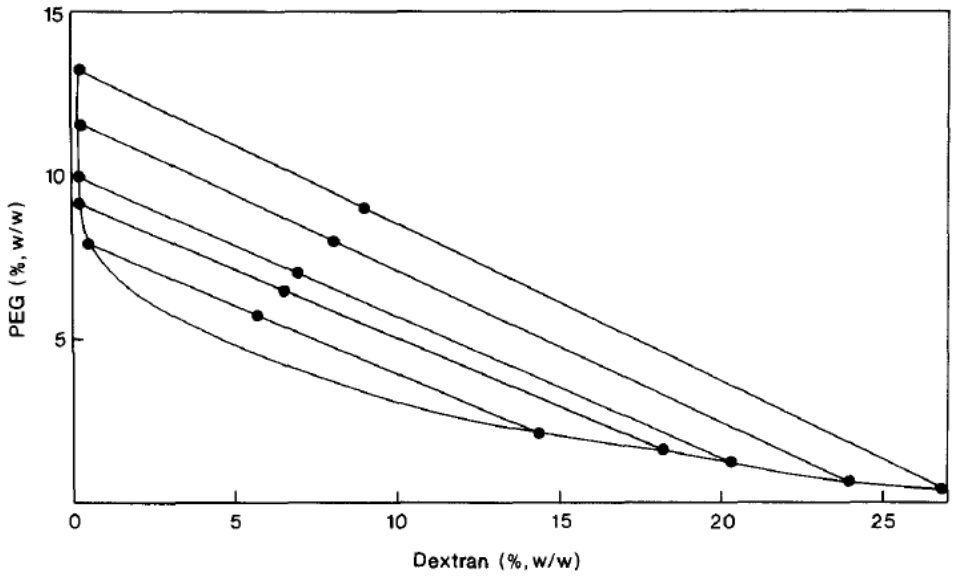


Figure 1.3: Phase diagram for the dextran T500/PEG 3350/water system at 0 °C. [From Harry Walter and Gote Johansson. Aqueous two-phase systems. *Methods in Enzymology.* 228, 5 (1994)].

Considering the advantages of the extraction such as, decrease in the process time, input energy saving (Kroner *et al.,* 1982) many enzymes have been purified using ATPE like

peroxidases from horse radish (Miranda *et al*., 1994) and leaves of *Ipomea palmetto* (Srinivas *et al*., 2000), lipase (Menge *et al*., 1992), beta-galactosidase (Veide *et al*., 1983) and amylo-glycosidase (Tanuja *et al*., 1997).

Organism Name	Protein	Reference
Bacteria		
Bacillus subtilis	Alpha-amylase	(Andersson *et al*., 1985)
Escherichia coli	Cellulase; L-Asparginase	(Ramadas *et al*., 1996)
Fungi		
Aspergillus niger	L-Asparaginase	(Jiang *et al*., 1999)
Plants		
Radish	Horse-raddish peroxidase	(Miranda *et al*., 1994)
Animals		
*Rabbit (*muscle)	Lactate-dehydrogenase	(Johansson *et al*., 1987; Johansson *et al*., 1986)
*Porcine (*kidney)	d-Amino-acid-oxidase	(Boland *et al*., 1989)
*Bovine (*liver)	Catalse	(Boland *et al*., 1989; Tjerneld *et al*., 1986).

Table 1.1: Proteins extracted by ATPS from different sources.

The potential of ATPE for small molecular weight biomolecules like amino acids have been demonstrated. The amino acids and peptides are much simpler than the larger protein molecules. The partition of the amino acids by ATPE, helped in understanding the fundamental thermodynamics of the partition of more complex proteins (Diamond *et al*., 1989; Zaslavsky *et al*., 1982) have concluded that the hydrophobic property of the protein is the determining factor for separation. Some amino acids like lysine, glutamic acid (Chu *et al*., 1990), tryptophane and phenylalanine have partitioned by ATPE (PEG/salt system).

The application of ATPE has attracted the most interest in biotechnology industry as a primary recovery operation for the isolation of proteins from crude feed stocks over conventional protein purification methods, such as membrane systems, chromatographic methods and centrifugation methods. The advantages include:

1. High bio-compatibility due to
    a. The fact that both the phases contain 85-95% water
    b. Very low interfacial tensions
    c. Phase components (polymers) contribute to protein stability by lowering the decomposition of labile biomolecules
2. Good resolution and yields
3. Easy to scale up
4. Volume reduction
5. Eco-friendly.

Even though ATPE has many advantages, some of the factors limited the use of ATPE for various applications. Some of the factors limiting the ATPE are as follows

1. High cost of phase forming polymer for large scale protein recovery
2. Slow demixing of the phases
3. Viscosity problems
4. Poor understanding of the mechanisms of partitioning

5. The lack of engineering design correlations
6. The reluctance of the biotechnology industry to incorporate novel techniques in protein purification.

1.3.3. Aqueous two-phase partition for the recovery of biological products

Aqueous two-phase systems provide a suitable environment to maintain biological activity and protein solubility (Walter, 1985)**.** Aqueous two-phase systems (ATPS) have been extensively exploited for the recovery of biological products to overcome the limitations of conventional organic-aqueous extraction, since both the phases contain more than 80% of water thus rendering a biocompatible environment for the biomolecules (Walter *et al.,* 1985; Albertsson, 1986; Zaslavsky, 1995). It has been established that ATPS are formed when combinations of hydrophilic solutes (polymers or polymer and certain salts) display incompatibility in aqueous solution above critical concentrations. In general, the research in ATPS can be divided into two major areas. The elucidation of the mechanistic molecular understanding of solute partitioning in ATPE is the main focus of one of the major areas of research, whilst the other is concerned with the practical implementation of the technique to process development. The area that addresses the molecular understanding of partitioning generally involves the use of model systems to establish and predict the behaviour of solutes in ATPS. For example characterization with the purified target product in an aqueous environment. Although, advances have been achieved with respect to solute partition in ATPE, additional knowledge is needed to fully understand the phenomena. On the other hand, it has been proved that the practical application of ATPE for the recovery of biological products from different sources [Table 1.1] generate robust, easy to scale-up and biocompatible

extraction processes. Such processes produce fractions from a variety of biological suspensions in a state suited for further purification and extraction of selected products (e.g. target enzymes). Development of ATPE process for the recovery of biological products most simply involves the design of extraction steps. A one-stage ATPS process is characterized by an extraction step that yields a bottom phase containing particles (cells or cell debris) and contaminants (e.g. RNA, carbohydrates, lipids) and a top polymer-rich phase containing the target product. The potential commercial value of the product is easily compromised by the relative high concentration of polymer in the top phase. Therefore, further processing of the top phase, for example ultra-filtration is required to remove the polymer from this stage. In case of a two-stage ATPS process, the first extraction eliminates the bottom phase particles and contaminants from the feedstock and generates a top phase enriched in the target soluble product. In the second extraction stage (back extraction), the product of interest is partitioned to a bottom salt-rich phase which enables re-use of the polymer-rich top phase. Further processing of the bottom phase by ultra-filtration yields a product concentrate. The practical application of aqueous two-phase partition for process development has been exploited for the recovery of biological products for more than 30 years. Unfortunately, this has not resulted in an extensive use of the technique in commercial processes (Hart *et al*., 1994)**.** This is attributed to several factors including the cost of phase forming polymers, a lack of knowledge of the technique, poor understanding of the mechanism governing phase formation and solute partition. Although, successful exploitation of ATPS for the recovery of desired products have been proved, the factors which disadvantage the technique for commercial adoption, need to be addressed.

Critical analysis of reports (Alves *et al*., 2000; Andrews *et al*., 1996; Bim *et al*., 2000; Harris *et al*., 1997; Johansson *et al*., 1998; Marcos *et al*., 1998; Minami *et al*., 1998; Persson *et al*., 1998; Pietruszka *et al*., 2000; Rito-Palomares *et al*., 1998; 2000a; 2000b; Venancio *et al*., 1996; Walker *et al.,* 1996) dealing with the use of ATPE in biochemicals recovery exhibited that, the application of ATPS for the recovery of proteins has resulted in processes designed as primary purification operations. Such processes have been characterized by single or multi-staged operations. From the total product recovery and economic view point, it is clear that the definition of one-stage ATPE primary recovery processes are preferred (affinity partitioning of proteins). Furthermore, the majority of multi-staged operation processes developed has exploited two-stage systems. In this context, three types of ATPS (i.e. polymer–salt, polymer–polymer and others) have been traditionally used. In the polymer–salt systems, polyethylene-glycol (PEG)–phosphate ATPS are commonly used due to several process advantages including: low cost and wide range of system pH (from 6 to 9) under which the ATPS are stable. In these extraction systems, the product of interest is concentrated in a phase that contains predominantly water and increased concentration of one of the phase forming components, which in the majority of the cases is PEG. As a representative example of the successful development of an extraction process that exploit ATPS in the extraction and purification of potato polyphenol oxidase (PPO) from the potato tuber (*Solanum tuberosum*) with partition coefficient (*"K"*) of 32.3, purification factor of 15.7 and a 97% yield of enzyme activity in the top phase (Bhalchandra *et al*., 2006)**.** Processing of an *Aspergillus niger* culture filtrate for the recovery of the extra-cellular enzyme β-

glucosidase had also resulted in a top phase with the protein concentrated up to 700 times (Johansson *et al*., 1998). The total product recovery for β-glucosidase was in the range of 85–95% with a concentration factor of 60–720 times. An additional case of the application of ATPS is the recovery of the recombinant apolipoprotein A1 expressed in *Escherichia coli* (Persson *et al*., 1998)**.** To process the filtrate from *E. coli* fermentation, thermoseparating polymers (ethylene oxide (EO) and propylene oxide (PO)) and starch were used. Apolipoprotein A1 was partitioned to the top EO–PO rich phase and the contaminant proteins to the bottom starch phase. The recoveries of the recombinant apolipoprotein were in the range of 85–90% with a purification factor of 2.5–2.7. Recently the non-optimized recovery of viral coat L1 protein (e.g. 65%) produced by recombinant E. *coli* was reported using a one-stage ATPS process (Rito-Palomares *et al*., 2002)**.**

Furthermore, the type of protein products that have been recovered with acceptable process yield (i.e. 65–100%) using ATPS varies from low to high-value proteins (e.g. BSA, pyruvate kinase, porcine insulin and apolipoprotein) which demonstrates the flexibility and potential generic application of this technique at bench scale. In this context, one of the major advantages of ATPS is their suitability to process suspensions with high concentration of biomass (up to 50% w/v) without compromising capacity or resolution. Furthermore, the complex nature of the biological sources (biological suspensions, fermentation broths, commercial sources, etc.) and the processes exploiting ATPS demonstrated the robustness and generic application of the technique. The success of ATPE in the efficient generation of bench-scale prototype processes with potential

commercial application has been proved by the existence of numerous reports dealing with the recovery of a large number of biological products. However, a very well known characteristic of this novel technique is the lack of large-scale ATPS as a part of downstream commercial processes. This may be attributed to the fact that the knowledge of the mechanism of solute partitioning in ATPS is limited. Consequently, industries show a reluctance to practice this technique at a process scale. Though reports dealing with commercial scale practice of ATPS are uncommon, it has been ably demonstrated at a 10 M^3 fermentation scale for the recovery of periplasmic IGF-I (Hart *et al*., 1994)**.**

The disadvantages of batch operations, the complications associated continuous mode of operation and the lack of suitable process equipment may explain the absence of ATPS being practiced at commercial scale. Furthermore, an important limitation of this technique is the absence of "commercial kits" (as in the case of conventional technologies), that facilitate the evaluation of ATPS processes at bench-scale. Consequently the process developments mostly rely on "in house" designs, which may raise issues of process reproducibility and robustness. This is an important aspect of the technique that needs to be addressed. Additionally, the relative high need for chemicals to form working ATPS has saddled this technique with an unfavorable economic image (Rito-Palomares *et al*., 2000a; 2000b)**.** Thus implementation of ATPS at process scale not only depends on technical feasibility, but also on process economics.

Practical strategies to design ATPE processes are needed to overcome the poor understanding of the molecular mechanism governing the behaviour of solute in ATPS. A practical approach favours the predictive design of extraction stages using this technique. Traditionally in ATPS process development, for each extraction process, operating conditions need to be empirically established. The application of practical strategies minimizes the necessary time for the design of ATPS extraction stages. As an initial step, general process conditions can be selected based upon experience in the partitioning of solute in ATPS. However, the accumulation of certain experience in ATPE represents a major disadvantage for the generic and wide application of this technique. It is clear that researchers interested in the use of ATPS need to become experts in the area before starting the design of defined experiments. Such a situation is explained by the lack of reports detailing the steps necessary for the application of ATPS.

Consequently, it is suggested that as a first step for process development, PEG–phosphate ATPS could be the phase of choice since, it is widely exploited and reported. It is necessary to augment the knowledge accumulated using these systems (Rito-Palomares *et al*., 2000; 2000b). Low molecular weight PEG (e.g. 1000 or 1450 Da) is initially preferred to concentrate the majority of cells, debris and contaminants in the lower phase. Once the general selection of the ATPS has achieved, a phase diagram is needed. This can be constructed using the cloud point method (Hustedt *et al*., 1985) or it can be obtained from other reports (Rito-Palomares *et al*., 2000)**.** The phase diagram is essential for evaluating the influence of system parameters (i.e. tie line length (TLL), phase

volume ratio (Vr) and pH) upon recovery of the target product and to define the operating conditions for the ATPE process.

Extraction usually comes in the purification process for a bioproduct and would precede a high resolution step such as chromatography. Proteins are generally obtained in very dilute solutions. Hence, the first step in their recovery is usually the reduction of large volumes. An aqueous two phase extraction can be used to concentrate samples by choosing the specific phase composition. A single or multi-step procedure may be used depending on the partition characters of the desired and undesired substances (Kaul *et al*., (2000).

Traditionally, purification is done in steps involving clarification using solid/liquid separation techniques like centrifugation or filtration, followed by concentration. ATPE has attracted interest mainly in the recovery and isolation of proteins from crude feed stock. Pilot-scale studies of the PEG/salt systems for extraction of enzymes (superoxide desmutase) from bovine liver tissue have been carried out (Closs *et al*., 1998). Aqueous two phase extraction (ATPE) has been recognized as an economical and efficient down stream process method and offers many advantages, such as low process time, low energy consumption and a biocompatible environment (Srinivas *et al*., 1999).

1.3.4. Selection of phase components for ATPE

Initially PEG-dextran systems were the materials of choice for ATPS due to their desirable physical properties and non-toxicity (Kula *et al*., 1982). A major limitation in

such systems with respect to industrial scale operations were the high cost, high viscosity and desired molecular weight of dextran. This favored the choice of PEG/salt system for industrial use. These systems also have the advantage that the phases have a lower viscosity, thus needing a shorter time for phase separation. Depending on the nature of the feed, as much as 50-90% PEG recycling has been achieved by repeated use of the final polymer phase that remains after the second extraction of protein into the salt rich bottom phase (Van-Berlo *et al*., 1998)**.** Processing of PEG phase, prior to recycling, increases the cost, therefore it is avoided. Different approaches for clean up of PEG phase have been de-proteination, desalting by ultra-filtration and extraction of PEG from the phase with an organic solvent followed by evaporation.

Many efforts have been put into replacement for the fractionated dextran by less expensive polymers such as crude dextran, starches, maltodextrins or pullulans as the bottom phase components (Hustedt *et al*., 1985; Sikdar *et al*., 1991) and galactomannan composed polysaccharides like guar gum (Simonet *et al*., 2000) and xanthan (Chethana *et al*., 2006).

1.4. Phase systems

1.4.1. Polymer/Polymer/Water systems

The most thoroughly studied polymer–polymer is the PEG-Dextran system (Albertson, 1986; Walter *et al*., 1994). In these systems both the phases contain 70 to 80% water. The polymers (PEG & Dextran) used in this systems have different molecular weights are water soluble, well characterized and commercially available. PEG-Dextran systems have

been used for a variety of applications based on mechanisms that determine the partition of biomolecules in ATPS (Albertsson, 1986; Zaslavsky, 1992). Initially PEG-Dextran systems were used to isolate cell membranes, organelles from chloroplasts and mainly in protein partitioning. PEG-Dextran systems have also been used for the purification of biological material on the laboratory scale. In biochemistry and cell biology, ATPE is a standard tool for plasma membrane and nucleic acid preparation (Larsson, 1994).

The PEG-dextran systems are suitable for study of salt effects, hydrophobicity in phase partitioning and have been used as an analytical tool for study of protein surfaces (Berggren *et al*., 2000a). This system has not been used on a large scale due to high cost of dextran. To introduce ATPS on a large scale, crude dextran has been used (Kroner *et al.,* 1982a). The results showed a nearly identical partitioning in the systems containing crude dextran compared with fractioned dextran. Many researchers tried to replace dextran in ATPS with cheap polymers e.g. Starch polymers like HP-Starch, CM-Cellulose, Ficoll etc (Sturesson *et al*., 1990; Tjerneld *et al*., 1986). HP-Starch was used as a model cheap polymer in a large scale application where it was shown to be suitable substitute for dextran. The partition behaviour of cutinase, α-lactalbumin and β-lactalbumin (bovine whey) with PEG-HP starch aqueous two phase system have shown encouraging results (Ortin *et al*., 1991). The HP-starch used in extraction of cutinase is purified and not very economical. Efforts have also been put into replacement of the fractionated dextran by less expensive polymers, such as crude dextran, starches, maltodextrins or pullulans as the bottom phase component, which greatly reduces the processing cost (Hustedt *et al*., 1985; Sikdar *et al*., 1991).

Low cost of the chemicals that form ATPS has become the dominant factor for large scale protein recovery with removal of cell debris. Hence, the use of inexpensive phase components and their recycling is vital to this process. It is equally important that these systems have lower viscosity and shorter de-mixing times to ensure good phase separation. Earlier reports have dealt almost exclusively with purified carbohydrates or their derivatives and have neglected the large amount of gums and carbohydrates already used in the paper, food or textile industries. Thus aqueous two phase polymer systems based on such commercial polymers may have a disadvantage because of the lower degree of purity as compared with purified phase polymers (Venancio *et al*., 1993). The dextran polymer can be replaced with the very hydrophilic synthetic polymeric materials like polyvinyl alcohol, polyvinyl pyrrolidone and polyacrylate as the bottom phase forming polymer (Johansson *et al*., 2008; Albersson, 1958; Saravanan *et al*., 2006) to create an environment for biomolecules that does not cause their denaturation. The advantage with polyacrylates is that they can interact strongly with proteins having a net positive charge (Chenming *et al*., 2005).

Ficoll is a neutral, highly branched, hydrophilic polysaccharide with a high molecular mass, which dissolves readily in aqueous solutions. The molecular radius of Ficoll range from 2-7nm. It is prepared by reaction of sucrose with epichlohydrin (poly sucrose polymer). The PEG-Ficoll polymer phase system is one that has been over looked in the past for biotechnology applications. In the system PEG 6000-Ficoll, up to 90% of the amylase and plasmid DNA was retained in the bottom phase (Viara *et al.,* 2001). Ficoll is

a highly polydisperse showing different peaks when subjected to gel permeation chromatography (GPC). As a result, the PEG-Ficoll system is blurred and the binodials obtained through turbidometric titrations differ significantly. Therefore PEG-Ficoll systems don't show model predictable behavior which prevents its use as a system for purification of specific biomolecules (Nielsen *et al.,* 2003).

The PEG polymer can be replaced with a random ethylene oxide propylene oxide (EOPO) copolymer as the top phase forming polymer (Harris *et al*., 1991). The EOPO polymer has thermoseparating properties, i.e. over a certain temperature; the polymer phase separates in to a water phase and a dense polymer phase. The advantage with EOPO polymer is that the protein can be partitioned to an almost polymer free water phase that facilitates interaction with subsequent purification techniques (Alred *et al., 1993*; Harris *et al*., 1991; Pesson *et al*., 1998; 1999; Cecillia *et al*., 2004). The EOPO-Dextran and PEG-Dextran phase diagrams are similar in their protein partitioning behavior (Berggren *et al*., 1999; Colleen *et al*., 2001b). Another advantage of the EO & PO polymers is by varying their subunits the temperature for phase separation can also be modulated (Persson *et al*., 2000b)**.** This latter factor can be applied when different thermostable proteins are partitioned. The bottom phase forming dextran can be replaced with a low cost hydroxypropyl starch or amylopectins which lowers the cost significantly on a larger scale (Person *et al.,* 1998; Sturresson *et al.,* 1990; Tjernald *et al*., 1986).

1.4.2. Polymer/Salt/Water systems

PEG/Salt/Water is a very well defined phase system. Such systems are generally based on salting out of the polymer. Many polymer/salt/water systems were developed by different researchers from different laboratories throughout the world. For industrial applications, polymer/salt systems are preferred over polymer/polymer systems because they are cost effective, easy to prepare and easy to handle. Furthermore, they require less time to demix (Fauquez *et al.*, 1985). The polymer/salt type ATPS are suited for handling soluble as well as insoluble matter probably due to their relatively high values of interfacial tension (0.1 to1.0 dyne/cm). The suitability of polymer/salt type ATPS for primary separation has been demonstrated on a pilot scale for recovery of biomolecules (Kroner *et al*., 1982b; Kroner *et al.,* 1982; Stranderg *et al.,* 1991; Veide *et al.,* 1983; Kepka *et al.,* 2003). It is often observed that low molecular weight substances partition fairly evenly between the phases. However weak interactions between one of the phase forming polymers and the partitioned material can cause an uneven partitioning. Polymer-salt systems have substantial difference between the physical properties of the individual phase components whereby the partitioning of biomolecules is unequal and favours one of the phases.

Different salts give different phase diagrams. The difference in phase diagram is due to the larger differences in density, greater selectivity and lower viscosity. These systems have considerable advantages over the aqueous polymer-polymer systems (Persson *et al*., 2000b; Tubio *et al*., 2006). The phase partitioning in homogeneous mixtures containing a polyelectrolytes depends highly on both the ionic strength and type of ions present.

Different salts in the phase system cause "*K*" values to be either increased or deceased. The order in which different ions affect partitioning in a Dextran-PEG system is indicated [Table 1.2] e.g. the target protein is isolated in the top PEG rich phase while contaminants such as host cell DNA and other proteins are discarded to the bottom phase.

The protein partition coefficient, K, can be written as the product of two factors, K0 and Kz. K0 is related to the relative solvation of the protein in the phases. K z depends on the net charge, Z, of the protein molecule and its interaction with the phases, which depends on the electrolytes present. This relation is expressed in Eq. [10]:

$$K = Ko\ Kz \qquad [10]$$

but is often used in its logarithmic form [Eq. 12]:

$$\log K = \log K_0 + \log K_z \qquad [12]$$

Furthermore,

$$\log Kz = \gamma Z$$

where γ, is a factor determined by the types and concentrations of polymers used, the types of electrolytes and their concentrations, and the temperature. It should be noted that the K_0 value may vary with the pH of the system. With certain salts, and depending on the polymer type and temperature of the phase system with y = 0, the charge dependence of partitioning is eliminated and the K observed for the protein is equal to its K_0 value. It has been shown that different inorganic salts like potassium phosphate, ammonium sulphate (Albertsson, 1986), sodium phosphate, sodium sulphate, potassium citrate

(Vernau *et al*., 1990) and $MgSO_4$ (Rasa *et al*., 2008) are capable of forming phases along with polymers.

Ions	
Anion	**Specific Values (γ^-)**
$H_2PO_4^-/HPO_4^{2-}$	**79**
SO_4^-	**77**
F -	**69**
Acetate	**56**
Cl^-	**50**
Br^-	**44**
I^-	**34**
SCN^-	**30**
ClO_4^-	**28**
Cations	**Specific Values (γ^+)**
K^+	**58**
Na^+	**56**
NH_4^+	**49**
Li^+	**43**
$(C_2H_5)_4N^+$	**32**
$(C4H_9)_4N^+$	**06**

Table 1.2: Specific Values, γ+ and, γ- ,for anion and cation for calculation of relative, γ values [From Harry Walter and Gote Johansson. Aqueous Two-Phase Systems. Methods in Enzymology 228, 32 (1994)].

The protein of choice normally partitions towards to PEG-rich top phase, from where the target protein can be back extracted into the fresh salt phase. This makes the system suitable for large scale extractions e.g. PEG-salt systems have been used for the

purification of metal ions (Rogers *et al*., 1996) and metallo-proteins such as hemoglobin (Albertsson, 1986). The drawback of these systems is high concentrations of PEG in the top phase and salt in the bottom phase which creates problems in susequent purification steps (Harry, 1994; Kula *et al*., 1999). These aqueous PEG-salt mixtures have the disadvantage of low solubility for amphiphilic proteins, which have a high tendency to aggregate in presence of high salt concentrations (Haghtalab *et al*., 2004).

Affinity ligands, using electrostatic interactions are not efficient in PEG-Salt systems. The high salt concentration in the solution will shield electrostatic interaction between the target molecule and the affinity ligand (Johansson *et al*., 1983) e.g. affinity bound target protein phosphofructokinase can be back extracted from a PEG-ligand phase into a PEG-Dextran system by extraction with a salt phase. However the problems can be solved by derivatization of PEG, which has affinity to proteins and can form a two phase system with the chosen salts e.g. PEG-Benzoate-Sodium sulphate and PEG-Phenylacetamide-Sodium sulphate systems were used for aqueous two phase affinity partitioning of penicillin acylase from *E. coli* into the top phase (Mahesh *et al*., 2003).

1.4.3. Surfactant based aqueous two phase system

Recently detergents and detergents with reversed solubility were considered as a possible means of separation of macro-molecules especially for the separation of hydrophobic proteins. One such detergent used relates to a nonionic surfactant which has been described as an alternative to standard polymer-polymer and polymer–salt systems. The phase forming surfactants used are polyethylene base pentaethylene-glycol mono n-

dodecyl ether ($C_{12}EO_5$), Triton X-114, Triton X-305 and Triton X-405 (Braunstein *et al.,* 2000). The basis of such type of ATPS is the temperature dependant and reversible hydration of the polar ethylene oxide head groups. The temperature at which the phase separation occurs is referred to as the cloud-point and is suited for the extraction of amphiphilic biomolecules. The potential of such type of two phase systems for separating membrane bound proteins from cytosolic and peripheral membrane proteins was first demonstrated by Bordier *et al*. (Bordier *et al*., 1981). The selective extraction of hydrophobic substances is because of lamellar structures formed in the miscibility groups of polyglycerol ether-water systems (Heusch *et al*., 1988). Detergent based ATPS have several specific properties e.g., extreme small density differences between the two liquid phases (0.003-0.005g/cm^3), low interfacial tensions (5-10μN/m) and complex rheological behavior of the product contained by the detergent rich phase, which makes processing difficult (Kula *et al*., 1999). Scale-up of detergent based systems has proved to be successful in extraction of cholestrol oxidase from *N. rhodochrous* (Minuth *et al*., 1997) and peptide tagged cutinase from whole broth of recombinant *Saccharomyces cerrvisiae* expressing cutinase variants carrying a (wp) 4tag (Rodenbrock *et al*., 2000).

When a desired fermentation product is to be recovered by this extraction process, a specific surfactant is selected on the basis of "Hydrophilic Lypophilic Balance" (HLB) values of least 15. The HLB is a quantitative measure of the overall hydrophilic nature of a given surfactant with higher values of HLB for increasing hydrophilicity of surfactant. This is a term can be calculated as follows.

The HLB for a nonionic polyhydric alcohol fatty acid is defined as:

$$HLB = 20(1-S/A)$$

Where S = Saponification number of the ester = mg KOH to neutralize 1 g fat.

A= acid number of the acid = mg KOH to neutralize 1 g acid or

$$HLB = (E+P)/5$$

Where E = weight percentage of oxyethylene content

P=weight percentage of polyhydric alcohol content

This has been extended to other hydrophilic and hydrophobic groups [Table 1.3].

HLB = 7 + sum (*hydrophilic + hydrophobic group numbers*)

Group	Group Number
Hydrophilic	
--SO_4 Na	38.7
--COOK	21.1
--sulfonate	11.0
--sorbitan ester	6.8
--free ester	2.4
--COOH	2.1
--OH	1.9
ether	1.3
tertiary amine	9.4
Hydrophobic	
-CH-,-CH_2-,CH_3-,-CH-	0.47
derived	
-(CH_2--CH_2--O)--	0.33
-(CH_2--CH_2--CH_2--O)-	-0.15

Table 1.3: Group numbers of different hydrophilic and hydrophobic groups used to derive the HLB values (Table from United States Patent 6105786 August 22, 2000).

where as the hydrophilic nature of the desired product increases the surfactant selected may require an increased HLB (>15) to maximize the yield. Generally surfactants of higher HLB will increase extraction yields for most proteins but will also result in less selective extraction e.g. recovery of alkaline protease (hydrophilic enzyme) needs a surfactant having HLB x 12.

1.5. Properties effecting the partitioning

Choice of polymer strongly influences phase formation in ATPS. Besides, several other factors like polymer concentration, polymer molecular weight, polydispersity of the polymer, pH, salt, hydrophobicity, viscosity, density and temperature affect the formation of immiscible phases (Albertsson, 1986). However, the composition of the systems can be modified to affect the partitioning. There is no specific single mechanism that has been clearly understood to influence phase formation and partitioning.

1.5.1. Effect of pH

Generally protein partitioning depends on the pH of the system. The variation of the *"K"* value of a protein in a specific system over a pH range is influenced by the ionic composition. At specific pH the net charge of the protein is zero, this is known as isoelectric point (PI). A protein has no net charge at its isoelectric point (pI), but if the pH is increased or decreased the net charge of the protein will become negative or positive respectively. This net charge can be used to direct the protein partitioning in the system. It has been shown that at pH values very close to pI, a protein partition coefficient in a system is the same irrespective of the salt used (Albertson *et al*., 1970; Johnsson *et al*.,

1970; 1974). The change in partition coefficient with change in pH is because of conformational changes in the protein thereby causing its denaturation. The denatured molecule has greater surface area in the phase environment where hydrophobic sites are exposed (Walter *et al*., 1985).

Addition of charge along with change in pH is often used to alter the partition of biomolecules. The *"K"* of a biomolecules varies exponentially with the electrochemical potential difference between the phases and the net charge of the partition biomolecules. This is postulated as follows

$$InK = InK_0 + \frac{FZ\Delta\Phi}{RT} \qquad [13]$$

where K_0 is the value of this coefficient in the absence of an electrochemical potential, "*F"* is the Faradays constant, "*Z*" is the charge on the biomolecules, "*T*" is the temperature and "*ΔΦ*" is the potential created by the salt in the system. A plot of In *"K"* verses the charge *Z* should be a straight line where slope is proportional to *ΔΦ*. However a change in pH may also affect the value of *ΔΦ*, the composition of the phases and the interactions between the ions of the salt. Therefore deviation from the linear behavior is significant (Gunduz *et al*., 2000a; Walter *et al*., 1994; Georgina *et al*., 2007). A strong diminution of porcine pancreatic lipase's (PPL) "*K*" value is observed depending upon PEG molecular mass as the pH increases from 6 to 8. At a pH close to the isoelectric point (pI) of the PPL (pI-5.18), the hydrophobic component (K_0) is greater than the

electrostatic forces. Thereby PEG of low molecular mass interacts with the PPL showing decrease in the partition coefficient "*K*".

1.5.2. Effect of salt concentration

The addition of salts influences the partition of proteins. This change in partitioning is mainly due to the unequal partitioning of ions between the phases thereby giving rise to an electrical potential difference between the phases and proteins, where partitioning depends upon the net charge (Johnsson *et al*., 1970; 1974; Albertson, 1986). This effect has been used in many studies to modify the partition coefficient value of a desired protein in favor of top or the bottom phases in ATPS. Partition coefficient is increased quite dramatically when higher concentrations of NaCl are added to ATPS. This effect has been attributed to a possible hydrophobic interaction.

Schimidt *et al*., 1994, have used NaCl 0.2-2M w/w concentration to induce partitioning of amylase in favor of the polyethylene glycol rich phase and thus to increase the amylase recovery. Myoglobin was partitioned close to its pI value and was thus affected very little by the change in salt composition. The most prominent effects were observed with β-galactosidase and BSA partitioned to the upper phase and lysozyme to the lower phase by the ClO_4^- and I^- ions (Min *et al*., 1995). The change in value of "*K"* is a function of the different salts used in the ATPS. Partition coefficient of phycoerythrin in a PEG-Dextran system in 0.005M potassium phosphate at pH 7 with different electrolytes (NaCl, KCL, cesium chloride, NH_4Cl, LiCl, KBr, ammonium sulphite, sodium sulphate, ammonium nitrate, sodium nitrate, sodium citrate, ammonium acetate) has been reported by

Albertsson (Albertsson, 1986). Ammonium sulphate or citrate had little effect on the value of "*K"* while potassium chloride and sodium nitrate reduced the "*K*" values that caused protein to migrate to the bottom phase. This demonstrates the ability of the different salts to change the value of "*K"*. Depending upon the charge on the partitioned substance, the *"K"* value will decrease or increase by salt addition according to the Hofmeister series [Figure 1.4] (Baldwin, 1996).

$$\mathbf{PO_4^{3-} > SO_4^{2-} > Cl^- > Br^- > NO_3^- > ClO_4^- > I^- > SCN^- > Mg^{2+} >}$$

$$\mathbf{Li^+ > Na^+ > K^+ > NH_4^+}$$

Figure 1.4: Hofmeister series for anions and cations.

Ions with low charge density e.g. ClO_4^- will partition more strongly to the more hydrophobic phase than ions with high charge density e.g. SO_4^{2-}. A specific salt will dominate the phase separation if its concentration is 10 times higher than the other salts present. The dominating salt will determine the protein partition while the influence of the other salts will be negligible (Johansson *et al*., 1974). The alternate way to introduce charges into the system is to use polymers as carriers for charged groups, for instance poly (N–Isopropylacrylamide vinyl imadizole (NIPAM) copolymers are positively charged at a pH below 7.0. Thus the partitioning of a protein in a system containing NIPAM-VI can be altered by simply changing the pH of the system (Person *et al*., 2000).

When the non-polar groups of a protein are embedded in the interior of the molecule, there is no contact with surrounding solution. Under such circumstances, no change in

partition coefficient *"K"* is observed (Baskir *et al*., 1989). Small change in surface properties can be used to effect separations which otherwise may not be recognized by a specific ligand e.g. palmitate–PEG is used to detect and magnify partitioning based on confirmational changes in human α-2-macroglobulin. The partition behavior is also sensitive to the phase system properties (Polymers and Salt concentration), nature of biomolecules and surface charge.

1.5.3. Surface properties

It is well known that the protein structure and surface influences the partitioning between the phases (Albertsson, 1986). Protein surface alterations can be monitored by partitioning in aqueous two phase systems. This method has proved to be sensitive in the detection of protein association and dissociation phenomenon (Middaugh *et al*., 1980). In general it has been observed that biomolecules behave differently when they are in contact with solution. Globular proteins are composed of both polar and non-polar amino acids. The majority of the polar groups are exposed on the exterior surface of the particles which are in contact with charged side groups in the surrounding solution (Cantor *et al*., 1980). Protein structure can be changed by adding a denaturing agent e.g. urea (Creignton *et al*., 1993) or pH / ligand induced structure changes in proteins (Ichikuwa *et al*., 1988; Hansen *et al*., 1988) e.g. Ramsch showed that by adding different concentrations of urea to a system composed of PEG and Na_2SO_4, the structure of T4 lysozyme mutants was changed and hydrophobic amino acids were exposed, thereby increasing the partition coefficient of the protein (Ramsch *et al.,* 2000). Furthermore, increase in partitioning coefficients of chymotrypsin inhibitor 2 (CI-2) is related to confirmation change from a

folded to an unfolded state at lower pH values (Umakashi *et al*., 2000). The electrostatic potential difference (ΔΨ), caused by an uneven distribution of ions, such as phosphate ions, clearly reveals sensitivity of ATPS to ΔΨ. Thus in the case of positively charged liposomes prefer the bottom dextran rich phase which is negatively charged.

1.5.4. Biomaterial concentration and size

In general, partitioning is not affected by concentration of the biomolecules in the ATPs. However at higher concentrations of individual or a mixture of biomolecules, some of the properties may change due to aggregation and precipitation. This small change could be induced to increased concentration of polymer or salt. With high concentration of BSA the partition isotherm is linear up to about 5% serum albumin in the bottom phase and deviates only slightly at higher concentration with Dextran-48/PEG 6000 in presence of Na_2HPO_4 or NaCl (Albertsson, 1986).

Small change in the partition coefficient also occur with the change of polymer in ATPS. Increase in BSA concentration at lower range (2-18 mg per 100g) in the above phase system results in increase of protein concentration in the top phase. However, further addition of protein to the phase did not result in further increase in protein concentration in the top phase (Chetana *et al*., 2006).

Albertson (1986) observed differences in protein partitioning as a function of molecular weight of polymer. The difference was small for small proteins (10000 Dalton), but increased substantially for larger proteins (up to molecular weight of 2500000 Dalton).

Even though no strict correlation has been found between the molecular weight of protein and their *"K"* values in Dextran–PEG systems, the *"K"* value are often smaller and larger for proteins having small and large molecular weights respectively. Sasakawa *et al*., 1972 observed such a correlation of relationship between *"K"* and molecular weights for papain, trypsin, α-amylase, BSA and β-Galactosidase. The partitioning of cells and cell homogenate is also of interest because proteins migrate to one of the two phases based on their molecular size; however the cells generally partition either almost entirely to one of the phases or at the interfaces. Thus it is possible to combine the separation of soluble biomolecules from the cells/cell debris thereby reducing the number of unit operations (Abbot *et al*., 1990).

1.5.5. Hydrophobicity of polymers

In a polymer-polymer two phase system a correlation exists for difference in hydrophobicity between the two polymers and their tendency towards phase separation. Pairing of hydrophobic polymer with a hydrophilic polymer or a salt solution will lead to faster phase separation. The partition coefficient of protein in PEG-Dextran-Water system is strongly influenced by the addition of hydrophobic PEG derivatives (Shanbhag *et al* ., 1974; Shanbhug *et al*., 1975; Axelsson *et al*., 1978; Shanbhag *et al* ., (1979); Johansson *et al*., 1984) e.g PEG esters (palmitate, deoxycholate) of fatty acids containing 2 to 20 carbon atoms. The presence of hydrophobic groups on a polymer system will improve the partition of molecules with hydrophobic sites (Banik *et al*., 2003). Shanbhag and Axelsson measured the partition coefficient for a series of proteins (bovine serum, albumin, beta lactoglobulin, hemoglobulin and cytochrome C) in a PEG-Dextran system

containing PEG bound fatty acids. It was observed that differences in partition coefficients (ΔK) were greater for each of the above proteins when fatty acid chain length was longer than eight carbon atoms. The electrical effects are multiplied by changing the salt composition of the phase whereby the difference in electrical potential of phases becomes zero (Diamond *et al*., 1992). Recently lactate dehydrogenase (LDH) when fused with hydrophilic penta-peptides, were partitioned in a hydrophobic EO30PO70-Dextran system (Fexby *et al*., 2004).

1.5.6. Polydispersity of polymers

Polydispersity is a measure of the distribution of molecular mass in a given polymer solution. The distribution of individual molecular masses in a batch of polymers is indicated by Polydispersity index (PDI) .The PDI values are always greater than 1. The polymers used in the study of phase equilibrium in polymer solutions are not of single molecular weight (mono dispersed). The Flory-Huggins theory for a mono dispersed polymer system in a solvent was first extended to polydisperse polymer systems by Scott (1945). Phase separation in solution containing polydisperse macromolecules affects their phase diagrams, but not always sharp change from a single phase to a two phase system. There is also a sharp change from a one phase system to a two phase system with polydispersed polymers. This is expected at the critical composition, where the two phases theoretically become equal and no polymer fractionation takes place (Koningsveld & Staverman, 1968).

It has been experimentally observed that in a liquid–liquid equilibrium of two polymer systems, the molecular weight distributions of the same polymers in the different phases can differ significantly from each other and from that of the initial feed (Lau *et al*., 1986).

Starch and cellulose always remain in the same phase while others such as chlorella or erythrocytes, either change phase or become adsorbed at the interface when the molecular weight of one polymer is changed. The model developed by Posses *et al*, successfully describes liquid–liquid phase equilibrium in aqueous two phase system and partitioning coefficient of biological molecules. It was observed that polydispercity hardly affects the calculation of the phase equilibrium but significantly affects the calculations of partition coefficients (Posses *et al*., 2004).

1.5.7. Effect of polymer molecular weight

The molecular weight of the polymer used influences the partition of proteins. Greater the molecular weight of the polymer, lower is the concentration required for phase separation. Larger the difference in molecular weight between two polymers, greater is the difference between the compositions of the two phases (Albertsson, 1986). This was confirmed by taking 16 different aqueous two phase PEG / dextran systems which were permutations and combinations of four PEG molecular weights (4000, 6000, 10000 and 20000) and the four dextran molecular weights (10000, 40000, 111000 and 500000) (Forciniti *et al*., 1991a). Increase in molecular weight of phase forming polymer will cause a protein to partition more towards the phase opposite to counter phase. This is used to adjust the partition coefficient, since “K” is affected by polymer concentration

(Albertsson *et al*., 1987). This is an entropic effect due to decrease in the total number of molecules in the phases with increase in molecular mass of polymer. Here the weight percentage of polymer is kept constant. It was shown that the "K" of pullulanase changed from 1.3 to 0.1, as PEG molecular weight was increased from 1000 to 40000 (Hustedt *et al*., 1978).

1.5.8. Effect of polymer concentration

The concentration of polymer in ATPS affects the partitioning of biomolecules. With increase in polymer concentration the partition coefficient of the biomolecules may increase or decrease depending on the properties of the molecule partitioned. An increase in polymer concentration usually favours the partition of cells at the interface or into the lower phase. This is ascribed to the increase in the number of molecules per unit weight of phase (Forciniti *et al*., 1991; Zaslavsk *et al*., 1995). Higher yield of chitinase in ATPS with 2% w/w PEG and 5% w/w dextran was obtained than with other systems having low concentrations of the polymer (Chen *et al*., 1994; 1995). The partition coefficient of C-Phycocyanin in PEG 4000-Potassium phosphate ATPS at pH 6 decreases with increase in polymer concentration (Ganapathi *et al*., 2007).

1.5.9. Influence of temperature

Partitioning of biomolecules in aqueous two phase polymer systems is dependent on the phase temperature (Albertsson, 1986). The temperature sensitivity is different for different types of two phase systems. Novel aqueous two phase systems have been developed which contain only one polymer to induce the phase separation (Johansson *et*

al., 1993; 1995; 1997). In this system a thermoseparating polymer was used (HMEOPE) i.e. a polymer which has a decreased solubility in water above a critical temperature. Two macroscopic phases (one polymer-enriched bottom phase and one water-rich top phase) can be obtained by heating the solution of the polymer, a few degrees above the cloud point. The cloud point is the temperature at which phase separation starts. At this temperature the solution becomes turbid or cloudy due to the formation of polymer rich emulsion droplets. The cloud point is dependent on the polymer concentration. The lowest cloud point is called the lower critical solution temperature (LCST) (Saeki *et al*., 1976). Among the two phase systems used for partitioning of biomaterials, the PEG-Dextran, Poly (Ethylene oxide)-Dextran, Ucon-Dextran [Ucon being a random copolymer of Poly (ethylene oxide)-Poly (propylene oxide)], EOPO-Starch and Water/HM-EOPO are the most frequent.

In the phase diagrams of these systems, the binodal curve will be lowered by decreasing temperature. In general, for those phase compositions far away from the critical point, temperature has a minimal effect on protein partitioning (Albertsson *et al*., 1986). However thermoseparating systems have stronger dependence on the temperature e.g. Apolipoprotein A1 could be back extracted from the HM-EOPO phase in to the new water phase by raising the temperature in water-HM EOPO aqueous two phase system (Johnsson *et al*., 1999).

1.6. ATPs for recombinant protein or extractive fermentation products

1.6.1. Affinity two phase partitioning

Conventional two phase systems often have a comparatively low selectivity for enrichment of biomolecules. As a result, two phase partitioning has low purification factors in batch procedures, unless the extraction steps are repeated several times. One way to increase the selectivity is to introduce affinity ligands. This process is generally known as affinity two phase partitioning. The general idea of affinity two phase partitioning is to partition the bulk of material (recombinant proteins from cell lysate or proteins from fermentation broth) into one of the phases and then to redistribute the material of interest into the other phase by interactions with an affinity ligand coupled to the phase polymer. PEG has been modified with hydrophobic ligands such as palmitic acid to extract human serum albumin in a PEG-dextran system (Shanbhag *et al*., 1974). The ligand can also be a compound that mimics the enzyme substrate or a coenzyme. Triazine dyes such as Cibacron blue or Procian yellow (mimics of coenzyme NAD^+) have been used to purify dehydrogenases and kinases (Biellmann *et al*., 1979; Kopperschläger *et al*., 1982; Nondek *et al*., 1984; Johansson *et al*., 1986). Inhibitors have also been used; thus P-amino benzamidine which is a strong inhibitor of trypsin when attached to PEG was able to extract trypsin using ATPS (Takerkart *et al*., 1974). Different types of erythrocytes have been separated in aqueous two phase system by using IgG coupled to PEG (Stocks *et al*., 1988; Sharp *et al*., 1986).

