AQUEOUS TWO-PHASE EXTRACTION FOR THE DOWNSTREAM PROCESSING OF ENZYMES

A thesis submitted to the

UNIVERSITY OF MYSORE

for the award of the degree of

DOCTOR OF PHILOSOPHY

In

Biotechnology

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DECLARATION

I Hereby declare that the thesis entitled "Aqueous Two-Phase Extraction for the Downstream Processing of Enzymes" submitted to the University of Mysore, for the award of the degree of Doctor of Philosophy in Biotechnology, is the result of the research work carried out by me in the Department of Food Engineering, Central Food Technological Research Institute, Mysore, under the guidance of Dr. KSMS Raghavarao, during the period April 1997 - August 2000.

I further declare that the results of this work have not been previously submitted for any other degree or fellowship.

CERTIFICATE

I Hereby certify that this thesis entitled "Aqueous Two-Phase Extraction for the Downstream Processing of Enzymes" submitted by Mr. N.D. Srinivas for the degree of Doctor of Philosophy in Biotechnology, University of Mysore, is the result of the research work carried out by him in the Department of Food Engineering, Central Food Technological Research Institute, Mysore, under my guidance and supervision during the period April 1997 - August 2000.

(Dr. KSMS. RAGHAVA RAO)

Date: 03.08.2000
Place: Mysore

ACKNOWLEDGEMENT

It is pleasure to express my gratitude to Dr. KSMS. Raghavarao for his valuable guidance, friendly support and constructive criticism, which were essential in the fulfillment of this task. I am also indebted to him for introducing me to the field of Biochemical Engineering.

It is a privilege to express my thankfulness to Dr. V. Prakash, Director, CFTRI for his invaluable suggestions as well as providing all the facilities to work in this institute.

My grateful acknowledgements are to Mr. A. Ramesh, Head, Food Engineering and Mr. H. Krishnamurthy, Food Engineering for allowing me to carry out the research work successfully in this department.

My special thanks are to Mr. R. Subramanian and Dr. G. Manoj for good advice and motivation during the course of this study. The help extended by Dr. N.G. Karanth, Additional Director, CFTRI, Mr. R. Subramanian and Mr. N.K. Rastogi during my Ph.D. registration deserves special mention.

I express my thanks to the whole staff of Food Engineering Dept, for creating an inspiring working atmosphere to fulfil my job. The help extended by Mr. Shanta Kumar (Glass blowing section), Mr. T. Ramesh, Mr. Jayaprakash, and Mr. V. Kumar in designing and fabrication of various instruments deserve a special note here. In addition, the help rendered by Mr. K.Venkatesh Murthy for using the facilities of Food Engineering Pilot Plant and by Mr. V.Ravichandran in computational work are gratefully acknowledged.

It is with pleasure that I cherish the memorable association with my colleagues in the Department. I heartily thank Ms. S. Nandini, Mrs. K.R. Rashmi, Mr. R.S. Barhate, Dr. P. Krishnamurthy, Ms. Lakshmi, Ms. Rajashri and Mr. P. Nagesh for their constant support and encouragement given during the preparation of the thesis.

I would be failing in my duties if I do not thank my fellow researchers Mr. S. Venkatesh, Mr. S. Harikrishna, Mr. B.S. Dattatreya, Mr. K.R. Kiran, Mr. Y.Y. Khan, Mr. Hema Kumar, Mr. S. Joshi, Mr. G.V. Chowdary and Mr. Devaraj Gowda for their friendly support which made my life in CFTRI a memorable one.

It is my duty to sincerely thank the authorities of CSIR, New Delhi for the financial support in the form of fellowship.

Finally, I wish to place on record the indebtedness to my beloved parents Mr. N.D. Venkatesh and Mrs. N.D. Lakshmi and brother Mr. N.D. Sridhar for their affectionate support and ceaseless inspiration.