1.6.2. Affinity tags for protein partitioning

To make a partitioning procedure specific for a target protein, from a protein mixture, one approach used by researchers is to add affinity tags to the target protein by genetic engineering (Kohler *et al*., 1991; Collen *et al*., 2001; Rodenbrock *et al*., 2001). There are

many different affinity tags available like histidine tags, tryptophan tags, FlAG tag and streptavidine peptide tag (Hopp *et al*., 1998; Brizard *et al*., 1994; Keefe *et al*., 2001)**.** A tag consisting of 6 histidine residues is commonly used. The histidine tag can interact with nickel, copper, zinc or cobalt ions coupled to iminodiacetate or nitrilo-acetic acid attached to a matrix (Porath *et al*., 1975)**.** Histidine tagged proteins have been purified in ATPS by coupling metal ion binding groups to one of the polymer (Franco *et al*., 1997)**.**

Proteins can also be used as fusion tags. β-Galactosidase has been used as a fusion partner in ATPS due to its strong partitioning behavior in PEG rich phases. The strong partitioning of beta-galactosidase towards the PEG phase is believed to be due to its high tryptophan content. This is the genesis of tryptophan tags being used in aqueous two phase partitioning (Kohler *et al.,* 1991).

The partitioning depends on several factors including choice of ligand–carring polymer, its molecular weight and concentration of the phase polymer. The partitioning behavior of a particle complexed to a ligand–polymer conjugate is determined to a large extent by the partitioning of the conjugate itself (Kopperschlager *et al*., 2000). One way of increasing the partitioning of a particle–ligand complex would therefore be to increase the partitioning of the ligand-polymer conjugate concentration. The temperature has a significant effect on binding protein to a ligand and changing the phase composition. Different salt and their concentrations also have effect on the partitioning of target molecule. Johansson clearly revealed affinity partitioning of phospho-fructokinase in PEG-Dextran system at pH 7 (Johansson *et al.,* 1983). The pH also influences the

partition coefficient (K). This is true with triazine dye ligands, which bind mainly to proteins with decreasing pH. The significant effect has been observed in the affinity partitioning of colipase (Erlanson-Albertsson *et al*., 1980; Johansson *et al*., 1984; Birkenmeier *et al*., 1984).

1.7. New materials/Polymers in ATPS

Since 1956, ATPS based on either two incompatible polymers or one polymer and a salt in a aqueous solution have proved to be highly efficacious for the gentle separation of cell membranes and organelles from crude cell lysate, as well as for enzymes from protein mixtures or cell extract (Walter *et al.*, 1994; Agasoster *et al*., 1998; Albertsson, 1958; 1986; Gunduz *et al*., 2000; Palomares *et al*., 2004; Roobol-Boza *et al*., 2004). The mild process conditions and fast phase separation prevent protein denaturation or enzyme inactivation. However despite the versatile advantages of ATPS, the aqueous nature of these systems entails some drawbacks with respect to purification of industrially interesting enzymes and proteins.

There has been a continuous search for ATPS compositions with new low-cost polymers, solvents with a high preference for the target biomolecules and process methods for improving commercial viability.

1.7.1. Ionic liquids

Recently room temperature ionic liquids (IL) have received extensive attention for their use as green solvent and as possible replacements for traditional volatile organic solvents

(Nakashima *et al*., 2003; Zhou *et al*., 2003; Kim *et al*., 2003). Ionic liquid are liquids containing only ionic salts whose melting point is relatively low (below 100^{o}C). They are frequently colorless fluids with negligible vapor pressure, high solubilizing power and broad range of chemistry (Armstrong *et al*., 2001). There are few examples where ionic liquids are used in biological processes.

Examples:

1. [C_4 mim] BF_4 (1-butyl-1-3-methylimidazolium tetra-fluraborate (Yangyang Jiang *et al*., 2007).
2. [C_4 min] PF_6 (1-butyl-1-3-methylimidazolium hexa-fluraphosphate (Yangyang Jiang *et al*., 2007).
3. [C_4 min] C_1 (1-butyl-1-3-methylimidazolium chloride (He *et al*., 2005).
4. Ammoeng 110^{TM} (Oligo ethylene glycol).

Rogers and coworkers (Gutowski *et al*., 2003) reported that some hydrophilic ionic liquids form aqueous two phase system (IL-ATPS) when contacting with concentrated solution of water–structuring salts. With the aid of buffer salts IL-ATPS could extract various solutes into the ionic liquid rich phase efficiently (Gutowski *et al*., 2003). For example [C_4 mim] C1/K_2HPO_4 ionic liquid was used in IL-ATPS to separate testosterone and opium alkaloids (He *et al*., 2005; Li *et al*., 2005). Recently, Liu and coworker used a [C_4 min] BF_4/Na_2HPO_4 IL-ATPs to extract penicillin (Liu *et al*., 2006) and drugs, Papaverine and Morphine (Li *et al*., 2005).

The main advantage of IL-ATPs is that they are an environment friendly replacement for conventional solvents. Ionic liquids would prevent the emissions associated with volatile organic compounds, the major source of environmental pollution and minimize the water usage in the ATPS process. All IL's are not green. Some are extremely toxic but they can be designed to be environment friendly. The main drawback of IL-ATPs lies in the difficulty in recycling ionic liquids from the substrate containing aqueous phase.

1.7.2. Thermoseparating polymers

Many thermoseparating polymers are basically composed of ethylene oxide monomers (EO). The polymers differ in structure of monomer units, molecular mass, branching hydrophobicity and their physical properties. The thermo-separating copolymers are generally hydrophobic. The polymers can be homo, random, block or grafted polymers [Figure 1.5].

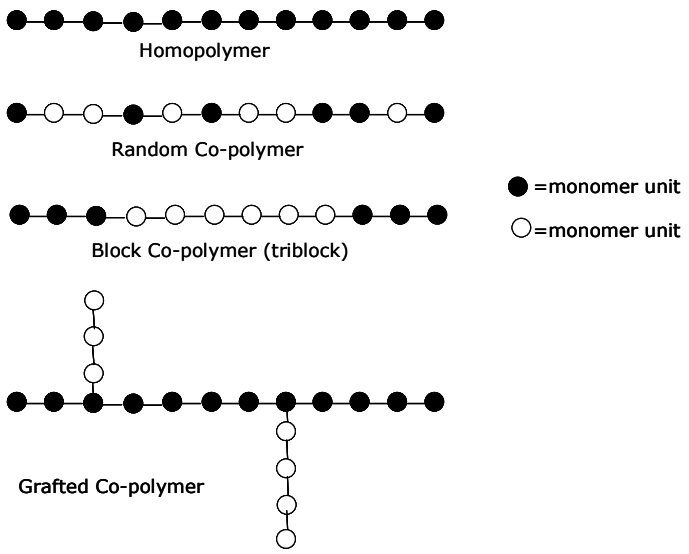


Figure 1.5: Schematic diagram of different types of polymers.

A thermo-separating polymer has an important property of decreasing solubility in water above a critical temperature. The polymer solution when heated a few degrees above the cloud point forms two macroscopic phases viz. one polymer–enriched bottom phase and one water rich top phase. At this temperature the solution become turbid or cloudy due to the formation of polymer-rich emulsion droplets. New aqueous two phase systems have been developed which contain only one polymer (thermoseparating polymer) and where no salt is necessary to induce the phase separation. In 1991, an ATPS was introduced containing linear thermoseparating EOPO copolymer and either dextran or a starch derivative (Harris *et al* 1991). Alred *et al*., 1994 and Berggren *et al*., 1995; 1999 reported the use of an EOPO copolymer with an equal weight percentage of EO and PO, EO50PO50 and dextran or hydroxylpropyl starch as phase forming polymers. Water solutions of thermoseparating copolymers upon heating above cloud point form two phases with water. The random copolymers of EOPO are those most often used in aqueous two phase systems (Alred *et al*., 1994; Berggren *et al*., 1995; 1999; Johansson *et al*., 1996; Person *et al*., 1998; 1999; 2000). Recently Bolognese *et al.,* (2005) have developed an ATPS composed of maltodextrin and copolymer of ethylene and propylene oxides for the partitioning of three proteins i.e. lysozyme, trypsin and BSA. In these systems the target protein partitions into the EOPO-rich phase of an ATPS system.

In Polymer-polymer ATPS systems difficulties are usually encountered in separating target molecules from the polymer solution. These systems would be more cost efficient if the polymer could be readily recycled without having to recourse to costly ultrafiltration or chromatographic steps for product recovery or phase purification. In

thermoseparating polymer ATPs, the proteins are quantitatively partitioned to the water phase and the copolymer can be recycled (Harris *et al.,* 199l). The thermoseparating ATPS (water/ EOPO) has also been used to study partitioning of amino acids and peptides (Johansson *et al*., 1995; 1997). Partitioning in these systems depends strongly on amino acids and peptide hydrophobicity, net charge and on salt added. The disadvantage of the water/EOPO system is that high concentration of EOPO polymer is not suitable for the partitioning of native globular proteins.

1.7.3. Plant polysaccharides

Initially ATPS technique was used for analytical work and phase systems composed of PEG/dextran and salt became widespread. However for work on large scale (10-1000 liters) the use of ATPS has been hampered by the high cost of dextran, unacceptably high viscosity of some polymers and high ionic strength which impose limitations on their use (Kroner *et al.,* 1984). There are several natural plant polysaccharide polymers that are used by many researchers around the globe as constituents of ATPS with and without PEG. The affordable price and bulk availability of starch, cellulose products and galactomannans has resulted in their use as polymer phases.

1.7.3.1. Starch polymers

Starches are composed of one or more α-glucan units. Starches are water soluble polymers that produce viscous dispersions and solutions at room temperature. The starches are mainly composed of amylase and amylopectin. Amylose is an essentially linear polysaccharide composed of (1-4) linked alpha-D-glucopyranosyl units with a

small amount of branching consisting of very short and very long branches. The amylopectin molecules have a branch-on-branch structure. They are composed of chains of (1-4) linked alpha-D-gluco pyranosyl units of various lengths joined via unequal spaced alpha-D (1-6) linkages (Whistler, 1993). The characteristics of a starch can be modified by chemical, physical and/or enzyme treatment to enhance or repress its intrinsic properties. This capability for modification has been a necessary factor in developing new applications for starch.

Ling *et al.,* 1989, initially demonstrated the use of modified starch in ATPS for large scale work. It had been previously shown that hydroxypropyl starch polymers (Reppel) are suitable for use in ATPS (Blennow *et al*., 1994; Miranda *et al*., 1997; Tjerneld *et al*., 1986; 1987; 1995). The starch polymers show the same behaviour as dextran in ATPS i.e. protein partitioning, salt effects and phase behaviour (Sturesson *et al*., 1990; Tjerneld *et al*., 1986). The main advantage of use on a preparative scale is the price of the polymer as exemplified by the introduction of modified starch, cellulose and polyacrylates on preparative scales (Hughes *et al*., 1998). As all these starch polymers are of technical grade and polydisperse in nature, they will not show distinct zones of separation in two phase systems. Therefore the two phase system is unstable at polymer concentrations close to the transition region. At higher polymer concentrations, the system is robust and the effect of batch variation is small.

1.7.3.2. Cellulose polymer

Cellulose is a linear natural homo-polysaccharide of D-glucose monomers with β(1-4) glycosidic linkages. Cellulose is chemically resistant and water insoluble. Cotton is an almost pure form of cellulose. Solubilization of cellulose by etherification (alkyl ethers), methylation (methylcellulose, hydroxyethyl cellulose) and other derivatives make it either hot or cold water soluble. Due to similarity between the dextran and cellulose with their physical and chemical composition, derivatized cellulose was a logical replacement to dextran in ATPS composed of PEG-Dextran systems. Albertsson successfully used methyl cellulose in polyvinylpyrrolidone/Methyl cellulose/hydroxylpropyl dextran, Na-Carboxymethyl cellulose/PEG/Salt and Na-Dextran sulfate/Na-Carboxymethyl cellulose systems (Albertsson, 1986).

Several factors influence the partition behaviour of proteins in two phase systems (Zaslavsky *et al.,* 1983). These include the properties of the phase forming polymer, the interactions of polymers with each other, salts and with the proteins. Introduction of charged polymers in the two phase systems creates a potential difference between the phases. The potential difference is dependent on the charge density of polymers. The electric potential difference between the phases should be comparably small as the partition of proteins is mainly dependent on other factors (Dissing *et al*., 1999). Initially poly (ethyleneimine) and hydroxyethyl cellulose were reported for partitioning of BSA, lactate dehydrogenase and myoglobin. In such ATPS systems pH and ionic species play an important part and affect the value of partition coefficient.

For increasing efficiency and selectivity, new ATPS are formed using block polymers L64, F68 or P105 and dextran (Svensson *et al.,* 1995). Skuseetal *et al.* used block polymers for the partition of proteins in systems formed by hydroxypropyl cellulose and P105 (Skuseetal *et al*., 1992).

1.7.3.3. Non-Cellulose polymers

Non-cellulosic polysaccharides like galactomannans, xyloglucans and glucurono-arabinoxylans are widespread components of the primary cell wall of many plants (Bacic *et al*., 1988). All galactomannans (GGMs) are characterized by a backbone of beta-D-Glcp-(1-4) and beta-D-Manp-(1-4) residues; the latter substituted at O-6 with D-Galp residues. Furthermore, recently small amounts of arabinose and xylose were shown to be present in GGMs, attached at O-6 of the -4)-beta-D-Manp-(1- redues e.g. Guar gum, locust bean, taragum and fenugreek gum.

The xyloglucan families of polysaccharides are major energy reserves in a range of seeds such as those of the tamarind tree (Kooiman *et al*., 1960; Meier *et al*., 1982). Tamarind seed powder also known as tamarind kernel powder (TKP) contain a polysaccharide composed of (1-4)-beta-D-glucan back bone substituted with side chains of alpha-D-xylopyranose and beta-D-galactopyranosyl-(1-2)-alpha-xylopyranose linked (1-6) to glucose residues.

The galactomannans (guar gum) and xyloglucan (TKP) are discussed in detail in chapter 2 and 6**.** This thesis mainly addresses the use of guar gum, derivatives of guar gum, TKP

powder, derivatives of TKP powder and their feasibility in formation of new aqueous two phase systems for protein partitioning.

Chapter 2

Partition of proteins in aqueous two phase system based on Hydroxypropyl-guar gum derivative-Dextran: Effect of salt and pH

2.1. Guar gum

2.1.1. General information

Guar gum is an edible natural polysaccharide which is extracted from the seeds of leguminious plants like *Cyanmposis tetragonolobus* and *Psoraloids* (Whistler, 1969). Guar crop is grown in India and Pakistan for centuries and is consumed by both humans and animals. In the south western United States of America, it is a cash crop grown with modern farm technologies where as in developing countries; it is still grown using hand labor. This plant needs very little surface water during its growing season; therefore it is grown in semi arid regions. Guar gum is produced from guar seeds. Guar seed is dicotyledonous, having a diameter of approximately 8mm. It consists of 14-17% hull, 43-47% germ and 35-42% endosperm. Pure guar gum is extracted from endosperm which separates the hull and embryo. Powdered endosperm is usually sold as guar gum. In 1976, the world wide consumption of guar products for food and industrial applications was estimated ~57million kilogram annually.

2.1.2. Chemical structure and physical properties

Guar gum is a carbohydrate polymer containing galactose and mannose as the structural building block. The ratio of the two components may vary slightly depending on the origin of the seed but the gum is generally considered to contain one galactose unit for every two units of mannose.

2.1.2.1. Structure

Guar gum is a linier chain of (1-4) linked beta-D-mannopyranose units, substituted by single unit side chains of alpha-D-galactopyranose (Dea *et al*., 1975). The alpha-D-galactopyranosyl units are linked (1-6) with the main chain [Figure 2.1] (Whistlera *et al*., 1951). In the pure polysaccharide guar, the ratio of D-galactose to D-mannose units is 1:2 to 1:6. The molecular weight of guar is approximately 2.28 x 10^5.

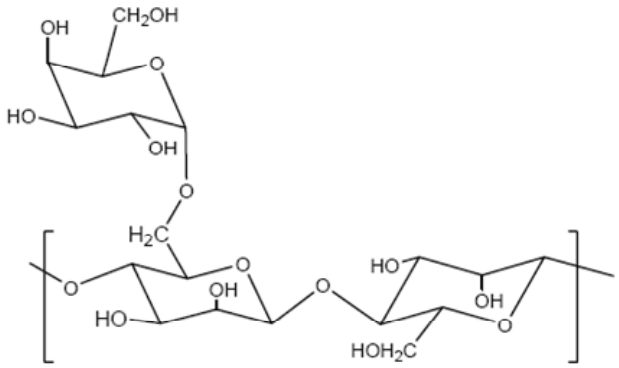


Figure 2.1: Structure guar gum.

2.1.2.2. Solubility

Guar gum is a cold water selling polymer. The particle size of the products represents the rate of thickening and final viscosity. Guar gum is one of the most highly efficient water thickening agents. It can tolerate limited concentration of water miscible solvents such as alcohols, but dimethyl-formide and dimethyl-sulphate are solvents for guar and also for most other polymers.

2.1.2.3. Viscosity

Guar gum at low concentrations, when dissolved in water forms highly viscous solutions. Heating a guar gum solution reduces the time to attain its full viscosity. The viscosity of guar solution can be measured with rotational shear-type viscometers. The viscosity of

fully hydrated 1% guar gum solution varies almost directly with the temperature (20-80^{o}C). Guar gum is stable over a wide pH range because of its non-ionic nature. Hydrated guar gum (3%) converts into gel by cross-linking and acts as random coil-polymer while exhibiting thickening properties.

Solvents	Non-Solvents
Water Liquid ammonia Hydrazine Formamide Ethylene diamine	Hydrocarbons Alcohols Ketones High Molecular weight glycol ethers Di-methyl sulfoxide (DMSO)

Table 2.1: Solvents and Non-solvetns for guar gum.

2.1.2.4. Rheology

Solutions of guar gum and its derivatives are non-Newtonian, classed as psedoplastic. They thin reversibility when heat is applied and degrade irreversibility with time when an elevated temperature is maintained. Solutions resist shear degradation when compared to other water soluble polymers, but they degrade with time under high shear.

2.1.3. Derivatives

Huang has observed that with in a few hours of guar gum solution preparation, biodegradation starts by the action of microorganisms or enzymes by hydrolyzing the linkages between the galactose and mannose, followed by oxidation (Huang, 1985). This

gives significant solution properties to guar gum solution. To make it more significant for various applications, derivatization of hydroxyl groups on the guar molecule can be made. Modifications of guar gum by derivatization include carboxy-methylation, ethylation oxidation, phosphatation and sulfatation (Bajpai *et al*., 1988)**.** The guar gum graft polymerization with acrylnitrile, methamethacrylate by microwave for significant adsorption of metal ion has been synthesized (Vandana *et al*., 2003)**.** These modified guar polymers have better properties and applicability when compared to unmodified guar gum.

There is an increasing demand for clarified guar solutions with alcohol solubility and improved thermal stability. This led the development of a number of chemically modified guar gums. The maximum theoretical degree of substitution (DS) in such molecule is '3', which represents the hydroxyl groups available for derivatization. The substitution of hydroxyl groups with ether, such as hydroxypropyl will allow side groups extraction which may change the solubility and other characteristics of the guar gum. The molar substitutes (MS) is defined as the average number of hydroxyl bearing substitutes per sugar unit and can also exceed three due to the additional availability of hydroxyl groups (Moorhouse *et al*., 1998)**.** The most widely known derivatives of guar gum are represented [Table 2.2].

2.1.3.1. Hydroxy-Propyl guar gum

Guar molecules have a tendency to aggregate during the hydraulic fracturing process, mainly due to intermolecular hydrogen bonding. Therefore, low-residue derivatives of

guar gum have been developed in attempts to eliminate this nature. Treatment of guar gum with propylene oxide and chloroacetic acid in alkaline medium, results in the formation of hydroxypropyl guar and carboxymethyl guar respectively. Substituted guar is more soluble in water than native guar (Gulbis, 1987)**.** Degree of substitution by propylene oxide on a hydroxyl groups along the guar backbone improved the guar gum properties. It is a measure of the total number of moles of propylene oxide that have been added to the guar polymer chain along the guar back bone. The solution property of derivatized guar gum changes with degree of DS. When hydroxypropyl groups are added to guar, the substitution occurs at any of the hydroxyl groups on the chain, either on the back bone or on the alpha-galactose side groups [Figure 2.2]. The modification sterically blocks the hydrogen bonding sites on the guar backbone and reduces the intermolecular hydrogen bonding between polymeric molecules (Cheng *et al*., 2002)**.** The HP-GG chain stiffens as the degree of substitution increases.

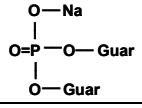
Type of derivative	**Structure of constituent**	**Ionic charge**
Hydroxypropyl guar gum (HP-GG)	$-CH_2-CH(OH)CH_3$	Non-ionic
Carboxymethyl guar gum (CM-GG)	-CH2-COO-Na-	Anion
Phosphoguar gum (P-GG)		-

Table 2.2: Guar gum substitution pattern.

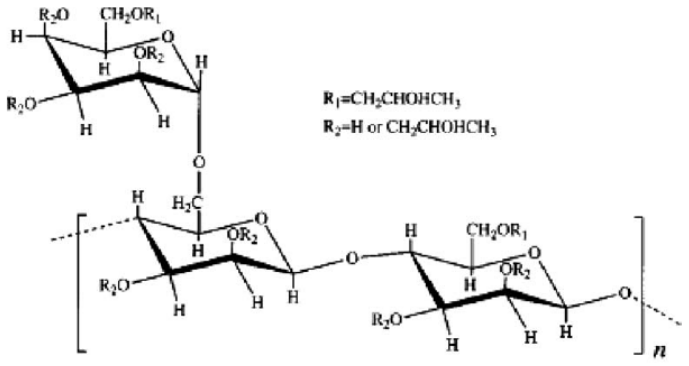


Figure 2.2: Molecular structure of HPGG.

2.1.3.2. Carboxy-Methyl guar gum

Chemical modifications of polysaccharides by etherification, esterification, oxidation and hydroxy-propylation are prepared generally for custom made derivatives, having desirable functionality. Carboxymethylation increases the hydrophilic nature, clarity of the polysaccharide solution and better soluble in aqueous systems. Carboxymethylation of guar gum is carried out by using monochloro-acetic acid under the catalytic influences of $NaHCO_3$ in dry state [Figure 2.3]. This is characterized by FTIR and ^{13}C NMR spectra (Parvathy *et al*., 2005).

Rhelogical properties of CM-GG solution showed non-Newtonian pseudoplastic behavior, regardless of the % N. At a constant rate of shear, the apparent viscosity of CM-GG solution decrease with the increase in %N (Sharma *et al*., 2004)**.**

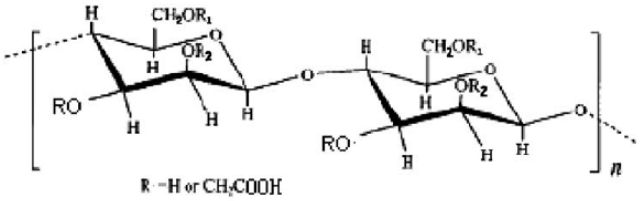


Figure 2.3: Molecular structure of CM-GG.

2.1.4. Applications

Guar gum being a high thickening agent, it is used as a viscosity builder and water binder in many industries like oil and gas, textile, paper, food, explosives, mining and aqueous partitioning of bio-molecules.

2.1.4.1. Food

Guar gum is used in the food industries for its ability to bond and adsorb large amount of water, there by contributing to viscosity and inhibiting ice crystals formation in frozen products along with smooth chewy texture to the product (Glodenstein *et al*., 1959)**.** Guar is given in ayurvedic formulations for diabetics to increase the fiber content in the daily meals.

2.1.4.2. Paper and Printing

In paper making process, guar gum adsorbs on hydrated cellulosic surfaces. It is a wet-end additive that promotes dry strength and improves sheet formation. Based on the viscosity property of guar gum solution, it is used in printing and dyeing of fibers and carpets.

2.1.4.3. Mining

The guar gum adsorbs hydrated mineral particles by hydrogen bonding followed by agglomeration as a result of bridging.

2.1.4.4. Explosives

Guar gum and hydroxyl-propyl guar gum are used to thicken and to gel blasting agents and explosive slurries. This is also used in cartridge explosives, by mixing with dry explosive ingredients as water-blocking agent (Maier *et al*., 1993).

2.1.4.5. Cosmetics

Cationic guar derivatives add substance to hair without building up after successive applications as do other cationic polymers. They are therefore used as thickeners in shampoos and conditioners.

2.1.4.6. Oil and Gas

Guar gum and its hydroxylpropyl and carboxymethyl ethers are used in the petroleum industry as additives for aqueous and water-methanol based fracturing fluids (Maier *et al*., 1993)**.** Guar gum in aqueous fluids is used in drilling shallow wells**.** These applications utilize the gums properties to increase viscosity, reduce fluid loss and decrease fluid friction. Kao Soap Company of Japan prepared liquid absorbents for use in disposable diapers and sanitary napkins**.** The particulate amorphous substances were used to adjust pH (pH 8-12) for smoothing the surface of semiconductor wafers**.**

2.1.5. Phases with GG/Dextran polymers and other natural polymers

The purification of high value proteins and other macromolecules that are produced in high concentrations by microbial fermentation have necessitated the use of new bioseparation methods that show greater selectivity towards their partitioning behavior, at the same time maintaining the molecular conformation of the product. Aqueous two-phase system (ATPs) provides such a useful technique for separation and purification of macromolecules, cells and cell organelles (Walter *et al.,* 1985). ATPs can easily scaled-up without an appreciable change in the nature or efficiency of the process. Moreover, phases are compatible with biomolecules and very little time is required to bring most two-phase systems into equilibrium. The system has flexibility of operation and is an attractive alternative to existing operations such as centrifugation, filtration and chromatographic separation process. The partition coefficient of biomolecule usually depends on the molecular weight, structure and charge of the particle, the ionic composition of the phase system, nature and molecular weight and concentration of the phase forming polymers. This applies to the partition of proteins in given ATPs.

The most widely used system for protein extraction consists of dextran and poly (ethylene glycol) (Johansson *et al.,* 1984; Tjerneld *et al.,* 1986). However, the high cost of fractionated dextran has limited the use of this system for large-scale separations.

As an alternative, for large scale enzyme processing, the PEG-salt systems have been used (Albertson *et al.,* 1990). Although this system is inexpensive, the high salt concentration in both phases of this system limits its usefulness. PEG-salt phase forms

only at high ionic strength, which may cause the denaturation of sensitive biological structures and the dissociation of most ligand-protein complexes (Kula, 1989).

PEG-salt system has the additional problem of waste disposal of salt. ATPs based on dextran, starch derivatives and cellulose derivatives have the advantage of their biodegradability (Sturesson *et al.,* 1990).

Therefore, Developing new aqueous two-phase systems based on cheaper polymers have been a challenging research goal (Pietruszka *et al.,* 2000). Recently studies have been performed on biphasic systems containing polysaccharides, i.e. guar/amylopectin (Closs *et al.,* 1998, 1999) and guar/dextran (Simonet *et al*., 2000).

The aim of present work was to develop new phase system based on gum derivative and to understand the main physico-chemical parameters that affect protein partitioning. We focused on partition behavior of two-model proteins viz. BSA and lysozyme and their comparison in following two ATPs systems.

1. Guar gum -Dextran 2,000,000 (ATPs 1)
2. Hydroxyl propyl guar gum -Dextran 2,000,000 (ATPs 2)

2.2. Materials and Methods

2.2.1. Materials

Guar gum and Hydroxypropyl Guar gum was gifted by Dabur India Limited (Alwar, India) Dextran produced by *Leuconostoc mesenteroides*; strain number B-512 with an average MW 2,000,000 and Bovine Serum Albumin (BSA) was procured from Sigma-

Aldrich, (USA). The lysozyme from chicken egg white (lyophilized with average molecular weight 14600) was procured from Hi-media chemicals India limited, (India).

2.2.2. Preparation of phases

All solutions were prepared on weight/weight basis with (0.02%) sodium azide used as a biocide. Phosphate and citrate buffers were used for maintaining a particular pH of the system. Solutions of Hydroxypropyl guar (5%) and Guar gum (1.25%) were prepared in distilled water under strong magnetic stirring at room temperature for 1h to prevent the formation of clumps and thereafter were allowed to hydrate over night at 10^0C. Dextran solution (10%) was prepared at 20^0 C under magnetic stirring for 2h.

2.2.3. Phase diagrams

2.2.3.1. Two-phase systems

The phases were prepared from the stock solutions of polymers in water. The polymer solutions were weighed and mixed with desired buffer of 0.1 M. The total weights of phases were 4g. Phases were vortexed vigorously for 2 minutes and allowed to settle for 8 h (ATPs 1) and 24 h (ATPs 2) at, 25°C ± 2°C [Table 2.3] to form two separate phases with a clearly visible interphase. The binodial curve was drawn as the frontier between the compositions. The protein partition experiments were also performed at 25°C ± 2°C by mixing the selected phases with a 100 μg of 10% protein solution. The study of protein partitioning was carried out at pH 5.0, 6.0, 7.0 and 8.0. Phases containing different salt concentrations were prepared by adding NaCl stock solution directly into the systems at pH 7.

Partition coefficient (K) was determined by taking the ratio of protein concentration in the top phase (gum-rich) to the protein concentration in the lower phase (Dextran rich). A proper material balance of protein in the two phases was made to ensure that no protein accumulation occurred at the inter phase.

2.2.3.2. Phase densities and viscosities

Top and bottom phases were separated using separating funnel without disturbing the interphase. Phase densities of separated phases were measured by taking ratio of mass to its volume. Phase viscosities were measured using a torque measuring, cone and plate rheometer (model CAP2000+, Brookfield Laboratories Inc; USA). Less than 0.5 ml solution containing polymer was carefully poured on a plate, run time adjusted for 30 sec at 25^0C and viscosity determined using spindle no. 01.

2.2.4. Protein partitioning

BSA and lysozyme were selected as standard proteins for partition studies in aqueous two-phase system, formed using guar gum (ATPs1) and hydroxyl propyl guar gum (ATPs2). Once clear phases had formed, the top and bottom phases were carefully separated using a micropipette and weighed. Samples of the top phase were diluted in water, and analyzed for protein concentration. Protein concentration in the top and bottom phase was determined according to Folin and Ciocalteu's method using a UNICAM spectrophotometer at 500nm. Protein concentration was determined for a set of

two independent systems. BSA and lysozyme were used separately as standards for protein estimation for both the phases.

2.3. Results and Discussion

2.3.1. Phase diagrams

The physical state of the blends after 8 h and 24 h are shown [Figure 2.4 & 2.5] respectively where the guar gum and HP-GG concentrations in initial mixtures (before phase separation) are plotted as a function of the dextran concentration in the initial mixtures. The binodial line demonstrates the areas between mono and biphases. The monophasic area is comparatively narrow and under our experimental conditions, no phase separation was observed at dextran and guar gum concentrations approximating 2.0 and 0.0625% respectively [Figure 2.4] and 1.5%, 0.6% respectively for Dextran and HP GG concentrations of approximately [Figure 2.5].

2.3.2. Phase densities and viscosities

Viscosities of separated phases were measured using a torque-measuring, cone and plate rheometer at 500 rpm. Rheological properties of both ATP systems are shown [Table 2.4]. While bottom phase viscosities are comparable to other systems values, reported in the literature, upper phase viscosities are much lower than those reported for purified polymers or commercial guar gum derivatives (Tjerneld *et al.,* 1986). Phases formed with crude hydroxypropyl starch (Venaneio *et al.*, 1993) or hydroxypropyl cellulose (Skuse *et al.*, 1992) exhibit higher values ranging from 1000 cp to 3500 cp respectively. The lower

viscosities for phases reported could be attributed to the lower polymer concentrations required to form two-phase systems.

2.3.3. Effect of pH on protein partitioning

ATPs 1 and 2 [Table 2.3] were prepared using different buffers of pH 4 to 8. Effect of pH on partition behavior in ATPs 1 and 2 of both BSA and lysozyme is presented [Figure 2.6 & 2.7]. Although a small change in K was value observed for both proteins for different pH, both favored partitioning into the bottom dextran rich phase irrespective of surface charge. On the other hand HPGG being a more hydrophobic polymer was observed to contribute markedly to partition of BSA and lysozyme on account of its hydrophobicity. The maximum 'K' value for BSA were at pH 5 (close to its pI values) while an opposite trend was observed for lysozyme, where 'K' value increased with increase in pH.

2.3.4. Effect of salt on protein partitioning

The effect of NaCl concentration on partition coefficient of BSA and lysozyme is shown [Figures 2.8 and 2.9]. Partitioning of BSA and lysozyme were studied in ATPs 1 and ATPs 2 phase system in presence of NaCl concentration. The recovery of BSA was maximum for HPGG phase for NaCl concentrations in the range of 0.4 to 0.6 M. Further increase in NaCl concentration lowered the K value for the chosen proteins [Figure 2.8], due to salting out from top guar gum rich phase. A similar trend of partitioning was observed for lysozyme in presence of NaCl in ATPs 2 [Figure 2.9]. Although no dependence of NaCl concentration on K value was observed for BSA, both proteins were concentrated in HPGG rich phase with 5-10 fold increase in partition coefficient.

2.4. Conclusion

The HPGG rich phase showed greater enrichment of proteins in solution as compared to GG rich phase. Effect of NaCl concentration on protein partitioning was more prominant in the HPGG-Dextran phases than in guar gum-Dextran phases. Guar gum is a low cost polymer and since low polymer concentrations are needed to form ATPs, the implications in cost reduction are significant in making at guar based aqueous two phase two phase separation an alternative to existing phase systems.

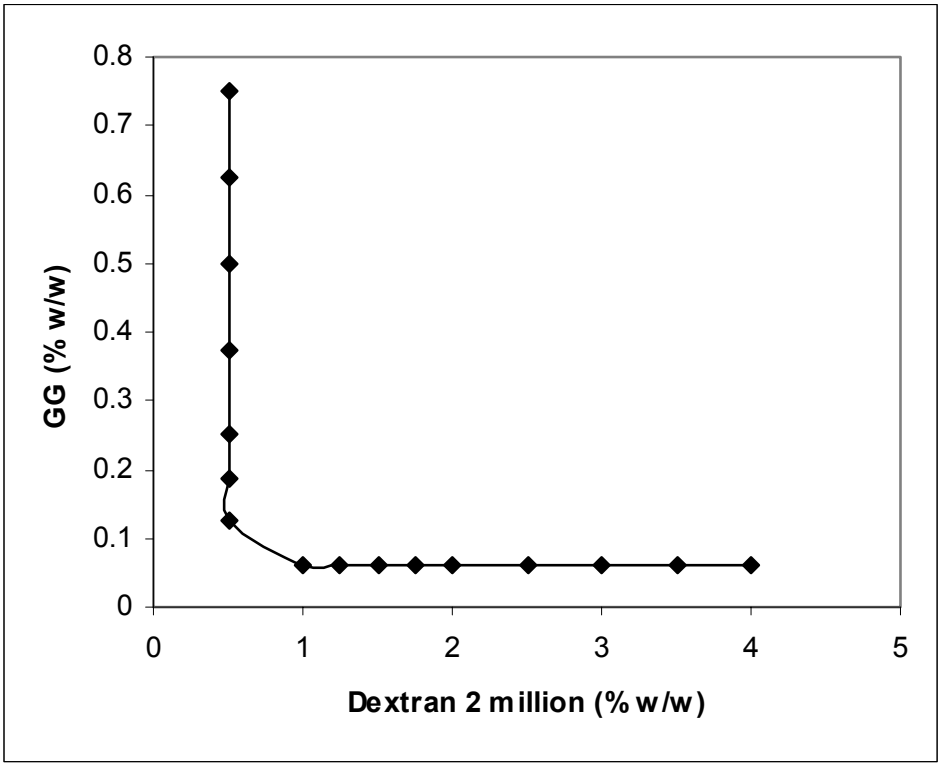


Figure 2.4: Phase diagram of Guar gum/Dextran 2 million at 28^0C, pH 7 (0.1M) in water.

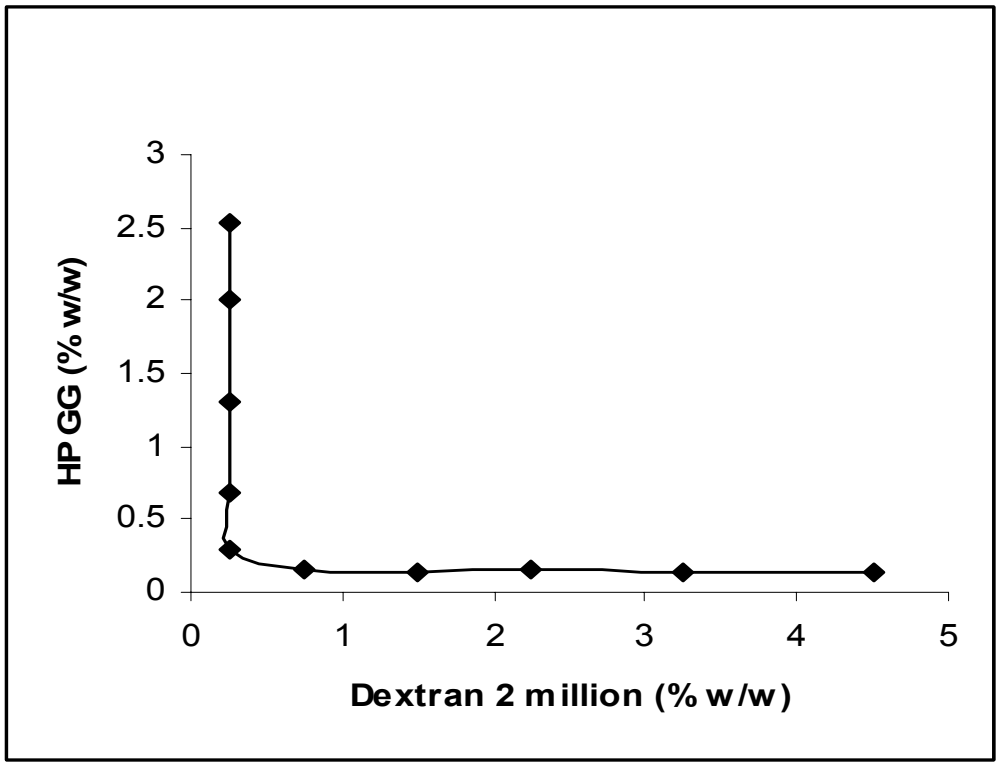


Figure 2.5: Phase diagram of hydroxypropyl guar gum /dextran at 28^0 C, pH 7 (0.1M) in water.

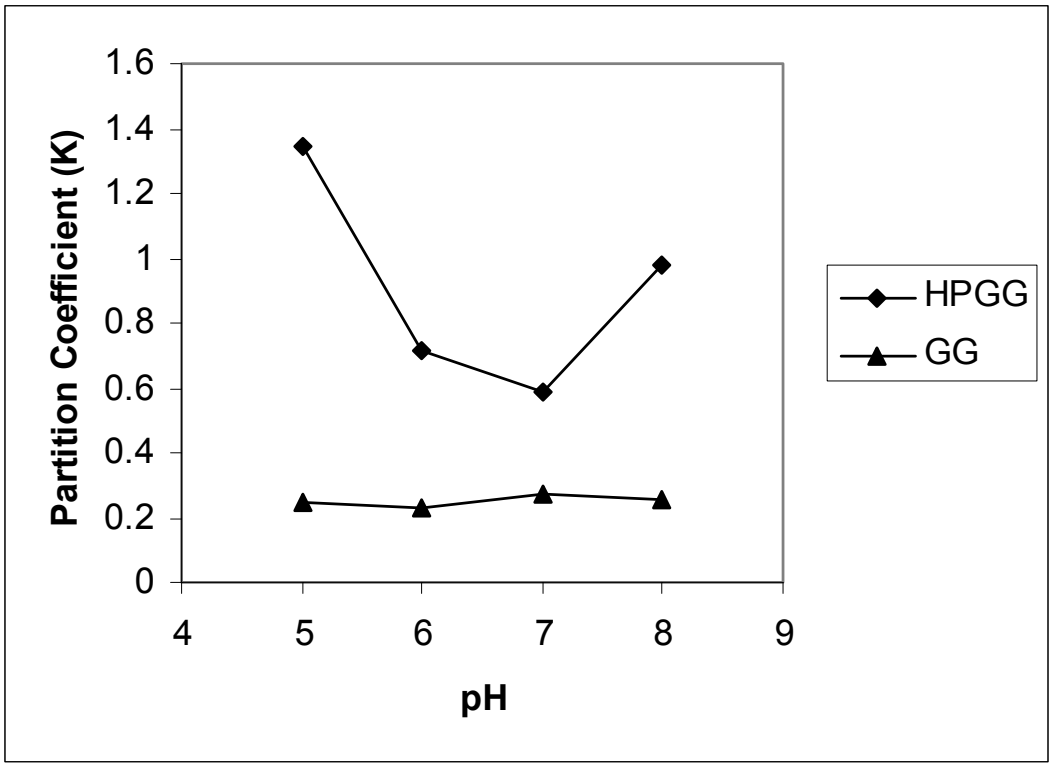


Figure 2.6: Effect of pH on BSA partitioning with 0.28 (% w/w) GG / 1.75 (% w/w) Dextran 2 million two-phase system and 1.25 (% w/w) HP GG and 2.0 (% w/w) Dextran 2 million at 28 ^{0}C.

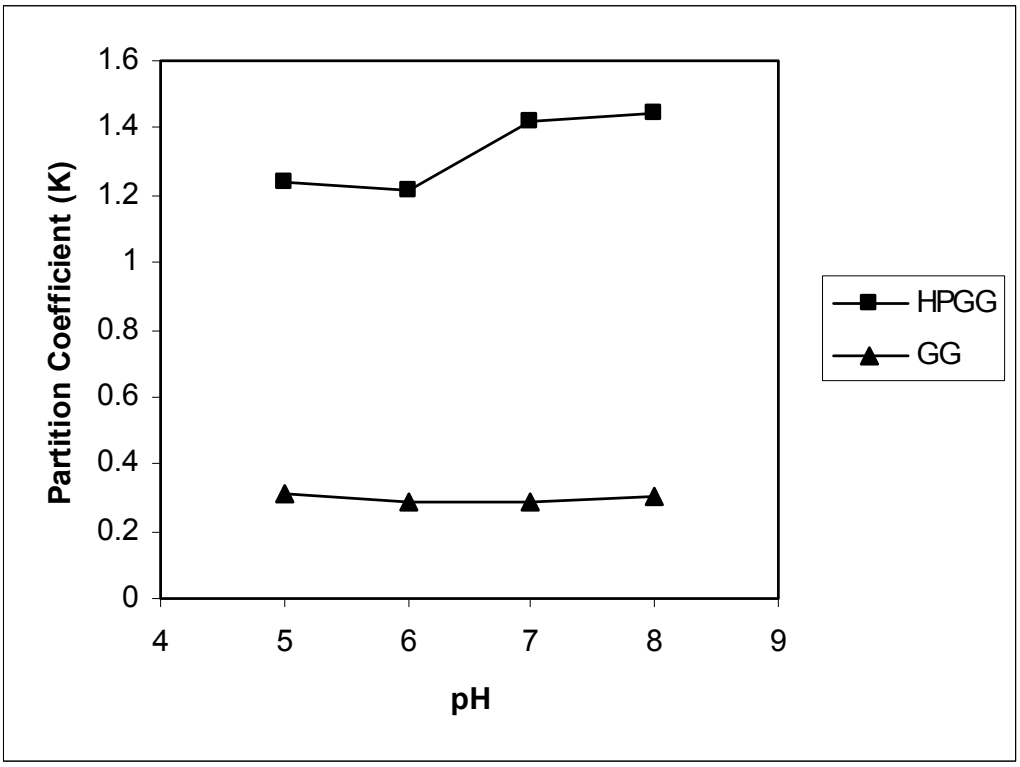


Figure 2.7: Effect of pH on Lysozyme partitioning with 0.28 (% w/w) GG / 1.75 (% w/w) Dextran 2 million two-phase system and 1.25 (% w/w) HP GG and 2.0 (% w/w) Dextran 2 million at 28^0C.

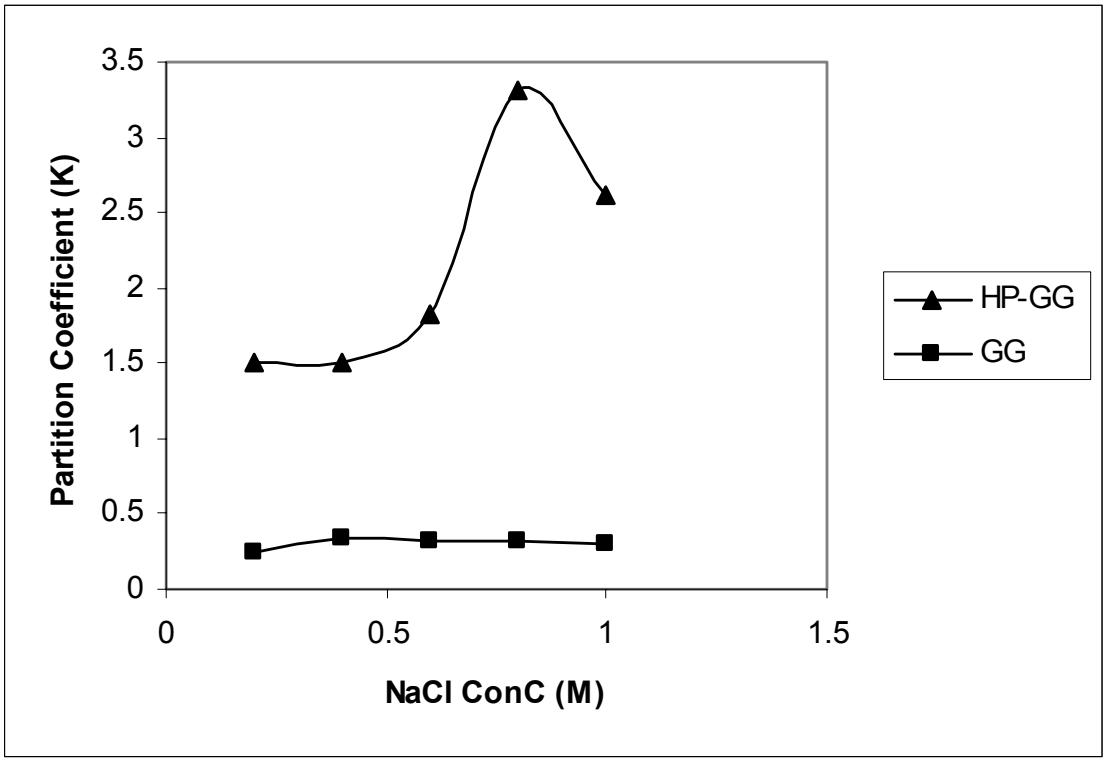


Figure 2.8: Effect of NaCl concentration on BSA partitioning with 0.28 (% w/w) GG / 1.75 (% w/w) Dextran 2 million two-phase system and 1.25 (% w/w) HP GG and 2.0 (% w/w) Dextran 2 million at 28^0C and pH 7.

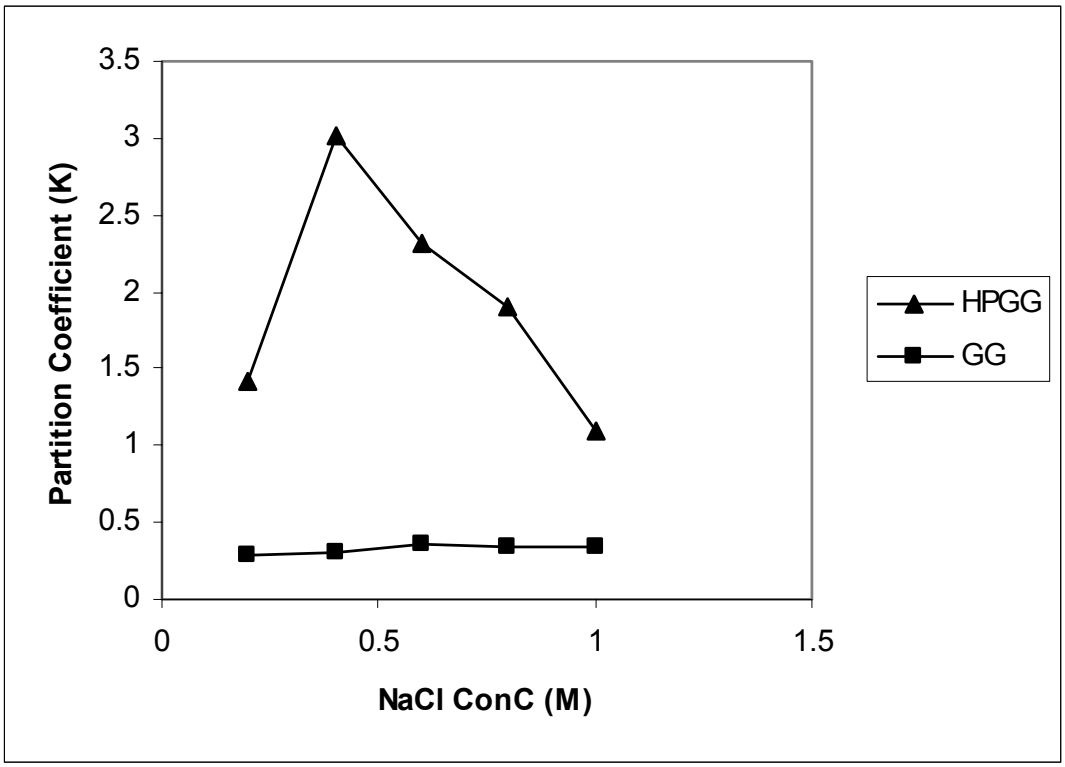


Figure 2.9: Effect of NaCl concentration on Lysozyme partitioning with 0.28 (% w/w) GG 1.75 (% w/w) Dextran 2 million two-phase system and 1.25 (% w/w) HP GG and 2.0 (% w/w) Dextran 2 million at 28^0C and pH 7.

Systems	Polymer 1	Polymer 2
ATPs 1	Guar gum 0.28 % (w/w)	Dextran 1.75 % (w/w)
ATPs 2	HPGG 1.25 % (w/w)	Dextran 2.00 % (w/w)

Table 2.3: Aqueous two phase system composition of ATPs 1 and ATPs 2.

ATP System	Upper phase		Bottom phase	
	Density (Kg m^{-3})	Viscosity* (cp) %Torque	Density (Kg m^{-3})	Viscosity * %Torque (cp)
1) 0.28%guar gum-1.75% Dextran	0.77	11.3 3.0	0.984	4.5 1.2
2) 1.25% HPGG-2.0% Dextran	0.986	23.6 6.3	0.998	12.0 3.2

Table 2.4: Density and Viscosity of the Guar gum-Dextran and HPGG-Dextran systems.

Chapter 3

α- Galactosidase hydrolyzed guar based aqueous two phase system

3.1. Alpha Galactosidase

Glycosidases are also known as carbohydrolases. O-Glycoside hydrolases (E.C.3.2.1.-) are a widespread group of enzymes, which hydrolyses α or β glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (The non-carbohydrate component is known as the aglycone and carbohydrate component is called the glycone [Figure 3.1].

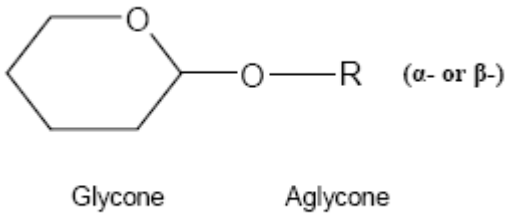


Figure 3.1: Back Bone structure of α-galactoside.

There are many examples of glycosidases, α-galactosidase, β-galactosidase, invertase, maltase, β-glucosidase, amylase etc. The enzymes responsible for hydrolyzing galactosidic linkages are termed as galactosidases. There are two types of galactosidases viz., α-galactosidases and β-galactosidases depending on the configuration of the anomeric carbon atom of galactose in the substrate molecule on which they act. α-D-Galactosidase (Alpha-D-galactoside galactohydrolase EC 3.2.1.22) is an enzyme capable of breaking down oligo-saccharides such as stachyose, melibiose, raffinose (Gdala *et al.,* 1997), polysaccharides like galactomannans [Figure 3.2] and glycoconjugates such as glycoproteins and glycolipids (Dey *et al.,* 1977).

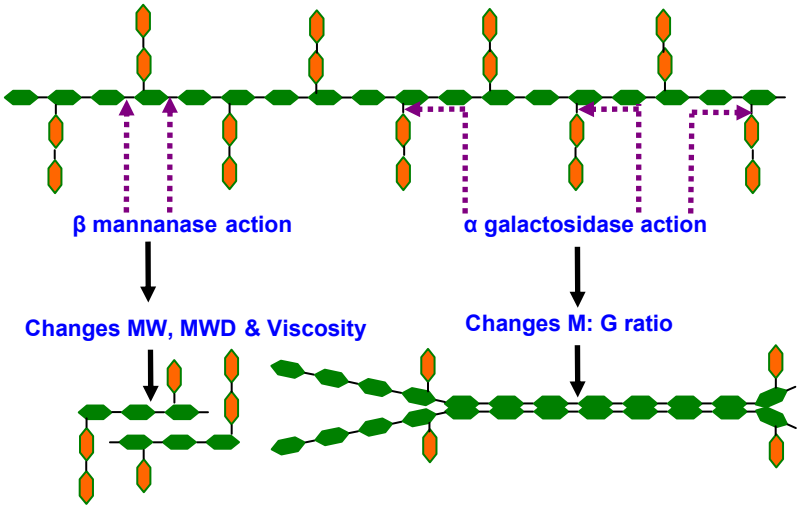
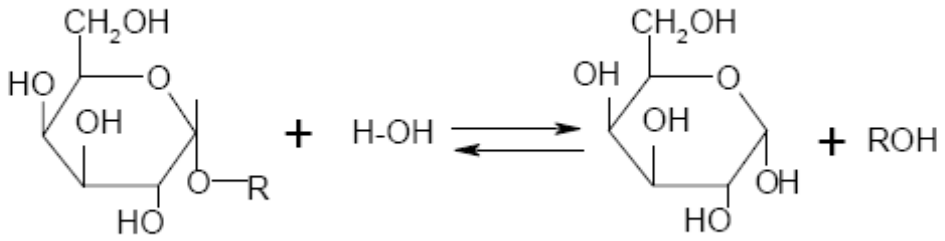


Figure 3.2: Schematic of Enzymatic Action on Guar Molecule.

Fischer was one of the first to isolate α-galactosidase from bottom yeast. Because of its action on melibiose it was named as melibiase, catalyzing the following reaction (Fischer & Lindner, 1895).



Where R is an alkyl or aryl group, or a glycosyl (mono or oligo) residue or group.

3.1.1. Occurrence and physiological functions

α-Galactosidases are widely distributed in nature among plants, animals and microorganisms. It is ubiquitous in many legume plants (seeds) (Helferich *et al.,* 1932). The presence of α-galactosidase was also reported from human: spleen, placenta, plasma, blood cells, bone marrow and liver (Bishop, *et al.,* 1981; Dean *et al.,* 1979; Monis *et al.,*

1967; Szmigielski *et al.,* 1966). Among microorganisms α- galactosidase activity was first detected in brewers yeast (Fischer *et al.,* 1895), subsequently it was also detected in *Saccharomyces carlsbergensis* (Lazo *et al.,* 1977), *Pichia guilliermondii* (Church *et al.,* 1980), *Candida javanica* (Cavazzoni *et al.,* 1987), *Aureobasidium pullulans* (Kremnicky *et al.,* 1997) etc.

The main physiological role of α-galactosidase in microorganisms could be in the hydrolysis of galactose containing oligo and polysaccharides present in the growth medium or in the natural environment, liberating an assimiable end products like galactose, glucose, mannose, which serve as a carbon source for growth of organism. In plants, α-galactosidase is believed to be involved in a variety of processes, most importantly in the hydrolysis of oligo-saccharides such as raffinose and stachyose during the early germinative period, resulting in the liberation of free sugars, which may serve as a ready energy source for the growing plant (Dey *et al.,* 1972). In animals, enzyme is reported to be involved in the hydrolysis of galacto-lipids. The deficiency of enzyme has been implicated in the manifestation of a disorder named Fabry's disease in humans. Fabry's disease belongs to the group of hereditary lysosomal storage disease and is caused by a reduced activity of α-galactosidase-A (Feldt-Rasmussen *et al.,* 2002), as α-galactosidase A and B are present in normal tissues. The enzyme may have a synthetic function, as transgalactosylation reactions have been reported for α-galactosidases (Dey *et al.,* 1979). Its role has been implicated in the removal of toxic accumulants and to hydrolyze phenolic glycosides (Strobel *et al.,* 1974). The α-galactosidase enzyme also occurs in brain tissues with possible involved in the hydrolysis of mono-galactosyl-di-glycerides and di-galactosyl-di-glycerides (Subba-Rao *et al.,* 1970).

3.1.2. Applications of α-galactosidases

The important property of α-galactosidase is the hydrolysis of α-1,6 linked D-galactosyl residues from galactose containing oligo and polysaccharides, has many potential applications in biotechnology and medicine. These applications have been reported by using α-galactosidases from several microbial and plant sources (Dey *et al.,* 1979; Bakunina *et al.,* 1988; Somiari *et al.,* 1995; Mulimani *et al.,* 1995; Ganter *et al.,* 1988; McCutchen *et al.,* 1996; Ohtakara *et al.,* 1987; Chiba *et al.,* 2002).

3.1.2.1. Industrial applications

1. The enzyme from the raffinose utilizing strain *Mortierella vinaceae* strain (Suzuki *et al.,* 1972) and *Aspergillus niger* (produced by Novo Nordisk A/S, Denmark) (Knap *et al.,* 2001) is used in beet sugar industry. D-Raffinose elimination by the action of α-galactosidase in beet syrups facilitates crystallization and consequently improves the yield of the sucrose in beet sugar industry (Yamane *et al.,* 1971).

2. Galactomannan occurs in varying amounts in the endosperm of a wide range of leguminous seeds (Buckeridge *et al.,* 1995). These polysaccharides from different species have different proportions of D-galactose and D-mannose, but always consist of a β-(1-4) mannan backbone with single D-galactose stubs linked with α-(1-6) bond. The complete hydrolysis of galactomannan requires the action of three enzymes: α-galactosiase, β-annosidase and exo-β-mannanse [Figure 3.2 and 3.3] (Duffaud *et al.,* 1997). Many investigators have developed processes for the modification of galactomannan polymers by using plant and microbial α-galactosidases (Halstead *et al.,* 2000; McCutchen *et al.,*

1996; Joersbo *et al.,* 2001; Ademark *et al.,* 2001; Bulpin *et al.,* 1990). Hence, the potential applications of guar gum are limited. Owing to the relatively high cost of locust

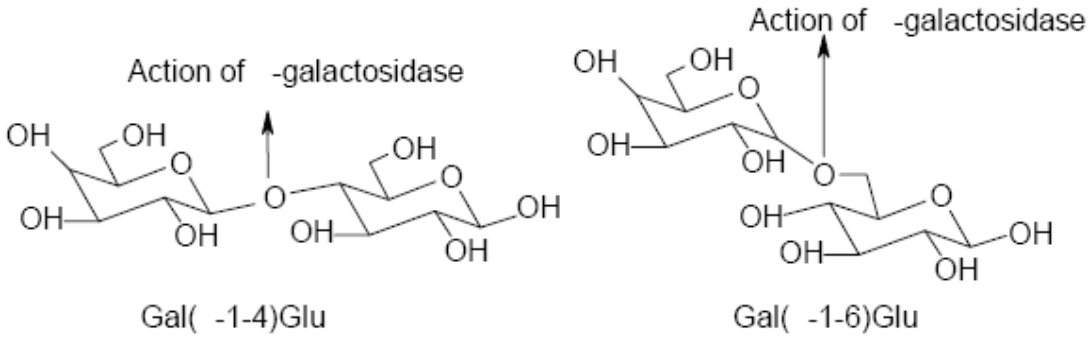


Figure 3.3: Molecular action of α-Galactosidase on Guar Molecule

bean gum, it is of commercial interest to exploit the guar gum by enzymatic modification. To convert guar galactomannan into a locust bean galactomannan equivalent, some of the side chains (1-6)-α-linked-D-galactosyl residues need to be removed without significant cleavage of the galactomannan back bone (Pai *et al.,* 2002). This can be achieved by partial enzymatic hydrolysis of guar gum by α-galactosidase (Cronin *et al.,* 2002).

3. Transgalactosylation activity of α-galactosidases has frequently been used for the synthesis of new saccharides (Ajisaka *et al.,* 1989).

4. In paper and pulp industry α-galactosidases could enhance the bleaching effect of β-1, 4 mannanase on soft wood and Kraft pulp (Clarke *et al.,* 2000).

5. Many α-galactosidase preparations that are available commercially found use as a dietary supplement in human's diets (Beano, Terrainzyme, Jarro-Zymes-Plus, EZ-Gest,

Bean-Zyme etc,) and animal feed processing. Among the *leguminosae*, soy in particular is used in large quantities for feeding pigs, cattle and poultry. The use of exogenous enzymes extracted from microbes has expanded recently and different enzymatic additives are being used to improve the nutritive value of monogastric diets (Marquardt *et al.,* 1996). Many attempts have been made to assess the effect of adding a high dose α-galactosidase to cereal-soybean-pea diets on the performance and improved digestive efficiency of growing pigs and broilers (Kidd *et al.,* 2001; Pan *et al.,* 2002).

6. When undigested sugars pass into the large Intestine, they are fermented anaerobically by α-galactosidase producing bacteria, resulting in production of gas and gastro-intestinal distress (Cristofaro *et al.,* 1974; Suarez *et al.,* 1999). The absence of α-galactosidase in human intestinal track prevents the hydrolysis of complex galactose containing sugars (Gitzelmann *et al.,* 1965). Enzymatic treatment of soybean or legume carbohydrates by α-galactosidase offers a promising solution for elimination of these oligo-saccharides, especially in soymilk (Cruz *et al.,* 1982).

7. Treatment of Fabry's disease: Fabry's disease is a recessive, X-linked, lysosomal storage disorder, caused by a deficiency of the enzyme α-galactosidase-A (α-Gal A) (Peters *et al.,* 2001), leading to an accumulation of the glycosphingolipid, mainly globotriaosylceramide (GL-3) in most tissues of the body, causing multi-system disease. Since Fabry disease cannot be cured, at present clinical management is symptomatic. Enzyme replacement therapy (ERT) with recombinant alpha-Gal A has been introduced as a new therapeutic option for the treatment of Fabry patients. Enzyme replacement

therapy mediated by gene transfer may become a promising alternative treatment strategy in the future (Breunig *et al.,* 2003; Breunig *et al.,* 2003a).

8. Blood group transformation: Type "O" blood is considered "universal" and may be used for transfusion in any individual of blood type A, B, AB and O. In order to increase the supply of type O blood, efforts have been made to develop methods for converting group A and B blood to group O blood (Goldstein *et al.,* 1989). Enzymatic conversion of type B to O erythrocytes with Coffea (coffee bean) α-D-galactosidase was first described by Harpaz and Flowers (Harpaz *et al.,* 1975) and subsequently adopted by Goldstein *et al* (Goldstein *et al.,* 1982).

3.1.3. α-Galactosidase: Microbial production

Microorganisms in particular have been regarded as treasure sources of useful enzymes. In recent years, there has been a phenomenal increase in the use of enzymes as industrial catalysts. These enzymes offer advantages over the use of conventional chemical catalysts for numerous reasons**:** they exhibit high catalytic activity, a high degree of substrate specificity, can be produced in large amounts, highly biodegradable, pose no threat to the environment and are economically viable.

The microorganisms preferred for commercial production of α-galactosidase are *Circinella muscae, Absidia griseol* and *Absidia hyalospora* by Hokkaido Sugar Co. Ltd., Tokyo, Japan (Narita *et al.,* 1975; Narita *et al.,* 1976), *Mortierella vinacea* (Suzuki *et al.,* 1974) and *Bacillus stearothermophilus by* Monsanto Company, St. Louis, Mo (Delente *et*

al., 1974). Recently it is being produced commercially by Novo Nordisk A/S, Bagsvaerd, Denmark using *Aspergillus niger* (Knap *et al.,* 2001).

Aspergillus awamori has been used industrially for the production of enzymes such as glucoamylase, a-amylase and protease (Ward *et al.,* 1996). The work published on *A. awamori* is focused on strain improvement for a specific product. Much effort has been made on the possible use of this specie for the production of other enzymes such as xylanase (Carlsen, *et al.,* 1995; Trinci *et al.,* 1970), protopectinase (Hours *et al.,* 1994) and chymosin (Berka *et al.,* 1991). For the industrial production of enzymes using fungi, often complex media containing solid substrates are used. The use of a complex medium may influence morphology, growth kinetics and end product formation. Scale up and optimization of economical bioprocess products demand optimal media and culture conditions, therefore, medium optimization study is very important.

3.1.4. Fermentation

The submerged fermentation for aerobic microorganisms is now well known and widely used method for the production of α-galactosidase (Suzuki *et al.,* 1974; Delente *et al.,* 1974a; Olivieri *et al.,* 1984). Although several α-galactosidase preparations are produced by solid-state fermentation method (Kotwal *et al.,* 1998; Annunziato *et al.,* 1986), this fermentation technique has not yet been exploited industrially for α-galactosidase production. Both the methods of fermentation have advantages and disadvantages. However the relative yield and ease of operation are deciding factors to choose the fermentation method.

3.1.5. Production of α-galactosidase submerge fermentation

Microorganisms in submerged fermentation (SmF) thrive in a liquid environment. Generally, the submerged fermentation processes are carried out in shake flasks or aerated and agitated fermentors equipped with controls of fermentation parameters. Submerged fermentation has several advantages over solid state fermentation such as**:** it requires less man power, gives higher yield and productivity, lower cost, less contamination and better temperature control during fermentation (Frost *et al.,* 1987).

A classical example of production of α-galactosidase by submerged fermentation method has been the batch fermentation by thermophilic and aerobic *Bacillus sp* JF_2 strain (Jin *et al.,* 2001). The design of fermentation system utilized in above said process [Figure 3.4].

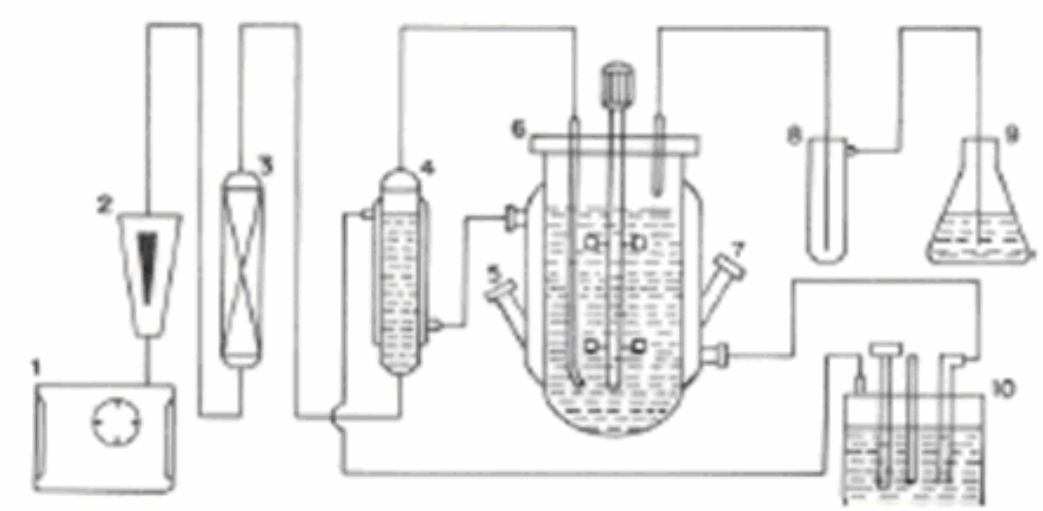


Figure 3.4: Schematic diagram of submerged fermentation process; (1) air pressure, (2) air flow meter (3) air filter (4) water vapour saturation (5) pH sensor (6) fermentor (7) take sample (8) air buffer (9) dilutent sulphuric acid (10) temperature control unit [Courtesy: Jin *et al.,* 2001].

Under submerged fermentation conditions various factors such as physical, chemical and nutritional parameters are known to have significant effect on productivity of α-galactosidase.

3.1.6. Medium composition

To obtain a suitable medium for enzyme production, effect of various carbon and nitrogen sources in combination with mineral salts on growth and enzyme production must be studied to achieve maximal product formation (Bridson *et al.,* 1970). The carbon source employed in microbial enzyme production is one of the most important factors in determining feasibility of the process. For α-galactosidase production, glucose, galactose, lactose, melibiose, raffinose and stachyose have been used at laboratory scale as well as at commercial scale (Narita *et al.,* 1975; Olivieri *et al.,* 1984; Roy *et al.,* 1991; Ikura *et al.,* 1987; Suzuki *et al.,* 1974). Cheap agriculture residues like, wheat bran or wheat flour, rice bran, soy flour or soybean cake, sorghum, corn, millet etc, are also used for enzyme production (Coombs *et al.,* 2001; Jin *et al.,* 2001; Kotwal *et al.,* 1995; Suzuki *et al.,* 1972). Optionally, an inducer; raffinose or melibiose can be added to induce the α-galactosidase formation, when agricultural residues were used as a carbon sources in the medium (Delente *et al.,* 1974). In few cases, waste effluent and waste byproducts have been utilized for cultivating the organism and producing α-galactosidase in the fermentation medium (Arnaud *et al.,* 1976; Wong-Leung *et al.,* 1993). In addition to above carbon sources, few microorganisms were found to assimilate complex carbohydrate containing substrates, like guar gum, locust bean gums and

polygalacturonate to synthesize α-galactosidase (McKay *et al.,* 1991; Duffaud *et al.,* 1997; Dey *et al.,* 1993).

The type of nitrogen source to be used depends essentially upon the nutritional requirement of the organism. A variety of nitrogen sources including both organic and inorganic have been evaluated for the production of α-galactosidase. *Bacillus stearothermophilus* (Delente *et al.,* 1974), *Bacillus sp* (Jin *et al.,* 2001), *Lactobacilli* (Mital *et al.,* 1973), *Streptomyces sp* (Oishi *et al.,* 1971) utilize exclusively organic nitrogen sources like tryptone, peptone, polypeptone, yeast extract, beef extract, casamino acids, soybean meal etc for their growth and α-galactosidase production. Since the soybean meal acts both as a source of carbon as well as nitrogen. It could be economical substrate for commercial enzyme production (Delente *et al.,* 1974). The inorganic nitrogen sources like ammonium sulfate, ammonium nitrate, ammonium acetate, urea etc have been used in the fermentation medium for the growth of *Azotobacter* (Wong *et al.,* 1990) and *Trichoderma reesei* (Zeilinger *et al.,* 1993). Many times combination of organic and inorganic nitrogen sources has been practiced (Cavazzoni *et al.,* 1987; Wong *et al.,* 1986).

Apart from carbon and nitrogen sources, many other essential elements such as phosphorus, magnesium, calcium and numerous trace elements such as iron, copper, cobalt, zinc, manganese may be required in the medium to support active cellular function. Trace elements often serve as cofactors in enzymatic reactions. However, very

little information is available regarding effect of many of these trace elements on α-galactosidase production.

3.1.7. Culture parameters

The principal physical parameters, which affect α-galactosidase production in submerged fermentation are pH, temperature, aeration and agitation. The pH is particularly critical for microbial growth and enzyme production. Control of pH is important to maintain an optimal environment for growth and product formation (Frost *et al.,* 1987). Most of the bacterial fermentations for α-galactosidase production are carried out in the pH range 6.0-7.5. To maintain this pH, phosphate buffer or phosphate ions have been added in the fermentation medium (Delente *et al.,* 1974; Jin *et al.,* 2001). For production of α-galactosidase by alkalophilic organisms; pH of the medium was adjusted to 9~11, by sodium carbonate (Akiba *et al.,* 1978; Ikura *et al.,* 1987). Most of the fungal fermentations for α-galactosidase production have been carried out in the acidic pH range 4.5-6.0 (Kotwal *et al.,* 1995; Kaji *et al.,* 1969).

In order to obtain optimal yields, fermentations must be carried out at constant temperature. The microorganisms are broadly classified in to three groups: psychrophiles, mesophiles and thermophiles depending on their temperature requirement for growth. Therefore incubation temperature for production of enzyme must depend on the type of organism used. The optimum incubation temperature reported for α-galactosidase production ranges between 30-37 ºC (Xiao *et al.,* 2000; Rios *et al.,* 1993) and 40-60 ºC (Puchart *et al.,* 2001; Talbot *et al.,* 1990).

Agitation speed and aeration rate on microbial enzyme production are important factors affecting successful progress of fermentation. Aeration could be beneficial for the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, product and oxygen (Brown *et al.,* 1970). Agitation is also an important parameter for adequate mixing, mass and heat transfer. In laboratory, most common practice is to employ shake flask cultures. Small jar fermentors provided with the mechanical agitators and air spargers are also used. There are few reports (Wong *et al.,* 1986; Zapater *et al.,* 1990), describing the conditions of aeration and agitation for the production of α-galactosidase. The conditions of agitation and aeration vary from strain to strain and the type of fermentors used. Delente *et al* have employed aeration rate of 0.8 vvm (volume of air per volume of medium) and agitation at 400 rpm to produce maximum α-galactosidase from *Bacillus stearothermophilus* in a 14 L fermentor (Delente *et al.,* 1974). In another case the aeration rate of 0.5 vvm and agitation at 240 rpm was used by Narita e al (Narita *et al.,* 1975), to produce α-galactosidase from *Circenella musae* in a 20 L fermentor. When *Corticium rolfsii* was grown for enzyme production in 200-liter fermentor, 1 vvm was provided (Kaji *et al.,* 1969). On the other hand, agitation creates shear forces causing morphological changes in the cells, variation in their growth and product formation and in most cases damage the cell coat structure. This effect was clearly seen in *Lactobacillus fermenti*, where biomass was higher for agitated cultures due to the improved mixing conditions and better oxygen transfer. However, higher enzyme activity was observed for static cultures. Lower activity in agitated culture was attributed to the effect of shear (Schuler *et al.,* 1985).

3.1.8. Production of α-Galactosidase in solid-state fermentation

Solid-State Fermentation (SSF) is the cultivation of microorganisms on water saturated solid raw materials, such as grains, beans or wheat bran in the absence of free flowing water. This is an alternative to the cultivation of microorganisms in a submerged medium. SSF has been used for centuries and is still being used to produce foods, such as soy sauce and mushrooms. In many cases, the microorganisms used in SSF are fungi, because they are ideally suited to colonize and penetrate solid particles. The advantage of SSF for fungal enzyme production are lower capital investment, high product concentration, reduced expenditure on down stream processing, minimized waste disposal, simpler reactor design with a minimum of controls (Mitchell *et al.,* 1992; Lonsane *et al.,* 1985). Other advantages of SSF technique include: (a) a simplified procedure for inoculum development, (b) easier scale up of process, (c) reduced solvent requirement for product recovery etc (Lonsane *et al.,* 1992). There have been numerous reports on the production of α-galactosidase by solid state fermentation processes (Annunziato *et al.,* 1986; Srinivas *et al.,* 1993). The first report on α-galactosidase production by *Mortierella vinacea* using a "koji" method appeared in 1969 (Suzuki *et al.,* 1969). The organisms reported to be producing α-galactosidase in wheat bran based solid-state fermentation are *Aspergillus awamori* (Silman *et al.,* 1980), *Aspergillus oryzae* (McKay *et al.,* 1991) and *Aspergillus niger* (Somiari *et al.,* 1992). Cruz *et al.,* (1982) and Kotwal *et al.,* (1988) reported production of α-galactosidase in SSF system by *Aspergillus oryzae* and *Humicola sp*, respectively. Its application in the hydrolysis of galacto-oligosaccharides present in soybean milk has also been demonstrated. Furthermore Srinivas *et al* (1994) described use of Plackett-Burman design for rapid

screening of several nitrogen sources, growth/product promoters, minerals and enzyme inducers for the α-galactosidase production by *Aspergillus niger* MRSS 234 in solid state fermentation system.

3.1.9. Strain improvement

The hallmark of all industrial fermentation processes is the over production of commercial enzymes by improved microbial strains. Conventionally strain improvement has been achieved through mutation and selection and also by genetic modification or genetic recombination (Frost *et al.,* 1987)**.** The use of mutation and selection to improve the productivity of culture has been strongly established for more than many years and is still being used as a valuable tool for industrial strain improvement. Delente *et al*., (1974) used a UV derepressed α-galactosidase to isolate mutant of B. stearothermophilus. In contrast to conventional techniques, recombinant DNA technology enables the incorporation of genes from different and unrelated species. Such modified organisms referred to as transgenic strains. To improve the productivity-to-cost ratio, it is now possible to modify genes for increase in enzyme productivity in microorganisms.

3.1.10. Purification of α-galactosidase

The first step in the purification of enzyme is the isolation of enzyme from cultured cells. A number of fungal, bacterial and yeast α-galactosidases are reported to be intracellular, In such cases recovery of intracellular α-galactosidase was carried out either by "French press" cell homogenizer (Church *et al.,* 1980; Wong *et al.,* 1986; Duffaud *et al.,* 1997; Rios *et al.,* 1993) or by grinding with abrasives (Galas *et al.,* 1996). Ultrasonication has

been also used to disintegrate the cells (Schuler *et al.,* 1985; Mital *et al.,* 1973; Kocabas *et al.,* 1999). In addition to this, organic solvents have been used for the extraction of intracellular α-galactosidase (Kotwal *et al.,* 1999). However, under submerged fermentation conditions, number of microorganisms secreting α-galactosidase outside the cell and is harvested simply by filtration or centrifugation (Talbot *et al.,* 1990; Berg *et al.,* 1980; Hashimoto *et al.,* 1991; Zapater *et al.,* 1990). Purification of proteins usually entails a series of independent steps in which the various physiochemical properties of the proteins of interest are utilized to separate it progressively from other unwanted constituents. The characteristics of the proteins that are utilized in purification include solubility, ionic charge, molecular size, adsorption properties, and binding affinity to other biological molecules (Robert *et al.,* 1982). α-Galactosidases from various sources have been purified by multi-step conventional purification procedures. Majority of procedures involved concentration of culture filtrate either by ammonium sulfate fractionation, ethanol or acetone precipitation or by membrane filtration (Shibuya *et al.,* 1995; Kotwal *et al.,* 1999; Garro *et al.,* 1996). Subsequently combination of chromatographic steps including ion exchange, gel filtration and hydrophobic interactions were used to obtain purified α-galactosidase (Church *et al.,* 1980; Puchart *et al.,* 2001; Duffaud *et al.,* 1997; Zapater *et al.,* 1990; Varbanets *et al*., 2001; King *et al*., 2002). In addition to above techniques, preparative gel electrophoresis (Pederson *et al.,* 1980), isoelectric focusing (Zeilinger *et al.,* 1993; Berg *et al.,* 1980) or chromatofocusing (Talbot *et al.,* 1990; Gherardini *et al.,* 1985) were used to achieve the highest purity of enzyme. The above methods have also been used to separate the multi-molecular forms

of α-galactosidases (Talbot *et al.,* 1990; Pederson *et al.,* 1980). Purification by more advanced tool like FPLC has also been reported (Fridjonsson *et al.,* 1999).

Apart from the conventional methods, in several instances, affinity chromatography has proved to be a successful technique in the purification of human (Mapes *et al.,* 1973), plant (Bom *et al.,* 1998; Berry *et al.,* 1991; Porter *et al.,* 1991) and microbial α-galactosidases (Li *et al.,* 1963). Moreover, in few cases, affinity chromatography has been used effectively for the separation of multi-molecular forms of plant α-galactosidases (Campillo *et al.,* 1982). Recently some of the recombinant α-galactosidases have been produced as fusion proteins with affinity tags, which enabled simpler one step purification, by its respective affinity ligand (Ishiguro *et al.,* 2001; Halstead *et al.,* 2000).

In general, medium optimization by the traditional 'one-factor-at-a time' technique was used (Gouka *et al.,* 1996). This method is not only laborious and time consuming but also often leads to an incomplete understanding of the system behavior.

Response surface methodology (RSM) can evaluate the effects of multiple parameters, alone or in combination, on response variables (Ward *et al.,* 1996). It has been successfully applied for optimizing conditions in food, chemical and biological processes (Carlsen *et al.,* 1995; Trinci *et al.,* 1970; Hours *et al.,* 1994), but has been rarely reported on for optimizing the production of alpha-D-galactosidase.

The present work describes the production of alpha-galactosidase by *Aspergillus awamori* (NCIM-1225) and the optimization of medium composition for galactosidase production with different substrates, guar, soya bean meal, yeast extract, ammonium nitrate, were performed by central composite design (CCD) and response surface methodology. Special interest has been paid to the development of higher productivity and a simple culture medium using cheap ingredients in order to reduce the cost for enzyme production. This study also will assist further in enzyme hydrolysis of guar gum for the formation of aqueous two phase system.

3.2. Materials and Methods

Part A

3.2.1. Preparation of primary inoculum and culturing of the microorganism (*Aspergillus awamori 1225*)

Culture *Aspergillus awamori (NCIM 1225)* was procured from the NCIM, National Chemical laboratory and it has been maintained on PDA (potato dextrose agar). Primary inoculum was prepared in a medium containing galactose 5g/l, Ammonium nitrate 5g/l, Magnesium sulphate 1g/l, Di potassium hydrogen phosphate 3g/l, di-hydrogen potassium phosphate 1.5g/l, Yeast extract 3g/l and it was kept at 28^{o}C for 48 h on an orbital shaker at 200 rpm. After the growth of microorganism it has been transferred to the growth medium containing everything same as primary inoculum medium except guar instead of galactose, and it has been kept at 28^{o}C for 96 h at 200rpm.

3.2.2. Selection of carbon and nitrogen source for the alpha galactosidase production

Microorganism *Aspergillus awamori* were grown on the two different carbon source (guar and kurma), inorganic nitrogen source (Ammonium nitrate; Ammonium sulphate) and organic nitrogen sources (soya bean meal; wheat bran; yeast extract) were optimized for the maximum production of alpha Galactosidase.

3.2.3. Enzyme assay for alpha galactosidase

Alpha galactosidase was assayed by reacting with 10mM *p*-nitrophenyl-α-d-galactopyranoside (*p*NP_Gal) as a substrate at 60^0 C for 5 minute in 100mM Na-Acetate buffer pH4.5. One unit of the α-d-galactosidase activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute. After five minute reaction was terminated by adding 3ml 2% sodium carbonate and produced paranitrophenyl was measured at 410nm by spectrophotometer.

3.2.4. Experimental design

In the factorial design we deliberately change one variables at a time to see the effect of it but it takes too much of time to optimize any media composition, but in case of RSM we can change more than one variable at a time to find the effect of these variable in combination, this software based technique is easier and less time consuming. For determination of optimum concentration of the variables we have chosen the central composite design (CCD) consisting of 30 runs, for four variable n= 4 the total no of experiment was 30 according to formula: 2^n (16 factor point) + 2n (8 axial point) + 6

(central point). These four variables are guar, soybean meal, yeast extract and ammonium nitrogen. These factors were initially optimized by the factorial design before doing RSM.

3.2.5. Empirical model fit to the experimental data

Quadratic model

A four variable experimental design involving a total number of 30 experiments including eight star and six center points [Table. 3.1] was employed for the optimization of the parameters. The second degree polynomials (Eq.1) were calculated with the statistical package (Design expert 7.1.3, Stat-Ease Inc., USA) to estimate the response of the dependent variable.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 - \beta_{11} x_1^2 - \beta_{22} x_2^2 - \beta_{33} x_3^2 - \beta_{44} x_4^2 - \beta_{12} x_1 x_2 - \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 \quad (1)$$

where Y is predicted response, X_1, X_2, X_3, X_4 are independent variables, β_0 is offset term, β_1, β_2, β_3, β_4 are linear effects, β_{11}, β_{22}, β_{33}, β_{44} are squared effects and β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} are interaction terms.

3.2.6. RSM matrix for optimization of culture media

The relation between the coded value of the input variable and the actual valve of the carbon and nitrogen sources are selected for optimization, where Factor guar (X_1); soybean meal (X_2); yeast extract (X_3); ammonium nitrate (X_4)) [Table 3.2].

The formula to calculate the real value from coded value

$$\chi_i = \frac{(A_i - A_0)}{\Delta A} \qquad (2)$$

Where X_i = coded value; A_i = actual value variables; A_o = actual value of the same variables at the center point; ΔA = difference between the variables.

Part B

3.2.7. Hydrolysis of GG and HPGG

GG and HPGG powders were purified by 70 % ethanol (5 volumes) precipitation and were dried in a vacuum drier for 24 hrs at 70 ^{o}C. The stock solutions of GG (1.25 % wt) and HP-GG/ (5 % wt) were prepared by dispensing purified GG and HPGG powders in water at 80 ^{o}C under magnetic stirring for 3 h. Enzymatic degradation of GG and HP GG were preformed using α-galactosidase, a fermentatively produced (from the above experiments) by *Aspergillus owamori (NCIM 1225)*. Enzyme hydrolysis was performed on 0.1, 0.25 and 0.5 wt % of GG and HPGG solutions and were incubated at 40 ^{o}C in shaking water bath. The samples were with drawn every 30 minutes and samples were analyzed for viscosity immediately.

For the optimization of enzyme units needed for the optimal degradation of HPGG, the pre-equilibrated HP-GG (0.25%) solutions are added with different enzyme volumes containing 17, 34, 52, 69 and 87 U/ml. The solutions were incubated at 40 ^{o}C in a shaking water bath for 90 minutes. The samples were withdrawn every 30 minutes and

samples were estimated for viscosity. Samples were measured for viscosity at 25 C using a plate and core viscometer. The cone was calibrated by using Brook Field standards.

3.2.8. Purification of the enzyme hydrolyzed HPGG

One liter enzyme hydrolysis reaction containing 2.5g of HPGG was performed at 40 ^{o}C for 90 minutes under magnetic stirring and pH 6.5. The enzyme hydrolyzed HPGG (EH-HPGG) was purified through ethanol precipitation and was washed with 80 and 95 % ethanol. The samples were finally purified by enzyme hydrolyzed HPGG was dried in vacuum oven at 60 ^{o}C for 24 h.

3.2.9. Preparation of Phase diagram

Stock solution of enzyme hydrolyzed HPGG (5%) and PVP K_{90} were prepared by dispensing in water at 25 ^{o}C by magnet stirring for 3h. The EH–HPGG and PVP K_{90} solutions were mixed in glass tubes in different proportions and volumes was adjusted with water. The tubes were sealed and incubated overnight at ambient temperature (25 ^{o}C). The phase diagram was plotted in the form of binodial curve according to Albertsson *et al* (Albertsson *et al*., 1977).

3.2.10. Partitioning of BSA

BSA was selected as standard protein for partition studies in enzymatic hydrolyzed HPGG and PVP K_{90}. ATPs was homogenously mixed with the aid of the vortex. The system was allowed to form two clear phases. The top and bottom phases were carefully separated by micropipette and the weight of the phases were noted. The top and bottom

phases were analyzed for protein concentration. The BSA concentration in the top and bottom phases was determined according to Bradford method (Bradford, 1976) using UNICAM spectrophotometer at 595nm.

Partition coefficient was detrmined as:

$$K = C_T / C_B$$

Where C_T and C_B are top and bottom phase concentrations respectively.

3.3. Results and Discussion

Part A

3.3.1. Choice of carbon source

The present strain of *A awamori* was tested for their stability to produce alpha-galactosidase in a guar gum containing medium and selected for further investigation. A moderate cell growth was observed in a medium containing guar gum (carbon source) and ammonium nitrate (nitrogen source), which resulted to produce 15.051 U/ml of alpha-galactosidase [Figure 3.5]. Therefore to increase the cell growth and there by increase the alpha-galactosidase activity, the medium was optimized with different carbon and nitrogen sources, for carbon source organism was grown on the two different carbon source such as guar (0.5%w/v) and kurma (1%w/v) along with basal media instead of galactose and we found that guar is giving the maximum alpha galactosidase activity as compared to kurma so that it has been selected for the further optimization.

3.3.2. Choice of nitrogen source

To further increase the alpha galactosidase activity organism was grown on the guar medium supplemented with different inorganic and organic nitrogen source. Inorganic nitrogen source which had been used is Ammonium nitrate (0.5%w/v) and ammonium sulphate (0.5%w/v) along with guar medium; out of this we got good activity with ammonium nitrate as compared to ammonium sulphate [Figure 3.6 & 3.7], this media composition has been used for further optimization with organic nitrogen source such as soya bean meal (1% w/v) and wheat bran (1% w/v) along with guar medium and ammonium nitrate and we got maximum enzyme activity in soya bean meal composition The statistical approach of medium optimization was carried out by using this guar soya bean meal medium.

3.3.3. Optimization of media

Response surface methodology (RSM) can evaluate maximum production based on a few sets of experiments in which alone or in combination or all the factors were varied within chosen range. This method has been successfully applied in the optimization of medium compositions (22–24) and fermentation processes (26). Hence a proper design that provides equal precision of estimation in all directions for the production of alpha-galactosidase is employed. The points for the design were selected from a single-parameter study. In optimizing the effect of four variables; guar gum, soya bean meal, yeast extract and ammonium nitrate on alpha-galactosidase production from *Aspergillus awamori (NCIM 1225)*, a 2^4 factorial design was applied. Out of them guar and Soya bean meal had shown stronger effect on alpha-galactosidase production and hence it was assigned significant levels.

For the development of a significant process, media optimization studies were carried out, which highly influences the production of alpha-galactosidase. For the optimization, Central composite experimental (CCD) design method was used. Total of 30 experimental runs were set with different combinations of guar gum, soybean meal, yeast extract and ammonium nitrate. The samples were withdrawn for every 24h for 96h and were analyzed for Alpha-glactosidase activity. The maximum galactosidase activity was observed after 96 h fermentation.

3.3.4. Statistical analysis

Appropriate experimental design was used by using the results for analysis of variance (ANOVA). ANOVA indicated that the quadratic model depicted from RSM could adequately used to describe the adequate production of alpha-galactosidase (sum of square, p-valve etc) and the results are shown in table. 3.

The response surface represents the relationships between responses and process factors. These are generated by plotting responses versus two variables.