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LIST OF ABBREVIATIONS

DSP Downstream processing

LLE Liquid-liquid extraction

ATPS Aqueous two-phase systems

A TPE Aqueous two-phase extraction

PEG Polyethylene glycol

MDX Maltodextrin

L.casei Lactobacillus casie

KL_a Over all mass transfer coefficient

HRP Horseradish peroxidase

ED Fractional dispersed phase hold up

V_s Dispersed phase velocity

DX Dextran

VI' Volume of top phase

Vb Volume of bottom phase

m Partition coefficient

mPa s Millipascal seconds

mN/m Millineuton per meter

IDA Imino-diacetic acid

pI Isoelectric point

EO Ethylene oxide

PO Propylene oxide

PODs Peroxidases

C_T Equilibrium concentration of enzyme in top

Phase

C_B Equilibrium concentration of enzyme in bottom

Phase

nm Nanometer

mM Millimolar

μl Microliter

% Percent

Km Michaelis kinetic constant

V mIX Maximum reaction velocity

J.LM Micromolar

SSF Solid state fermentation

MHz Megahertz

D Droplet diameter

Δp Density difference between phases

g Acceleration due to gravity

V Droplet rise/fall velocity

KLa Mass transfer coefficient

V d Dispersed phase velocity

BSA Bovine serum albumin

Ho Dispersion height

H Continuous phase height

L Flow rate of dispersed phase

V_o Dispersion volume

C_j Inlet concentration of HRP in PEG-rich dispersed phase

Co Outlet concentration of HRP in PEG-rich dispersed phase

C_s Concentration of HRP in salt-rich continuous phase

V d Static drop volume

γ Interfacial tension

D_N Nozzle diameter

V_F Drop volume

CONTENTS

SYNOPSIS

In the recent years there have been tremendous efforts by research and industrial community for the production of biochemicals through application of fermentation technology and cell culture. However, the technology for downstream processing (DSP) of biological products from the media/broth has not kept pace with the advances in upstream operations involving bioreactors, despite the fact that in many cases DSP contributes major share of the final product cost. The separation of many biochemicals from the product stream is still performed by batch mode small-scale processes such as column chromatography, salt and solvent precipitation and electrophoresis for which scale-up poses considerable problems, making them uneconomical at large-scale unless the product is of high value. Affinity-based chromatographic separations though have excellent selectivity and have been carried out in large scale, for the most part such systems operate discontinuously and is very expensive, which in turn makes the product also expensive. The economy of scale has not often been realized. Therefore, current research in the area of DSP is directed towards the development of efficient and scalable alternative bioseparation processes with potential for continuous operation.

Liquid-liquid extraction (LLE) is a traditional chemical engineering unit operation for which the design and scale-up of both batch as well as continuous processes are already accomplished. Unlike affinity chromatography and precipitation, LLE is well known to be operative on continuous mode at large scale with high throughputs. The process is easy to operate and high flexible. LLE using organic/aqueous phase has been employed in many chemical industries. However, this technique with all its advantages has not gained wide industrial recognition in the field of biotechnology, mainly due to the poor solubility of proteins in organic solvents and the tendency of organic solvents to denature the proteins.

In recent years LLE using the aqueous two-phase systems (ATPS) has been recognized as a superior and versatile technique for DSP of biomolecules, and a wealth of information has been reported in the literature on various aspects of aqueous two-phase extraction (A TPE) for the isolation and concentration of proteins, enzymes and other biological materials. The major advantages of A TPE include high capacity, biocompatible environment, low interfacial tension, high yield, lower process time as well as energy, and high selectivity. Further, it offers ease of scaleup, continuous operation and most importantly, allows easy adaptation of the equipment and the methods of conventional organic-aqueous phase extraction used in the chemical industry. However, ATPE is not selective enough to provide the extreme purity usually desired. It is recognized as a primary purification step in the overall process of enzyme/protein purification in which the final purification is achieved by methods such as chromatography, or crystallization. This technique is also effective in the removal of contaminating material such as polysaccharides and nucleic acids.

The subject matter of this thesis is organized into four chapters.

CHAPTER 1 - comprises the general introduction and scope of the present investigation. Literature pertaining to

fundamentals, factors affecting the extraction and purification of various enzymes, large-scale extraction of various enzymes, mathematical modeling, recent developments and recent applications of ATPSs are discussed.

CHAPTER 2 - Deals with the partition behaviour of two selected enzymes in ATPSs and their purification approach by ATPE coupled with other techniques such as gel filtration and ultrafiltration under two sections.

Section A - In this section, Partition behaviour of a plant peroxidase extracted from the leaves of Ipomoea palmetta was studied in aqueous two-phase systems. Influence of various parameters such as system pH, phase composition, phase polymer molecular weight and concentration of NaCI on the partitioning behaviour of the enzyme was evaluated. Desirable conditions for differential partitioning of the enzyme and cell debris were found in systems having polyethylene glycol of molecular weight 1550, where enzyme partitioned to the bottom phase and cell debris partitioned to the top phase. In contrast, in systems having polyethylene glycol of molecular weight 6000 and 4000, both cell debris and enzyme partitioned to the bottom phase. Optimum values of partition coefficient and purity for the enzyme in the unfiltered extract were observed in PEG (l550)/KH2P04 system having 2% NaCl. Hence using this system a larger-scale (l liter scale) extraction, concentration and purification of the enzyme was performed in combination with ultrafiltration to obtain about 76% recovery, 6.0 fold purity and 8.8 fold activity concentration of the enzyme. By coupling ATPE with gel-filtration (on Sephadex G-I00 column), the enzyme was purified to about 49 fold and this approach was shown to be superior to conventional approach with 3 times enhancement in purification fold.