Final Equation in Terms of Actual Factors:

$$\text{Enzyme activity (Y)} = 30.96967 + 3.18392\,x_1 + 3.31275\,x_2 + 2.05267x_3 + 2.50733x_4 - 0.7613x_1^2 - 3.81338x_2^2 - 3.23975x_3^2 - 2.31525x_4^2 - 1.29350x_1\,x_2 - 0.67550x_1\,x_3 + 1.90450\,x_1\,x_4 + 1.15175x_2\,x_3 + 1.30075x_2\,x_4 + 0.64750x_3\,x_4$$

The calculated F-value (29.33) indicates that the model was significant at a greater confidence level. The probability of P-valve was also very low (P=0.0008) which coincides with the agreement for significance of the model. The parity plot showed a satisfactory correlation between the experimental and predicted valves of alpha-galactosidase [Figure 3.8]. The points cluster around the diagonal lines which indicate the good fit of the model. Since the deviation between the experimental and predicted valves was comparatively less. The coefficient of determination of R^2 implies that the sample variation of 92% for production and is attributed to the independent variables *viz* guar gum, soya bean meal, yeast extract and ammonium nitrate. This CCD-quadratic model was not able to explain 7.9% of the total variation. The multiple correlation coefficient for the production of alpha-galactosidase is 0.96. This significant difference between the experimental; and predicted values explains the goodness of the model.

The prediction of the optimum values of medium composition was analyzed by applying the regression analysis to the equation-1.using Design Expert 7.1.3. The predicted medium composition was performed and repeated 3 times. The predicted and actual medium composition for optimum alpha-galactosidase was represented in the tab.1. The resulting 3D plots generated the effect of guar gum, soya bean meal, yeast extract, ammonium nitrate and the concentrations on the production of alpha-galactosidase by *Asp. awamori* [Figure 3.9 - 3.12]. The graphs demonstrated that the response surface had maximum point. An increase in the guar gum with yeast extract up to the optimum point increased the alpha-galactosidase production to a maximum level and further increase of guar gum with yeast extract, remained constant production of alpha-galactosidase.

The combined effect of guar, soya bean meal, yeast extract and ammonium nitrate was shown in [Figure 3.9 - 3.12], represents that an increase in the concentration of soya bean meal along with yeast extract, enhanced the production of alpha-galactosidase but more significant when compared with guar gum and yeast extract. The effect of the guar and soya bean meal concentration on the production of alpha-galactosidase is shown in [Figure 3.11], clearly indicates higher production of alpha-galactosidase. An increase in the guar gum concentration with soya bean increased the alpha-galactosidase production gradually but after it reaches an optimum level, the trend remains constant. The 3D graphs appear like parabolic.

3.3.5. Experimental verification of the predicted model

The predicted optimum levels of the media compositions were GG (6g/L), soya bean meal (1.255g/L), YE (0.3g/L.), and ammonium nitrate (1g/L).Verification of the predicted values of medium composition was taken in to consideration for the optimum level production of alpha-galactosidase. The maximum production obtained with experimental medium composition is 36.549, which is in good agreement with an activity of 36.716 as predicted by the theoretical model. The results clearly indicate that there is no significant difference between experimental and predicted responses. The model which is verifiable by experiments, derived from RSM can be proved to be used for the production of alpha-galactosidase by *Asp. awamori*.

3.3.6. Scale up studies

In an attempt to fermentative production of alpha-galactosidase was carried out at 2L scale to explore whether the predictive model from RSM could be applied to large scale production. The fermentation was carried out at optimum conditions for the production of the alpha-galactosidase enzyme. The alpha-galactosidase production was 36.560. The results indicated no significant difference between large and small scale synthesis. The results reassured the values obtained in small sale for industrial scale production of alpha-galactosidase.

3.3.7. Enzymatic modification of HPGG (EM-HPGG)

Figure 3.13 shows a typical viscosity and time plots of HPGG (0.5, 0.25 and 0.1 wt %) solutions hydrolyzed by α-galactosidase enzyme (17 U/ml) at 40 ^{o}C and pH 6.5. Results corresponding to different extent of hydrolysis (0 to 120 minutes) showed significant reduction in viscosity during the course of the enzymatic hydrolysis when compared to non-degraded HPGG. The α-galactosidase which cleaves the galactose side chains reduced the viscosity of HPGG solutions. This reflects the cleavage of only side chain linkages by the α-galactosidase. Since the β-mananase has large effect on guar degradation (Duffaud *et al.,* 1997). In Figure 3.13a, 0.5 % of HPGG has significant reduction of viscosity when compared to 0.25 wt % [Figure 3.13b] and 0.1 wt % [Figure 3.13c]. In all the three cencentrations of HPGG 0.5%, 0.25% and 0.1%, there is decrease in viscosity up to 60 minutes. However there is no considerable change in the viscosity from 60 to 120 minutes.

To optimize the enzyme concentration, HPGG solutions (0.25 wt %) were hydrolyzed with varying concentrations of α-galactosidase for different exposure time under magnetic stirring at 40^{o}C and pH 6.5 [Figure 3.14]. HPGG solutions were hydrolyzed with 17, 34, 52, 69 and 87 U/ml of α-galactosidase enzyme. The viscosities observed after 30 minutes showed drastic reduction in viscosity when compared to un-hydrolyzed HPGG. As the concentration of enzyme increase from 17 to 87 U/ml, there is a change in lowering of viscosity which relates to un-dydrolyzed HPGG (maximum up to 90 minutes). High substrate concentration and viscosity of polymer solution effect hydrolysis of polymer using enzymes (Cheng *et al.,* 2000). As a result lower substrate concentration samples (0.25 % wt) were selected for optimization of enzyme concentration for the hydrolysis of HPGG.

3.3.8. Phase diagram

Binodial curve for PVP/EM-HPGG/H_2O system at 25 ^{o}C is shown in [Figure 3.15]. This curve was determined by fitting a sigmoid equation to the experimental data. It could be seen that there is a significant effect of EM-HPGG concentration in the formation of ATPs. It can be seen that this system form two phases with lower polymer concentration. The EM-HPGG polymer was enriched in denser bottom phase, while PVP found in the upper phase.

3.3.9. Protein partitioning

In order to find the partitioning of proteins in the PVP/EM-HPGG/H_2O systems, a standard protein BSA was tried. The distribution of BSA in PVP/EM-HPGG/H_2O system

was studied by addition of protein solutions to the stock solutions of the both the phases. The partitioning coefficient (K) of the BSA is 4.9. The experimental K values obtained shows that BSA is concentrated in the top phase.

3.4. Conclusion

Media components are optimized for the production of alpha-galactosidase by using RSM, with the help of *Asp. awamori*. A highly significant quadratic polynomial obtained by the CCD was very useful in determining the significant effects on alpha-galactosidase production. The model predicted the media composition for the optimal production of alpha-galactosidase. The experiments were conducted by utilizing the media composition of the predicted values for the optimum production of alpha-galactosidase. This showed the significant model for the industrial production of alpha-galactosidase. This is the first report on the production of alpha-galactosidase by RSM with reference to guar gum, yeast extract, soya bean meal and ammonium nitrate. Thus RSM proved to be a powerful tool in optimizing alpha-galactosidase production by *Asp.awamori*. The hydrolysis of guar and hydroxylpropyl-guar gum was investigated. The enzyme hydrolyzed guar gum was investigated for the formation of PVP/EM-HPGG/H_2O aqueous two phase system. The partitioning coefficient (K) of BSA is 4.9 reveles that BSA is concentrated in the top phase.

Experiment No.	Factor X_1	Factor X_2	Factor X_3	Factor X_4	Experimental value	Predicted value
1	-1	-1	-1	-1	12.27	12.82
2	1	-1	-1	-1	23.041	19.32
3	-1	1	-1	-1	22.062	17.13
4	1	1	-1	-1	18.054	18.45
5	-1	-1	1	-1	16.336	14.68
6	1	-1	1	-1	17.805	18.47
7	-1	1	1	-1	24.201	23.59
8	1	1	1	-1	24.403	22.21
9	-1	-1	-1	1	10.317	10.13
10	1	-1	-1	1	25.586	24.24
11	-1	1	-1	1	22.258	19.64
12	1	1	-1	1	29.31	28.58
13	-1	-1	1	1	16.924	14.58
14	1	-1	1	1	23.433	25.99
15	-1	1	1	1	27.35	28.69
16	1	1	1	1	37.43	34.93
17	-2	0	0	0	18.49	21.56
18	2	0	0	0	33.025	34.29
19	0	-2	0	0	8.511	9.09
20	0	2	0	0	18.588	22.34
21	0	0	-2	0	9.772	13.91
22	0	0	2	0	21.916	22.12
23	0	0	0	-2	13.106	16.69
24	0	0	0	2	25.978	26.72
25	0	0	0	0	29.844	30.97
26	0	0	0	0	31.606	30.97
27	0	0	0	0	31.704	30.97
28	0	0	0	0	31.018	30.97
29	0	0	0	0	30.823	30.97
30	0	0	0	0	30.823	30.97

Tables 3.1: Operating variables, predicted and experimental data used in the Design of experiment (DOE).

Factors g/L	Coded values				
	-2	-1	0	+1	+2
X_1	0.15	0.3	0.45	0.6	0.75
X_2	0.0	0.5	1.0	1.5	2.0
X_3	0.0	0.125	0.25	0.375	0.5
X_4	0.15	0.3	0.45	0.6	0.75

Table 3.2: Experimental range and level of the factors.

Alpha-galactosidase production					
Source	**Sum of squares**	**d.f**	**Mean square**	**F-*ratio***	**P-*Valve***
Model	**1568.73**	**14**	**112.05**	**12.49**	**0.0001**
Residual ***Lack of fit*** ***Pure error***	**134.61** **132.36** **2.26**	**15** **10** **5**	**8.97** **13.24** **0.45**	**29.33**	**0.0008**
R^2	**0.9210**				

Table 3.3: ANOVA and regression analysis for the production of alpha-galactosidase.

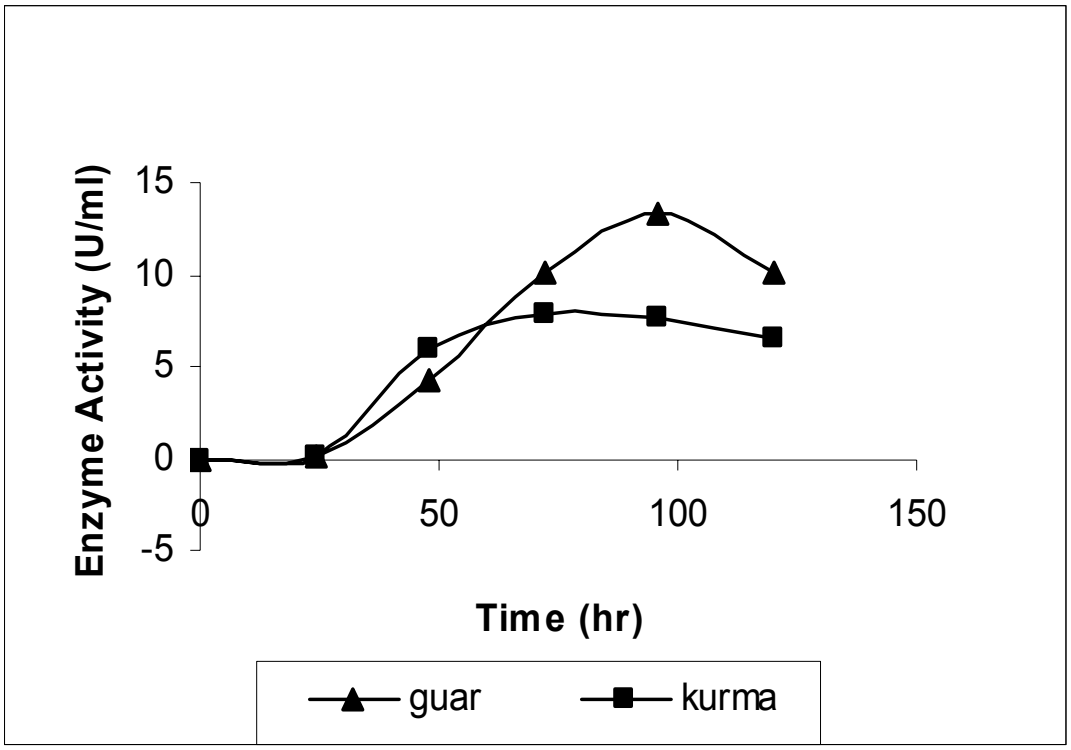


Figure 3.5: Effect of two different carbon sources (guar; kurma) on alpha-galactosidase production by *Asp.awamori.*

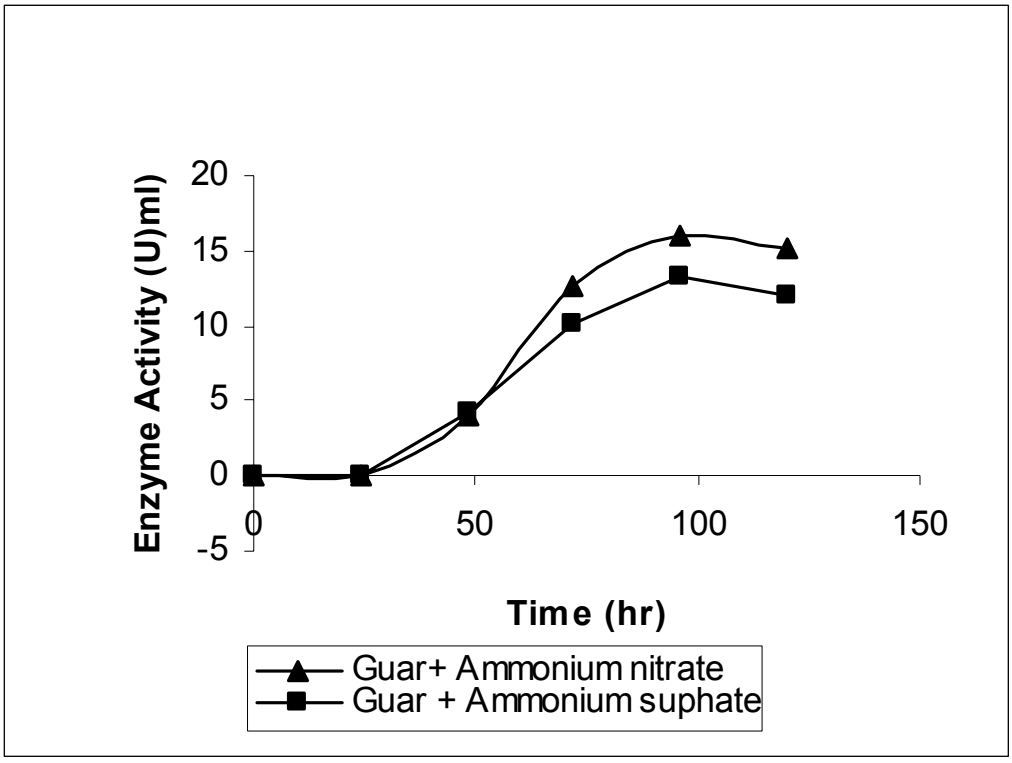


Figure 3.6: Effect of two different inorganic nitrogen sources (ammonium nitrate; ammonium -sulphate) on alpha-galactosidase production by *Asp.awamori***.**

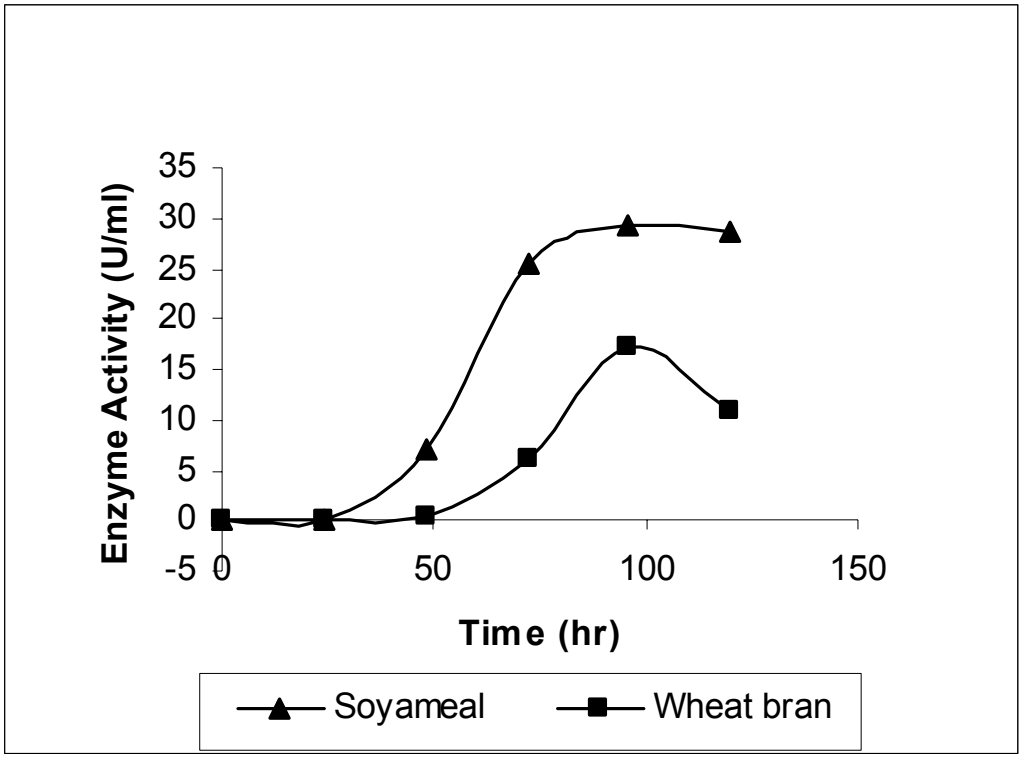


Figure 3.7: Effect of two different organic nitrogen sources (soya bean meal; wheat bran) on alpha-galactosidase production by *Asp.awamori*.

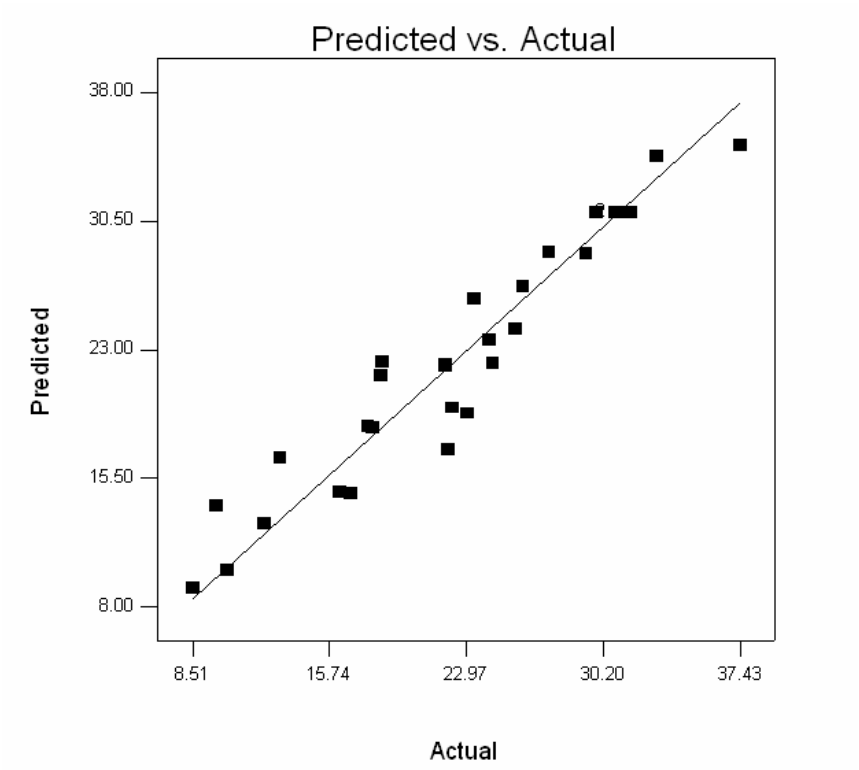


Figure 3.8: Parity plot showing the distribution of experimental *vs*. predicted valves of alpha-Galactosidase production**.**

.

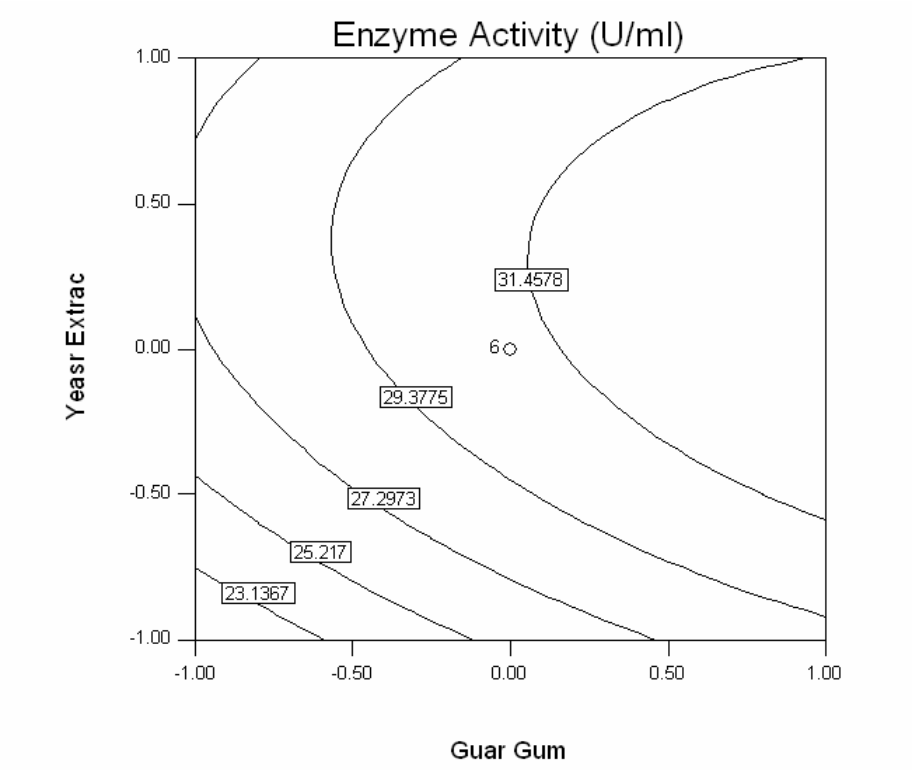


Figure 3.9: Counter plot of yeast extract vs guar on alpha-Galactosidase production.

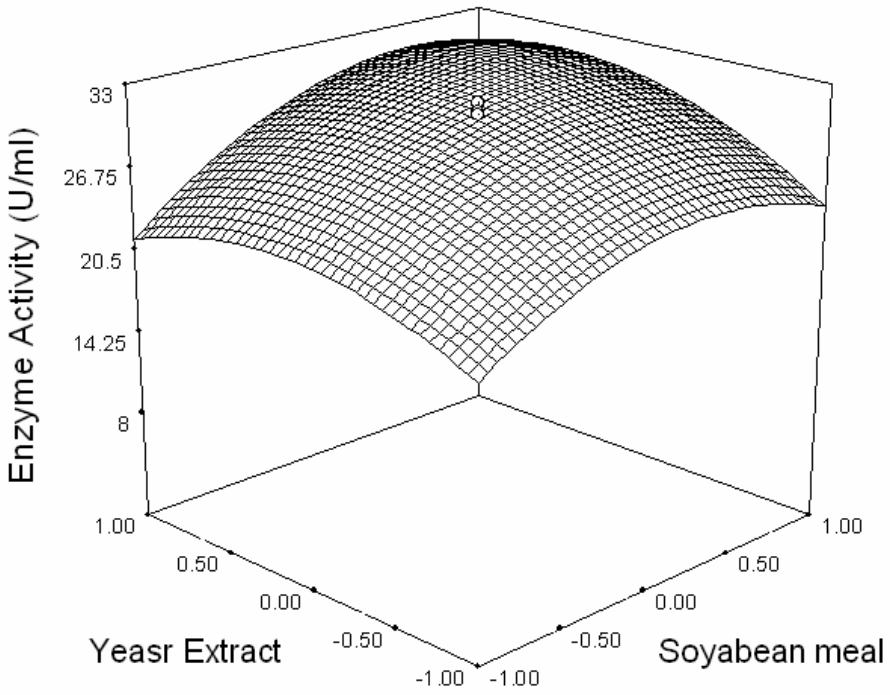


Figure 3.10: Response surface and counter plot of yeast extract vs soya bean meal on alpha-Galactosidase production**.**

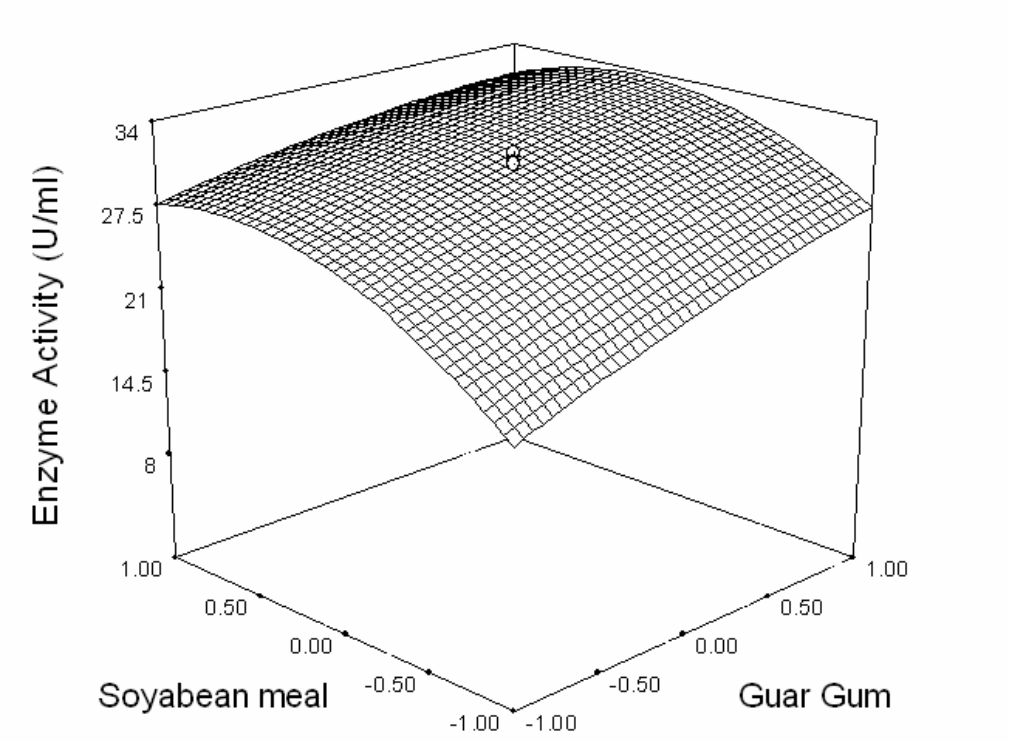


Figure 3.11: Response surface and counter of plot soya bean meal vs guar on alpha-Galactosidase production.

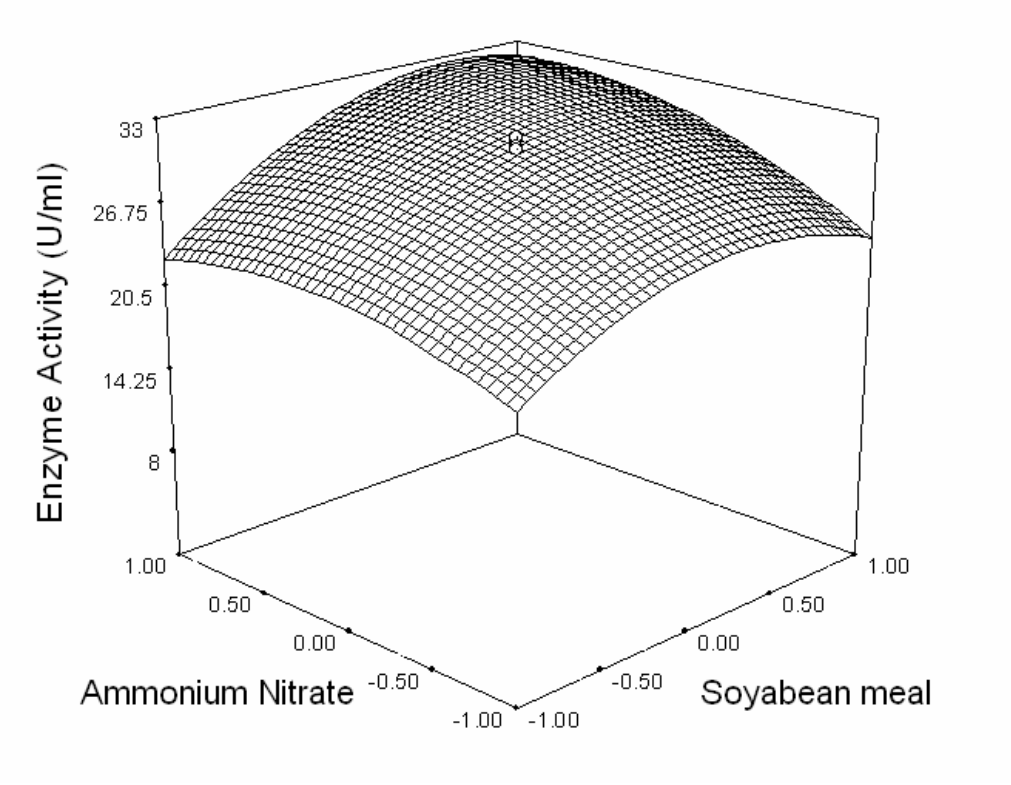


Figure 3.12: Response surface and counter plot of ammonium nitrate vs soya bean meal on alpha-Galactosidase production**.**

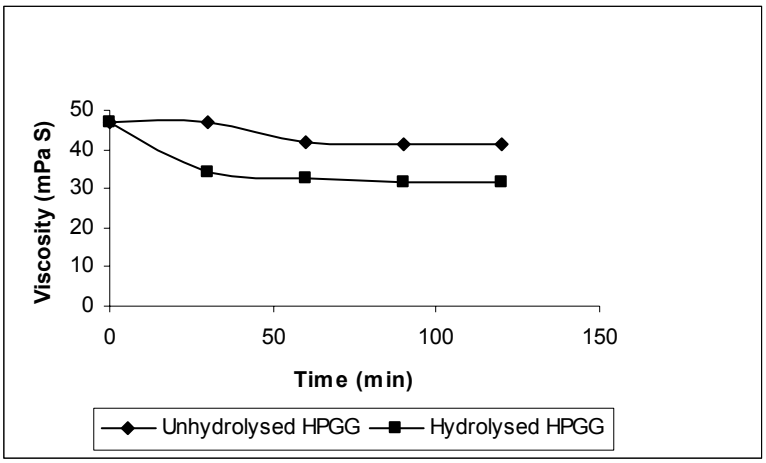


Figure 3.13a : Typical plot of viscosity as a function of time for Un-hydrolysed and hydrolysed HPGG (0.5 % wt) solutions at 40 ^{o}C.

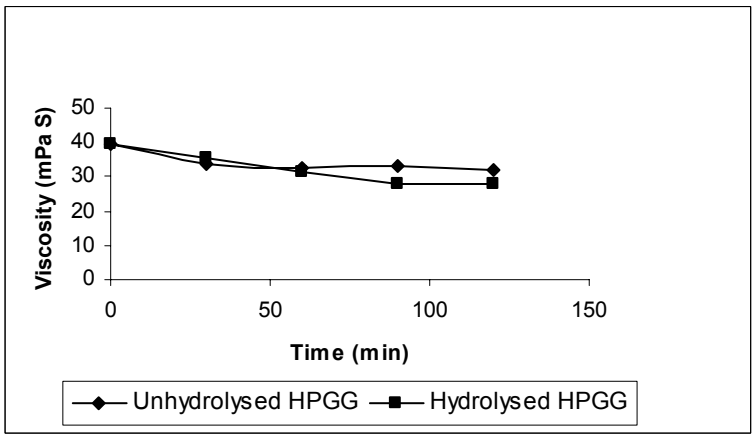


Figure 3.13b: Typical plot of viscosity as a function of time for Un-hydrolysed and hydrolysed HPGG (0.25 % wt) solutions 40 ^{o}C.

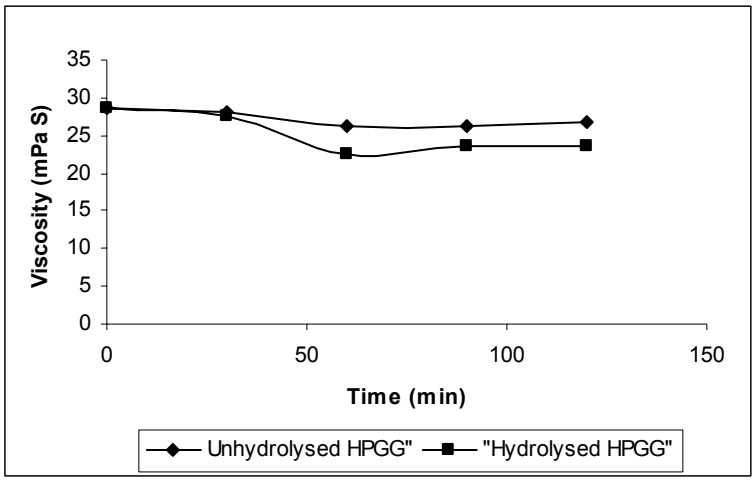


Figure 3.13c: Typical plot of viscosity as a function of time for Un-hydrolysed and hydrolysed HPGG (0.1 % wt) solutions 40 ^{o}C.

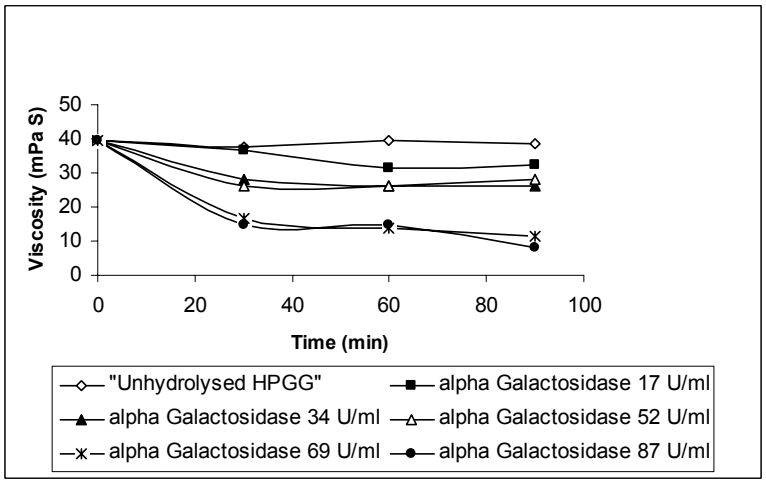


Figure 3.14: Effect of enzyme concentration on kinetics of reduction in viscosity for enzymatically degraded Un-hydrolysed and hydrolysed HPGG (0.25% wt) solutions 40 ^{o}C.

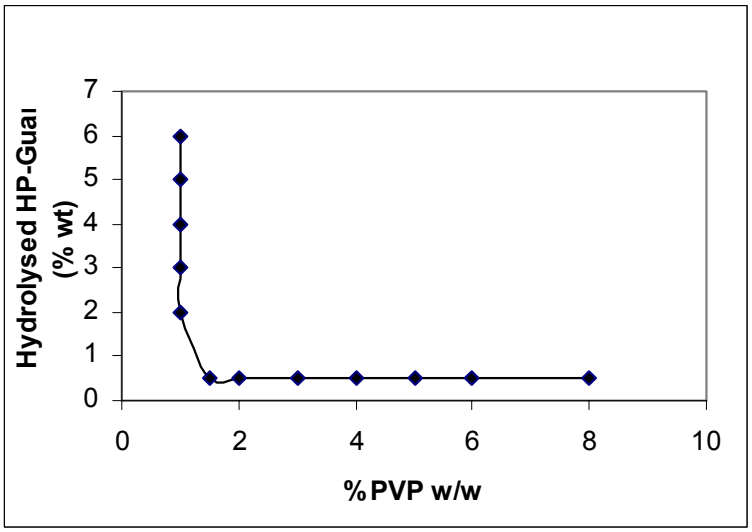


Figure 3.15: Phase diagram for enzyme hydrolysed-HPGG and polyvinyl pyrrolidone (PVP K_{90}) at 25 °C.

Chapter 4

Protein partition on Phospho-Guar gum aqueous two phase system

4.1. Introduction

Aqueous two phase systems (ATPs) have been widely used for the separation of various biomolecules especially proteins by virtue of their partitioning in the two phases (Albertsson, 1986; Walter *et al*., 1985)**.** This unit operation has made the down stream processing inexpensive and easily scalable for enzymes (Kula *et al*., 1982)**.** Although, its biotechnological applications are known for more than 30-years, the ATPs technique has not been used extensively in the industries. This is because the choice of the polymers used in ATPs was mainly restricted to poly (ethylene glycol) (PEG) and dextran or hydrophobically modified starch, e.g. hydroxypropyl starch (Reppal PES 200) etc (Ling *et al*., 1989)**.** However, high cost of the fractionated dextran and denaturation of the biomolecules with high concentration of salts has limited the use of this system for large scale isolations (Kula *et al*., 1989; Johansson *et al.,* 1984; Tjerneld *et al.,* 1986). Therefore, a lot of interest has been generated on biocompatible, mild and low cost protein partitioning systems. Polyvinyl pyrrolidone (PVP)/ dextran and polyvinyl alcohol/dextran have been investigated as cheaper alternatives (Albertsson, 1986). Several polymers like malto-dextrin (Szlag & Giuliano, 1988), starch derivatives (Venancio *et al.,* 1993) cellulose derivatives (Skuse *et al.,* 1992), guar (Simonet *et al.,* 2000) and cashew nut gums (Sarubbo *et al.,* 2000) etc. have been studied. Still there is a need to develop new aqueous two-phase systems, which are less expensive with high or equal partitioning properties similar to fractionated dextran (Pietruszka *et al.,* 2000). Guar gum and its derivative hydroxylated guar gum (trade name solvitose gum) are used in textile industry. Use of this polymer in ATPs leads to three times increase in the partition coefficient of the bovine serum albumin (Venancio *et al.,* 1995).

Guar galacto-mannan is a neutral water-soluble polysaccharide extracted from the seeds of *Cyamopsis tetragonoloba*. Guar is a hydrophilic polymer with outstanding thickening property and wide applications (Robinson *et al.,* 1982**;** Dea & Morrison, 1975). It consists of a linear backbone of *beta*-1-4 linked mannose units and is stabilized by randomly attached *alpha*-1-6 linked galactose units as side chains. The ratio of mannose / galactose units ranges from 1.6:1 to 1.8:1. In aqueous solution, guar acts as a random-coil polymer. High viscosity of guar solutions arises due to its high molecular weight (up to 2 million) (Vijayendran & Bone, 1984). Guar gum has ability to modify the properties of aqueous environments by its capacity to swell, emulsify, stabilize, chelate, encapsulate, flocculate, film/membranes and gel formation. Guar gum derivatives are prepared by cross linking with non-toxic linker molecules. This chemical modification is done to meet requirement of specific industrial applications**.** Swelling property of native gum can be decreased with specific charge on molecule (Gliko-Kabir *et al.,* 2000). Guar gum have been modified by reacting it either with borax or glutaraldehyde (Rubinstein & Gliko-Kabir, 1995**;** Gliko-Kabir *et al.,* 1998) or trisodium trimetaphosphate (STMP) to reduce GG swelling properties (Gliko-Kabir *et al.,* 2000a). Due to presence of extensive intermolecular association (hyper entanglement) through hydrogen bonding, guar gum and its derivatives are excellent thickeners, which are useful for many applications (Lapasin, & Pricl, 1995)**.** Water soluble Phospho-guar gum is obtained by reacting guar gum with an aqueous phosphate solution and oxidizing the resulting phosphate ester in the presence of alkali and at a temperature of about 130^{o}C. The product whose viscosity in aqueous solution can be set very accurately is used as thickening agent, particularly in

the paper industry. This chemical modification may be useful for development of new polymer aqueous two-phase system.

In the present work, phase separation systems were formed with a system containing simple synthetic neutral polymer "polyvinyl pyrrolidone" and a natural polymer "Phospho-Guar gum". The system was used to study the partitioning of sample proteins having different characteristics. The protein partitioning study of both the proteins was carried at different pH values.

4.2. Materials and Methods

4.2.1. Polymers and Chemicals

Polyvinyl pyrrolidone K_{90} (PVP) from S.D Fine-chemicals Ltd (India), phospho-guar gum (PGG) from Dabur India Ltd (India), Bovine serum albumin (BSA) and L-DOPA (L- 3, 4, Dihydroxy phenyl alanine) from Hi-media Co (India) were used. Polyphenol oxidase (PPO) was extracted from mushroom. All other chemicals for partition study were of analytical grade.

4.2.2. Aqueous two phase system preparation

Stock solutions of polyvinyl pyrrolidone 20% (*w/w)* and Phospho guar gum 1.5% (*w/w)* were prepared in aqueous solution containing 0.05% *(w/v)* sodium azide. Predetermined volumes of the solutions were added together; its volume was adjusted to required level with water, mixed well and incubated at 25°C for 4 h to form aqueous two phases. pH of

the system was maintained at 5, 6, 7 and 8 with 100 mM sodium acetate and 100 mM sodium phosphate respectively.

4.2.3. Phase diagram

By mixing two polymers in water, phase formation occurs above a certain concentration whereas other mixtures gave a homogeneous phase. This behavior is observed by using a phase diagram where concentration of one polymer is plotted against the other which generally yields a curved line. All the compositions of the polymers represented by points above the line show phase formation whereas, mixtures represented by points below the line gave a homogeneous phase. This line dividing the area in phase forming and homogeneous solution is called bionodial.

In the present case phase diagram was constructed by thoroughly mixing two polymers in varying concentration after adjusting the volume with water. The system pH was maintained by 0.1 M Phosphate buffer saline pH-7.

4.2.4. Determination of Tie lines

Tie lines were obtained by joining two pairs of points lying on the binodial curve. Any total composition represented by point on the tie line gives phase system with same phase composition but with different volumes of the two phases. For determining the tie line compositions, the top and bottom phases were separated carefully by micropipette. The separated phases were dried for determination of % weight of water, in vacuum oven at 50°C till constant weight. The dried phases were re-dissolved in water and precipitated

with alcohol. The % weight of PGG in the bottom phase as well as in the top phase was estimated. The precipitated polymer was vacuum dried. % PVP was calculated by balancing the mass of total weights of top and bottom phases, % water and % PGG.

4.2.5. Tie line length

The percent tie line length (TLL) was calculated by the equation:

$$TLL\% = \sqrt{(\Delta\ PGG)^2 + (\Delta\ PVP)^2}$$

Where 'Δ PGG' (*% w/w*) is the difference in concentration of the predominant top phase forming polymer between top and bottom phases, and 'Δ PVP' (*% w/w*) is the difference in concentration of the predominant top phase forming polymer between top and bottom phases.

4.2.6. Protein partitioning

Protein partitioning study was conducted by adding two individual proteins (BSA and PPO) in PVP/PGG/H_2O ATPs. For maximum protein partitioning the phases were incubated for 4 h at 25^oC. The clear top and bottom phases were carefully separated by micropipette and weighed separately. Separated top and bottom phases were diluted in buffer, if necessary, and analyzed for protein concentration and enzyme activity in top and bottom phases separately. The protein concentration in top and bottom phases was determined by modified Folin-Lowery method (Lowry *et al.,* 1951). The PPO activity in top and bottom phases was determined. Partition coefficient (K) was defined as ratio of

top phase and bottom phase concentration of protein or enzyme activity. BSA was used as standard protein.

4.2.7. Preparation of crude PPO enzyme

Polyphenol oxidase was extracted by homogenizing fresh mushroom in ice-cold phosphate buffer pH 7 with 10mM cysteine. To the filtered homogenate $(NH_4)_2SO_4$ was added to make 25% saturation and centrifuged at 10,000×*g* for 20 min. The resulting pellet was dissolved in 50mM phosphate buffer pH 7 and desalted by dialyzing against the same buffer. This dialysate was freeze dried and the dry enzyme powder was used for ATPs studies (Vaidya *et al.,* 2006).

4.2.8. Estimation of PPO activity

Polyphenol oxidase (PPO) activity was determined by using 3, 4-dihydroxy-L-phenylalanine (L-DOPA) as the substrate (Pathak & Ghole, 1994) using a "chemito visible sectrophotometer" at 470 nm**.** One unit of enzyme activity was defined as the amount of enzyme, which causes an increase in absorbance by 0.001 min−1 at 25°C, which corresponds to the conversion of 0.01μmol of substrate to product (Dopachrome) (Vaidya *et al.,* 2006).

4.2.9. Phase densities and Viscosities

The top and bottom phases were separated carefully by a micropipette. Densities were measured by using 10 ml pycnometer at 25°C. The pycnometer was weighed on analytical balance (Mettler), then the bulk densities of both top and bottom phase were

measured by taking ratio of mass to its volume ratio. Individual phase viscosities of top and bottom phases were measured using Brookfield viscometer **(**CAP 2000+, cone-plate viscometer), at 25^{o}C.

4.3. Results and Discussion

4.3.1. Phase diagram

Phase diagram for PVP/PGG/H_20 ATPs is shown in the Figure 4.1. The binodial curve was plotted on the basis of phase forming nature of two polymers, when mixed in different concentrations. The compositions above the critical point formed the aqueous two phases, whereas compositions the below the point remained homogenous. At 1.75 to 4 (*% w/w*) concentration of PVP, the binodial almost joins the PVP axis, indicating that PGG is practically excluded from the PVP- rich phase. Phase separation was observed in a mixture containing 1.75*% w/w* of PVP and 0.01*% w/w* of PGG, represents the limit for visual observation. This agrees with the fact that the binodial is generally shifted toward the axis of the lower molecular weight polymer and also corresponding to guar concentrations (<1*% w/w*) in galactomannan/dextran mixtures (Simonet *et al.,* 2000; Garnier *et al.,* 1995). The phase diagram indicated that as molecular weight of PGG is very high, only a small amount of it is required to form aqueous two-phase system. If the concentration is greater than 1% *w/w* PGG, the curve did not follow a vertical asymmetry. Thus it is shifted away from the vertical axis and displayed a typical curvature at higher PGG concentration. These peculiar properties of the phase diagram may be due to the high viscosity of the medium. Being bottom phase of PGG and having large molecular weight, there is no formation of droplets which may interfere in the

formation of aqueous two phases. In fact, it is well known that concentrated guar solutions exhibit a very high viscosity. This type of curvatures coincides with the results (Albertsson, 1986)**.** The phase diagram shows that PVP is compatible with PGG for aqueous two-phase system.