Section B - Aqueous two-phase extraction (ATPE) in combination with Ultrafiltration was employed for concentration and purification of amyloglucosidase produced by solid state fermentation. After extraction (with water) from dry moldy bran the dilute enzyme extract was concentrated by A TPE in a polyethylene glycol (pEG)/maltodextrin (MDX) system. The enzyme in the top PEG rich phase was then extracted into a Na_2HP0_4 rich bottom phase and further concentrated by ultrafiltration. The partitioning behavior of amyloglucosidase was examined in PEG/MDX, PEGlNa2S04, PEGlNa2HP04, PEG/ $KH2P0_4$ aqueous two-phase systems. Effect of buffering salts such as NaCI, Na_2HP0_4, KH_2P0_4 and Na_2S0_4 on the partitioning behavior of enzyme was studied in PEG/MDX system. Maximum partitioning of amyloglucosidase was seen with KH_2P0_4 (m= 18.1). A two stage A TPE employing PEG/MDX (buffered with KH_2P0_4) and PEGINa2HP0$_4$ systems, followed by ultrafiltration has resulted in an overall recovery of 78.4% with 3.1 fold purification and 9.4 fold concentration of the enzyme.

CHAPTER 3 - Aqueous two-phase extraction has been recognized as a versatile downstream processing technique for the recovery of biomolecules. A major deterrent to its industrial exploitation is the slow demixing of the two aqueous phases after extraction, due to their similar physical properties. This chapter, under three subheadings addresses the slow demixing problem of ATPSs by employing external fields such as acoustic and microwave.

Section A - A method to decrease the demixing times of PEG/salt ATPSs, employing travelling acoustic wave field is reported. Effects of phase composition and microbial cells on demixing in PEG/potassium phosphate two phase system are studied in detail. As phase composition increased, demixing time decreased gradually. Phase volume ratio was found to have a significant effect on demixing time at low phase compositions. However, at intermediate and high phase compositions, only a little effect on demixing time was observed. The effect of phase composition and volume ratio on demixing behavior was explained based on the collision rate and size of the dispersed phase droplets. At all the phase compositions studied, the acoustically assisted process decreased the demixing time by 17-60% when compared to demixing under gravity alone. Increasing the cell concentration increased the demixing time markedly in case of yeast. However, it remained practically constant in case or" L. easei. Application of acoustic field reduced the demixing times up to 60% and 40% in case of yeast and L. easei, respectively. Visual observations indicated that ultrasonication has caused a mild circulation currents in the phase dispersion enhancing droplet-droplet interaction, which in turn enhances the rate of coalescence that eventually enhanced the rate of demixing.

Section B - In this section, Acoustic demixing was employed for the first time to decrease the demixing time in polymer/polymer (PEG/maltodextrin) system. Acoustically assisted process employed here significantly decreased the demixing time. In these systems up to about 2 fold decrease in demixing time was observed. Ultrasonication has induced mild circulation currents in the phase dispersion, which has enhanced the rate of droplet coalescence, eventually resulting in decreased demixing time. The process was found to be simple, economical (low current and high voltage device), easily scalable and furthermore readily available ultrasonic transducers could be employed. In polymer/polymer systems, phase demixing was found to depend greatly on which of the phases is continuous and viscosity of the continuous phase was observed to have a strong influence on the movement of the droplets and hence the phase demixing. Addition of NaCl decreased the density difference between the phases, which in turn increased the demixing time.

Section C - In this section, microwave field was explored as an external field to enhance the demixing rates in A TPSs. In PEG/salt systems about 2 fold and in PEG/maltodextrin systems about 1.5 fold decrease in demixing time was observed when exposed to microwave field. The process was optimized in such a way that minimum amount of heat is generated in the system.

CHAPTER 4 - This chapter has been categorized into two sections with section A dealing on mass transfer aspects in A TPSs and section B on drop dynamics in ATPSs.