The tie lines were plotted with the analysis of constituent compositions of both the top and bottom phases. Total composition of the top and bottom phases of the system is given in Table 4.1. The phase composition indicates that the carbohydrate polymer was enriched in the denser bottom phase while PVP was found in the top phase. The effect of temperature on the phase diagram was conducted at three different temperatures (25°C, 35°C and 40°C). At higher temperatures like 35°C and 40°C, clear distant phase formations were not observed.

4.3.2. Physical characteristics properties of top and bottom phases

The phase density and viscosities of the top and bottom phases of PVP/ PGG/H_20 systems are given in Table 4.2. The density and viscosity values of both top and bottom phases are very close, that results in enhanced demixing time (4 h). Generally it is expected that higher the molecular weight of the polymers, higher the viscosity and higher the viscosities lower the concentration of polymers required.

4.3.3. Protein partitioning

BSA was analyzed as standard protein and polyphenol oxidase (PPO) as test protein in PVP/PGG/H_2O aqueous two-phase system. Protein partitioning of BSA and PPO at two

different concentrations of PGG in PVP/PGG/H_2O ATPs was estimated. Partition coefficient for both the proteins was >1, that indicated partitioning of both proteins in the top phase.

4.3.4. Effect of pH on the partition coefficient (K) of BSA

In order to know the influence of pH on BSA partitioning (K), experiments were performed at pH and different concentrations of PGG in PVP/PGG/H_2O (i.e. 0.40% *w/w* and 0.80% *w/w*) aqueous two-phase system. For both the proteins (BSA and PPO) different partition coefficients were obtained at different pH. As BSA is stable in the pH range of 5 to 8, experiments were restricted to that pH range and results are shown in Figure 4.2**.** The partition coefficient for BSA in 3% *w/w* PVP/0.40 % *w/w* PGG/H_20 aqueous two phase system at pH 5.0 was lowest (i.e 8.94) whereas at pH 8, partition coefficient increased to 21.67. This may be due to the migration of most of the protein (BSA) to the PVP rich top phase at higher pH. Thus with change in pH of the system, individual proteins showed different partition behavior. This behavior may be due to two reasons first, net charge (positive or negative) of the protein and second, due to surface properties of the proteins.

With increase of PGG concentration from 0.40% to 0.80% *w/w*, maximum partition coefficient was observed at pH 6 (K=11.331) which declined with increase of pH to 8 in PVP/PGG/H_20 ATPs. Thus BSA exhibit different property may be influenced by conformation and specific interactions with phase forming polymers. Similar results were obtained for other polymer-polymer systems (Almeida *et al.,* 1998**;** Christian *et al.,* 1998;

Kishida *et al.,* 1998)**.** The free volume in top phase remained constant with an increase in pH, for a given tie line length and this trend (extent of increase) remained almost constant for all tie lines.

4.3.5. Effect of pH on the partition coefficient (K) of polyphenol oxidase

Experiments for partitioning of a test protein "polyphenol oxidase" were conducted to confirm suitability of the new PVP/PGG/H_2O aqueous two-phase system. Since PPO is stable in the pH range of 5.0 to 8.0, the effect of pH on partitioning were studied in this range only. From the Figure.4.3, it is observed that PPO is partitioned in the PVP rich top phase and with an increase in pH in 3% *w/w* PVP/0.40 % *w/w* PGG/ H_20 ATPs. At pH 5.0 partitioning coefficient was 3.1, which increased to 5.26 at pH 8. This may be due to net charge on the protein surface. PPO partitioning showed less variation as compared to BSA with the change in system pH from 5 to 8 in 3% *w/w* PVP/0.80 % *w/w* PGG/H_20 ATPs.

4.3.6. Influence of Phospho-guar gum (PGG) concentration on the protein partitioning

From Figure 4.2 & 4.3, effect of the tie line length (TLL) on the protein partitioning in PVP/PGG/H_2O ATPs at pH 5.0 to 8.0 for BSA and PPO is seen. With increase of TLL decrease in the K value of both proteins were observed. The results indicated that there was a significant change in the BSA and PPO partition coefficients with increase in tie-line length. This might be because of different molecular sizes and surface properties of BSA and PPO. The addition of water-soluble polymers to a solution of proteins is known

to precipitate the proteins because of biospecific affinity. Polymers used in aqueous two phase system should permit high solubility of proteins in order to be effective over a large concentration range. Since protein solubility decreases with increase in PGG concentration, this could be the reason for decrease in K with respect to tie line compositions.

4.4. Conclusion

Due to lower cost of polyvinyl pyrrolidone/ phospho-guar gum system provides a potential alternative to expensive fractioned dextran for aqueous two-phase systems. Use of PVP/PGG/H_2O ATPs for separation of BSA and PPO was investigated on the basis of net surface charge, conformation of the protein and bio-specificity of individual proteins. The partitioning of two proteins, BSA and PPO, with different characteristics showed similar trend with the influence of pH and tie-line. The results obtained showed that it was possible to change the degree of partitioning by selecting the right conditions like pH and tie line. The new aqueous two-phase system is developed and is expected to facilitate the recovery of phospho-guar gum.

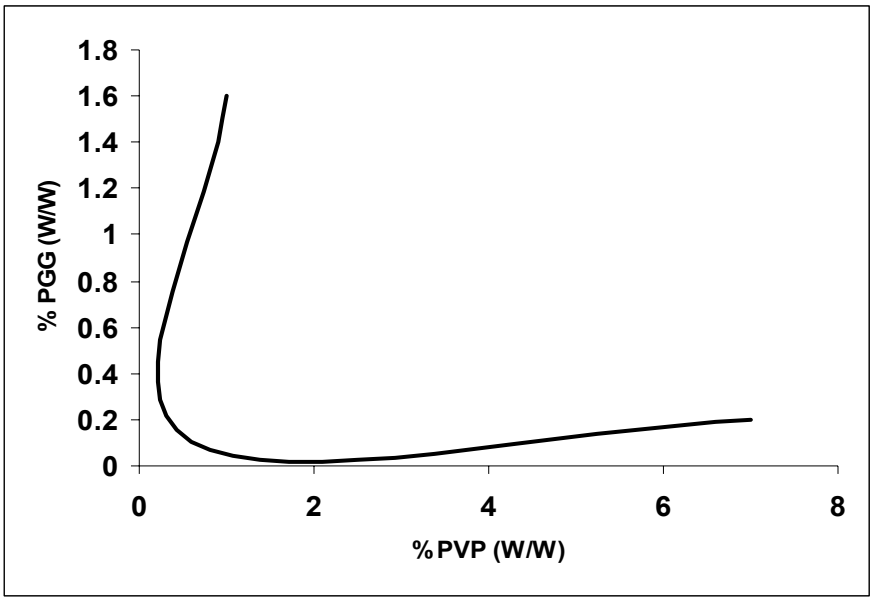


Figure 4.1. Phase diagram for Phospho guar gum and Polyvinyl pyrrolidone (PVP) at 25^{o}C.

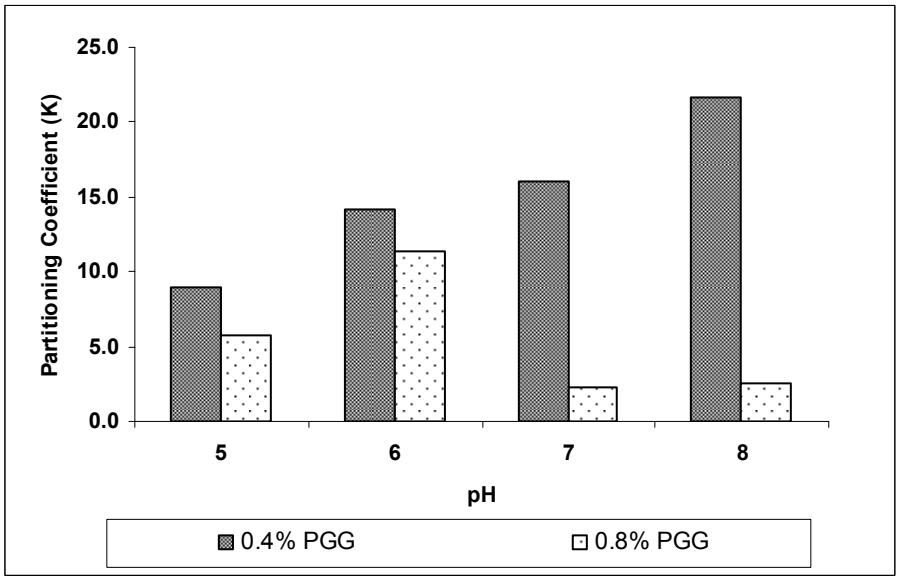


Figure 4.2. Partitioning of standard protein (BSA) at different pH with 0.4% and 0.8 % concentrations of phospho-guar gum and 3 % concentration of polyvinyl pyrrolidone (PVP) respectively at 25^{o}C.

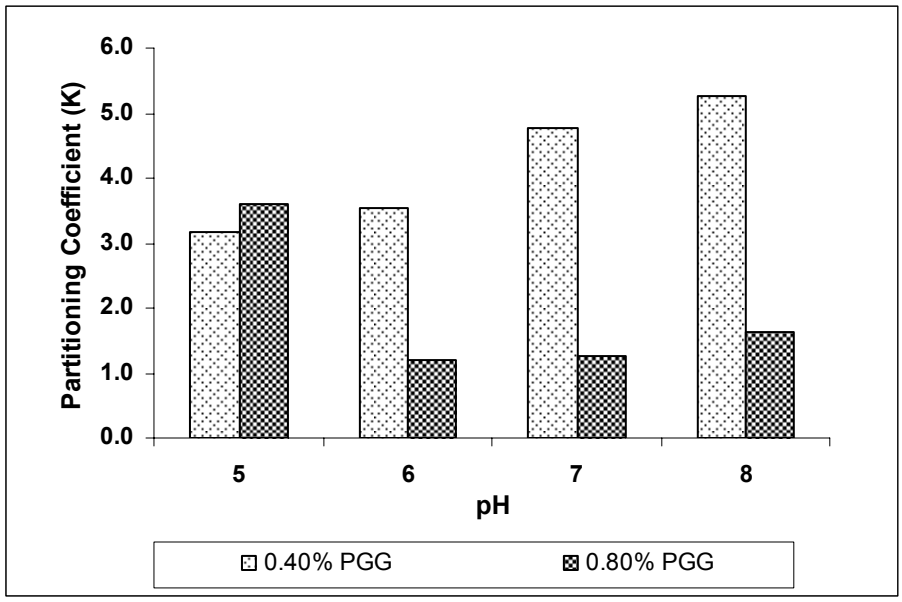


Figure 4.3. Partitioning of poly phenol oxidase (PPO) at different pH with 0.4% and 0.8 % concentrations of phospho-guar gum and 3% concentration of polyvinyl pyrrolidone (PVP) respectively at 25^{o}C.

Tie Line		
	1	**2**
Total Composition		
Phospho-Guar gum	0.4	0.8
PVP K_{90}	3	3
Water (H_20)	96.6	96.2
Top Phase (%)		
Phospho-Guar gum	0.02	0.06
PVP K_{90}	3.37	3.23
Water (H_20)	96.61	96.71
Bottom Phase (%)		
Phospho-Guar gum	1.404	2.32
PVP K_{90}	2.661	2.196
Water (H_20)	95.935	95.484

Table 4.1. Tie line composition (w/w) of Phospho-Guar/ PVP system at 25°C
1) 0.40 % Phospho-Guar gum 2) 0.80 % Phospho-Guar gum.

System (% w/w)	**Density (Kg/m^3)**		**Viscosity (mPa S)**	
PGG/PVP/H_20	**Top**	**Bottom**	**Top**	**Bottom**
0.4 / 3 / 96.6	**0.991**	**0.996**	**88.7**	**225.0**
0.8/ 3 / 96.2	**0.992**	**1.002**	**90.0**	**296.8**

Table 4.2. Physical characteristics of Phospho-Guar/ PVP systems at 25°C**.**

Chapter 5

Vinyl-2-pyrrolidone derivatized guar gum based aqueous two phase system

5.1. General ntroduction

Aqueous two phase systems (ATPs) have found use in biochemical research for separation and purification of biomolecules (Walter *et al.,* 1986; Kamihira *et al.,* 1992) ATPs not only provide a gentle environment for bioactive proteins but also offer unique possibilities for their down stream processing. It has been established that ATPs are formed when combinations of hydrophilic solutes (polymers or polymer and certain salts) display incompatibility in aqueous solution above critical concentrations (Albertsson, 1971). Besides physical extraction, a reactive extraction may also be employed by covalent binding of a ligand to one of the phase polymers (e.g. PEG). It is possible to bind the ligand to a third polymer that preferentially favors one of the phases and has the advantage of being precipitated by the change in pH, temperature or some other physical properties (Kamihira *et al.,* 1992).

Guar gum (GG) is a gum obtained from the seeds of *Cyamopsis tetragonalobus,* which belongs to *Leguminaceae* family. The galactomannan of guar gum has a backbone of β (1-4)-linked D-mannopyranose units to which, at intervals, are attached with single units of D-galactopyranose by α (1-6)-glycosidic bonds (Ahmed *et al.,* 1950; Dea *et al.,* 1975). The mannose/galactose ratio is about 1: 6. In aqueous solution, guar acts as a random-coil polymer (Robinson *et al.,* 1982) and exhibits thickening properties that are widely used, particularly in the food and textile industry.

Native guar gum as well as its derivatives are commercially important and find use in diverse applications like oil-well drilling, paper and textile sizing, as a binding agent for

explosives, food, etc. Graft reactions like grafting of acrylamide, (Behari *et al.,* 1999; Deshmukh *et al.,* 1987; Bajpai *et al.,* 1988) acrylic acid, (Taunk *et al.,* 2000) methyl methacrylate (Chowdhury *et al.,* 2001) on to guar gum have been reported.

Redox reactions are well documented for grafting on various polymers. Activation energies of redox polymerization reactions are typically ~15kcal/mole. These are usually highly selective to form specific primary radical species (Cao *et al.,* 2002). The special significance of redox reactions in aqueous solutions is that they proceed at high rates at relatively low temperatures, whereby high molecular weight polymers are obtained. Moreover, transfer and branching reactions are less prominent. Ceric ammonium nitrate (CAN) is a very efficient catalyst for grafting vinyl monomers on to natural polymers at low temperatures (Kubota *et al.,* 1995 ; Okieimen *et al.,* 1996 ; Farag *et al.,* 2002; Athawale *et al.,* 2000; Lutfor *et al.,* 2001; Tripathy *et al.,* 1999; Shah *et al.,* 1995; Cao *et al.,* 2002; Castellano *et al.,* 1997; Athawale *et al.,* 1997; Athawale *et al.,* 1998). In presence of peroxydisulfate, radicals can often be produced at a convenient rate from peroxides, at temperatures lower than those at which they are thermally decomposed. The mode of attack in redox reactions is usually highly selective, leading to well defined primary radical species (Sarac *et al.,* 1999; Misra *et al.,* 1982).

Guar has a highly branched structure than dextran which allows for modification reactions under modest reaction conditions. In addition, guar is a relatively cheap and abundant material. We synthesized cerium(IV) mediated grafting of 1-vinyl-2-pyrrolidone on to guar gum for protein partitioning in aqueous two phase system with

dextran as a counter polymer. A new aqueous two-phase extraction protocol was established using the derivatized guar and dextran mixture. A phase diagram was constructed based on the experimental phase separation shown by the system. The influence of ionic salt (sodium chloride, NaCl) was investigated on the partitioning of Standard Bovine serum albumin (BSA) system.

5.2. Materials and Methods

5.2.1. Materials

Guar gum was obtained as a gift from Dabur India Ltd. 1-vinyl-2-pyrrolidone (1-VP; Fluka), Potassium peroxydisulfate ($K_2S_2O_8$; Merck India Ltd.) and Ceric ammonium nitrate (CAN: Qualigens Ltd.) were used. Dextran (2-million from Sigma-Aldrich) was used for partition studies. All other reagents used were of analytical grade. Methanol and acetone were distilled twice before use.

5.2.2. Derivatization

1.5% w/w aqueous GG solution was homogenized at 80^{o}C in a round bottom flask for 30 min. The solution was cooled to room temperature and 1.8% w/w 1-VP was added with constant stirring. The grafting reaction was initiated by adding $K_2S_2O_8$ (5 mol % of GG) followed by aqueous CAN (4.8 mol % of GG) in an inert atmosphere with constant stirring at an ambient temperature and pressure. The reaction was continued overnight and terminated by adding hydroquinone (1000 ppm). The product was precipitated in methanol and freed off unreacted substrates by giving several washings of water, dried, and weighed. For isolating PVP homopolymer, washings were concentrated under low-

pressure. The concentrated mass (PVP homopolymer) was precipitated in acetone and further purified by giving several washings of water. PVP-homopolymer was dried in a vacuum oven till constant weight and weighed.

5.2.3. FTIR analysis

A Shimadzu 8300-Fourier transform infrared spectrophotometer (FTIR) with a resolution of 1 cm^{-1} in the transmission mode, was used. Polymers were milled (2 mg each), mixed with potassium bromide (100 mg), and pressed into a solid disk of 1.2 cm diameter prior to the infra-red measurement.

5.2.4. Scanning electron microscopy

Surface morphology was carried by mounting both GG and derivative GG on stubs and sputter-coated with gold to be able to visualize the particles. Micrographs were taken on a JEOL JSM-5200 scanning electron microscope.

5.2.5. Preparation of aqueous two phase system

Stock solutions of both polymer phases were prepared in an aqueous medium (10% w/w vinyl-2-pyrrolidone-guar gum and 20% w/w Dextran). The dextran and V-GG solutions were mixed in glass tubes in varying proportions and volume was adjusted with water. The tubes were sealed and incubated overnight at ambient temperature (25^{o}C). The phase diagram was plotted in the form of a binodial curve.

5.2.6. Phase diagram

10 gm of aqueous two-phase systems were prepared for plotting phase diagram according to method reported by Albertson (Albertson, 1971). Solutions of two polymers were weighed in tubes in twenty different proportions, mixed and their volume was adjusted with water. The tubes were sealed and left overnight at room temperature (25^{o}C) for separation of phases. The clearly visible two phases were separated carefully with the aid of the micropipette and the wet-weights of the bulk top and bottom phases were noted. Both the bulk top and bottom phases were dried under vacuum at 50^{o}C till constant weights. The dry mass of both phases were weighed to determine the content of water. The dried polymer was dissolved in water for determination of dextran. The equilibrium concentrations of dextran in top and bottom phases were determined by dextranase enzyme hydrolysis assay. The dextran in top and bottom phase samples were treated with dextranase enzyme, the samples were incubated for 30 min at 37 °C. This leads to hydrolysis of dextran to maltose. This maltose released was estimated by DNSA method (Miller, 1959). The DNSA reagent was prepared by dissolving 1.0 gm of 3, 5-dinitrosalicylic acid, 200 mg phenol, 50 mg sodium sulfite, 20 gm sodium potassium tartrate tetrahydrate in 100 ml of 2% NaOH. 1ml of the above reagent was added to 1ml samples obtained by hydrolysis of dextran; incubated for 15 min in boiling water bath and cooled to room temperature. 10 mL water was added to reaction mixture and its absorption was measured at 540 nm. Isomaltose is used as a standard; a calibration curve was plotted under similar conditions (Janson and Porath, 1966). The concentrations of dextran in the samples of both the top and bottom phase were calculated according to the standard calibration curve. The content of the derivative guar is determined by

subtracting weight of dextran, weight of water from total wet weights of the respective phases.

5.2.7. Phase viscosities and densities

The top and bottom phase were separated care fully by a micropipette. Densities were measured by using 10 ml pycnometer at 25°C. pycnometer was weighed on analytical balance (Mettler), then the bulk densities of both top and bottom phase were measured by taking ratio of mass to its volume ratio. Experiment was performed in triplicate. Viscosity of both top and bottom phases were measured at 25^{o}C using a plate and cone viscometer (Brookfield-CAP 2000+, cone-plate viscometer). The cone was calibrated by using water and Brookfield standard as calibrants.

5.2.8. Protein partitioning

5.2.8.1. Partition coefficient (K)

For partitioning experiments, 1.66% dextran (2 million) / 1.66 % V-GG/ 96.68 % H_2O was selected for the protein partitioning study. Pre-weighed polymers were mixed with sample protein i.e. BSA, LO and Phytase and subjected to a protein partitioning procedure as described above. Phases were isolated separately.

The protein partition coefficient (K) was estimated as:

$$K = C_T/C_B$$

Where, C_T and C_B are top and bottom phase concentrations respectively.

5.2.8.2. Protein estimation

For determination of BSA concentration in the phases, samples were withdrawn from both the phases and were diluted with a known amount of distilled water, and its concentration was determined by Bradford method (Bradford, 1976). Diluted sample is incubated for 5 min after adding 5 ml of Bradford reagent and mixed the samples thoroughly. The absorbance is measured at 595 nm. The sample concentration was compared with standard curve. BSA is used as standard protein.

5.2.8.3. Lactate oxidase assay

The partitioning of lactate oxidase from a partially purified cell lysate of *Aerococcus sp* was carried out with the above aqueous two phase systems. The cell lysate was added and well mixed to form a homogenous solution allowing lactate oxidase to partition between the two phases. The lactate oxidase assay was performed according to A. Toda (Toda *et al.,* 1998), at pH 7.4 with phosphate buffer and 10mM L (+) lactic acid was preheated at 37°C for 5 min; 0.10 ml of both the top and bottom phases containing the enzyme solution was then added. After incubation for 10 min at 37°C, 0.5 ml of 1mM 2, 4-dinitrophenylhydrazine was added and the mixture was further incubated at 37°C for 20 min. The reaction was terminated by addition of 5ml of 0.4 M NaOH and the brown color developed was measured spectrophotometrically at 515nm. One unit of activity was defined as the amount of enzyme that caused the formation of 1.0 μmole of pyruvate per minute under test condition.

5.2.8.4. Phytase assay

Phytase measurements were carried out at 37°C on a shaking water bath in triplicate. The

enzymatic reaction was initiated by adding of 0.10 ml enzyme extract to the assay mixture consisting of 350µl of 0.1 M sodium acetate buffer, pH 4.5, containing 500 nmol sodium phytate and was incubated for 30 min; the liberated inorganic phosphate was measured by a ammonium molybdate method (Ullah et al., 1987). One milliunit (mU) was defined as one nmole of phosphate liberated per minute per milliliter.

5.3. Results and Discussion

5.3.1. Grafting

N-vinylpyrrolidone (NVP) possess some important properties like readily copolymerization with many acrylics and susceptibility for grafting reactions. NVP increases the hydrophobicity of grafted gum and allows phase formation and reduces the viscosity of grafted gum as compared to normal guar. Due to reduced viscosity of grafted guar gum it forms gel-free solution even at 2% w/w solution in water which is not seen with normal guar gum. Another important aspect is, these polymers are known to be biodegradable and biocompatible. It is miscible with water in all proportions. It owes its water compatibility not to hydroxyls, but to the amide structure, which is also present in polypeptides/biopolymers.

Percent yield of the polymer was 67%. The analysis of homopolymer revealed very low concentration (<3 %) of PVP homopolymer, which in turn indicates a very high graft efficiency of the polymer.

The percent grafting (% G) was calculated by following formula:

$$\% G = \frac{W_d - W_g}{W_g} \times 100 \qquad (A)$$

where, W_d is weight of grafted copolymer and W_g is weight of GG.

The percent grafting efficiency (% GE) was calculated by following formula:

$$\% GE = \frac{W_d - W_g}{W_h} \times 100 \qquad (B)$$

where, W_d is weight of grafted copolymer, W_g is weight of GG and W_h is weight of homopolymer (PVP).

From the calculations, it was observed that the %G was 70.1 and the %GE was 56.0. Grafting reaction usually takes place by free radical mechanism and Ce^{3+} in conjunction with peroxydisulfate, forms free radicals. The detailed mechanism of the reaction is described elsewhere (Fakhrul-Razi *et al.,* 2001).

5.3.2. FTIR spectroscopy

Figure 5.1 illustrates the comparison of derivatized and underivatized guar gum. With respect to original guar gum, additional absorption peaks appeared in the spectrum of guar gum-vinyl-2-pyrrolidone [Figure 5.1], such as a C=O stretching band at 1660 cm^{-1}, and a C-N stretching band at 1384.8 cm^{-1}, corresponding to a carbonyl group and C-N bond of poly(N-vinyl-2-pyrrolidone). Additionally the product formation was confirmed by the disappearance of O-H bending vibration indicating the mechanism of O-H side

grafted reaction. This IR analysis indicates that vinyl-2-pyrrolidone is grafted onto GG.

5.3.3. SEM analysis

The derivatized guar gum polymer power was characterized by scanning electron micrography. The particles appear homogenous with smooth surface and non porous on cross linking. Two facts are to be mentioned: first, normal guar gum particles were small and having the rough surface morphology, which would help in attaining highly viscous aqueous solution. On derivatization with 1-vinyl-2-pyrrolidone gave soft surface texture and blocked the hydrophilic sites. Secondly the viscosity of the solution decreases as compared to normal guar gum. SEM analysis revealed the uniform morphology without microphase separation of the derivatized product [Figure 5.2]. The derivatized guar was then evaluated for use as counter polymer in polymer-polymer aqueous two phase system.

5.3.4. Phase diagram

Phase diagram for the system Dextran 2 million/ V-GG/ H_2O is shown in [Figure 5.3]. It is clear from the figure that phases are forming over a wide range of concentrations of V-GG and dextran. The grafted polymer was enriched in the denser bottom phase while dextran was found in the top phase. At dextran concentrations above 10% w/w, the binodal does not join the dextran axis, indicating that beyond a specific concentration of V-GG and dextran the polymers are immicisible. Clearly visible phase separation was observed in a mixture containing 2.5% w/w of dextran and V-GG as low as 1.25% w/w. This is in agreement with the observation that the binodal attains equilibrium parallel to

the X-axis phenomenon contrary to that reported by *Simonet* (Simonet *et al.,* 2000). At higher guar concentrations (5% w/w), the curve follows a vertical asymptote.

This peculiar property of the phase diagram is a result of high viscosity of the phases. It is well known that concentrated guar solutions show a very high viscosity: for instance, it is impossible to prepare guar solutions above 2% w/w but grafting of guar gum resulted in a decrease in viscosity. The V-GG rich phase was enriched in the lower phase while dextran rich phase was found in the top phase *Simonet* (Simonet *et al.,* 2000) have reported entrapment of dextran in a guar phase where dextran has to be removed by centrifugation. In the present case, need of the centrifugation was obviated.

The top and bottom phase density and viscosity of the individual Dextran 2 million / V-GG/ H_2O phase systems are presented in [Table 5.1]. The Dextran 2 million/ V-GG system has less density and viscosity values when compared to normal-GG aqueous two phase systems (results not shown). We found that slightly higher viscosities attained by the systems are because of higher molecular weight of unfractioned V-GG. This result agrees with the fact that lower concentrations of the polymers with large molecular weights are required for the phase separation. As shown in [Table 5.1], the densities and viscosities of all the bottom phases are higher than the respective individual top phases. The density and viscosity values of the top and bottom phases are very close, thus resulting in an enhanced demixing time of the phases.

5.3.5. Protein partitioning

5.3.5.1. Partition coefficient (K)

Three proteins BSA fraction-V, partially purified intracellular lactate oxidase from cell-lysate of *Aerococcus viridians* and partially purified extra cellular phytase from fermentation broth of *Aspergillus niger* (NCIM 516) were used as test proteins for the newly derivatized Dextran 2 million/ V-GG/ H_2O system. The criteria for selecting the lactate oxidase and phytase in ATPs was, both the proteins have almost equal isoelectric point (pI) i.e. 4.7, and lactate oxidase is an intracellular enzyme while phytase is an extracellular enzyme. The partition coefficient of the model proteins are reported in [Table 5.2]. When partition coefficients of BSA, LO and Phytase were plotted, it was observed that BSA was partitioned in to the bottom phase with the K value <1, while LO and phytase partitioned to top phase with the K values >1. The difference in partition coefficients was because, in a polymer-polymer two phase system, a correlation exists for the difference in hydrophobicity between the two polymers and their tendency towards phase separation.

5.3.5.2. Effect of neutral salt on protein partitioning

It has been demonstrated that it is mainly the ratio between the ions and total ionic strength, which determines the partition (Walter *et al.,* 1986). Previously many researchers showed that BSA partitioning is effected with the increase of NaCl concentration (Lu *et al.,* 1995). The partition of proteins depends on the type of charge on the protein used in a particular system. To test the efficiency of the Dextran 2 million/ V-GG/ H_2O system, standard protein, BSA, was chosen as sample protein. The distribution of BSA in Dextran/V-GG system was studied by the addition of protein solution to the

phase system. The experimental results showed that protein was concentrated in the bottom phase. The partition experiments were repeated to investigate the effect of salt, NaCl [Figure 5.4]. It was observed that BSA partition coefficient without salt was 0.479 [Table 5.2]. We studied the effect of salt concentration from 0 – 2.5 M NaCl. In the range of 0-0.85 M NaCl there was no appreciable change in the partition coefficient. However when NaCl concentration was increased beyond 1.0 M, the value of K showed a significant decline with BSA, Lactate oxidase (LO) and phytase [Figure 5.5].

The unequal distribution of the ions in the phases is significant as it creates a partition potential between the phases. This potential in turn affects the distribution of the charged particles, such as proteins. The large number of charges on these macromolecules affects the partition potential. The mechanisms that cause the uneven distribution of biomolecules are poorly understood and the widespread use of ATPS has been limited because of the complexity of predicting the partitioning of substances**.** By analyzing the system composition, at low salt concentrations, it is suggested that accumulation of the chloride ion in the dextran-rich phase can occur, causing it to become more negatively charged as compared to the lower phase. Thus, the negatively charged substances are partitioned preferentially into the lower phase.

5.4. Conclusion

An effective method of grafting VP on guar matrix was developed. It gave the advantage of high graft efficiency and yield. The aqueous two phase extraction protocol was established using BSA as a standard protein. Dextran 2 million/ V-GG/ H_2O formed

phases over a wide range of polymer concentrations of the phases. The optimum phase system was used to study the protein partitioning of BSA, LO and phytase. Effect of salt on BSA partitioning was investigated and found to improve partition at lower concentration.

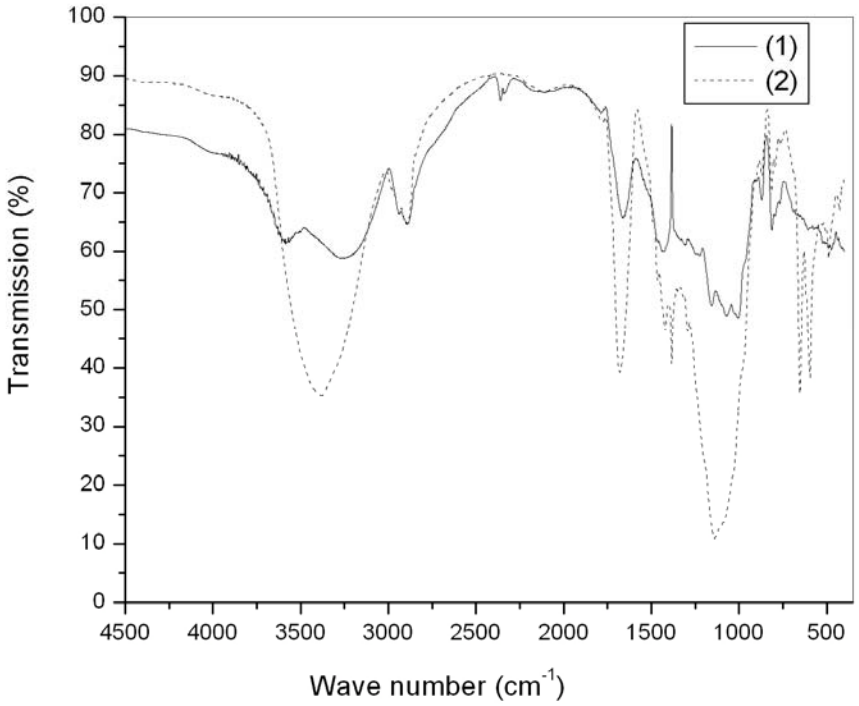
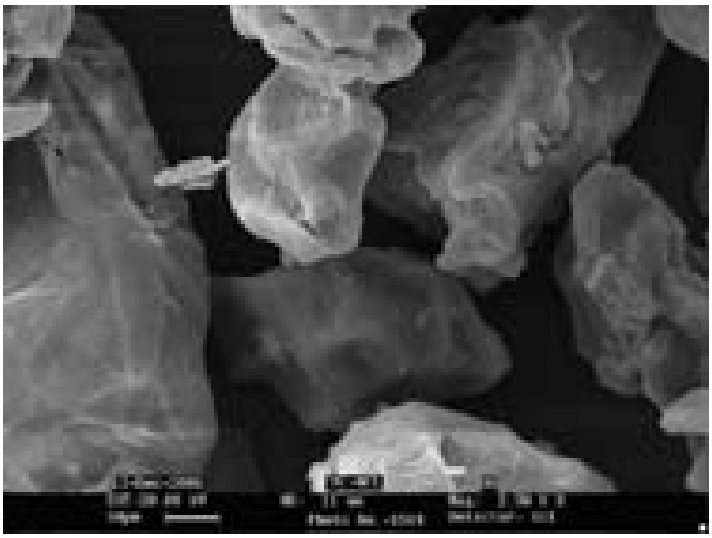


Figure 5.1: IR spectrum of original guar and grafted guar. (1): Original guar gum; (2): Vinyl pyrrolidone grafted guar gum.

[a]



[b]

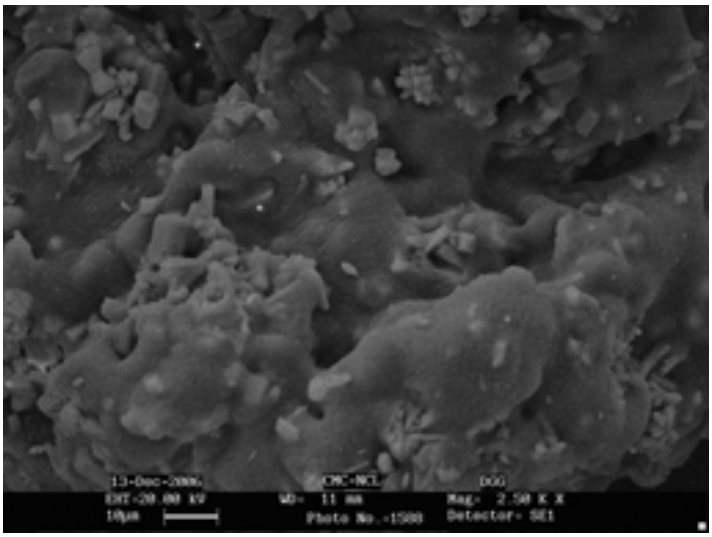


Figure 5.2: SEM photographs showing [a] guar gum and [b] vinyl-2-pyrrolidone-guar gum. Absence of microphase separation in the derivatized product indicates the uniform grafting.

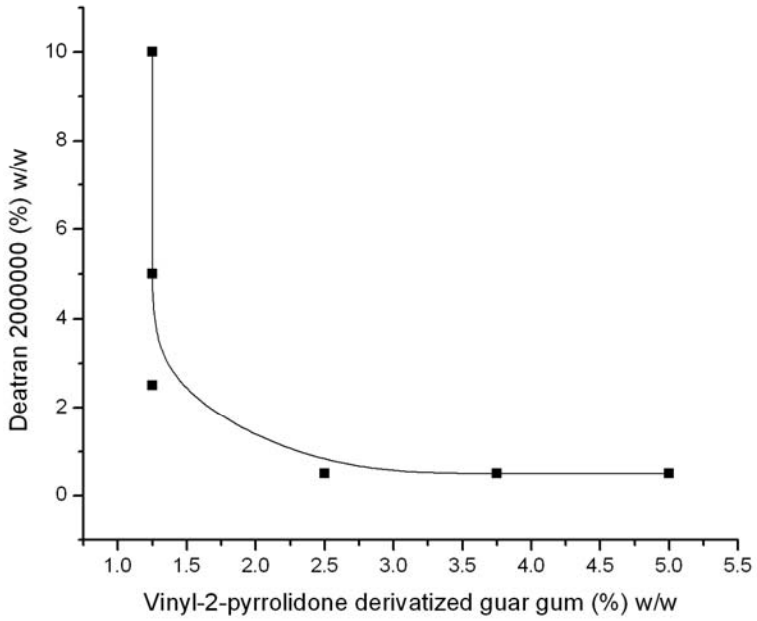


Figure 5.3: Phase diagram for the Dextran 2 million/ V-GG/ H_2O system at room temperature (25^{o}C).

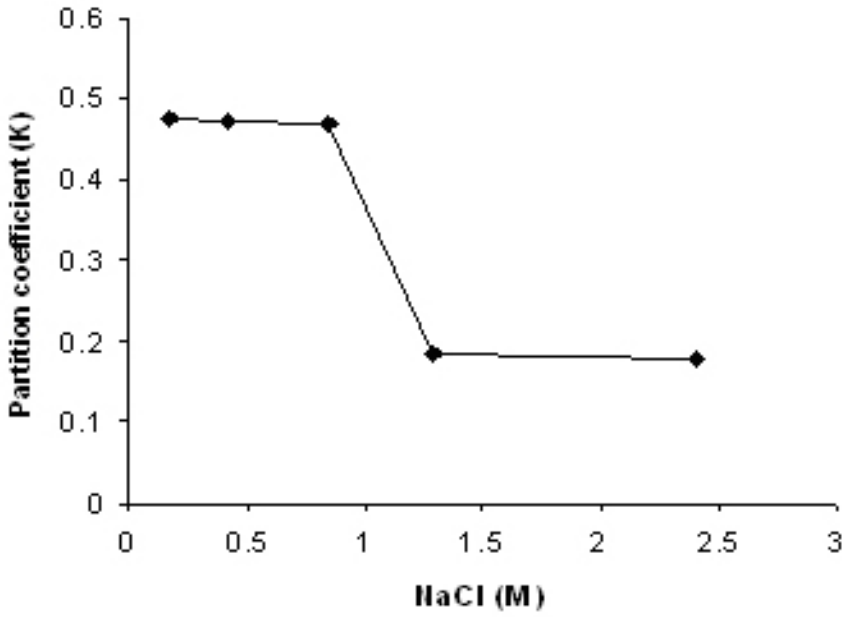


Figure 5. 4: Effect of NaCl addition on BSA partitioning coefficient (K) in 1.66% Dextran 2 million/ 1.66% V-GG / 96.68 H_2O (% w/w) system at 25^{o}C.

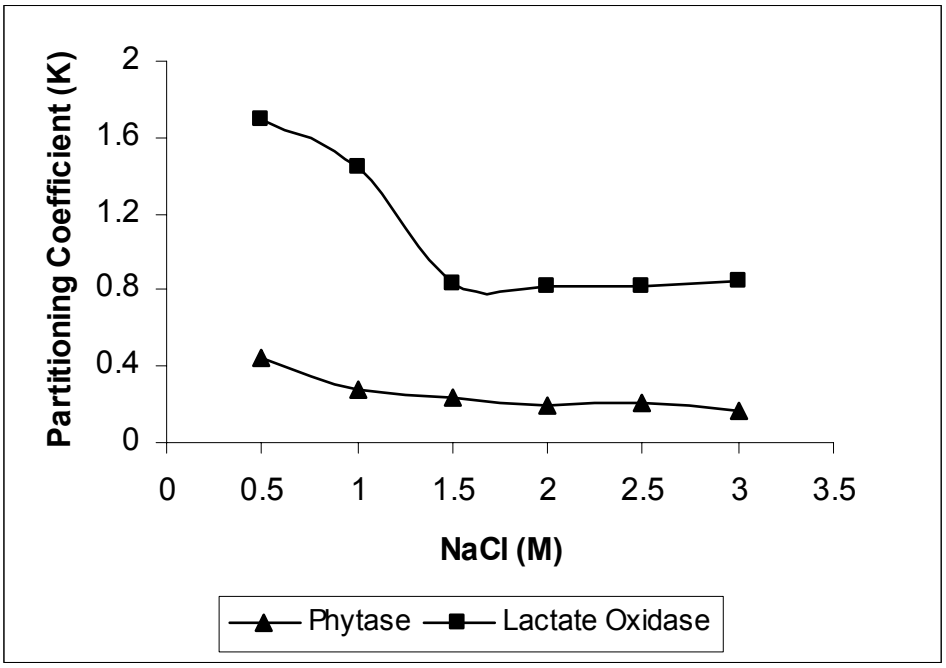


Figure 5.5: Effect of NaCl addition on Lactate oxidase (LO) and Phytase partitioning-coefficient (K) in 1.66% Dextran 2 million/ 1.66% V-GG / 96.68 H_2O (% w/w) system at 25^{o}C.

System (% w/w)	Density (Kg/m^3)		Viscosity (mPa S)	
Dextran/VGG//H_20	Top	Bottom	Top	Bottom
1.66/0.83/97.50	1.018	1.024	31.80	32.65
1.66/1.66/96.68	1.021	1.038	32.85	35.65
1.66/2.50/95.84	1.022	1.064	33.75	44.05
1.66/3.33/95.01	1.025	1.080	34.81	59.05

Table 5.1: Physical characteristics of Dextran 2 million/ V-GG/ H_2O Systems at 25°C.

Protein/Enzyme Coefficient	Partition (K)
BSA (Fraction-V)	0.479
Lactate Oxidase(LO)	1.981
Phytase	2.586

Table 5.2: Partition coefficients of BSA, LO and Phytase without NaCl in1.66% Dextran 2 million/ 1.66% V-GG / 96.68 H_2O (%w/w) system at 25°C.

Chapter 6

A new aqueous two phase system based on Tamarind kernel powder

6.1. Tamarind kernel powder

Tamarind Kernel Powder (TKP) is a natural polysaccharide obtained from the seeds of *Tamarindus indica* (Tamarind tree), a tree found in tropical countries. Commercial production of TKP commenced in 1943, after research conducted in the forest research institute, Dehradun, India found the gum to be a useful replacement for starch in desizing applications in the textile and paper industry.

6.1.1. Source

Tamarindus indica is a common forest and cultivated particularly southern parts of India and Africa. Fruits are in the form of large 10-15 cm long pods, containing 55% pulp, 33.9% seed and 11.1% shell and fiber (Whistler, 1973)**.** TKP is purified from endosperm of tamarind seeds.

6.1.2. Chemical and Physical properties

The principal constituent of TKP is a polymer composed of glucose, xylose and galactose [Figure 6.1]. It has a main chain of β-D-(1-4) linked gluco-pyranosyl units, to which D-gluco-pyranosyl units are attached through an alpha-D-(1-6) linkage, at every second, third and fourth glucose residue in the main chain (Kooiman *et al*, 1961). One D-galacto-pyranoside unit is attached to one of the xylopyranaside unit thought a beta-D-(1-2) linkage.

The ratio of glucose: xylose: galactose in TKP has been reported as 3:2:1 (Hazmi *et al*.,

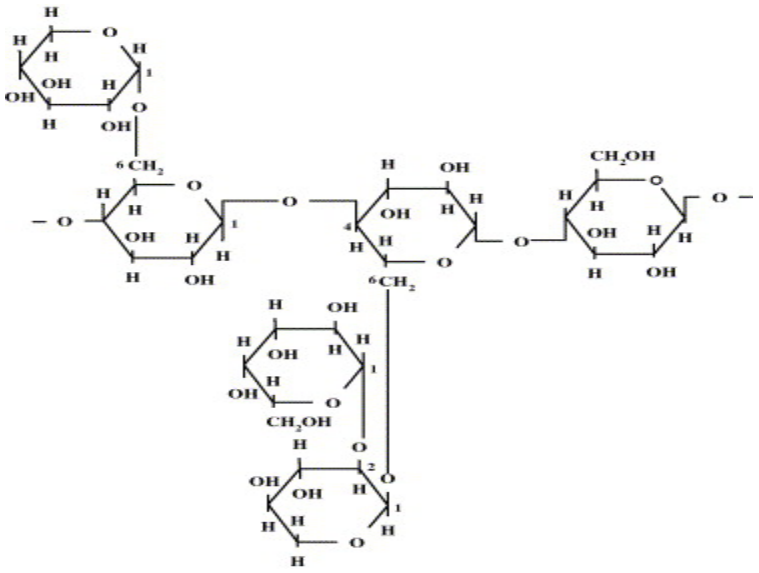


Figure 6.1: Structure of TKP.