Section A - Often multistage contactors is needed to achieve the desired degree of mass transfer. One such contactor is spray column, which can be conveniently used to isolate and purify proteins using A TPE technique. We have studied the effect of orifice size and addition of NaCl on the over all mass transfer coefficient (KLa) of horse radish peroxidase (HRP) and fractional dispersed phase hold up (ϵD) in a simple spray column using PEG/phosphate A

TPS. KLa and ϵD increased with an increase in dispersed phase velocity (Vs) as well as capillary diameter. Presence of NaCI has increased KLa. The overall mass transfer coefficient for HRP normalized for dispersed phase hold up, were found to be independent of Vs. This result was expected since true mass transfer coefficients do not vary with phase velocity.

Section B - Mass transfer is crucial for the efficient extraction of biomolecules in spray columns using ATPSs. Therefore a knowledge of the interfacial area of contact (available during drop formation) when one aqueous phase is injected into another through an orifice in a column is necessary to estimate the mass transfer rates in ATPSs. Existing models available in the literature for prediction of drop volume during formation are all on liquid-liquid systems containing organic-aqueous phases. The difference between the phases of conventional organic-aqueous systems and aqueous two-phase systems is that the physical properties in the latter are extreme. A review of the published information indicates that the dispersed phase viscosity in the ATPS is significantly higher than LLE. Similarly, in LLE, the difference in density is at least twice that in the case of the A TPS. In addition, the interfacial tension is very low in A IPS when compared LLE. Hence, we have modified the model developed for aqueous-organic systems to suit for ATPSs at low flow rates. This model is based on the force balance analysis for predicting the drop volume during formation in ATPSs. The model predictions has shown a good agreement with the experimental results carried out using polymer/salt ATPS at low flow rates.

At the end of this thesis, suggestions for future work was proposed followed by References.

CHAPTER 1

General Introduction

1. 1. AQUEOUS TWO-PHASE SYSTEMS

Recent developments in biotechnology have opened new avenues toward the production of many biomolecules of importance for research, pharmaceutical, clinical, and industrial usage. Downstream processing is an integral part of any product development, and the final cost of the product depends largely on the cost incurred during overall recovery process. The conventional technique of filtration for solid liquid separation is not suitable for the bioseparation, in which the size of the microorganisms to be separated is small, especially when the cells are broken to release the intracellular components causing increase in the viscosity of the system (Huggins, 1978; Mosqueira, 1981). Precipitation and column chromatography are not only expensive but also result in lower yields. Centrifugation and other modem methods like electrophoresis have enormous scale-up problems, making them uneconomical unless the product is of high value. Hence, there is a need to develop efficient, cost effective, fast, simple, and eco-friendly downstream processing methods for recovery of biomolecules produced by biotechnological means. Extraction using aqueous two-phase systems (ATPSs) is one such method. Although this technique was developed during mid-1950's by Albertsson, its importance and applications have been realized only in the recent past.

Liquid-liquid extraction using organic-aqueous phase systems is extensively used in the chemical industry. However, with all its advantages, this method has not gained wide industrial recognition in the field of biotechnology due to the poor solubility of proteins in the organic solvents and the tendency of the organic solvents to denature them. Aqueous two-phase extraction (ATPE) has been successful to a large extent in overcoming the limitations of conventional organic-aqueous extraction, since it employs two aqueous phases. ATPE has been recognized as a superior and versatile technique for the extraction and purification of biomolecules (Walter et al., 1985; Albertsson, 1986; Zaslavski, 1995). Few other liquid-liquid extraction methods used in the extraction and purification of various biomolecules are reverse micellar extractions (Luisi and Magid, 1986), cloud point extractions (Hinze and Pramauro, 1993), extractions using thermo-separating polymers (Johansson et al., 1995) and micellar extractions (Scamehom et al., 1988).

Aqueous two-phase systems are by well known for their utility in the extraction and purification of biological materials such as enzymes/proteins, nucleic acids, viruses, cell organelles etc. These applications are the subjects of severaI books and review papers (Kula et al., 1982; WaIter et al., 1985; Albertsson, 1986; Diamond and Hsu, 1992; Zaslavski, 1995; Raghavarao et al., 1998). The major advantages of A TPE include high capacity biocompatible environment, low interfacial tension, high yield, lower process time and energy, high selectivity and ease of scale-up. Further, it offers continuous operation and most importantly, the equipment and the methods of conventional organic-aqueous phase extraction can be easily adapted. However, ATPE is not selective enough to provide the

extreme purity usually desired. It is recognized as a primary purification step in the overall process of protein purification (Abbot et al., 1990; Sikdar et al., 1991) in which the final purification is achieved by methods such as chromatography, or crystallization. This technique is also effective in the removal of contaminating materials such as polysaccharides and nucleic acids (Kula et al., 1982).

There are mainly two types of ATPSs; Polymer-polymer and Polymer-salt.

Tables 1.1 and 1.2 shows the typical salts and polymers used for the formation of ATPSs. An ATPS is formed by the addition of two water-soluble polymers or a polymer and salt, to aqueous media above their critical concentrations. In this way two aqueous phases are formed without the involvement of an organic solvent.

Among polymer/salt systems, the most popular system is polyethylene glycol/potassium phosphate and among polymer/polymer systems extensively studied one is polyethylene glycol/dextran (PEG/DX). For industrial applications, polymer/salt systems are preferred over polymer/polymer systems because they are easy to prepare, require lesser time to separate and show better selectivity for protein extraction. In addition, recovery of the proteins from the salt phase can be easily accomplished by simple dialysis.

Each A TPS is characterized by an exclusive phase diagram, which indicates the equilibrium phase composition for that particular system. Figure 1.1 shows a typical phase diagram for PEG (8000)/maltodextrin two-phase system at 27 °e. The binodal curve in the figure delineates one phase region from two-phase region. Phase diagrams for a number of systems are reported in various books and reviews (Walter et aI., 1985; Albertsson, 1986; Diamond and Hsu, 1992; Zaslavsky, 1995) and various methods for the construction of a binodal curve have been discussed by Bamberger et al. (1984). In Figure 1.1 the line that links two different points on the binodal, passing through the point Al (a typical total phase composition point) is called a tie line. Points T 1 and B I represent the compositions of the separated top and concentration of polymer required is around 8-16% (w/w) and salt around 10% (w/w), which typically exceeds 2M (Raghavarao et al., 1998).

1. 1. 1. Factors affecting aqueous two-phase systems

Molecular weight, hydrophobicity and concentration of the polymers, concentration of the salts, type and concentration of the externally added salts and temperature affects the formation of A TPS. There is a critical concentration of the phase forming solutes for each A TPS, above which only phase separation occurs. Below this critical concentration, the system exists in the homogenous form as indicated by the point H in Figure 1.1. The critical concentration of the phase forming solutes always lies on the binodai. Higher the molecular weight of the polymer, lower the concentration required for phase separation and vice-versa (Diamond and Hsu, 1989a,b). Higher the hydrophobicity of the polymers in polymer/polymer systems, lower is the tendency toward phase formation (Albertsson, 1986). Temperature has a considerable effect on phase diagram (Walter et aI., 1985). At lower temperature, polymer/polymer systems need only lower concentrations of the phase forming solutes to separate into two phases. However, polymer/salt systems require higher concentrations of the phase forming solutes to separate

into two phases at lower temperature (Zaslavski, 1995).

1. 1. 2. Physical properties of aqueous two-phase systems

Physical properties of ATPS, such as density, viscosity and interfacial tension determine the phase separation rate and also contribute to the biomolecule partitioning (Raghavarao et al., 1995). Physical properties of a system increase with an increase in the tie line length/phase composition. Various methods for the determination of these physical properties, especially for interfacial tension have been reported (Bamberger et al., 1984; Walter et al., 1985; Albertsson, 1986). A systematic measurement of these physical properties in PEG/salt systems has been carried out and reported (Snyder et al., 1992; Wu et al., 1996). However, for polymer/polymer systems such a compilation of data still appears to be unavailable.

In case of polymer/polymer systems, viscosity values ranges from 10-1000 mPa s (Sawant et al., 1990) and in case of PEG/salt systems viscosity of the top phase ranges from 10-100 mPa s and that of bottom phase is very less, close to that of water (Snyder et aI., 1992). The density difference between the two phases lies in the range of 10-100 kg/m^3 (Sawant et aI., 1990). It is less in case of polymer/polymer systems when compared to polymer/salt systems. Interfacial tension between the two phases in A TPS in general is very low when compared to organic-aqueous two-phase systems. Its value lies in the range of 0.0001 to 0.1 mN/m in polymer/polymer systems and in the range of 0.1-2.0 mN/m in case of PEG/salt systems (Albertsson, 1986).