1985). Hydrolysis of the TKP produced D-glucose, D-xylose and D-galactose in the ratio of 4:3:1.3 suggesting that the main chain is cellulose like (Taylor *et al*., 1985)**.** The molecular weight of the purified TKP, based on the measurement of viscosity**,** osmometry and copper number has been shown to be 52350, 54600, and 55600 respectively. Tamarind gum has been hydrolyzed with fungal enzymes, as well as with acids (Rao *et al*., 1973)**.** The alkyl and hydroxyalkyl derivativess of tamarind gum have improved swelling properties in water.

6.1.3. Physical properties

TKP disperses rapidly in cold water but a uniform solution is quickly obtained on heating the mixture or by dispersing the powder in hot water. Maximum viscosity is usually obtained after boiling the dispersion for 20-30min. Solutions are Non-Newtonian and show marked pseudoplasticity on being subjected to shear. It is insoluble in most organic solvents. Viscosities of tamarind solutions are higher than those of starch and lower than

guar gum dispersions of equivalent concentration. Peak viscosity of 5% tamarind dispersion is more than 1600 Brabender units (BU), as compared with 300 BU for a cooked corn starch dispersion of equal concentration. Maximum viscosity of TKP occurs at pH 7-7.5.

Vigorous stirring rate has an adverse effect on the ultimate viscosity of starch sols. However, tamarind gum sols do not become thin on agitation. This dissimilarity between the two sols has been attributed to a basic difference in their dispersion mechanisms. The size of tamarind gum particles depend on the extent and type of grinding. Soluble calcium, sodium and magnesium salts have a minimum viscosity effect on particle size pastes.

6.1.4. Applications

As suggested by forest research Institute, TKP is a good substitute for starch in textile and paper industry. Its application has been extended to sizing of jute**,** formulation of oil drilling fluids, food ingredients and cosmetics. Owing to the low cost of the gum, it has shown excellent market penetration in adhesion and film-forming applications. Food applications of tamarind polysaccharides and its enzyme hydrolyzed include jams, jellies and as a stabilizer in ice cream (Meer *et al*., 1975)**.** Based on the emulsifying and thickening properties, it could be used in cosmetic formulations.

6.1.4.1. Polyphenol oxidase

Phenol oxidases are oxido-reductases that catalyze oxidation of phenolic compounds (Durain *et al*., 1997). They are subdivided in to two subclasses, tyrosinase and laccase. Tyrosinase is an enzyme with a dinuclear copper center, which is able to insert oxygen in a position ortho- to an existing hydroxyl group in an aromatic ring, followed by the oxidation of the diphenol to the corresponding quinine. PPO is ubiquitously distributed in animals, plants, fungi and bacteria**.** Purification of phenol oxidases and PPO is a major challenge on account of its susceptibility to rapid denaturation.

6.1.5. Why TKP is used?

The recovery of microbial gums or exo-polysaccharides is largely dependent on their cost and end use (Smith *et al*., 1982). Recovery of these gums involves many unit operations such as solid removal, concentration, alcohol precipitation etc. and plays an important role in the economics of their production. Currently it is performed by precipitation with low molecular weight aliphatic alcohols such as ethanol or isopropanol (Gonzals *et al.,* 1989). The amount of alcohol required is 2-3 times the volume of the fluid to be processed (Hawking, 1986). Tamarind kernel powder (TKP) possesses properties which are comparable to those of microbial gums. Due to its rheological properties, water solubility, thickening properties, low cost and ease of purification, tamarind kernel powder has very attractive applications that will be comparable or even better than those of microbial gums and polysaccharides. TKP has similar structural characteristics to those of starch. Starch and its derivatives are used extensively in ATPS.

The object of the present work is to form a new ATPS with TKP; carboxymethyl TKP; Hydroxypropyl TKP /PVP/H_2O and to examine its feasibility for partitioning of BSA and other proteins.

6.2. Material and Methods

Bovine serum albumin (BSA), PVP, PVA was obtained from (Fluka) Sigma-Aidrich, (USA), Crude TKP, HP-TKP and CM-TKP obtained from Mysore starch Ltd, Mysore, India and Dextran T-500 from Amersham Biosciences, Uppsala (Sweden). All other chemicals were of analytical grade**.**

6.2.1. Purification of TKP

TKP, CM-TKP and HP-TKP were purified by precipitation with 5 volumes of ethanol (Rodrigues *et al.,* 1993). The precipitate was further subjected to concentrated ethanol twice and filtered. The precipitate was dried under vacuum in a hot air oven at 40^oC till constant weight.

6.2.2. Preparation of solutions

Solutions of TKP (%), CM-TKP (%), HP-TKP (% w/w) were prepared by cold dispersion of the powder for 2 h in distilled water containing 0.05% of sodium azide (NaN_3) as a bactericide, followed by heating at 80^oC for 60min under stirring. A solution of polyvinyl pyrollidone (PVP) (20%) was prepared by dissolving the sample in distilled water containing 0.05% NaN_3 at room temperature under stirring for 1h.

6.2.3. Phase diagram

The phase diagram was determined at 25^{o}C according to Albertson's procedure (Albertson, 1981). Different weights of TKP, CM-TKP and HP-TKP solutions were mixed with defined weights of PVP $K_{90,}$ PVA and dextran T-500 solutions in test tubes. The mixtures were vortexed and left at 25^{o}C for 8h for phase separation. By mixing two polymers in water, phase formation occurred only beyond a critical concentration of the individual polymers, whereas other mixtures gave a homogeneous phase. The demarcation between PVP-TKP, PVP-CMTKP and PVP-HPTKP compositions showing monophasic and biphasic behaviour was selected by direct observation of two phase formation. Systems that displayed a distinct phase/phase interface were considered biphasic. The top and bottom phases are separated carefully. A phase diagram was formulated by plotting a graph of concentration of one polymer against the other which generally yielded a curved line. All the compositions of the polymers represented by points above the line show phase formation whereas, mixtures represented by points below the line gave a homogeneous phase. This line dividing the area into phase forming and non-phase forming solutions is called the binodial.

The amount of carbohydrate polymer in the bottom as well as top phases was estimated by precipitating with ethanol. The filtrate was dried and weighed to estimate the amount of PVP present. All the experiments with TKP, CM-TKP and HP-TKP were performed separately.

6.2.4. Analysis of phases

6.2.4.1. Determination of phase composition

Aqueous two phases obtained by tie-line combinations were analyzed for phase polymer composition. For determining the tie line compositions, the top and bottom phases were separated carefully by a micropipette. The separated phases were dried in vacuum oven at 70 °C till constant weight, for determination of % weight of water. The dried phases were re-dissolved in water and precipitated with alcohol. The % weight of HPTKP in the bottom phase as well as in the top phase was estimated. The precipitated polymer was vacuum dried. % PVP was calculated by balancing the mass of total weights of top and bottom phases, % water and % HPTKP.

6.2.4.2. Phase densities and Viscosities

Densities were measured using a 10 ml pycnometer at 25°C. The pycnometer was weighed on an analytical balance (Mettler), and the bulk densities of both top and bottom phase were measured by taking its mass to volume ratio. Individual phase viscosities of top and bottom phases were measured using a Brookfield viscometer (CAP 2000+, cone-plate viscometer), at 25^{o}C.

6.2.5. Protein partition

6.2.5.1. Effect of Tie line

Protein partitioning study was conducted by adding BSA to different tie line compositions of HPTKP ranging from 1.4 % wt to 2.2 % wt and 3 % wt PVP. The tubes were well mixed and incubated for 8 h at 25^{o}C. The clear top and bottom phases were carefully separated using a micropipette and weighed separately. Separated top and

bottom phases were diluted where necessary with buffer and analyzed for BSA concentration and PPO activity in top and bottom phases separately.

6.2.5.2. Effect of salts

The aqueous two phase systems (3 wt %PVP /1.44 wt % HPTKP/H_2O) were prepared from stock solutions of PVP (20 % wt) and HPTKP (5 % wt) polymers in water. To the two phase systems was added salts to give a final molarity of 0.13 M to 1.25 M [sodium chloride (NaCl), potassium chloride (KCl), di-potassium phosphate (K_2HPO_4) and ammonium sulphate $(NH4)_2SO_4$). BSA and PPO protein solutions were added to the two-phase systems, the solutions were well mixed equilibrated at 25^oC for 8h to allow for protein partitioning. The same sets of experiments were conducted for PPO partitioning.

6.2.5.3. Effect of pH

Different weights of the polymer stock solutions of HPTKP (5 % wt) and PVP (20 % wt) were mixed to prepare aqueous two phase systems (3 wt %PVP /1.44 wt % HPTKP/H_2O) using sodium phosphate buffer (pH 5.8, 6.2, 6.6, 7.0, 7.4, 7.8 and 8.2) to investigate the influence of pH on partitioning of protein. The buffer concentration was maintained at100 nM. The total weight of the system was 4g. The systems were mixed and incubated for 8 h in order to obtain two clear phases. The partition experiments were performed at 25°C by mixing systems with a 100μg of a 5 mg ml^{-1} protein solution. The study of protein partitioning was carried out at 5.8, 6.2, 6.6, 7.0, 7.4, 7.8 and 8.2 for different tie-line compositions. The same sets of experiments were conducted under identical conditions for PPO partitioning.

6.2.5.4. Effect of BSA concentration

The effect of BSA concentration ranging from 2.5 to 50 mg/ml was added to tubes containing 3 % wt PVP/1.44 % wt/HPTKP/H_2O at pH 7. The tubes were well mixed and incubated for 8 h at 25 ^oC. After the formation of clear distinct phases the phases were separated and analyzed for BSA partitioning.

6.2.6. Partition coefficient

The protein concentration in top and bottom phases was determined by the method of Bradford (1976). BSA was used as standard protein. Partition coefficient (K) was defined as ratio of top phase and bottom phase concentration of protein or enzyme activity.

$$K= C_T/C_B$$

Where C_T and C_B are the equilibrium concentrations of protein in the top phase and bottom phase respectively (Albertsson, 1986).

6.2.7. Polyphenol oxidase assay

Polyphenol oxidase (PPO) activity was determined by using 3, 4-dihydroxy-L-phenylalanine (L-DOPA) as the substrate (Pathak & Ghole, 1994) at 470 nm using a Chemito visible spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme, which causes an increase in absorbance by 0.001 min−1 at 25°C, which corresponds to the conversion of 0.01μmol of substrate to product (Dopachrome) (Vaidya *et al.,* 2006).

6.3. Results and Discussion

6.3.1. Phase diagram

The binodial was determined by visual observation. Phase diagrams for the systems PVP/TKP and T-500/TKP ATPs are shown in the [Figure 6.2a & 6.2b]. The binodial curve was plotted on the basis of phase forming nature of two polymers, when mixed in different concentrations. The compositions above the critical point formed the aqueous two phases, whereas compositions below the point remained homogenous. The effect of increase in concentration of TKP on the binodial curve was as expected. An increase in the amount of polymer was necessary for the formation of two phase system (Albertsson *et al*., 1990). It was observed that with the exception of PVA, separate phases were obtained for all the counter polymers tested with TKP polymer. The phases appeared indistinct to the naked eye. This could be ascribed to the presence of impurities and the high polydispersity of TKP, which gives rise to indistinct phases. Different combinations of PVP/CMTKP (Carboxy-methyl TKP), PVA/CMTKP and T-500/CMTKP did not form ATPs. All the carboxy-methyl based compositions gave homogenous solutions even after 72 hr of incubation at 25^{o}C and pH 7.

Hydroxy-propyl TKP (HPTKP) formed two phase systems with PVA, T-500 and PVP, where the HPTKP phase forming polymer is same in all the systems studied. The phase separation shows that HPTKP is incompatible with PVA, T-500 and PVP. In all these n compositions, HPTKP was observed to be enriched in the bottom phase. **P**hase separation was also observed with minimum concentration of PVA (1.875 % wt) and HPTKP (0.625 % wt) [Figure 6.2c]. The phases were viscous because of the PVA. The phase diagram for the dextran T-500/HPTKP phase system at 25^{o}C is shown in Figure 6.2d. A

significant improvement in phase clarity and separation between top and bottom phases was observed. HPTKP was enriched in the denser bottom phase while T-500 was predominantly in the upper phase.

Phase separation was observed in a mixture containing 2.5 to 10 % wt concentration of PVP. The binodial curve of PVP/HPTKP/H_2O doesn't join the HPTKP (0.625 % wt) axis, indicating that HPTKP is incompatible with PVP. Values of 2.5 % wt of PVP and 0.625 % wt of HPTKP represent the limit for visual detection of phase separation [Figure 6.2e]. The tie line compositions of both top and bottom phases are given in [Table 6.1].
The phase diagram indicated that though the molecular weight of HPTKP was not very high, contrary to expectations, only a small amount (0.625% wt) of it is required to form an aqueous two-phase system, moreover it is well known that concentrated TKP solutions exhibit a very high viscosity; when compared to guar and guar derivatives which have 10 fold higher molecular weight of polymer, where lower polymer concentration was required to form ATPS. One crucial factor for anaomalous behaviour of phase compositions may be due to the viscosity effect of polymer. Since HPTKP normally migrates to the bottom phase, there is lack of droplet formation, a phenomenon which has been observed to interfere in the formation of aqueous two phases (Ganapathi *et al*., 2007).

6.3.2 Physical characteristics properties of top and bottom phases

The phase density and viscosities of the top and bottom phases of PVP/ HPTKP/H_20 systems are given in [Table 6.2]. The viscosity values of both top and bottom phases

show considerable difference, which is reflected in reduced demixing time (8h). Generally it is expected that polymers with higher molecular weight show increase in viscosity. In turn, higher the polymer viscosities, lower is the concentration of polymers required.

6.3.3. Protein partitioning

6.3.3.1. Effect of HPTKP concentration on K of BSA

BSA was analyzed as standard protein and polyphenol oxidase (PPO) as test protein in PVP/PGG/H_2O aqueous two-phase system. Protein partitioning of BSA at five different concentrations of HPTKP in PVP/HPTKP/H_2O ATPs at pH 7.0 was estimated. The partition coefficient of BSA with increased concentration of HPTKP (1.44 - 2.2 % wt) is given in a [Table 6.3]. Partition coefficient for BSA was >1, which indicates partitioning of BSA in the top phase. With increase in HPTKP concentration (1.44-2.2 %wt), the trend is towards decline in partition coefficient (K). Polymers used in aqueous two phase system should permit high intrinsic solubility of proteins in order to be effective over a large concentration range. Since the intrinsic protein solubility decreases with increase in HPTKP concentration, this could explain the decrease in K value for higher concentrations of HPTKP.

6.3.3.2. Effect of salts on BSA and PPO partitioning

The effect of different concentration of NaCl and KCl in ATPS were studied for the partitioning of BSA [Figure 6.3a and 6.3b]. The partitioning of BSA was studied at pH 7 and 25^oC with PVP/HPTKP/H_2O. The partitioning of BSA is maximum (K=2.3) for a

NaCl concentration of 0.25 M, while K=1.7 for KCl at 0.1M. As the morality of the system increases, the K value decreases and most of the BSA tend to precipitate. The same trend of partition was observed for PPO [Figure 6.4]. Higher the valency of the anion, lower the concentration required to form a two-phase system. This trend has also been observed for aqueous two phase systems of PEG with different salts. Anions with a higher valence are better salting-out agents than anions with a lower valence because anions with higher valency show greater hydration than those with lower valency, thus decreasing the amount of water available to hydrate HPTKP. When the anion and cation of a salt have different relative affinities for phase-forming polymers, the requirement of electroneutrality in each phase results in a Donnan-type electrostatic potential difference between the phases. This potential difference appears to have a pronounced effect on partitioning of charged solutes (such as proteins). Therefore the choice of salt has an important influence on the partitioning of a target biomolecule. The more hydrophobic anions or cations will drive the partitioning of their counterions into the more hydrophobic phase.

The partitioning of BSA with K_2HPO_4 and $(NH4)_2SO_4$ were also studied in PVP/HPTKP/H_2O system at 25^oC and pH 7 with in a range of 0.05 to 0.5 M concentration [Figure 6.5a and 6.5b]. The K values for BSA in both the salts are >1 at 0.05M. However, as the salt concentration increases, the K value decreases for $(NH_4)2SO4$ while no significant change in K value is observed for K_2HPO_4. The partitioning of PPO with K_2HPO_4 and $(NH4)_2SO_4$ is <1 which clearly shows that it is partitioned into the bottom phase. As the molarity of the salts increases from 0.05 to 0.5

M, there is no significant change in the K value. The partitioning of molecules between the two phases is a complex phenomenon because of the involvement of many interactions between the solute and the phase forming components. This makes the molecular mass, chemical properties of the polymer, the size and the chemical properties of the partitioned solute extremely important. Due to the complexity of the partitioning phenomenon, it is difficult to predict protein behaviour and select separation conditions for a rational planning of experiments**.**

6.3.3.3. Effect of pH on the partition coefficient (K) of BSA and PPO

The effects of pH on the partitioning of BSA and PPO are shown in [Figure 6.6]. In order to know the influence of pH on BSA partitioning (K), experiments were performed over a wide range pH (5.8 to 8.2) in PVP/HPTKP/H_2O aqueous two-phase system of different polymer concentrations (i.e. 3 % wt and 1.44 % wt). For both the proteins (BSA and PPO) different partition coefficients were obtained at different pH. As BSA and PPO are stable in the pH range of 5 to 8, experiments were restricted to pH range (5.8 to 8.2). The pH dependence on K appears to be stronger for BSA when compared to PPO. The observed pH dependence suggests that for BSA partitioning PVP/HPTKP/H_2O is perhaps a better choice. The partition coefficient for BSA and PPO were found to vary with the increase of pH. At optimum pH 7, maximum K value for BSA is 2.4. This may be due to the migration of most of the protein (BSA) to the PVP rich top phase at higher pH. Thus with change in pH of the system, individual proteins showed different partition behavior. This behavior may be due to two reasons first, net charge (positive or negative) of the protein and second, due to surface properties of the proteins.

Experiments for partitioning of a test protein "polyphenol oxidase" were conducted to confirm suitability of the new PVP/HPTKP/H_2O aqueous two-phase system. Since PPO is also stable in the pH range of 5.0 to 8.0, the effect of pH on partitioning were studied in this range only. With increasing pH from 5.8 to 8.2 [Figure 6.6], there was no discernable change in the value of *K*. The extent of pH dependence of *K* depends on the type of protein and is perhaps affected by thedegree of salvation of PPO. The pH also influences the ionizable group present on the solute (PPO) which in turn will alter solute surface charges and hence, its partition coefficient. Thus, PPO showed a different partitioning behaviour which may be influenced by its conformation and the specific interactions PPO has with phase forming polymer. Similar results have been reported for other polymer-polymer systems (Almeida *et al.,* 1998; Christian *et al.,* 1998; Kishida *et al.,* 1998).

6.3.3.4. Effect of BSA concentration on partitioning coefficient (K)

For a range of BSA concentrations studied (2.5–50 mg/100g), an increase in the protein concentration led to a proportional increase in the partition coefficient values from 0.92 to 1.20, indicating that the migration to bottom phase decreased [Figure 6.7]. The K value of BSA remained constant for values of 2.5 to 25 mg/100g for BSA concentrations from 50 mg/100g onwards, the K value increased to 1.2. This clearly demonstrates that higher concentration of BSA led to higher migration of BSA to the top phase.

6.4. Conclusion

Polyvinyl pyrrolidone and Hydroxypropyl tamarind kernel powder are low cost polymers which provide a potential alternative to expensive fractioned dextran for aqueous two-phase systems. A new aqueous two phase system, namely PVP/HPTKP/H_2O ATPs, is reported along with its phase diagram. Partitioning of the model proteins Bovine serum albumin (BSA) and Polyphenol oxidase (PPO) were studied in the new phase system. The partitioning of BSA and PPO in presence of NaCl, KCl, K_2HPO_4 and $(NH4)_2SO_4$ were investigated.

The results showed that it was possible to change the degree of partitioning by selecting the right kind of salt and its concentration. The influence of pH partitioning behavior of BSA and PPO was investigated. Hydroxypropyl tamarind kernel powder is used in aqueous two-phase system along with PVP, for the first time for the partition of proteins. The HPTKP is expected to be a major contributor in the recovery of proteins using aqueous two phases.

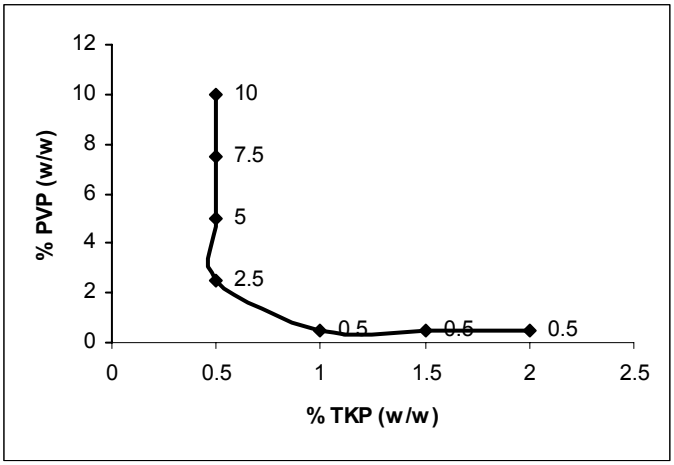


Figure 6.2a: Phase diagram for the PVP/TKP/ H_2O system at room temperature (25^oC).

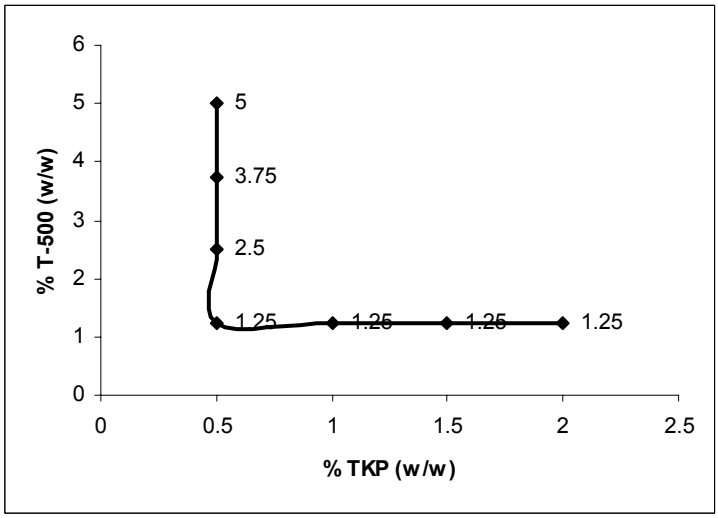


Figure 6.2b: Phase diagram for the T-500/TKP/ H_2O system at room temperature (25^oC).

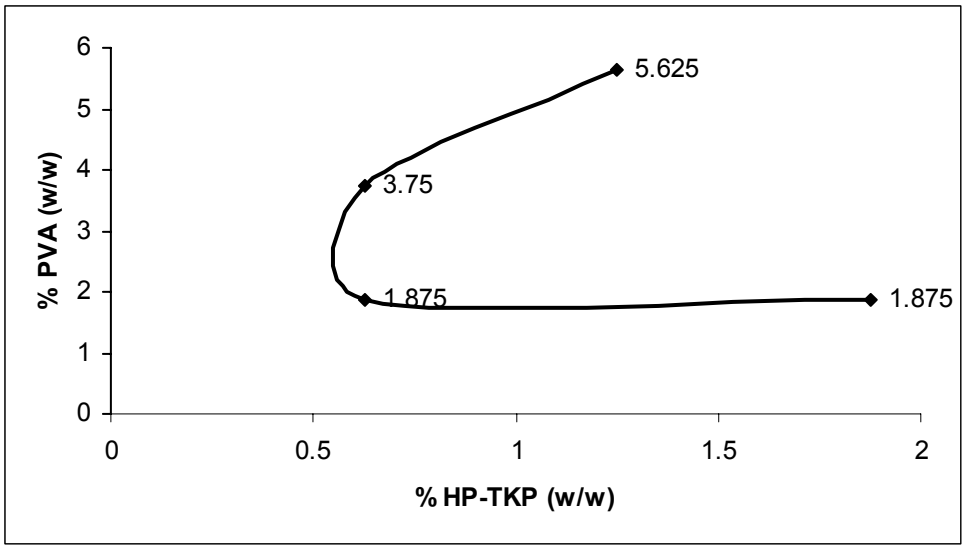


Figure 6.2c: Phase diagram for the PVA/HPTKP/ H_2O system at room temperature (25^oC).

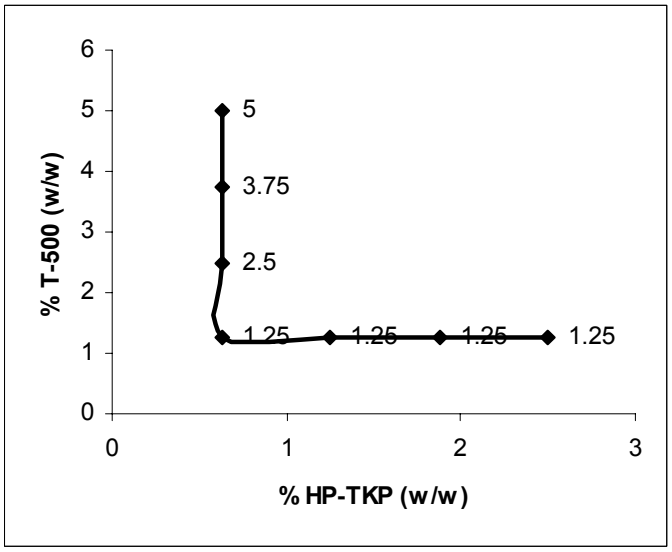


Figure 6.2d: Phase diagram for the T-500/HPTKP/ H_2O system at room temperature (25^oC).

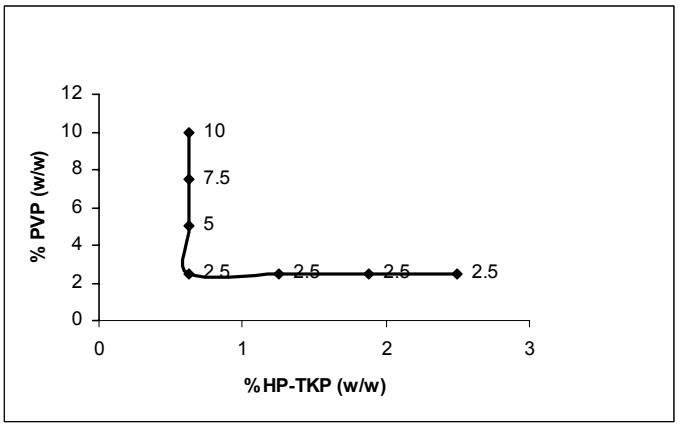


Figure 6.2e: Phase diagram for the PVP/HPTKP/ H_2O system at room temperature (25^oC).

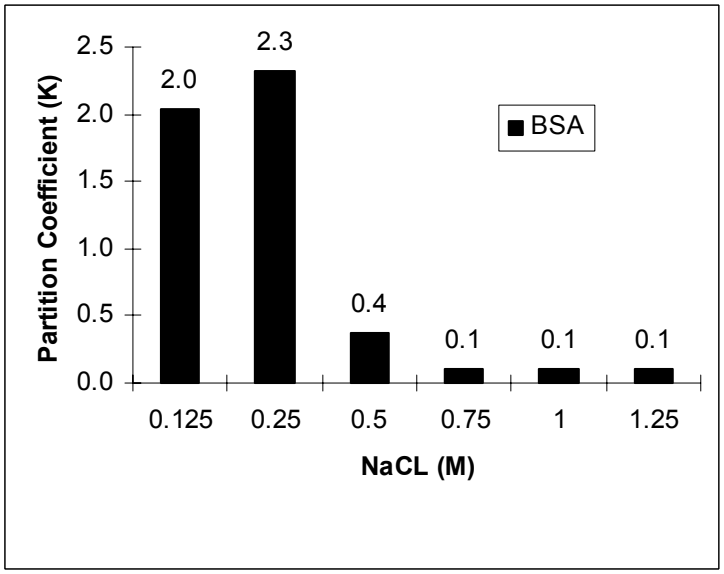


Figure 6.3a: Effect of sodium chloride on partition coefficient (K) of BSA in 3% PVP / 0.625 % HPTKP/H_2O (w/w) system at 25^oC.

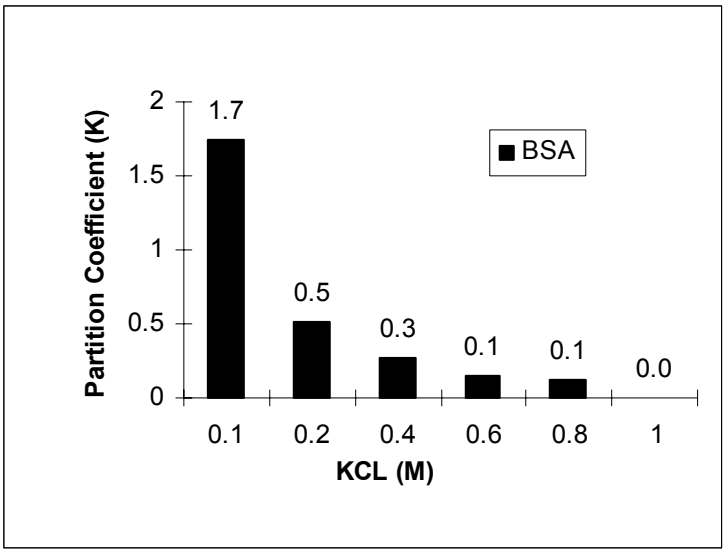


Figure 6.3b: Effect of potassium chloride on partition coefficient (K) of BSA in 3% PVP / 0.625 % HPTKP/H_2O (w/w) system at 25^oC.

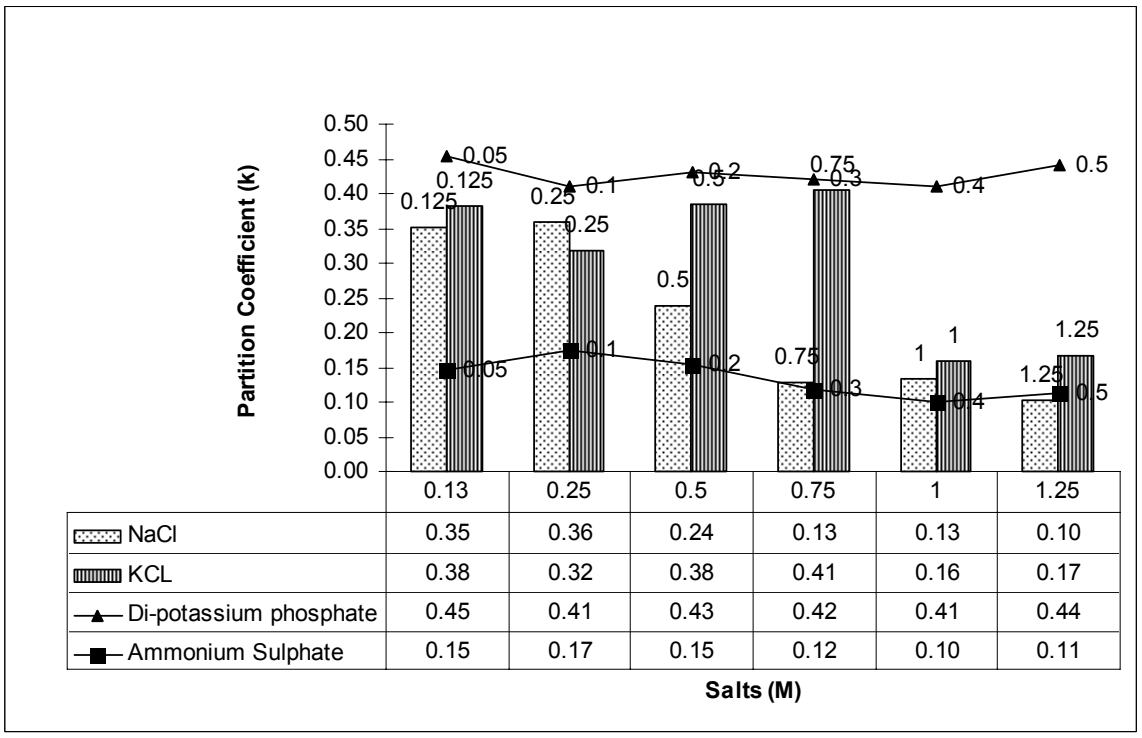


Figure 6.4: Effect of different salts on partition coefficient of PPO in 3% PVP/0.625% HPTKP /H_2O (w/w) system at 25^oC.

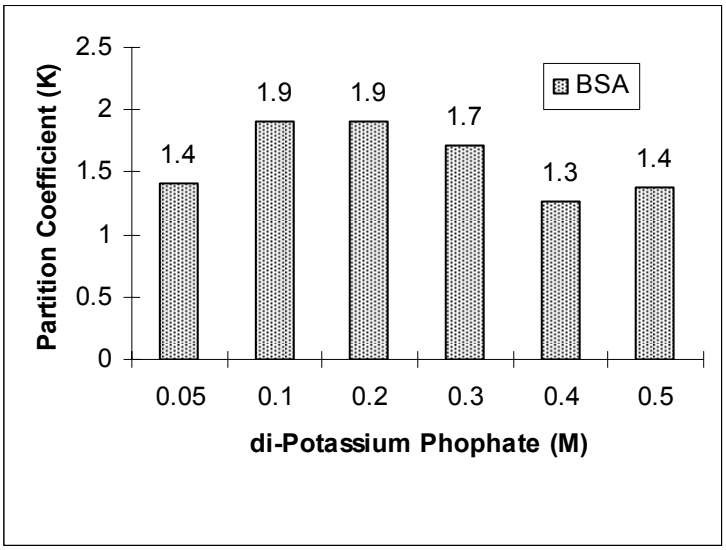


Figure 6.5a: Effect of di-potassium phosphate on partition coefficient (K) of BSA in 3% PVP / 0.625 % HPTKP/H_2O (w/w) system at 25^oC.

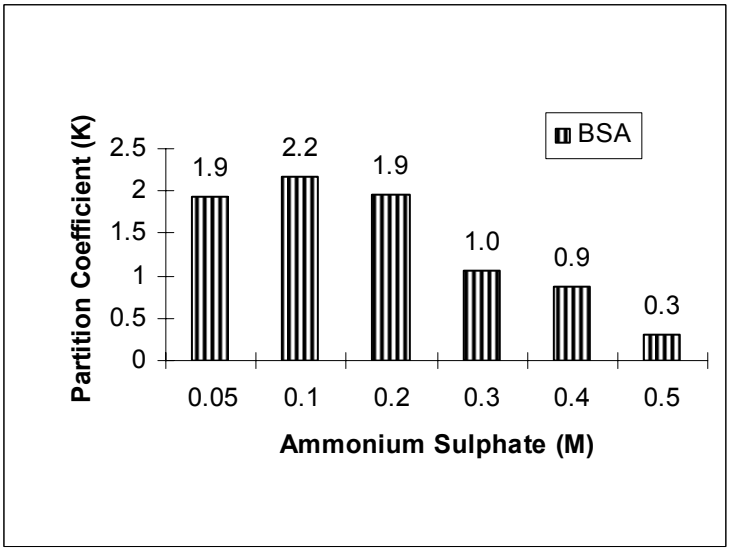


Figure 6.5b: Effect of ammonium sulphate on partition coefficient (K) of BSA in 3% PVP / 0.625 % HPTKP/H_2O (w/w) system at 25^oC.

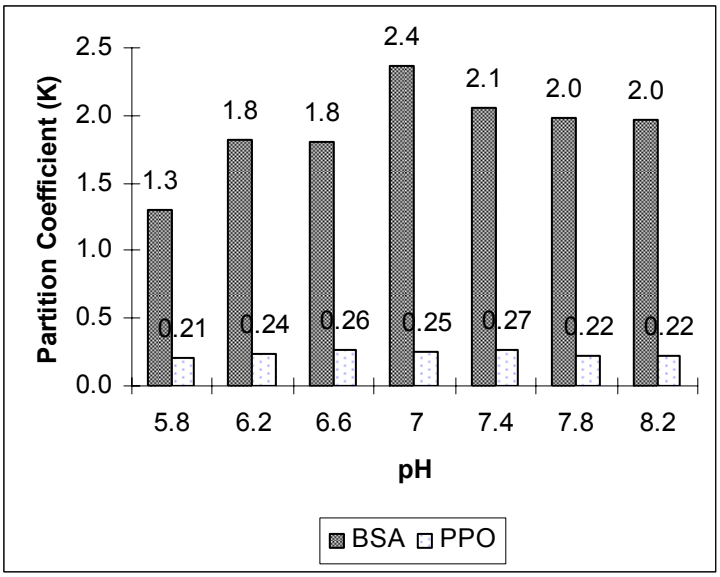


Figure 6.6: Effect of pH on partition coefficient (K) of BSA and PPO in 3% PVP / 0.625 % HPTKP/H_2O (w/w) system at 25^oC.

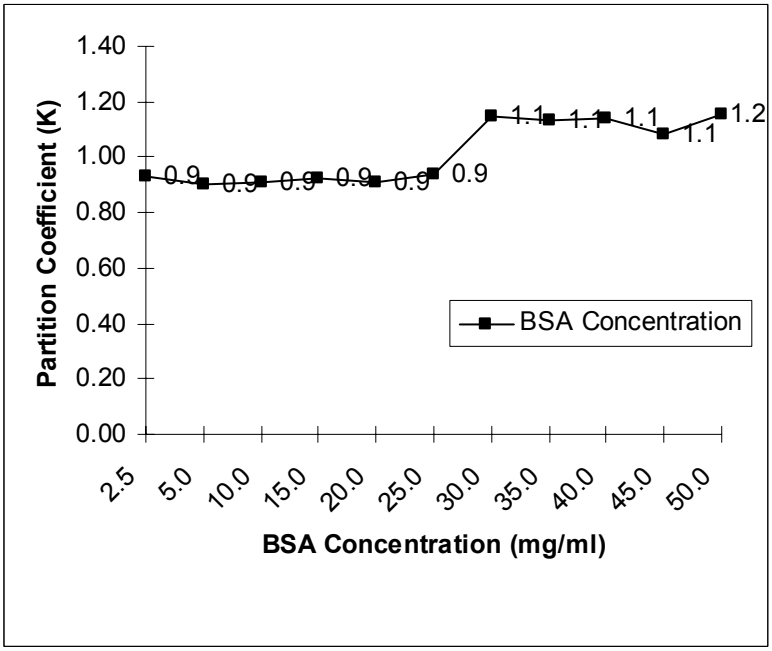


Figure 6.7: Figure 6.8: Effect of BSA concentration in 3% PVP / 0.625 % HPTKP /H_2O (w/w) system at 25^oC and pH 7.

Tie Line					
	1	**2**	**3**	**4**	**5**
Total Composition (% w/w)					
HP-TKP	1.4	1.6	1.8	2.0	2.2
PVP K_{90}	3	3	3	3	3
H_20	95.6	95.4	95.2	95	94.8
Top Phase (% w/w)					
HP-TKP	0	0	0	0	0
PVP K_{90}	0.25	0.33	0.5	0.5	0.6
H_20	99.75	99.67	99.51	99.5	99.4
Bottom Phase (% w/w)					
HP-TKP	4.16	4.4	4.75	5.89	6.46
PVP K_{90}	0	0	0	0	0
H_20	95.84	95.6	95.25	94.11	93.54

Table 6.1: Tie line compositions of PVP/HPTKP/ H_2O systems at 25^oC**.**

System (% w/w) PVP/HPTKP/H_20	Density (Kg/m^3) Top	Bottom	Viscosity (mPa S) Top	Bottom
3/1.4/95.6	1.08	1.00	61.8	335.6
3/1.6/95.4	1.02	1.04	59.5	324.3
3/1.8/95.2	1.03	1.02	60.0	322.5
3/2.0/95.0	1.02	1.06	61.8	298.1
3/2.2/94.8	1.03	1.06	60.0	283.1

Table 6.2: Physical characteristics of PVP/HPTKP/ H_2O Systems at 25°C.

System (% w/w) PVP/HPTKP/H_20	Partition Coefficient (K)
3/1.4/95.6	4.79
3/1.6/95.4	4.30
3/1.8/95.2	3.32
3/2.0/95.0	2.54
3/2.2/94.8	2.65

Table 6.3: Partition coefficient of BSA in PVP/HPTKP/ H_2O Systems at 25°C and pH 7.

REFERENCES

References

Abbot, N. L. and Halton, J. A. Liquid-Liquid extraction for protein Separations. *Chem. Eng .Prog*. 31-41 (1990).

Abbot, N. L., Blankschtein, D. and Hatton, T. A. Protein partitioning in aqueous polymer systems. *Bioseparation.* 20, 421–423 (1990).

Ademark, P., De-Vries, R. P., Hagglund, P., Stalbrand, H. and Visser, J. Cloning and characterization of *Aspergillus niger* genes encoding an α-galactosidase and a β-mannosidase involved in galactomannan degradation. *European Journal of Biochemistry.* 268, 2982-2990 (2001).

Ademark, P., Larsson, M., Tjerneld, F. and Stalbrand, H. Multiple α-galactosidases from *Aspergillus niger*: Purification, characterization and substrate specificity Enzyme. *Microb. Technol*. 29, 441-448 (2001).

Agasoster, T. Aqueous two-phase partitioning sample preparation prior to liquid chromatography of hydrophilic drugs in blood. *Journal of Chromatography B:Biomedical Sciences and Applications.* 716, 293-298 (1998).

Ahmed, F. and Whistler, R. L. The structure of guaran. *J. Amer. Chem. Soc.* 72, 2524 (1950).

Ajisaka, K. and Fujimoto, H. Regioselective synthesis of trisaccharides by use of a reverse hydrolysis activity of α-and β-galactosidase. *Carbohydr. Res.* 185, 139-146 (1989).

Akiba, T. and Horikoshi, K. Localization of α-galactosidase in an alkalophilic strain of *Micrococcus*. *Agri. Biol. Chem*. 42, 2091-2094 (1978).

Albertson, P. A., Johansson, G. and Tjerneld, F. Aqueous two-phase separations: separation processes in biotechnology. 287-318 (Marcel Dekker, New York, 1990).

Albertsson, P. A. Partition of cell particles and macromolecules. (2nd edition.), (Willey-Interscience, New york, 1971).

Albertsson, P. A. Aqueous polymer-phase systems: Partition of Cell Particles and Macromolecules. (3rd edition.), (John Wiley and Sons Inc, New York, 1986).

Albertsson, P. A. Chromatography and partition of cells and cell fragments. *Nature.* 177, 771-774 (1956).

Albertsson, P. A. Particle fraction in liquid two phase systems, the composition of some phase systems and the behaviour of some model particles in them: Application to the isolation of cell walls from microorganisms. *Biochem. Biophysics. Acta.* 27, 378 (1958).

Albertsson, P. A. Partition of Cell Particles and Macromolecules (2nd edition.), (Wiley-Interscience, New York, 1971).

Albertsson, P. A. Partition of cells particles and macromolecules. (3rd edition.), (Wiley Interscience, New York, 1986).

Albertsson, P. A., Cajarville, A., Brooks, D. E. and Tjerneld, F. Partition of protein aqueous two phase systems and the effect of molecular weight of the polymer. *Biochim. Biophys. Acta.* 926, 87-93 (1987).

Albertsson, P. A., Sasakawa, S. and Walter, H. Cross partition and isoelectric points of proteins. *Nature.* 228, 1329-1330 (1970).

Almeida, M. C., Venancio, A., Teixeira, J. A. and Aires-Barros, M. R. Cutinase purification on poly(ethylene glycol)-hydroxypropyl starch aqueous two-phase systems. *J. Chromatogr. B: Biomed. Sci. Appl.* 26, 151-159 (1998).

Alred, P. A., Kozlowski, A., Harris, J. M. and Tjerneld, F. Application of temperature-induced phase partitioning at ambient temperature for enzyme purification. *J. Chromatogr. A.* 659, 289-298 (1994).

Alred, P. A., Tjerneld, F. and Modlin, R. F. Partitinining of ecdysteroids using temperature-induced phase separation. *J. Chromatog*. 628, 205-214 (1993).

Alred, P. A., Tjerneld, F., Kozlowski, A. and Harris, J. M. Synthesis of dye conjugates of ethylene oxide-propylene oxide copolymers and application in temperature-induced phase partitioning. *Bioseparation.* 2, 363-373 (1992).

Alsop, R. M. Progress in industrial microbiology: *Microbial polysaccharides.* (Bushell, M. E. Editor) 18, (Elsevier, Amsterdam, 1993).

Alves, J. G. L. F., Chumpitaz, L. D. A., da-Silva, L. H. M. and Franco, T. T. Partitioning of whey proteins, bovine serum albumin and porcine insulin in aqueous two-phase systems. *J. Chromatogr. B.* 743, 235-239 (2000).

Andersson, E., Johansson, A. and Hahn, H. B. α-Amylase production in aqueous two-phase system with *Bacillus subtilis*. *Enzyme and Microbial Technology.* 7, 333–338 (1985).

Andrews, B. A., Nielsen, S. and Asenjo, J. A. Partitioning and purification of monoclonal antibodies in aqueous two-phase systems. *Bioseparation.* 6, 303 (1996).

Annunziato, M. E., Mahoney, R. R. and Mudgett, R. E. Production of α-galactosidase from *Aspergillus oryzae* grown in solid state culture. *J. Food Sci.* 51, 1370 (1986).

Armando, V., Catarina, A., Lucilia, D. and Teixeira, J. A. Protein partition on aderivative guar gum based aqueous two phase system. *Bioseparation.* 5, 253-258 (1995).

Armstrong, D., Zhang, L. K. He, L. and Gross, L. M. Ionic Liquids as Matrixes for Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *Anal. Chem.* 73, 3679-3686 (2001).