1. 2. EXTRACTIONS USING AQUEOUS TWO-PHASE SYSTEMS

Extractions using aqueous two-phase systems is mainly used as a primary purification step in the overall protein recovery train (Abbot et al., 1990; Sikdar et al., 1991). For a successful extraction of the desired biomolecule from fermentation broth or plant extract, the parameters have to be optimized in such a way that the cell debris along with some contaminating biomolecules partitions to one phase and the desired biomolecule partitions to other phase. In addition, to achieve optimal recovery and purity, one needs to pay attention towards the partition coefficient of the desired biomolecule, volume ratio of the system and concentration range of the broken cells (Hustedt et aI., 1985). Partition coefficient of a biomolecule is defined as the ratio of the equilibrium concentration of the protein in the top phase to that in the bottom phase. It determines the selective distribution of the desired product in an A IPS. The exact mechanism governing the partition coefficient is largely unknown. However, Raghavarao et al. (1995) have suggested that the molecules partitions in such a way that the maximum number of interactions are possible and the minimum energy state of the system is achieved. Factors that affect partition coefficient are size and charge of the protein, choice and molecular weight of the polymer, phase composition, pH of the system, type and concentration of the additives and temperature, which are discussed in detail by Raghavarao et al. (1995).

Isolation and purification of various proteins have been demonstrated using ATPSs (Alberts son, 1986; Walter et aI., 1985). Scale-up is found to be easy in ATPE than in other processes (Kula et aI., 1982; Tjerneld et aI., 1987; Hustedt et aI., 1988). It is important to note that partition coefficient is independent of initial protein concentration

over a fairly wide concentration range and scale of operation. Hence for the commercial applicability of ATPE, experiments can be conducted at small scale and the results obtained can be directly applied for large-scale extractions. Cordos and Kula (1986) and Schutte et al. (1983) have reported processes for the large-scale isolation and purification of formate dehydrogenase from yeast and lactate dehydrogenase from bacteria, respectively. Strandberg et al. (1991) reported a pilot scale extraction study of a recombinant protein in a PEG/salt system with an overall recovery of 37%. Many more large-scale purification studies of the enzymes/proteins (Kroner et aI., 1978; Viede et al., 1983; Kroner et aI., 1984; Boland et aI., 1991; Papamichael et aI., 1992) including the recent one from Hart et aI. (1994) for the isolation and purification of human insulin-like growth factor I have indicated the suitability of this technique for large-scale operations.

Apart from the large-scale extraction and purification of extracellular enzymes as well as recombinant proteins, A TPE also finds applications in many other fields namely: (i) extraction and purification of intracellular and membrane proteins (Sivars and Tjemeld, 1997) (ii) concentration and purification of viruses (Alberts son, 1986), nucleic acids (Cole, 1991; Walter et al., 1985) and plant proteins (Persson and Johansson, 1989; Vilter, 1990) (iii) partitioning and separation of microbial cells (Alberts son, 1986) as well as animal cells (Hamamoto et al., 1996) (iv) in food industry, for the clarification of cheddar whey (Chen, 1989) and isolation of high phytin containing particles from rice bran (Ogawa et al., 1975) (v) in the measurement of relative hydrophobicity and approximate isoe1ectric pH of biomo1ecules (Zaslavsky, 1995) and (vi) in bioremediation (Rogers, 1997)

After a successful extraction using ATPE, removal of polymer (pEG or DX) or salt with which the desired biomolecule is present is essential for further purification of the biomolecule. Separation of PEG from the protein can be achieved by addition of a new salt so as to form a new PEG/salt system (Hustedt et aI., 1985) wherein the desired biomolecule partitions to the salt phase in which the concentration of PEG is very low. Such a residual amount of PEG can sometimes be tolerable, or if desired can be removed along with the salt by ultrafiltration or diafiltration (Hustedt et aI., 1985; Hummel et aI., 1985). A simple dialysis can remove the salt from the biomolecule. PEG can aIso be removed by chromatographic adsorption on hydroxypatite or ion-exchange columns (Albertsson, 1986).

1. 3. MODELING IN AQUEOUS TWO-PHASE SYSTEMS

Mathematical modeling can be immensely exploited for the prediction of biomolecule partitioning in ATPS without measuring a number of parameters in order. Modeling of protein partitioning in ATPS is a complex problem since a number of parameters including electrostatic interactions, hydrophobic interactions, protein size etc., govern the partition behavior. Baskir et aI. (1989) and Diamond and Hsu (1989b) analyzed the existing models in their reviews and compared them with their own models. King et aI. (1988) and Kabir and Cabezas (1996) presented a deeper thermodynamic basis for such predictive models. Models available in A TPE can be broadly categorized into three types. (i) Lattice models, based on a lattice representation of polymer solutions within each of the co-existing phases. (ii) Virial expansion models, which explain the thermodynamic properties of the system, based on virial type

expansion in the concentration of the system components. (iii) A scaling thermodynamic approach utilizing the recently developed concepts aimed at describing polymer solution behavior (Abbot et aI., 1990). Despite the availability of many models in the literature, there is a need to develop many more models in this area (Raghavarao et aI., 1998). The interactions of the water with the phase forming polymers, buffering salts and proteins, which plays a key role in protein partitioning (Baskir et al., 1989) must be evaluated. The Flory-Higgins interaction coefficients need to be determined by enthalpic contributions from the chain segments (Gustafsson et al., 1986). Furthermore, models that examines the influence of protein surface properties such as surface charge and hydrophobicity needs to be developed.

1. 4. RECENT DEVELOPMENTS IN AQUEOUS TWO-PHASE SYSTEMS

Some recent developments in ATPE, which mainly focuses on the selectivity and scale-up aspects for the industrial exploitation of the technique, are affinity partitioning, electroextraction, extractive bioconversion, external field assisted demixing and temperature induced phase separation.

1. 4. 1. Affinity partitioning

Affinity partitioning involves the preferential/biospecific interaction between the biomolecule and affinity ligand, which is attached to one of the phase forming polymers. The interaction results in the formation of a biomolecule-polymer derivative ligand complex, which selectively partitions to one phase leaving behind the contaminants in the other phase. The nature of interaction determines the type of ATPS to be used. If the interaction is of electrostatic type then polymer/polymer system is preferred, since high salt concentration interferes with the electrostatic interaction. If the interaction between the ligand and protein is of hydrophobic type then polymer/salt system would be preferred, since high salt concentration enhances this type of interaction. (Walter et aI., 1991). Most of the reports available in the literature on affinity partitioning pertain to polymer/polymer systems (Diamond and Hsu, 1992) since protein-ligand interactions are predominantly of electrostatic in nature.

Cordes and Kula (1986) have purified formate dehydrogenase form Candida Biodinii cell homogenate in large scale using procion red HE3B as affinity ligand in a three step A TPE followed by ultrafiltration and lyophilization. Tjerneld et aI. (1987) have demonstrated the large scale affinity purification of lactate dehydrogenase from Pig muscle in a PEG/hydroxypropyl starch system using porcine red HE3B as affinity ligand. Affinity purification of a recombinant protein A (26 fold purity) was reported in PEG/hydroxypropyl starch two-phase system by employing an enteric coating polymer eudragit S-1 00 as ligand carrier and IgG as the ligand (Kamihira et aI., 1992). Metal chelates were shown to have great potential as affinity ligands. PEG bound imino-diacetic acid (IDA) were loaded with divalent ions of transition group and utilized for affinity extraction (KopperscWager and Birkenmeier, 1990; Plunkett and Arnold, 1990; Suh et aI., 1990; Arnold, 1991).

1. 4. 2. Extractive Bioconversions

This technique involves simultaneous production and extraction of the products in A TPS using biocatalysts such as enzymes or microorganisms. The biocatalyst partitions to one phase and the product is extracted to the other phase, thus facilitating its isolation and avoiding product inhibition. In case of substrates inhibiting the biocatalyst, the systems conditions were manipulated in such a way that the substrate and biocatalyst partitions to opposite phases (Lee and Chang, 1989). Mattiasson (1983) demonstrated the feasibility of carrying out enzymatic conversions in A TPS by performing the process in one of the phases and continuously removing the product to the other phase, thus providing a convenient a system which economically uses the soluble enzymes for the degradation of macromolecular substrates. Tjerneld et al. (1985a,b) investigated the semicontinuous hydrolysis of cellulose in a PEG/crude dextran system by employing cellulolytic enzymes from Trichoderma reesii. Planas et al. (1996) used this process to enhance the production of lactic acid using lactobacillus sp by reducing the end product inhibition. Many more applications of this process have been reviewed by Raghavarao et al. (1998). Most of the bioconversions have been performed in polymer/polymer systems. Lee and Chang (1989) were the first to successfully employ PEG/salt system for the production of acrylamide from acrylonitrile using brevibacterium. Selective partitioning of the product to top phase avoided the possible product inhibition thereby increasing the rate of production. Extractive bioconversions using ATPS was demonstrated to be economically viable and can improve certain existing processes (Mattiasson and Holst, 1991).

1. 4. 3. Electro-extraction

The charge directed transfer of charged biomolecules (proteins) to either one of the phases in ATPS by applying electric field constitutes electro-extraction. The electric field is applied in the direction perpendicular to the phase interface, which provides the stability against convection facilitating product recovery (Theos and Clark, 1995).