Arnaud, N. and Bush, D. α-Galactosidase production. US Patent 3,966,555 (1976).

Athawale, V. D. and Lele, V. Syntheses and characterisation of graft copolymers of maize starch and methacrylonitrile. *Carbohydr. Polym.* 41, 407 (2000).

Athawale, V. D., Lele, V. Graft copolymerization onto starch. II. Grafting of acrylic acid and preparation of it's hydrogels. *Carbohydr. Polym.* 35, 21 (1998).

Athawale, V. D., Rathi, S. C. Effect of chain length of the alkyl group of alkyl methacrylates on graft polymerization onto starch using ceric ammonium nitrate as initiator. *Eur. Polym. J.* 33, 1067 (1997).

Axelsson, C. G. Changes in structure and hydrophobic surface properties of betalactoglobulin determined by partition in aqueous two-phase polymeric systems. *Biochim. Biophys. Acta.* 533, 34-42 (1978).

Bacic, A., Harris, P. J. and Stone, B. A. The Biochemistry of Plants. 14, 297-371 (Academic Press, New York, 1988).

Backman, L. Binding of human carbonic anhydrase to human hemoglobin. *European Journal of Biochemistry.* 120, 257-261 (1981).

Bajpai, U. D. N. and Rai, S. Grafting of acrylamide onto guar gum using $KMNO_4$/oxalic acid redox system. *J. Appl. Polym. Sci*. 35, 1169 (1988).

Bakunina, I. Y., Sova, V. V., Nedashkovskaya, O. I., Kuhlmann, R. A., Likhosherstov, L. M., Martynova, M. D., Mihailov, V. V. and Elyakova, L. A., α-Galactosidase of the marine bacterium *Pseudoalteromonas* sp. KMM 701. *Biochem. (Mosco).* 63, 1209-1215 (1998).

Baldwin, R. L. How Hofmeister ion interactions affect protein stability. *Biophys. J.* 71, 2056-2063 (1996).

Bandmann, N., Collet, E., Leijen, J., Uhlen, M., Veide, A. and Nygren, P. A. Genetic engineering of the *Fusarium solani pisi* lipase cutinase for enhanced partitioning in PEG-phosphate aqueous two-phase systems. *J. Biotechnol.* 79, 161-172 (2000).

Banik, R. M., Santhiagu, A., Kanari, B., Sabarinath, C. and Upadhyay, S. N. Technological aspects of extractive fermentation using aqueous two-phase systems. *World Journal of Microbiology and Biotechnology.* 19, 337–348, (2003).

Baskir, J. N., Hutton, T. A. and Sutter, U. W. Protein partitioning in two phase aqueous polymer systems. *Biotechnol. Bioeng.* 34, 541-558 (1989).

Bassani, G., Farruggia, B., Nerli, B., Romanini. D. and Pico, G. Porcine pancreatic lipase partition in potassium phosphate-polyethylene glycol aqueous two-phase systems. *Journal of Chromatography B.* 859, 222-228 (2007).

Behari, K., Taunk, K. and Tripathi, M. Cu^{+2}/mandelic acid redox pair initiated graft copolymerization acrylamide onto guar gum. *J. Appl. Polym. Sci.* 71, 739 (1999).

Berg, J. O., Lindqvist, L., Nord, C. E. Purification of glycoside hydrolases from *Bacteroides fragilis*. Appl. *Environ. Microbiol*. 40, 40-47 (1980).

Berggren, K., Johansson, H. O. and Tjerneld, F. Effects of salts and the surface hydrophobicity of proteins on partitioning in aqueous two-phase systems containing thermoseparating ethylene oxide-propylene oxide copolymers. *Journal of Chromatography A.* 718, 67-79 (1995).

Berggren, K., Maarten, R., Egmond. and Tjerneld, F. Substitutions of surface amino acid residues of cutinase probed by aqueous two-phase partitioning. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology.* 1481, 317-327 (2000).

Berggren, K., Veide, A., Nygren, P. A. and Tjerneld, F. Genetic engineering of protein-peptide fusions for control of protein partitioning in thermoseparating aqueous two-phase systems. *Biotechnol. Bioeng.* 62, 135-144 (1999).

Berka, R. M. et al. *Aspergillus niger* var. *awamori* as a host for the expression of heterologous genes: *Applications of enzyme biotechnology* (Kelly, J. W. and Baldwin, T. O., Editors.), 273–292 (Plenum Press, New York, 1991).

Berry, M. J. Immunoaffinity purification of industrially relevant enzymes. *Anal. Process.* 28, 141-142 (1991).

Biellmann, J. F., Samama, J. P., Branden, C. I. and Eklund, H. X-Ray studies of the binding of Cibacron blue F3G-A to liver alcohol dehydrogenase. *Eur. J. Biochem*. 102, 107-110 (1979).

Bim, M. A. and Franco, T. T. Extraction in aqueous two-phase systems of alkaline xylanase produced by Bacillus pumilus and its application in kraft pulp bleaching. *J. Chromatogr. B.* 743, 349-356 (2000).

Birkenmeir, G., Usbeck, E. and Kopperschlanger, G. Affinity partitioning of albumin and alpha-fetoprotein in an aqueous two phase system using poly(ethylene)glycol-bound triazine dyes. *Anal. Biochem*. 136, 264-271 (1984).

Bishop, D. F. and Desnick, R. J. Affinity purification of α-galactosidase A from human spleen, placenta, and plasma with elimination of pyrogen contamination. Properties of the purified splenic enzyme compared to other forms. *J. Biol. Chem*. 256, 1307-16 (1981).

Blennow, A. Starch syntethase from potato starch tubers: Methods in Enzymology. (walter, H. and Johansson, G. Editors.) 228, 178-182 (Academic Press, London, 1994).

Boland, M. J., Hesselink, P. G .M. and Hustedt, H. Extractive purification of enzymes from animal tissue using aqueous phase systems. *Journal of Biotechnology.* 11, 337-352 (1989).

Boland, M. J., Hesselink, P. G. M., Papamicheal, N. and Hustedt, H. Extractive purification of enzymes using aqueous two phase: pilot scale studies. *J. Biotechnol.* 19, 19-33 (1991).

Bolognese, B., Nerli, B. and Pico, G. Application of the aqueous two-phase systems of ethylene and propylene oxide copolymer-maltodextrin for protein purification. *Journal of Chromatography B*. 814, 347-353 (2005).

Bom, I., Wassenaar, D. and Boot, J. Hybrid affinity chromatography of α-galactosidase from *Verbascum thapsus*. *J. Chromato. A*. 808, 133-139 (1998).

Bordier, C. Phase separation of integral membrane proteins in Triton X-114 solution. *J.Biol.Chem*. 256, 1604-1607 (1981).

Box, G. E. P. and Draper, N. R. Empirical model-building and response surfaces. (Wiley, New York, 1987).

Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 72, 248-254 (1976).

Braunstein, L. E., Rochester, N. Y., Nathaniel, T. B., Burlingame, C., Ganshaw, G. C., Tracy, C., Thomas, P. G. and Pacifica, C. Surfactant-based extraction process. US patent number: 6105786 (2000).

Breunig, F., Knoll, A. and Wanner, C. Enzyme replacement therapy in fabry disease: Clinical implications. *Curr. Opin. Nephrol. Hypertens.* 12, 491-5 (2003).

Breunig, F., Weidemann, F., Beer, M., Eggert, A., Krane, V., Spindler, M., Sandstede, J., Strotmann, J. and Wanner, C. Fabry disease: *Diagnosis and treatment. Kidney. Int. Suppl*. 84, 181-185 (2003a).

Bridson, E. Y. and Brecker, A. Design and formulation of microbial culture medium: Methods in microbiology. (Norris, J. R. and Ribbons, D. W. Editors.) 3A, 229-295 (Academic Press, London, 1970).

Brizard, B. L., Chubet, R. G. and Vizard, D. L. Immunoaffinity purifaction of FLAG epitode-tagged bacterial alkaline phosphatase using a novel monoclonal antibody and peptide elution. *BioTechniques.* 16, 730-735 (1994).

Brown, D. E. Aeration in the submerged culture of microorganisms: *Methods in Microbiology* (Norris, J. R. and Ribbons, D. W. editors.) 2, 137-174 (Academic press, London, 1970).

Buckeridge, M. S, Panegassi, V. R., Rocha, D. C. and Dietrich, S. M. C. Seed galactomannan in the classification and evolution of the leguminosae. *Phytochem.* 38, 871-875 (1995).

Bulpin, P. V., Gidley, M. J., Jeffcoat, R. and Underwood, D. R. Development of a biotechnological process for the modification of galactomannan polymers with plant α-galactosidase. *Carbohydrate Polymers.* 12, 155-168 (1990).

Campillo, D. and Shannon, L. M. An a-galactosidase with hemagglutinin properties from soybean seeds. *Plant Physiol*. 69, 628-631 (1982).

Cantor, C. R. and Schimmel, P. R. *Biophysical chemistry. (Freeman*, San Francisco, 1980).

Cao, Y., Qing, X., Sun, J., Zhou, F. nd Lin, S. Graft copolymerization of acrylamide onto carboxymethyl starch. *Eur. Polym. J.* 38, 1921 (2002).

Carlsen, M., Spohr, A. B., Nielsen, J. and Villadsen, J. Morphology and physiology of an α-amylase producing strain of *Aspergillus oryzae* during batch cultivations. *Biotechnol. Bioeng*. 49, 266–276 (1995).

Castellano, I., Gurruchaga, M. and Goni, I. The influence of drying method on the physical properties of some graft copolymers for drug delivery systems. *Carbohydr. Polym*. 34, 83 (1997).

Cavazzoni, V., Adami, A. and Craveri, R. α-Galactosidase from the yeast *Candida javanica. Appl. Microbiol. Biotechnol*. 26, 555-559 (1987).

Cecilia, K., Jenny, R., Lemmens, R., Tjerneld. F. and Gustavsson, P. E. Extraction of plasmid DNS from *Escherichia coli* cell lysate in a thermoseparating aqueous two phase system. *Journal chromatography A.* 1024, 95-104 (2004).

Chen, H. C., Chang, C. C. and Chen, C. X. Optimization of arachidonic acid production by *Mortierella alpina* Wuji-H4 isolate. *J. Am. Oil. Chem. Soc.* 74, 569-578 (1997).

Chen, J. P. and Lee, M. S. Enhanced production of *Serratia marcescens* chitinase in PEG/dextran aqueous two-phase systems. *Enzyme and Microbial Technology.* 17, 1021-1027 (1995).

Chen, J. P. and Lee, M. S. Simultaneous production and partition of chitinase during growth of *Serratia marcescens* in an aqueous two-phase system. *Biotechnol. Tech.* 8, 783–788 (1994).

Cheng, Y. U and Prudhomme, R. K. Enzymatic degradation of guar and substituted guar galactomannans. *Biomacromolecules*. 1, 782-788 (2000).

Cheng, Y., Brown, K. M., Robert, K. and Home, P. Characterization and intermolecular interactions of hydroxypropyl guar solutions. *Biomacromolecules.* 3, 456-461 (2002).

Chenming, Z., Medina-Bolivar, F., Buswell, S. and Cramer, C. L. Purification and stabilization of ricin B from tobacco hairy root culture medium by aqueous two-phase extraction. *Journal of biotechnology.* 117, 48 (2005).

Chethana, S., Rastogi, N. K. and Raghavarao, K. S. M. S. New aqueous two phase system comprising polyethylene glycol and xanthan. *Biotechnol. Lett.* 28, 25-28 (2006).

Chiba, Y., Sakuraba, H., Kotan, I. M., Kase, R., Kobayashi, K., Takeuchi, M., Ogasawara, S., Maruyama, Y., Nakajima, T., Takaoka, Y. and Jigami, Y. Production in yeast of a-galactosidase A, a lysosomal enzyme applicable to enzyme replacement therapy for Fabry disease. *Glycobiology.* 12, 821-828 (2002).

Chowdhury, P., Samui, S., Kundu, T. and Nandi, M. M. Graft polymerization of methyl methacrylate onto guar gum with ceric ammonium sulfate/dextrose redox pair. *J. Appl. Polym. Sci.* 82, 3520 (2001).

Christian, T. J., Manley-Harris, M. and Richards, G. N. A preliminary study of the use of larch arabinogalactan in aqueous two-phase systems. *Carbohydrate Polymer.* 35, 7-12 (1998).

Chu, I. M., Chang, S. L., Wang, S. H. and Yang, W. Y. Extraction of amino acids by aqueous two phase partition. *Biotech. Tech.* 4, 134-136 (1990).

Church, F. C., Meyers, S. P. and Srinivasan, V. R. Isolation and characterization of α-galactosidase from *Pichia guilliermondii. Dev. Ind. Microbiol.* 21, 339-348 (1980).

Clarke, J. H., Davidson, K. and Rixon, J. E. A comparison of enzyme aided bleaching of soft wood paper pulp using combination of xylanase, mannanase and α-galactosidase. *Appl. Microbiol. Biotechnol*. 53, 661-667 (2000).

Closs, C. B., Conde-Petit, B., Roberts, I. D., Tolstoguzov, V. B. and Escher, F. Phase separation and rheology of aqueous starch/galactomannan systems. *Carbohydrate polymers.* 39, 67-77 (1999).

Closs, C. B., Tolstoguzov, V. B., Conde-Petit, B. and Escher, F. Phase separation of aqueous starch/galactomannan systems: Influence of β-lactoglobulin addition. Gums and stabilizers for the food industry. (9th edition, Williams, P. A. and Phillips, G. O. Editors.), 231–238 (*The Royal Society of Chemistry*, Cambridge, 1998).

Collen, A., Ward, M., Tjerneld, F. and Stalbrand, H. Genetic engineering of the *Trichoderma reesei* endoglucanase I (Cel7B) for enhanced partitioning in aqueous two-phase systems containing thermoseparating ethylene oxide-propylene oxide copolymers. *Journal of Biotechnology.* 87,179-191 (2001).

Coombs, J. and Brenchley, J. E. Characterization of two new glycosyl hydrolases from the lactic acid bacterium *Carnobacterium piscicola* strain BA. Appl. *Environ. Microbiol*. 67, 5094-5099 (2001).

Cote, G. L. Low-viscosity α-D-glucan fractions derived from sucrose which are resistant to enzymatic digestion. *Carbohydrate Polymers.* 19, 249-252 (1992).

Coulson, J. M and Richardon, J. F. Chemical engineering (4th edition.), (Pergamon Press, Oxford, 1991).

Cramer, C. L., Weissenborn, D. L., Oishi, K. K., Grabau, E. A., Bennett, S., Ponce. E., Grabowski, G. A. and Radin, D. N. Bioproduction of human enzymes in transgenic tobacco. *Ann. N.Y. Acad. Sci*. 792, 62-71 (1996).

Creighton, T. E. *Proteins: Structures and molecular properties*, (2nd Edition.), (Freeman, W. H. and Company, New York, 1993).

Cristofaro, F. M. and Wuhrmann, J. J. Involvement of the raffinose family of oligo-saccharides in Flatulenc: *Sugar in Nutrition*. (Sipple, H.L and Mcnutt, K. W, Editors.), 313-336 (Academic Press, New York, 1974).

Cronin, C. E., Giannouli, P., McCleary, B. V., Brooks, M. and Morris, E. R. Formation of strong gels by enzymic debranching of guar gum in the presence of ordered xanthan. *Gums and Stabilisers for the Food Industry.* 278, 289-296 (2002).

Cruz, R. and Park, Y. K. Production of fungal α-galactosidase and its application to the hydrolysis of galacto-oligosccharides in soybean milk. *J. Food. Sci.* 47, 1973-1975 (1982).

Cube, M. C., Smith, J. C. and Harriot, P. *Unit operations in chemical engineering* (4th edition.), (MC-Graw Hill, New York, 1984).

Dea, I. C. M. and Morrison, A. Chemistry and interactions of seed galactomannons. *Advances in carbohydrate chemistry and biochemistry* 31, 241-312 (1975).

Dean, K. J. and Sweeley, C. C. Studies on human liver α-galactosidases. III. Partial characterization of carbohydrate-binding specificities. *J. Biol. Chem*. 254, 10006-10010 (1979).

Delente, J., Johnson, J. H., Kuo, M. J. and O-Connor, R. J. Process for the preparation of heat resistant α-galactosidase enzyme. U.S. Patent, 3846239 (1974a).

Delente, J., Johnson, J. H., Kuo, M. J., O-Connor, R. J. and Weeks, L. E. Production of a new thermostable neutral α-galactosidase from a strain of *Bacillus stearothermophilus*. *Biotechnol. Bioeng*. 16, 1227-1243 (1974).

Deshmukh, S. R. and Singh, R. P. Drag reduction effectiveness, shear stability and biodegradation resistance of guar gum-based graft copolymers. *J. Appl. Polym. Sci.* 33, 1963 (1987).

Dey, P. M. and Pridham, J. B. Biochemistry of α-galactosidases. *Adv. Enzymol*. 15, 91-130 (1977).

Dey, P. M. and Pridham, J. B. Biochemistry of α-galactosidases. *Adv. Enzymol.* 36, 91 (1972).

Dey, P. M. Transgalactosylation activity of sweet almond α-galactosidase: synthesis of saccharides. Phytochemistry. 18, 35 (1979).

Dey, P. M., Patel, S. and Brownleader, M. D. Induction of α-galactosidase in *Penicillium ochrochloron* by guar (*Cyamopsis tetragonobola*) gum. *Biotechnol. Appl. Biochem*. 17,361-71 (1993).

Diamond, A. D. and Hsu, J. T. Aqueous two-phase systems for biomolecule separation. *Adv. Biochem. Eng Biotechnol.* 47, 89-135 (1992).

Diamond, A. D. and Hsu, J. T. Fundamental studies of biomolecules partitioning in aqueous two-phase systems. *Biotech. Bioeng.* 34, 1000-1014 (1989).

Dissing, U. and Mattiasson, B. Integrated removal of nucleic acids and recovery of LDH from homogenate of beef heart by affinity precipitation. *Bioseparation.* 7, 221-229 (1998).

Dobry, A. and Boyek, F. Phase Separation in Polymer Solution. *Journal of Polymer Science.* 2, 90-100 (1947).

Duffaud, G. D., McCutchen, C. M., Leduc, P., Parker, K. N. and Kelly, R. M. Purification and characterization of extremely thermostable beta-mannanase, β-

mannosidase, and α-galactosidase from the hyperthermophilic eubacterium *Thermotoga neapolitana* 5068. *Appl. Environ. Microbiol*. 63, 169-77 (1997).

Durain, N. and Esposito, E. Lignin biodegradation and effluent treatment by ligninolytic fungi. *Microbiologia Ambiental.* (De-Melo, I. S. and Acevedo, J. L. Editors.) Chapter 12, 269 (CNPMA / EMBRAPA Publishers, S. P., Brazil, 1997).

Enfores, S. and Haggstrom, L. *Bioprocess technology and applications*. (Hogskoletryckeriet, Royal institute of technology, Stockholm, 2000).

Erlanson-Albertsson, C. Measurment of binding of colipase to a triacylglycerol substrate. *Biochim. Biophys. Acta.* 617, 371-382 (1980).

Fakhrul-Razi, A., Isam-Qudsieh, Y. M., An-Md-Zin-Wan-Yunus, W., Mansor-Ahmad, B. and Mohama-Zaki-Rahman, Ab. Graft copolymerization of methyl methacrylate onto sago starch using ceric ammonium nitrate and potassium per sulfate as redox initiator systems. *J.Appl. Polym. Sci*. 82, 1375 (2001).

Farag, S. and Afaleq, E. I. A. Preparation and characterization of saponified delignified cellulose polyacrylonitrile-graft copolymer *Carbohydr. Polym*. 48, 1 (2002).

Fauquez, P. F., Hustedt, H. and Kula, M. R. Phase equilibration in agitated vessels during extractive enzyme recovery. *J. Chem. Tech. Biotech,* 35, 51-59 (1985).

Feldt-Rasmussen, U., Rasmussen, A. K., Mersebach, H., Rosenberg, K. M., Hasholt, L. and Sorensen, S. A. Fabry disease a metabolic disorder with a challenge for endocrinologists? (Review*) Horm. Res*. 58, 259-265 (2002).

Fexby, S., Nilsson, A., Hambraeus, G., Tjerneld, F. and Bulow, L. Partitioning and characterization of tyrosine-tagged green fluorescent proteins in aqueous two-phase systems. *Biotechnol. Prog.* 20, 793-798 (2004).

Fischer, E. and Lindner, P. Uber die Enzyme einigen Hefen. *Chem. Ber*. 28, 3034 (1895).
Forciniti, D. and Hall, C. K. Influence of polymer molecular weight and temperature on phase composition in aqueous two-phase systems. *Fluid Phase Equilibria.* 61, 243-262 (1991a).

Forciniti, D., Hall, C. K. and Kula, M. R. Protein partitioning at the isoelectric point: Influence of polymer molecular weight and concentration and protein size. *Biotechnology and Bioengineering.* 38, 986-994 (1991).

Franco, T. T., Galaev, I. Yu., Hatti-Kaul, R., Holmberg, N., Bulow, L. and Mattiasson, B. Aqueous two-phase system formed by thermoreactive vinyl imidazole/vinyl caprolactam copolymer and dextran for partitioning of a protein with a polyhistidine tail. *Biotechnol. Tech.* 11, 231-235 (1997).

Fridjonsson, O., Watzlawick, H., Gehweiler, A., Rohrhirsch, T. and Mattes, R., Cloning of the gene encoding a novel thermostable α-galactosidase from *Thermus brockianus* ITI360. *Appl. Environ. Microbiol*. 65, 3955-63 (1999).

Frost, G. M. and Moss, D. M. Production of enzymes by fermentation: *Biotechnology*, Vol. 7a. (Ed. John F. Kennedy) 65 (1987).

Galas, E. and Miszkiewicz, H. Purification and some properties of α-galactosidase from *Mortierella vinacea* IBT-3. *Acta Microbiologica polonica.* 45, 143-154 (1996).

Ganapathi, P. and Raghavarao, K. S. M. S. Aqueous two phase extraction for purification of C-phycocyanin. *Biochemical Engineering Journal*. *34,* 156-164 (2007).

Ganter, C., Bock, A., Buckel, P. and Mattes, R. Production of thermostable recombinant α-galactosidase suitable for raffinose elimination from sugar beet syrup. *J. Biotechnol*. 8, 301-310 (1988).

Garnier, C., Schorsch, C. and Doublier, J. L. Phase separation in dextran/locust bean gum mixtures. *Carbohydrate Polymers.* 28, 313-317 (1995).

Garro, M. S., De-Valdez, G. F., Oliver, G. and De-Giori, G. S. Purification of α-galactosidase from *Lactobacillus fermentum*. *J. Biotechnol*. 33, 302-5 (1996).

Gavasane, M. R. and Gaikar,V. G. Aqueous two-phase affinity partitioning of *Penicillin acylase* from *E. coli* in presence of PEG-derivatives. *Enzyme and Microbial Technology.* 32, 665-675 (2003).

Gdala, J., Johansen, H. N., Bach, K. E., Knap, I. H., Wagner, P. and Jorgensen, O. R. The digestibility of carbohydrates, protein and fat in the small and large intestine of piglets fed nonsupplemented and enzyme supplemented diets. *Animal Feed Science Technology.* 65,15-33 (1997).

Georgina, B., Beatriz, F., Bibiana, N., Diana, R. and Guillermo, P. Porcine pancreatic lipase partition in potassium phosphate–polyethylene glycol aqueous two-phase systems. *Journal of Chromatography B.* 859, 222–228 (2007).

Gherardini, F., Babcock, M. and Salyers, A. A. Purification and characterization of two alpha-galactosidases associated with catabolism of guar gum and other alpha-galactosides by *Bacteroides ovatus*. *J. Bacteriol*. 161, 500-506 (1985).

Gitzelmann, R. and Auricchio, S. The handling of soy α-galactosidase by a normal and galactosemic child. *Pediatrics.* 36, 231-235 (1965).

Gliko-Kabir, I., Yagen, B., Baluom, M. and Rubinstein, A. Phosphated crosslinked guar for colon-specific drug delivery II. In vitro and in vivo evaluation in the rat. *Journal of Controlled Release.* 63, 129-134 (2000a).

Gliko-Kabir, I., Yagen, B., Penhasi, A. and Rubinstein, A. Low swelling, biodegradable guar and its potential use as colon specific drug carrier. *Pharmaceutical Research.* 15, 1019–1025 (1998).

Gliko-Kabir, I., Yagen, B., Penhasi, A. and Rubinstein, A. Phosphated crosslinked guar for colon-specific drug delivery I. Preparation and physicochemical characterization. *Journal of Controlled Release.* 63, 121-127 (2000).

Glodenstein, A. M. and Alter, E. N. *Industrial gums*. 321, (Academic, New york, 1959).

Gokhale, D. V., Patil, S. G. and Bastawde, K. B. Optimization of cellulase production by *Aspergillus niger* NCIM 1207. *Appl. Biochem. Biotechnol.* 30, 99-109 (1991).

Goldstein, J. Conversion of ABO blood groups. *Transfus. Med. Rev*. 3, 206–212 (1989).

Goldstein, J., Siviglia, G., Hurst, R. et al. Group B erythrocytes enzymatically converted to group O survive normally in A, B and O individuals. *Science*. 215, 168-170 (1982).

Gonzales, R., Johns, M. R., Greenfield, P. F. and Pace, W. Xanthan gum precipitation using ethanol. *Process. Biochem*. 24, 200-203 (1989).

Gouka, R. J., Hessing, J. G. M., Punt, P. J., Stam, H., Muster, W. and Van-den-Honde, C. A. M. J. J. An expression system based on the promoter region of the *Aspergillus awamori* 1,4-β-endoxylanase gene. *Appl. Microbiol. Biotechnol*. 46, 28-35 (1996).

Goyal, P., Kumar, V. and Sharma, P. Carboxymethylation of tamarind kernel powder. *Carbohydrate Polymers.* 69, 251-255 (2007).

Greenberg, N. A. and Slavin, J. L. Partially hydrolysed guar gum: Clinical nutrition uses. *Nutrition.* 19, 549-552 (2003).

Gulbis, I. In reservoir stimulation, economides. (M. J. and Noite K. G.), (Schlumberger Educational Services, Houston, 1987).

Gunduz, U. Partitioning of bovine serum albumin in an aqueous two-phase system: Optimization of partition coefficient. *Journal of Chromatography B: Biomedical Sciences and Applications.* 743, 259-262 (2000a).

Gunduz, U. Viscosity prediction of polyethylene glycol–dextran–water solutions used in aqueous two-phase systems. *Journal of Chromatography B:Biomedical Sciences and Applications.* 743, 181-185 (2000).

Gutowski, K. E., Broker, G. A., Willauer, H. D., Huddleston, J. G., Swatloski, R. P., Holbrey, J. D. and Rogers, R. D. Controlling the aqueous miscibility of Ionic Liquids: Aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations. *J. Am. Chem. Soc.* 125, 6632-6633 (2003).

Haghtalab, A. and Mokhtarani, B. The new experimental data and a new thermodynamic model based on group contribution for correlation liquid–liquid equilibria in aqueous two-phase systems of PEG and (K_2HPO_4 or Na_2SO_4). *Fluid Phase Equilibria.* 215, 151-161 (2004).

Halstead, J. R., Fransen, M. P., Eberhart, R. Y., Park, A. J., Gilbert, H. J. and Hazlewood, G. P. α-Galactosidase A from *Pseudomonas fluorescens* subsp. cellulose: Cloning high level expression and its role in galactomannan hydrolysis. *FEMS Microbiol. Lett*. 192,197-203 (2000).

Hansen, J. C. and Gorski, J. Conformational transitions of the estrogen receptor monomer. Effects of estrogens, antiestrogen and temperature. *J. Biol. Chem.* 261, 13990-13996 (1986).

Harpaz, N., Flowers, H. M. and Sharon, N. studies on B antigenic sites of human erythrocytes by use of coffee bean α-galactosidase. *Arch. Biochem. Biophys.* 170, 676–683 (1975).

Harris, P. A., Karlstrom, G. and Tjerneld, F. Enzyme purification using temperature-induced phase formation. *Bioseparation.* 2, 237-246 (1991).
Harry, W. and Johansson, G. Aqueous Two-Phase Systems. *Methods in Enzymology.* 228, 5 (1994).

Hart, R. A., Lester, P. M., Reifsnyder, D. H., Ogez,J.R. and Builder, S. E. Large scale: In situ isolation of periplasmic IGF-I from *E.coli. Biotechnology.* 12, 1113-1117 (1994).

Hashimoto, H., Goto, M., Katayama, C. and Kitahata, S. Purification and some properties of α-galactosidase from *Pseudomonas fluorescens* H-601. *Agr. Biol. Chem.* 55, 2831-2838 (1991).

Hawking, A. J. Economic Aspects of Biotechnology. 93-126 (Cambridge, University Press, Cambridge, UK, 1986).

Hazmi, M. I. A. L. and Stauffer, K. R. Gas chromatographic determination of hydrolyzed sugars in commercial gums. *Journal of Food Science.* 51, 1091-1092 (1986).

He, C., Li, S., Liu, H., Li, K. and Liu, F. Extraction of testosterone and epitestosterone in human urine using aqueous two-phase systems of ionic liquid and salt. *Journal of Chromatography A.* 1082, 143-149 (2005).

Helferich, B. and Appel, H. Emulsin-X. Z. *Physiol. Chem*. 237, 254 (1932).

Heusch, R. and Kopp, F. Structures in aqueous solutions of nonionic tensides. *Progress in Col-loid and Polymer Science*. 77, 77–85 (1988).

Hopp, T. P., Prickett, K. S., Price, V. L., Libby, R. T., March, C. J., Cerretti, D. P., Urdal, D. L. and Conlon, P. J. A short polypeptide marker sequence useful for recombinant protein identification and purification. *Bio. Technology.* 6, 1204-1210 (1998).

Hours, R. A., Katsuragi, T. and Sakai, T. Growth and protopectinase production of *Aspergillus awamori* in solid-state culture at different acidities. *J. Ferment. Bioeng*. 78, 426–430 (1994).

Hsu, S. C. Don, T. M. and Chiu, W. Y. Free radical degradation of chitosan with potassium persulfate. *Polymer Degradation and Stability.* 75, 73-83 (2002).

Huang, S. J. *Encyclopedia of polymer science engineering* (2nd Edition. Mark, H. F. et al. Editor.), 220-243 (Wiley-Interscience, New York, 1985).

Hughes, P. and Lowe, C. R. Purification of proteins by aqueous two phase partition in novel acrylic copolymers. *Enzyme. Microb. Technol*. 10, 115-122 (1988).

Hustedt, H., Kroner, K. H. and Kula, M. R. Applications of phase partitioning in biotechnology: *Theory, methods, uses and applications to biotechnology.* (Walter, H., Brooks, D. E. and Fisher, D. Editors.), 529-587 (Academic, Orlando, FL, 1985).

Hustedt, H., Kroner, K. H., Stach, W. and Kula, M. R. Procedure for the simultaneous large-scale isolation of pullulanase and 1,4-Œ-glucan phosphorylase from *Klebsiella pneumoniae* involving liquid-liquid separations. *Biotechnology and Bioengineering.* 20, 1989 (2005).

Ichikawa, K., Hashizume, K., Miyamoto, T., Nishii, Y., Yamauchi, K., Ohtsuka, H. and Yamada, T. Conformational transition of thyroid hormone receptor upon hormone binding: Demonstration by aqueous two-phase partitioning. *Journal of Endocrinology.* 119, 431-437(1988).

Ichikawa, K., Hashizume, K., Miyamoto, T., Nishii, Y., Yamauchi, K., Ontsuka, H. and Yamada, T. *J. endocrinology*. 119, 431 (1998).

Ikura, Y., Horikosh, I. K. Isolation and some properties of α-galactosidase producing Bacteria. *Agric. Bio. Chem*. 51, 243-245 (1987).

Ishiguro, M., Kaneko, S., Kuno, A., Koyama, Y., Yoshida, S., Park, G. G., Sakakibara, Y., Kusakabe, I. and Kobayashi, H. Purification and characterization of the recombinant *Thermus* sp. strain T2 α-galactosidase expressed in *Escherichia coli*. *Appl. Environ. Microbiol*. 67, 1601-1606 (2001).

Ivanova, V., Yankov, D., Kabaivanovaand, L. and Pashkoulov, D. Simultaneous biosynthesis and purification of two extracellular *Bacillus* hydrolases in aqueous two-phase systems. *Microbiological Research.* 156, 19-30 (2001).

Janson, J. C. Large-scale affinity purification-state of the art and future prospects. *Trends Biotechnol*. 2, 31-38 (1984).

Janson. and Porath, A. Bacterial dextranase. *Methods in Enzymology.* 8, 615 (1966).

Jiang, N. Q. and Zhao, F. S. L-Asparaginase release from *Escherichia coli* cells with aqueous two-phase systems. *Industrial Microbiology.* 29, 5–9 (1999).

Jiang, Y., Xia, H., Guo, C., Mahmood, I. and Liu, H. Phenomena and mechanism for separation and recovery of penicillin in ionic liquids aqueous solution. *Ind. Eng. Chem. Res.* 46, 6303-6312 (2007).

Jin, F., Li, y., Zhang, C. and Yu, H. Thermostable α-galactosidase production from the thermophilic and aerobic *Bacillus sp*. JF strain. *Process Biochem*. 36, 559-564 (2001).

Joersbo, Morten, Marcussen Jan, Brunstedt Janne. In vivo modification of the cell wall polysaccharide galactomannan of guar transformed with α-galactosidase gene cloned from senna. *Molecular Breeding.* 7, 211-219 (2001).

Johansson, G. and Anderson, M. Liquid-Liquid exraction of glycolytic enzymes from Baker's yeast using triazine dye ligands. *J. Chromatogr.* 291, 175-183 (1984).

Johansson, G. and Joelsson, M. Effect of polymer structure on affinity partitioning of lactate dehydrogenase in polymer-water two-phase systems. *J Chromatogr.* 411, 161-166 (1987).

Johansson, G. and Joelssondoi, M. Liquid-liquid extraction of lactate dehydrogenase from muscle using polymer-bound triazine dyes. *Applied Biochemistry and Biotechnology.* 13, 15-27 (1986).

Johansson, G. and Reczey, K. Concentration and purification of β-glucosidase from *Aspergillus niger* by using aqueous two-phase partitioning. *J. Chromatogr. B.* 711, 161-172 (1998).

Johansson, G. Effects of salts on the partition of proteins in aqueous polymeric biphasic systems. *Acta. Chem. Scand B.* 28, 873-882 (1974).

Johansson, G. Partition of salts and their effect in a dextran-poly(ethylene glycol)-water two phase system. *Biochem. Biophysics. Acta.* 221, 387-390 (1970).

Johansson, G. Partition of salts and their effects on partition of proteins in a dextran-poly (ethylene glycol)-water two-phase system. *Biochim. Biophys. Acta.* 221, 387-90 (1970).

Johansson, G. Partition of salts on partition of proeins in aqueous polymeric biphasic systems. *Acta. Chem. Sand B.* 28, 873-882 (1974).

Johansson, G., Anderson, M. and Akerlund, H. Counter-current distribution of yeast enzymes with polymer-bound triazine dye affinity ligand. *J. Chromatogr*. 298, 483-493 (1984).

Johansson, G., Kopperschlager, G. and Albertsson, P. A. Affinity partitioning of phospho-fructokinase from baker's yeast using polymer-bound Cibacron blue F3G-A. *Eur. J. Biochem.* 131, 589-594 (1983).

Johansson, H .O., Karstrom, G., Mattiasson, B. and Tjerneld, F. Effect of hydrophobicity and counter ions on the partitioning of amino acids in the thermoseparating UCON-water two phase systems. *Bioseparation.* 5, 269-279 (1995).

Johansson, H. O., Gunnar-Karlstrt, M. T. and Tjerneld, F. Experimental and theoretical study of phase separation in aqueous solutions of clouding polymers and carboxylic Acids. *Macromolecules.* 26*,* 4478-4483 (1993).

Johansson, H. O., Karlstrom, G. and Tjerneld, F. Temperature-induced phase partitioning of peptides in water solutions of ethylene oxide and propylene oxide random copolymers. *Biochim. Biophys. Act.* 1335, 315-325 (1997).

Johansson, H. O., Karlstrom, G., Mattiasson, B. and Tjerneld, F. Effect of hydrophobicity and the counter ions on the partitioning ogf amino acids in thermoseparating UCON-water two phase systems. *Bioseparation.* 5, 269-278 (1995).

Johansson, H. O., Karlstrom,G. and Tjerneld, F. Experimental and theoretical study of phase separation in aqueous two phase separation in aqueous solutions of clouding polymers and carbonic acids. *Macromolecules.* 26, 4478-4483 (1993).

Johansson, H. O., Lundh, G., Karlstrom, G. and Tjerneld, F. Effects of ions on partitioning of serum albumin and lysozyme in aqueous two-phase systems containing ethylene oxide/propylene oxide copolymers. *Biochim. Biophys. Acta.* 1290, 289-298 (1996).

Johansson, H. O., Magaldi, F. M., Feitosa, E. and Pessoa, A. Jr. Protein partitioning in poly(ethylene)glycol/sodium polyacrylate aqueous two-phase systems. *Journal of Chromatography A*. 1178, 145-539 (2008).

Johansson, H. O., Magaldi, F. M., Feitosa, E. and Pessoa, A. Jr. Protein partitioning in poly(ethylene glycol)/sodium polyacrylate aqueous two-phase systems. *J. Chromatogr. A*. 1178, 145-539 (2008).

Johansson, H. O., Persson, J. and Tjerneld, F. Thermoseparating water/polymer system: A novel one-polymer aqueous two-phase system for protein purification. *Biotechnol. Bioeng.* 66, 247-257 (1999).

Jones, K. A. Review of biotechnology and large scale affinity chromatography. *Chromatographia.* 32, 469-480 (1991).

Kaji, A. and Ichimi, T. Production and properties of galactosides from *Corticium rolfsii*. *Appl. Microbiol*. 18, 1036-1040 (1969).

Kamihira, M., Kaul, R. and Mattiasson, B. Purification of recombinant protein A by aqueous two-phase extraction integrated with affinity precipitation. *Biotechnol. Bioengg*. 40, 1381 (1992).

Karsten, K., Friedmann, T. and Boxman, A. The bioseparation needs for tomorrow. *Trends in Biotechnology.* 19, 438-441 (2001).

Katoh, S. Scaling-up affinity chromatography. *Trends in Biotech*. 5, 328 (1987).

Kaul, A. Aqueous two phase systems: *Methods and protocols.* 11-19 (Humana Press Inc, New Jersey, 2000).

Kawanura, S., Kasai, T. and Tanusi, S. Purification and properties of α-galactosidase from *Escherichia coli* subsp. communiorIAM 1272. *Agric. Biol. Chem*. 40, 641-648 (1976).

Keefe, A. D., Wilson, D. S. Seelig, B. and Szostak, J. W. One-step purification of recombinant proteins using a nanomolar-affinity streptavidin-binding peptide, the SBP-Tag. *Protein Expression and Purification.* 23, 440-446 (2001).

Kepka, C., Collet, E., Persson, J., Stahl, A., Lagerstedt, T., Tjerneld, F. and Veide, A. Pilot Scale extraction of an intracellular recombinant cutinase from *E.Coli* cell homogenate using a thermoseparating aqueous two-phase system. *J. Biotech.* 103, 165-181 (2003).

Kepka, C., Lemmens, R., Vasi, J., Nyhammar, T. and Gustavsson, P. E. Integrated process for purification of plasmid DNA using aqueous two-phase systems combined with membrane filtration and lid bead chromatography. *Journal of Chromatography A.* 1057, 115-124 (2004).

Kidd, M. T., Morgan, G. W. Jr., Zumwalt, C. D., Price, C. J., Welch, P. A., Brinkhaus, F. L. and Fontana, E. A. a-Galactosidase enzyme supplementation to corn and soybean meal broiler diets. *Journal of Applied Poultry Research.* 10, 186-193 (2001).

Kim, D. W. and Chi, D. Y. Polymer-supported ionic liquids: Imidazolium salts as catalysts for nucleophilic substitution reactions including fluorinations. *Angewandte Chemie International Edition.* 43, 483-485 (2003).

King, M. R., White, B. A., Blaschek, H. P., Chassy, B. M., Mackie, R. I., Cann, I. K. Purification and characterization of a thermostable α-galactosidase from

Thermoanaerobacterium polysaccharolyticum. *J. Agric. Food. Chem.* 50, 5676-5682 (2002).

Kishida, A., Nakano, S., Kikunaga, Y. and Akashi, M. Synthesis and funcionalities of poly (N-vinylalkylamide). VII. A novel aqueous two phase systems based on poly (N-vinylacetamide) and Dextran. *Journal of Applied Polymer Science.* 67, 255-258 (1998).

Knap, I. H., Hjort, C. M., Halkier, T. and Kofod, L. V. α.-Galactosidase enzyme. U.S Patent 6197566 (2001).

Kocabas, E. E. and Dizbay, M. Production of α-galactosidase Using *Bacillus stearothermophilus Var. Calidolactis* C953. *Tr. J. of Biology*. 23, 47-54 (1999).

Kohler, *et al*. Engineering proteins to enhance their partition coefficients in aqueous two-phase systems. (Kohler, et al., Editor) 9, 642-646 (New york, 1991).

Koningsveld, R. and Staverman, A. J. Liquid-liquid phase separation in multicomponent polymer solutions. I. Statement of the problem and description of methods of calculation. *J.Polym.Sci.* 6, 367 (1968).

Kooiman, P. On the occurrence of amyloids in plant seeds. *Acta Botanica Neerlandica.* 9, 208-219 (1960).

Kooiman, P. The constitution of *Tamarindus-amyloid. Rec. Trav. Chim. Pays-Bas*. 80, 849-865 (1961).

Kooperschlager, G. and Kirchberger, J. Dye ligand affinity of proteins in aqueous two phase systems, Methods and protocols. (Kaul. H. Editor.) 329 (Humana Press Inc, New Jersey, 2000).

Kopperschlager, G. and Johansson, G. Affinity partitioning with polymer-bound Cibacron blue F3G-A for rapid, large-scale purification of phosphofructokinase from baker's yeast. *Analytical Biochemistry.* 124, 117-124 (1982).

Kotwal, S. M., Gote, M. M., Khan, M. I., Khire, J. M. Production, purification and characterization of constitutive intracellular α-galactosidase from the thermophilic fungus *Humicola sp*. *J. Ind. Microbiol and Biotechnol*. 23, 661-667 (1999).

Kotwal, S. M., Gote, M. M., Sainkar, S. R., Khan, M. I. and Khire, J. M. Production of α-galactosidase by thermophilic fungus *Humicola* sp. in solid-state fermentation and its application in soya milk hydrolysis. *Process Biochem*. 33, 337-343 (1998).

Kotwal, S. M., Khan, M. I. and Khire, J. M. Production of thermostable α-galactosidase from thermophilic fungus *Humicola sp*. *J. Industrial. Microbiol*. 15, 116-120 (1995).

Kotwal, S. M., Khire, J. M. and Khan, M. I. purification and characterization of an extracellular α-galactosidase from the thermophilic fungus *Humicola sp*. *J. Biochem. Mol. Biol and Biophys.* 3, 9-17 (1999).

Kremnicky, L. and Biely, P. β-Mannanolytic system of *Aureobasidium pullulans. Arch. Microbiol*. 165, 350-355 (1997).

Kroner, K. H., Husteadt, H. and Kula, M. R. Evaluation of Crude Dextran as Phase-Forming Polymer for the Extraction of Enzymes in Aqueous Two Phase Systems in Large Scale. *Biotechnology and Bioengineering.* 24, 1015-1045 (1982a).

Kroner, K. H., Hustedt, H. and Kula, M. R. Extractive enzyme recovery: economic considerations. Process Biochemistry. 170-179 (1984).

Kroner, K. H., Stach, W., Schutte, H. and Kula, M. R. Scale-up of formate dehydrogenase by partition. *Journal of Chemical Technology and Biotechnology*. 32, 130-137 (1982b).

Kubota, H. and Suzuki, S. Comparative examinations of reactivity of grafted celluloses prepared by u.v and ceric salt-initiated graftings. *Eur. Polym. J.* 31, 701 (1995).

Kula, M. R. Large-scale protein recovery using aqueous phase systems: Separation using aqueous two-phase systems. (Derek, F. and Ian, A. Sutherland Editors), 295-297 (Plenum Press, New York, 1989).

Kula, M. R. and Selber, K. Protein purification and aqueous liquid extraction. *Encyclopedia of bioprocess technology: Fermentation, biocatalysts and bioseparation.* (Flickinger, M. C. and Drew, S. W. Editors.), 2179-2191 (John wiley and Sons, New York, 1999).

Kula, M. R., Kroner, K. H. and Hustedt, H. Purification of enzymes by liquid-liquid extraction. *Advances Biochemical Engineering.* 24, 73-118 (1982).

Kula, M. R., Kroner, K. H. and Hustedt, H. Purification of enzymes by liquid–liquid extraction: *In Advances in Biochemical Engineering.* (Fiechter. Editor) 24, 74–118 (Springer-Verlag, 1982).

Kula, M. R., Kroner, K. H. and Hustedt, H. Purification of enzymes by liquid-liquid extraction. *Advances in Biochemical Engineering.* 24, 74-118 (1982).

Kula, M. R., Kroner, K. H., Husted, T. H., Fisher, D. E. and Sutherland, I. A. *Separations using Aqueous Phase Systems.* 295-298 (Plenum, London, 1989).

Kumar, A., Galaev, I. Yu. and Mattiasson, B. Metal chelate affinity precipitation: a new approach to protein purification. *Bioseparation.* 7,185-194 (1999).

Lai, L. S. and Kokini, J. L. Physicochemical changes and rheological properties of starch during extrusion. *Biotechnol. Prog. 7*, 251-266 (1991).

Lapasin, R. and Pricl, S. *Rheology of industrial polysaccharides: Theory and applications.* (Chapman and Hall, London, 1995).

Lau, W. W. Y., Burns, C. M. and Huang, R. Y. M. Molecular weights and molecular weight distribution in phase equilibria of the polystyrene-poly(methylmethacrylate)-toluene system. *Journal of Applied Polymer Science.* 30, 1187-1194 (1986).

Lazo, P. S., Ochoa, A. G. and Gascon, S. α-Galactosidase from *Saccharomyces carlsbergensis*. Cellular localization, and purification of the external enzyme. *Eur. J. Biochem*. 77, 375-382 (1977).

Li, S. H., He, C. Y., Liu, H. W., Li, K. A. and Liu, F. Ionic liquid-salt aqueous two-phase system, a novel system for the extraction of abused drugs. *Chinese Chemical Letters.* 16, 1074-1076 (2005).

Li, S., He, C., Liu, H., Li, K. and Liu, F. Ionic liquid-based aqueous two-phase system, a sample pretreatment procedure prior to high-performance liquid chromatography of opium alkaloids. *Journal of Chromatography B.* 826, 58-62 (2005).

Li, Y. T., Li, S. C. and Shetlar, M. R. α-Galactosidase from *Diplococcus pneumoniae*. *Arch. Biochem. Biophys*. 103, 436-442 (1963).

Linden, J.C. Immobilized α-galactosidase in the sugar beet industry. *Enzyme microb.Technol.* 4, 130-136 (1982).

Linder, M., Fanni, J., Parmentier, M., Sergent, M. and Phan-Tan-Luu, R. Protein recovery from veal bones by enzymatic hydrolysis. *J Food Sci.* 60, 949-952 (1995).

Ling, T. G. I., Nilsson, H. and Mattiasson, B. Reppal-PES - A starch derivative for aqueous two- phase systems. *Carbohydrate Polymers.* 11, 43-54 (1989).

Liu, Q., Yu, J., Li, W., Hu, X., Xia, H., Liu, H. and Yang, P. Partitioning behavior of penicillin G in aqueous two phase system formed by ionic liquids and phosphate. *Separation Science and Technology.* 41, 2849-2858 (2006).

Lonsane, B. K., Ghildyal, N. P., Budiatman, S. and Ramkrishna, S. V. Engineering aspects of solid state fermentation. *Enzy. Microbial. Technol*. 7, 258-265 (1985).

Lonsane, B. K., Saucedo-Castaneda, G., Raimbault, M., Roussos, S., Viniegra-Gonzalez, G., Ghildyal, N. P., Ramakrishna, M. and Krishnaiah, M. M. Scale-up strategies for solid-state fermentation systems. *Process Biochem.* 27, 259-273 (1992).

Lowry, O. H., Roseburgh, N., Farr, A. L. and Randall, R. Protein measurement with the phenol reagent. *Journal of Biological Chemistry*. 193, 265-275 (1951).

Lu, M., Johansson, G., Albertsson, P. A. and Tjerneld, F. Partitioning of proteins in dextran/hydrophobically modified dextran aqueous two-phase systems. *Bioseparation.* 5, 351-358 (1995).

Luong, J. H. T. and Nguyen, A. L. Novel separations based on affinity interactions. *Advanced Biochemical engineering Biotechnology.* 47, 137-158 (1992).

Lutfor, M. R., Sidik, S., Wanyunus, W. M. Z., Rhman, M. Z. Ab., Mansoor, A. and Jelas, H. Preparation and swelling of polymeric absorbent containing hydroxamic acid group from polymer grafted sago starch. *Carbohydr. Polym.* 45, 95 (2001).

Mahesh, R., Gavasane. and Vilas, G. Gaikar. Aqueous two-phase affinity partitioning of *penicillin acylase* from *E. coli* in presence of PEG-derivatives. *Enzyme and Microbial Technology*. 32, 665-675 (2003).

Maier, H., Anderson, M., Karl, C. and Magnusun, K. *Industrial gums: Polysaccharides and their derivatives* (3rd edition.) 199, (1993).

Maier, H., Anderson, M., Karl, C. and Magnusun, K. *Industrial gums, polysaccharides and their derivatives* (3rd edition.) 204, (1993a).

Mapes, C. and Sweeley, C. C. Preparation and properties of an affinity column adsorbent for differentiation of multiple forms of α-galactosidase activity. *J. Bio. Chem*. 248, 2461-2470 (1973).

Marcos, J. C., Fonseca, L. P., Ramalho, M. T. and Cabral. J. M. S. Variation of penicillin acylase partition coefficient with phase volume ratio in poly(ethylene glycol)–sodium citrate aqueous two-phase systems. *J. Chromatogr. B.* 711, 295-299 (1998).

Marquardt, R. R., Brenes, A., Zhang, Z. and Boros, D. Use of enzymes to improve nutrient availability in poultry feed stuffs. *Animal Feed Science and Technology.* 60, 321-330 (1996).

Marquardt, R. R., Brenes, A., Zhang, Z. and Boros, D. Use of enzymes to improve nutrient availability in poultry feedstuffs. *Animal Feed Science and Technology.* 60, 321-330 (1996).

McCutchen, C. M., Duffaud, G. D., Leduc, P., Petersen, A. R. H., Tayal, A., Khan, S. A. and Kelly, R. M., Characterization of extremely thermostable enzymatic brekers (α-1,6-galactosidase and β-1,4-mannanase) from the hyperthermophilic bacterium *Thermotoga neapolitana* 5068 for hydrolysis of guar gum. *Biotechnol. Bioeng*. 52 , 332-339 (1996).

McKay, A. M. Production of extracelluar β-glucosidase and α-galactosidase during fungal growth on polygalacturonate. *J. Food Sci*. 56, 1749 (1991).

Meer, G., Meer, W. A. and Tinker, J. Water-soluble gums. *Food Technology*. 22-30 (1975).

Meier, H. and Reid, J. S. G. Reserve polysaccharides other than starch in higher plants. *Encyclopedia of Plant Physiology*. 13A, 418-471 (Springer, Berlin, 1984).

Menge, U. Extraction of lipases in aqueous two phase systems: The strategy for an optimization. *Frontiers in Bioprocessing II.* (Todd, P., Sikdar, S. and Bier, M. Editors.), 331-339 (Washington, 1992).

Middaugh, C. R. and Lawson, E. Q. Analysis of protein association by partitioning in aqueous two-phase polymer systems: Applications to the tetramer-dimer dissociation of hemoglobin. *Anal. Biochem.* 105, 364-368 (1980).

Miller, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*. 31, 426–428 (1959).

Min, L. and Johansson, G. Partitioning of proteins in dextran/ hydrophobically modified dextran aqueous two-phase systems. *Bioseparation*. 5, 351-358 (1995).

Minami, N. M. and Kilikian, B. V. Separation and purification of glucoamylase in aqueous two-phase systems by a two-step extraction. *J. Chromatogr. B.* 711, 309-312 (1998).

Minuth , T., Gieren , H., Pape , U., Raths, H. C., Thommes , J., Kula, M. R. Pilot scale processing of detergent-based aqueous two-phase systems. Biotechnology and Bioengineering. 55, 339–347 (1997).

Miranda, M. V. and Cacone, O. Partition of horse radish peroxidase in aqueous two phase systems containing polyvinylpyrollidone. *Bioseparation.* 7, 25-30 (1997).

Miranda, M. V. and Cascone, O. Partition behavior of Horse Radish Peroxidase iso-enzymes in aqueous two-phase poly(ethylene)glycol/ phosphate systems. *Biotech. Tech.* 8, 275-280 (1994).

Misra, G. S. and Bajpai, U. D. Redox polymerization. *Prog. Polym. Sci.* 8, 61 (1982).

Mital, B. K., Shallenberger, R. S. and Steinkraus, K. H. α-Galactosidase activity of *Lactobacilli*. *Appl. Microbiol*. 26, 783-788 (1973).

Mitchell, D. A. and Lonsane, B. K. Definition, characterstics and potential In solid substrate cultivation (Doelle, H. W., Mitchell, D. A. and Rolz, C. E. Editors.), (Elsevier Science Publishers, London, 1992).

Monis, B. and Wasserkrug, H. Histochemistry of glycosidases of Megakaryocytes and Platelets: A comparative study. *Histochemie.* 10, 363-368 (1967).

Moorhouse., Ralph., Harry., David, N., Merchant. and Uday. Guar gum : A better polysaccharide for colonic drug delivery. *Society of Petroleum Engineering.* 39531, 253-269 (1998).

Morimoto, J. Y., Irene, C. J. U. and Unrau, A. M. Chemical and physical properties and the enzymatic degradation of some tropical plant gums. *Journal of Agricultural and Food Chemistry*. 10, 134-137 (1962).

Mulimani, V. H. and Ramlingam. Enzymic hyrolysis of raffinose and stachyose in soymilk by α-galactosidase from *Gibberella fujikuroi*. *Biochem. Mol. Biol. Int*l. 36, 897-905 (1995).

Nakashima, T. and Kimizuka, N. Interfacial synthesis of hollow TiO_2 microspheres in ionic liquids. *Am. Chem. Soc.* 125, 6386-6387 (2003).

Narita, S., Naganishi, H., Yokouchi, A. and Kagaya, I. Method for manufacture of α-galactosidase by microorganisms. U.S Patent 3867256 (1975).

Narita, S., Naganishi, H., Yokouchi, A. and Kagaya, I. Method for manufacture of α-galactosidase by micro-organism. U.S Patent, 3957578 (1976).

Nielsen, L. K. Analysis of the phase behavior of the aqueous poly(ethylene glycol)-ficoll system. *Biotechnol. Prog.* 19, 1269-1273 (2003).

Nondek, L. and Ponec, R. Chemically bonded electron acceptors as stationary phases in high-performance liquid chromatography. *Journal of Chromatography A.* 294, 175-183 (1984).

Ofman, D., Slim, G. C., Watt, D. K. and Yorke, S. C. Free radical induced oxidative depolymerisation of chondroitin sulphate and dermatan sulphate. *Carbohydrate Polymers.* 33, 47-56 (1997).

Ohtakara, A. and Mitsutomi M. Immobilization of thermostable α-galactosidase from *Pycnoporus cinnabarinus* on chitosan beads and its application to the hydrolysis of raffinose in beet sugar molasses. *J. Ferment. Technol*. 65, 493-498 (1987).

Okieimen, F. E. and Ogbeifun, D. E. Graft copolymerizations of modified cellulose: Grafting of methyl acrylate, ethyl acrylate and ethyl methacrylate on carboxy methyl cellulose. *Eur. Polym. J.* 32, 311 (1996).

Olivieri, R., Pansolli, P., Fascetti, E. and Cuiffiolotti, P. Process for the production of α-galactosidase and uses of the enzyme thus obtained. US Patent 4431737 (1984).

Omya, T. and Tabuchi, K. Tamarind gum carboxymethyl ether alkali metal salts. *Japanese patent 60,* 166302 (1985).

Ortin, A., Cebrian, J. A., Lopez-Perez, M. J. and Johansson, G. An aqueous hydroxypropylstarch-poly(ethylene glycol) two-phase system for extraction of alpha-lactalbumin and beta-lactoglobulin from bovine whey. *Bioseparation*. 2, 197-205 (1991).

Pai, V. B. and Khan, S. A. Gelation and rheology of xanthan/enzyme modified guar blends. Carbohydr. Poly. 49, 207-216 (2002).

Pan, B., Li, D., Piao, X., Zhang, L. and Guo, L. Effect of dietary supplementation with a-galactosidase preparation and stachyose on growth performance, nutrient digestibility and intestinal bacterial populations of piglets. *Archives of Animal Nutrition.* 56, 327-337 (2002).

Parvathy, K. S., Susheelamma, N. S., Tharanatan, R. N. and Gaonkar, A. K. A simple non aqueous method for carboxymethylation of galactomannans. *Carbohydrates Polymers.* 62, 137-141 (2005).

Pathak, S. U. and Ghole, V. S. Affinity purification and properties of polyphenoloxidase from *Solanum tuberosum*. *Photochemistry.* 36, 1165-1167 (1994).

Patil, G. and Raghavarao, K. S. M. S. Aqueous two phase extraction for purification of C-phycocyanin. *Biochemical Engineering Journal.* 34, 156-164 (2007).

Pederson, D. M. and Goodman, R. E. Isozymes of α-galactosidase from *Bacillus stearothermophilus*. *Can. J. Microbiol*. 26, 978-984 (1980).

Persson, J., Johansson, H. O. and Tjerneld, F. Biomolecule separation using temperature-induced phase separation with recycling of phase-forming polymers. *Ind. Eng. Chem. Res.* 39, 2788-2796 (2000).

Persson, J., Johansson, H. O., Galaev, I., Mattiasson, B. and Tjerneld, F. Aqueous polymer two-phase systems formed by new thermoseparating polymers. *Bioseparation.* 9, 105-116 (2000).

Persson, J., Kaul, A. and Tjerneld, F. Polymer recycling in aqueous two-phase extractions using thermoseparating ethylene oxide–propylene oxide copolymers. *Journal of Chromatography B:Biomedical Sciences and Applications.* 743, 115-126 (2000b).

Persson, J., Kaul, A. and Tjerneld, F. Polymer recycling in aqueous two-phase extractions using thermoseparating ethylene oxide-propylene oxide copolymers. *J. Chromatogr. B:Biomed. Sci. Appl.* 743, 115-126 (2000).

Persson, J., Nystrom, L., Ageland, H. and Tjerneld, F. Purification of recombinant apolipoprotein A-1 Milano expressed in *Escherichia coli* using aqueous two-phase

extraction followed by temperature-induced phase separation. *J. Chromatogr. B.* 711, 97-109 (1998).

Persson, J., Nystrom, L., Ageland, H. and Tjerneld, F. Purification of recombinant and human apolipoprotein A-1 using surfactant micelles in aqueous two-phase systems: Recycling of thermoseparating polymer and surfactant with temperature-induced phase separation. *Biotechnol. Bioeng.* 65, 371-381 (1999).

Persson, J., Nystrom, L., Ageland, H. and Tjerneld, F. Purification of recombinant and human apolipoprotein A-1 using surfactant micelles in aqueous two-phase systems: Recycling of thermoseparating polymer and surfactant with temperature-induced phase separation. *Biotechnol. Bioeng.* 65, 371-381 (1999).

Pessoa-Filho, P. A. and Mohamed, R. S. Thermodynamic modeling of the partitioning of biomolecules in aqueous two-phase systems using a modified Flory–Huggins equation. *Process Biochemistry.* 39, 2075-2083 (2004).

Peter, F. S., Whitaker, A. and Stephen, J. H. Principles of fermentation technology. 301 (Aditya books (P) LTD, New Delhi, 1997).

Peters, F. P. J., Kho, T. L. and Vermeulen, A. Anderson-Fabry's disease: α-galactosidase deficiency. *The Lancet.* 357, 138-140 (2001).

Piculell, L., Bergfeldt, K. and Nilsson, S. Factors determining phase behaviour of multi component polymer systems: *Biopolymer mixtures* (Harding, S. E., Hill, S. E. and Mitchell, J. R.), 13–35 (Nottingham,1995).

Pietruszka, N., Galaen, I. Y., Kumar, A., Brzozowski, Z. K. and Matiasson, B. New polymers forming aqueous two-phase polymer systems. *Biotechnol. Prog.* 16, 408-415 (2000).

Porath, J., Carlsson, J., Olsson, I. and Belfrage, G. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature.* 258, 598-599 (1975).

Porter, E. J. and Ladisch, M., R. Ion exchange and affinity chromatography in the scaleup of the purification of α-galactosidase from soybean seeds. *Biotechnol. Bioeng*. 37, 356-363 (1991).

Prabhanjan, H. Studies on modified tamarind kernel powder. Part I: Preparation and physicochemical properties of sodium salt of carboxymethyl derivatives. *Starch.* 41, 409-414 (1989).

Puchart, V., Vrsanska, M., Bhat, M. K. and Biely, P. Purification and characterization of α-galactosidase from a thermophilic fungus *Thermomyces lanuginosus*. *Biochim. Biophys. Acta.* 1524, 27-37 (2001).

Ramadas, M., Halst, O. and Mattiasson, B. Production of amyloglucosidase by *Aspergillus niger* under different cultivation regimens. *World Journal of Microbiology and Biotechnology.* 12, 267–271 (1996).

Ramsch, C., Kleinelanghorst, L. B., Knieps, E. A., Thommes, J. and kula, M. R. Aqueous two phase systems containing urea: Influence of protein structure on protein partitioning. *Biotechnology Bioengineering.* 69, 83-90 (2000).

Rao, P. S. and Beri, R. M. Acetylation of tamarind seed jellose. *Proceedings of the Indian Academy of Sciences A.* 42, 199-203 (1955).

Rao, P. S. and Srivastava, H. C. Tamarind: *Industrial Gums.* (2nd edition. Whistler, R. L. Academic Press.) 369-411, New York (1973).

Rasa, H., Mohsen, N. M. and Modarress, H. Phase separation in aqueous two-phase systems containing poly(ethylene glycol) and magnesium sulphate at different temperatures. *The Journal of Chemical Thermodynamics.* 40, 573-579 (2008).

Rios, S., Pedregosa, A. M., Fernandez, M. I. and Laborda, F. Purification and molecular properties of an α-galactosidase synthesized and secreted by *Aspergillus nidulans*. *FEMS Microbiol. Lett*. 112, 35-41 (1993).

Rito-Palomares, M. and Lyddiatt. A. Practical implementation of aqueous two-phase processes for protein recovery from yeast. *J. Chem. Technol. Biotechnol.* 75, 632 (2000a).

Rito-Palomares, M. and Middelberg, A. Aqueous two-phase systems for the recovery of a recombinant viral coat protein from Escherichia coli. *J. Chem. Technol. Biotechnol.* 77, 1025 (2002).

Rito-Palomares, M. Practical application of aqueous two-phase partition to process development for the recovery of biological products. *Journal of Chromatography B.* 807, 3-11 (2004).

Rito-Palomares, M., Dale, C. and Lyddiatt. A. Generic application of an aqueous two-phase process for protein recovery from animal blood. *Process Biochem.* 35, 665 (2000b).

Rito-Palomares, M., Negrete, A., Galindo, E. and Serrano-Carreon, L. Aroma compounds recovery from mycelial cultures in aqueous two-phase processes. *J. Chromatogr. B.* 743, 403-408 (2000).

Rito-Palomares. M. and Hernandez, M. Influence of system and process parameters on partitioning of cheese whey proteins in aqueous two-phase systems. *J. Chromatogr. B.* 711, 81 (1998).

Robert, K. S. Protein purification, principles and practice: Advanced text in Chemistry (Charles, R. and Cantor, Editors.), (Springer, New York, 1982).

Robinson, G., Ross-Murphy, S. B. and Morris, E. R. Viscosity molecular weight relationships, intrinsic chain flexibility and dynamic solution properties of guar galactomannan. *Carbohydrate Research.* 107, 17-32 (1982).

Rodenbrock, A., Selber, K., Egmond, M. R. and Kula, M. R. Extraction of peptide tagged cutinase in detergent-based aqueous two-phase systems. *Bioseparation.* 9, 269-276 (2000).

Rodrigues, J. F., Paula, R. C. M., and Costa, S. M. Metodos de isolamento de gomas naturais: comparacao atraves da goma do cajueiro (*Anacardium occidentale L.*). *Polimeros: Ciencia e Tecnologia.* 1, 31-36 (1993).

Roe, S. *Protein purification techniques*. (2nd Edition.), (Oxford University Press, Oxford, 2001).

Rogers, R. D., Bond, A. H., Bauer, C. B., Zhang, J. and Griffin, S. T. Metal ion separations in polyethylene glycol-based aqueous biphasic systems: Correlation of partitioning behavior with available thermodynamic hydration data. *Journal of Chromatography B:Biomedical Sciences and Applications.* 680, 221-229 (1996).

Rollings, J. Enzymatic depolymerization of polysaccharides. *Carbohydrate Polymers.* 5, 37-82 (1985).

Roobol-Boza, M., Dolby, V., Doverskog, M., Barrefelt, A., Lindqvist, F., Oppermann, U. C., Kohler-Van-Alstine, K. and Tjerneld, F. Membrane protein isolation by in-situ solubilization, partitioning and affinity adsorption in aqueous two-phase systems: Purification of the human type 1-11[beta]-hydroxysteroid dehydrogenase. *Journal of Chromatography A.* 1043, 217-223 (2004).

Roy, D., Chevalier, P., Ward, P. and Savoie, L. Sugar fermented by *Bifidobacterium infants* ATCC 27920 in relation to growth and α-galactosidase activity. *Appl. Microbiol. Biotechnol*. 34, 653-655 (1991).

Rubinstein, A. and Gliko-Kabir, I. Synthesis and swelling dependent enzymatic degradation of borax modified guar gum for colonic delivery purposes, S.T.P. *Pharmaceutical Sciences.* 5, 41–46 (1995).

Saeki, S., Kuwahara, N., Nakata, M. and Kaneko, M. Upper and lower critical solution temperatures in poly(ethylene glycol) solutions. *Polymer.* 17, 685-689 (1976).

Sarac, A. S. Redox polymerization. *Prog. Polym. Sci.* 24, 1149 (1999).

Saravanan, S., Reena, J. A., Rao, J. R., Murugesan, T. and Nair, B.U. Phase equilibrium compositions, densities, and viscosities of aqueous two-phase poly(ethylene) glycol + poly(acrylic acid) system at various temperatures. *J. Chem. Eng. Data.* 51, 1246 -1249 (2006).

Sarubbo, L. A., Oliveira, L. A., Porto, A. L. F., Duartec, H. S., Carneiro-Leaoc, A. M. A., Lima-Filhob, J. L., Campos-Takakid, G. M. and Tambourgia, E. B. New aqueous two-phase system based on cashew-nut tree gum and poly (ethylene glycol). *Journal of Chromatography B*. 743, 79-84 (2000).

Sasakawa, S. and Walter, H. Partition behavior of native proteins in aqueous dextran-poly(ethyleneglycol)-phase systems. *Biochemistry.* 11, 2760-2765 (1972).

Schiavio, A. and Maderno, C. Soluble derivatives of the glucogalactoxylans of tamarind flour. *Italian patent* 574928 (1958).

Schmidt, A. S., Ventom, A. M. and Asenjo, J. A. Partitioning and purification of α-amylase in aqueous two-phase systems. *Enzyme and Microbial Technology.* 16, 131-142 (1994).

Schuler, R. Kinetic properties of α-galactosidase from *Lactobacillus fermenti. Enz. Microbial Technol*. 7, 207-211 (1985).

Schurr, G. *et al.,* Focusing powder technology on processing biologically produced products: *14th International congress of chemical and process engineering.* (Praha, Czech Republic, 2000).

Scott, R. L. The thermodynamics of high-polymer solutions II: The solubility and fractionation of a polymer of heterogeneous distribution. *Chem. Phys.* 13, 178-187 (1945).

Shah, S. B., Patel, C. P. and Trivedi, H. C., Ceric-induced grafting of acrylate monomers onto sodium alginate. *Carbohydr. Polym*. 26, 61 (1995).

Shanbhag, V. P. and Axelsson, C. G. Hydrophobic interaction determined by partition in aqueous two-phase systems. Partition of proteins in systems containing fatty-acid esters of poly(ethylene glycol). *Eur. J. Biochem.* 60, 17-22 (1975).

Shanbhag, V. P. and Johansson, G. Interaction of human serum albumin with fatty acids. Role of anionic group studied by affinity partition. *Eur. J. Biochem.* 93, 363-367 (1979).

Shanbhag, V. P. and Johansson, G. Specific extraction of human serum albumin by partition in aqueous biphasic systems containing poly(ethylene glycol) bound ligand. *Biochem. Biophys. Res. Commun.* 61, 1141-1146 (1974).

Sharma, B. R., Kumar, V. and Soni, P. L. Carbon oylethylation of guar gum. *Carbohydrate Polymers.* 58, 449-453 (2004).

Sharp, K. A., Yalpani, M., Howard, S. J. and Brooks, D. E. Synthesis and application of a poly(ethylene glycol)-antibody affinity ligand for cell separations in aqueous polymer two-phase systems. *Analytical Biochemistry.* 154, 110-117 (1986).

Shibuya, H., Kobayashi, H., Park, G. G., Komatsu, Y., Sato, T., Kaneko, R., Nagasaki, H., Yoshida, S., Kasamo, K. and Kusakabe, I. Purification and some properties of α-galactosidase from *Penicillium purpurogenum*. *Biosci. Biotechnol. Biochem*. 59, 2333-2335 (1995).

Shieh, C. J., Akoh, C. C., Koehler, P. E. Four-factor response surface optimization of the enzymatic modification of triolin to structured lipids. *J. Am. Oil. Chem. Soc.* 72, 619-623 (1995).

Shimohiro, Y., Ogawa, M. and Ohnishi, T. Thickening agent for paste. *European Patent Application.* 130385 (1983).

Shousun, C. S, Zon, G., Schneerson, R. and Robbins, J. B. Ultrasonic irradiation of bacterial polysaccharides. Characterization of the depolymerized products and some applications of the process. *Carbohydrate Research.* 152, 7-20 (1986).

Sikdar, S. K., Cole, K. D., Stewart, R. M., Szlag, D. C., Todd, P. and Cabezas, H. Jr. Aqueous two-phase extraction in bioseparations: An assessment. *Bio. Technol.* 9, 253-256 (1991).

Silman, R. W., Black, L. T., McGhee, J. E. and Bagley, E. B. Hydrolysis of raffinose in a hollow-fiber reactor using an unrefined mixture of α-galactosidase and invertase. *Biotechnol. Bioeng*. 22, 533-541 (1980).

Silva, L. H. M., and Meirelles, A. J. A. Phase equilibrium in polyethylene glycol/maltodextrin aqueous two-phase systems. *Carbohydrate Polymers*. 42, 273-278 (2000a).

Silva, L. H. M. and Meirelles, A. J. A. Bovine serum albumin, α-lactoalbumin and β-lactoglobulin partitioning in polyethylene glycol/maltodextrin aqueous-two-phase systems. *Carbohydrate Polymers*. 42, 279-282 (2000b).

Simonet, F., Garnier, C. and Doublier, J. L. Partition of proteins in the aqueous guar/dextran two-phase system. *Food Hydrocolloids.* 14, 591-600 (2000).

Singh, V., Sethi, R., Tewari, A., Srivastava, V. and Sanghi, R. Hydrolysis of plant seed gums by microwave irradiation. *Carbohydrate Polymers.* 54, 523–525 (2003).

Skuse, D. R., Norris-Jones, R., Yalpani, M. and Brooks, D. E. Hydroxypropyl cellulose/poly (ethylene glycol)-co-poly (propylene glycol) aqueous two-phase systems: System characterization and partition of cells and proteins. *Enzyme Microbial Technology.* 14, 785-790 (1992).

Smith, D. C. and Wood, T. M. Isolation of mutants of *Aspergillus awamori* with enhanced production of extracellular xylanase and β-xylosidase. *World. J. Microbiol. Biotechnol*. 7, 343-354 (1991b).

Smith, I. H., Pace, G. W. Recovery of microbial polysaccharides. *J. Chem. Tech. Biotechnol.* 32, 119-129 (1982).

Somiari, R. I, Balogh, E. Properties of an extracellular glycosidase of *Aspergillus niger* suitable for removal of oligo-saccharides from cow pea meal. *Enzyme. Microb. Technol*. 17, 311-316 (1995).

Somiari, R. I. and Balogh, E. Hydrolysis of raffinose and stachyose in cowpea (*Vigna unguiculata*) flour, using α-galactosidase from *Aspergillus niger*. *World J. Microbiol. Biotechnol*. 8, 564-566 (1992).

Srinivas, M. R. S., Chand, N. and Lonsane, B. K. Use of Plackett-Burman design for rapid screening of several nitrogen sources, growth/product promoters, minerals and enzyme inducers for the production of α-galactosidase by *Aspergillus niger* MRSS 234 in solid state fermentation system. *Bioprocess Eng.* 10, 139-144 (1994).

Srinivas, M. R. S., Padmanabhan, S. and Lonsane, B. K. Growth kinetics, α-galactosidase biosynthesis, and concomitant invertase production by *Aspergillus niger* NCIM 839 in a solid-state fermentation system. *Chem. Mikrobiol. Technol. Lebensm.* 15, 41-6 (1993).

Srinivas, N. D., Barhate, R. S. and Raghavarao, K. S. M. S. Aqueous two phase extraction coupled with ultrafiltration for downstream processing of a plant peroxidase. *J. Food. Eng*. 54, 1-6 (2000).

Srinivas, N. D., Rashmi, K. R. and Raghavarao, K. S. M. S. Extraction and purification of a plant peroxidase by aqueous two-phase extraction coupled with gel filtration. *Process Biochemistry.* 35, 43-48 (1999).

Stocks, S. J. and Brooks, D. E. Development of a general ligand for immunoaffinity partitioning in two phase aqueous polymer systems. *Analytical Biochemistry.* 173, 86-92 (1988).

Strandberg, L., Kiihler, K. and Enfors, S. O. Large-Scale fermentation and purification of a recombinant protein from *Escherichia coli. Process Biochemistry*. 26, 225-234 (1991).

Strobel, G. A. Toxin binding protein of sugar cane, its role in the plant. *Proc. Natl.* 71, 4234 (1974).

Sturesson, S., Tjerneld, F. and Johansson, G. Partition of macromolecules and cell particles in aqueous two-phase systems based on hydroxypropyl starch and poly(ethylene glycol). *Appl. Biochem. Biotechnol.* 26, 281-295 (1990).

Suarez, F. L., Springfield, J. F., Julie, K., Lohrmann, T. T., Kerr, P. S. and Levitt, M. D. Gas production in humans ingesting a soybean flour derived from beans naturally low in oligo-saccharides. *Am. J. Clin. Nutr*. 69, 135-139 (1999).

Subbarao, K. and Pieringer, R. A. Metabolism of glyceride glycolipids - IV : Enzymatic hydrolysis of monogalactosyl and digalactosyl diglycerides in rat brain. *J. Neurochem*. 17, 483 (1970).

Suzuki, H., Kobayashi, H., Ozawa, Y. and Kamibayashi, A. Method for manufacture of α-galactosidase by microorganisms. U.S. Patent 3795585 (1974).

Suzuki, H., Ozawa, Y. and Tanabe, O. Method of reducing raffinose content of beet molasses. U.S Patent, 3647625 (1972).

Suzuki, H., Ozawa, Y., Oota, H. and Yoshida, H. Stidies on the decomposition of raffinose by α-galactosidase of mold. Part-I α-Galactosidase formation and hydrolysis of raffinose by the enzyme preparation. *Agri. Biol. Chem*. 33, 506 (1969).

Svensson, M., Lime, J. P. and Tjerneldi, F. Phase behavior in aqueous two-phase systems containing micelle-forming block copolymers. *Macromolecules. 28,* 3597-3603 (1995).

Szlag, D. C. and Giuliano, K. A. A low cost aqueous two phase system for enzyme extraction. *Biotechnology Techniques.* 2, 277–282 (1988).

Szmigielski, S. An improved method for histochemical demonstration of β-glucuronidase and α-galactosidase activity in bone marrow and blood cells. *J. Lab. Clin. Med*. 67, 709 (1966).

Takerkart, G., Segard, E. and Monsigny, M. Partition of trypsin in two-phase systems containing a α-diamidino, ω-diphenylcarbamyl poly(ethylene glycol) as competitive inhibitor of trypsin. *FEBS Letters.* 42, 218-220 (1974).

Talbot, G. and Sygusch, J. Purification and characterization of thermostable β-mannanase and α-galactosidase from *Bacillus stearothermophilus*. *Appl. Environ. Microbiol.* 56, 3505-3510 (1990).

Tanuja, S., Srinivas, N. D., Raghavarao, K. S. M. S. and Gowthaman, M. K. Aqueous two-phase extraction for downstream processing of amyloglucosidase. *Process. Biochem.* 32, 635-641 (1997).

Taunk, K. and Behari, K. Graft copolymerization of acrylic acid onto guar gum. *J. Appl. Polym. Sci*. 77, 39 (2000).

Taylor, I. E. P. and Atkins, E. D. T. X-ray diffraction studies on the xyloglucan from tamarind (*Tamarindus indica*) seed. *FEES. Lett.* 181, 300–302 (1985).

Thomas, B. and Webb, J. A. Multiple forms of α-galactosidase in mature leaves of *Cucurbita pepo*. *Phytochem.* 16, 203-206 (1977).

Tjerneld, F., Alred, P. A., Modlin, R. F., Kozlowski, A. and Harris, J. M. Purification of biomolecules using temperature induced phase separation: Aqueous biphasic separations: Biomolecules to metal ions. (Rogers, R. D., Eitman, M. A. Editors.), 119-131 (Plenum Press, New York, 1995).

Tjerneld, F., Berner, S., Cajarville, A. and Johansson, G. New aqueous two-phase system based on hydroxypropyl starch useful in enzyme purification. *Enzyme and Microbial Technology*. 8, 417-423 (1986).

Tjerneld, F., Johansson, G. and Joelsson, M. Affinity liquid-liquid extraction of lactate dehyogenase on a large scale. *Biotech. Bioeng.* 30, 809-816 (1987).

Tjerneld, F., Persson, I. and Albertsson, P. A. Enzymatic hydrolysis of cellulose in aqueous two-phase systems I: Partition of cellulases from *Trichoderma reesei*. *Biotechnol. Bioeng*. 27, 1036-1043 (1985).

Toda, A., Nishiya, Y. Gene cloning, purification, and characterization of a lactate oxidase from *Lactococcus lactis* subsp. *cremoris* IFO3427. *J. Ferment. Bioeng.* 85, 507 (1998).

Trinci, A. P. J. Kinetics of apical and lateral branching in *Aspergillus nidulans* and *Geotrichum lactis. Trans. Br. Mycol. Soc*. 55, 17–28 (1970).

Tripathy, T., Pandey, S. R., Karmakar, N. C., Bhagat, R. P. and Singh, R. P. Novel flocculating agent based on sodium alginate and acrylamide. *Eur. Polym. J.* 35, 2057 (1999).

Tsuchiya, H. M., Hellman, N. N., Koepsell, H. J., Corman, J., Stringer, C. S., Rogovin, S. P., Bogard, M. O., Bryant, G., Feger, V. H., Hoffman, C. A., Senti, F. R. and Jackson, R. W. Factors affecting molecular weight of enzymatically synthesized dextran**.** J. Am. Chem. Soc. 77, 2412-2419 (1955).

Tubio, G., Pellegrini, L., Nerli, B. B. and Pico, G. A. Liquid-Liquid Equilibria of aqueous two-phase systems containing poly(ethylene glycols) of different molecular weight and sodium citrate. *J. Chem. Eng. Data.* 51, 209-212 (2006).

Ulezlo, I. V. and Zaprometova, O. M. Microbial α-galactosidase (a review). *Appl. Biochem. Microbiol*. 18, 1-12 (1982).

Ullah, A. H. J. and Gibson, D. M. Extracellular Phytase from *Aspergillus ficuum* NRRL, 3135: Purification and Characterization. *Preparative Biochemistry.* 17, 63 (1987).

Umakoshi, H., Persson, J., Kroon, M., Johansson, H. O., Otzen, D., Kuboi, R. and Tjerneld, F. Model process for separation based on unfolding and refolding of chymotrypsin inhibitor-2 in thermoseparating polymers two phase systems. *J. Chromatogr. B:Biomed. Sci. Appl.* 743, 13-19 (2000).

Vaidya, B. K., Suthar, H. K., Kasture, S. and Nene, S. Purification of potato polyphenol oxidase (PPO) by partitioning in aqueous two-phase system. *Biochemical Engineering Journal.* 28, 161–166 (2006).

Van, B. M., Luyben, K. C. and Van-der-Wielen, L. A. Poly(ethylene glycol)-salt aqueous two-phase systems with easily recyclable volatile salts. *J. Chromatogr. B: Biomed. Sci. Appl.* 711, 61-68 (1998).

Van-Berlo, M., Luyben, K., Ch, A. M. and van-der-wielen, L. A. M. Poly (ethylene glycol)–salt aqueous two-phase systems with easily recyclable volatile salts. *J. Chrom.* B. 711, 61-68 (1998).

Vandana, S., Rupali, S., Ashutosh, T., Vasundhara, S. and Rashmi, S. Hydrolysis of plant seed gums by microwave irradiation. *Carbohydrate Polymers.* 54, 523–525 (2003).

Varbanets, D. L., Malanchuk, V. M., Buglova, T. T., and Kuhlmann, R. A. *Penicillium sp*. α-galactosidase: Purification and substrate specificity. *Carbohydr. Poly*. 44, 357-363 (2001).

Veide, A., Smeds, A. L. and Enfors, S. O. A process for large-scale isolation of β-galactosidase from *E-coli* in an aqueous two-phase system. *Biotech. Bioeng*. 25, 1789-1800 (1983).

Venancio, A., Almeida, C. and Teixeira. J. A. Enzyme purification with aqueous two-phase systems: comparison between systems composed of pure polymers and systems composed of crude polymers. *J. Chromatogr. B*. 680, 131 (1996).

Venancio, A., Catarina, A., Lucilia, D. and Teixeira, J. A. Protein partition on aderivative guar gum based aqueous two phase system. *Bioseparation.* 5, 253-258 (1995).

Venancio, A., Teixeira, J. A. and Mota, M. Evaluation of crude hydroxypropyl starch as a bioseparation aqueous-phase-forming polymer. *Biotechnol. Prog.* 9, 635-639 (1993).

Vernau, J. and Kula, M. R. Extraction of proteins from biological raw material using aqueous polyethylene glycol–citrate phase systems. *Biotechnology and Applied Biochemistry.* 12, 397–404 (1990).

Viara, I., Dragomir, Y., Ludmila, K. and Dimitre, P. Simultaneous biosynthesis and purification of two extracellular Bacillus hydrolases in aqueous two-phase systems. Microbiological research. 156, 19-30 (2001).

Vijayendran, B. R. and Bone, T. Absolute molecular weight and molecular weight distribution of guar by size exclusion chromatography and low-angle laser light scattering. *Carbohydrate Polymers.* 4, 299-313 (1984).

Walker, S. G., Dale, C. J. and Lyddiatt, A. Aqueous two-phase partition of complex protein feedstocks derived from brain tissue homogenates. *J. Chromatogr. B*. 680, 91 (1996).

Walter, H. and Johansson, G. Partitioning in aqueous two-phase systems: *An overview. Anal. Biochem*. 155, 215 (1986).

Walter, H. and Johansson, G. Aqueous two-phase systems. (Walter, H. and Johansson, G. Editors.), (Academic Press, London, 1994).

Walter, H. and Johansson, G. aqueous two-phase systems. *Methods in Enzymology.* 228, 264 (1994).

Walter, H., Brooks, D. E. and Fisher, D. Partitioning in aqueous two phase systems: *Theory, methods, uses and applications to biotechnology*. (Academic Press, New York, 1985).

Ward, M. Production of calf chymosin by *Aspergillus awamori*: *Genetics and Molecular Biology of Industrial Microorganisms* (Hershberger, C. L., Queener, S. W., and Hegeman, G., Editors.), 288–294 (American Society for Microbiology, Washington, DC, 1996).

Whistler, R. L and Barkalow, D. G., Industrial gums, Polysaccharides and their derivatives. (3rd Edition.) 247, 579-600, (1993).

Whistler, R. L. and Barkalow, D. G. *Industrial gums, polysaccharides and their derivatives.* (3rd Edition.) 247, (1993a).

Whistler, R. L. *The encyclopedia of polymer science and technology.* 416, (John Wiley and Sons, New York, 1969).

Whistlera, R. L. and Durso, D. F. Isolation and characterization of two crystalline disaccharides from partial acid hydrolysis of guaran. *Journal of American Chemical Society.* 73, 4189-4190 (1951).

Willett, J. L., Millard, M. M. and Jasberg, B. K. Extrusion of waxy maize starch: Melt rheology and molecular weight degradation of amylopectin. *Polymer.* 38, 5983-5989 (1997).

Wong, H., Hu, C., Yeh, H., Su, W., Lu, H. and Lin, C. Production, purification and characterization of α-galactosidase from *Monascus pilosus*. *Appl. Environ. Microbiol*. 52, 1147-1152 (1986).

Wong, T. Y. Melibiose is hydrolyzed exocellularly by an inducible exo-α-galactosidase in *Azotobacter vinelandii*. *Appl. Environ. Microbiol*. 56, 2271-3 (1990).

Wong-Leung, Y. L., Fong, W. F. and Lam, W. L. Production of α-galactosidase by Monascus grown on soybean and sugarcane wastes. *World. J. Microbiol. Biotechnol*. 9, 529-533 (1993).

Xiao, M., Tanaka, K., Qian, X. M., Yamamoto, K. and Kumagai, H. High yield production and characterization of α-galactosidase from *Bifidobacterium breve* grown on raffinose. *Biotechnol. Lett*. 22, 747-751 (2000).

Yamane, T. Decomposition of raffinose by -galactosidase. An enzymatic reaction applied in the factory-process in Japanese beet sugar factories. *Sucr. Belge-Sugar Ind. Abstr.* 90, 345-348 (1971).

Zapater, I. G., Ullah, A. H. J. and Wodzinski, R. J. Extracellular α-galactosidase from *Aspergillus ficuum* NRRL3135 purification and characterization. *Preparative Biochem*. 20, 263-296 (1990).

Zaslavsky, B. Y. Bioanalytical applications of partitioning in aqueous polymer two phase systems. *Anal. Chem*. 64, 765-773 (1992).

Zaslavsky, B. Y. Aqueous two phase partitioning: *Physical chemistry and bioanalytical applications.* (Marcel Dekker Inc, New York, 1995).

Zaslavsky, B. Y., Mesetechkina, N. M., Miheeva, L. M. and Rogozhin, S. V. Measurement of relative hydrophobicity of amino-acid side chains by partition in an aqueous two phase system-hydrophobicity scale for nonpolar and ionogenic side-chains. *J. Chrom.* 240, 21-28 (1982).

Zaslavsky, B. Y., Mestechkina, N. M. and Rogozhin, S. V. Characteristics of protein-aqueous medium interactions measured by partition in aqueous Ficoll-Dextran biphasic system. *J. Chromatogr.* 260, 329-336 (1983).

Zeilinger, S., Kristufek, D., Arisan-Atac, I., Hodits, R., Kubicek, C. P. Conditions of formation, purification, and characterization of an α-galactosidase of *Trichoderma reesei* RUT C-30. *Appl. Environ. Microbiol*. 59,1347-1353 (1993).

Zhang, C., Lillie, R., Cotter, J. and Vaughan, D. Lysozyme purification from tobacco extract by polyelectrolyte precipitation. *Journal of Chromatography A*. 1069, 107-112 (2005).

Zhou, Y. and Antonietti, M. Synthesis of very small TiO_2 nanocrystals in a room-temperature ionic liquid and their self-assembly toward mesoporous spherical aggregates. *J. Am. Chem. Soc.* 125, 14960-14961 (2003).

OUT COME OF THE PRESENT WORK

Publications

Kumar Kautharapu, Narahari S. Pujari, Sandeep B. Golegaonkar, Surendra Ponrathnam, Sanjay N. Nene and D. Bhatnagar. Vinyl-2-pyrrolidone derivatized guar gum based aqueous two-phase system. *Separation and Purification Technology.* (Article in Press).

Papers Communicated

Kumar Kautharapu, Sandeep B. Golegaonkar, K.S.M.S. Raghavarao, D. Bhatnagar, Sanjay N. Nene. Protein partition in phospho-guar and polyvinyl pyrrolidone based new aqueous two phase system *(Communicated).*

Kumar Kautharapu, Sangita Kasturi, D. Bhatnagar and Sanjay S. Nene. Partition of proteins in Aqueous Two-phase Systems Based on Guar gum derivative-Dextran :Effect of salt and pH *(Communicated).*

Kumar kautharapu, S. Prabhakar, D. Bhatnagar and Sanjay Nene. Production and Optimization of Guar α-Galactosidase by *Aspergillus awamori* (NCIM 1225) *(Communicated).*

Kumar kautharapu, Amol Dive, D. Bhatnagar, Sanjay Nene, Raghavarao K. S. M. S. Novel Aqueous two phase sytem with Tamarind Kernel power and PVP K_{90}. *(Communicated)*

Publications *(Not relatated to thesis)*

Asavari Kulkarni, L. Pavithra, Shravanti Rampalli, Devraj Mogare, Kumar Babu, and Samit Chattopadhyay. HIV-1 integration sites are flanked by potential MARs that alone can act as promoters. *Biochemical and Biophysical Research Communications*. 322, 672-677 (2004).

Paper Communicated/Preparation *(Not related to thesis*)

Kumar kautharapu, Amol Dive, S. Prabhakar, D. Bhatnagar, Sanjay Nene Statistical optimization of lactate oxidase production by *Aerococcus viridans. (Manucript under preparation).*

Patents

Indian/Foreign Patent application: Kharul, U. K., Gadre, R. V., Jogdand, V. V., Chendake, Y. J., Karadkar, P. B., Kumbharkar, S. C., Kautharapu, K. B. A process for deacidification using membranes.