Lavine and collegues (Levine and Bier, 1990; Levine et aI., 1992) have reported that the electrophoretic mobility of proteins in ATPSs is greatly impeded in one direction. The protein transfer was easily achieved from its non-preferred phase to its preferred phase. However, from preferred to non-preferred phase transfer was not possible. On the contrary, Theos and Clark (1995) have shown that electrophoretic mobility of proteins can be easily achieved in both directions. Authors separated oppositely charged binary protein mixtures by transferring them into opposite phases of a ATPS by operating in between the pI's of two proteins. Electrophoretic mass transfer across the interface was hindered in the phase systems with high Donnan potential, whereas it was easily achieved in the phase systems with low Donnan potential. Electro-extraction appears to be a promising technique for the separation of charged proteins at the commercial scale owing to its advantages such as controlling starting composition, limiting convective mixing and facilitating product isolation over the conventional free solution method (Theos and Clark, 1995).

1. 4. 4. Field assisted phase demixing

Phase demixing is slow in ATPSs owing to similar phase densities, high individual phase viscosities and low

interfacial tension (Raghavarao et aI., 1995). Centrifugation enhances the phase demixing, but the process is uneconomical at the large-scale. Larsson and coworkers (Vikrostrom et al., 1987; Larsson, 1994) have explored the possibility of applying magnetic field externally to enhance the phase demixing in polymer/polymer ATPS. In this method magnetic particles or ferrofluids were introduced into the system and then magnetic field was applied. A semicontinuous three-step separation of enzymes has been developed using this method where separation was achieved in less than one hour (Vikrostrom et aI., 1987). Rahavarao et al. (1990, 1991) have reported an about 2-5 fold enhancement in the demixing rate in polymer/polymer systems, by the application of electric field. The observed enhancement in phase demixing was attributed to the increased mobility of phase droplets in the presence of electric field (Brooks and Bamberger, 1982) and was explained based on the electro-osmotic flow model (Raghavarao et al., 1998).

1. 4. 5. Temperature induced phase separation

One of the major bottlenecks that limit the industrial exploitation of ATPSs is the high cost of the phase forming polymers, which could be countered by the recovery and recycling of the polymers. Ultrafiltration, a method known for the recovery of the most commonly used polymer, PEG in ATPSs is expensive on large scale. Hence there is a need to approach this in a different way. Use of temperature sensitive phase forming polymers in ATPSs is one such approach. These polymers separate from water and forms into two phases when their aqueous solutions are heated to or above a certain critical temperature called the cloud point (Harris et al., 1991; Alred et al., 1993, 1994; Johansson et al., 1995; Berggren et al., 1999). The most commonly used water soluble thermo separating polymers in ATPSs are the random copolymers of ethylene oxide (EO) and propylene oxide (PO), henceforth collectively called as EOPO polymers. Recently Persson et al. (1999) demonstrated the purification of a recombinant protein, apolipoprotein A-I from E.coli fermentation broth using the thermo separating ATPS. The use of these EOPO polymers along with low cost polymers has improved the technique of A TPE and made the primary recovery economical. In addition, the recycling of polymers is important for the development of environmentally benign aqueous two-phase technique.

1. 5. NEW PHASE SYSTEMS

The technique of aqueous two-phase extraction has become more efficient in terms of partitioning and purification of biomolecules by the introduction of novel phase systems. For instance, in microemulsion phases biomolecule partitioning can be enhanced up to 3-4 orders of magnitude by manipulating the system parameters such as pH, ionic strength and surfactant concentration (Fisher and Sutherland, 1989). Formation of agarose beads in ATPSs is another strategy for enhancing the selective partitioning (Ling and Mattiasson, 1989). In this technique, top phase was formed with a co-polymer of PEG and polypropylene glycol and bottom phase constituted hydroxy ethylated agarose, which gels at low temperatures. Furthermore, this technique is not restricted to agarose alone, other polymers like alginate and carragenan have also been used. Stewart and Todd (1990) reported that saturated solution of NaCI form two-phase system with PEG at elevated temperature (60°C). This system was suitable for the

separation of amino acids, dipeptides and nucleotides form acid hydrolysates used in food industry and not suitable for protein separations due to high temperature required for phase formation and due to the protein denaturing property of high NaCl concentration. Glotova et al. (1993) reported a new phase system consisting of skim milk proteins/sodium salt of carboxymethyl cellulose in water. Terstappen et al. (1992) developed a detergent based ATPS for the extraction and purification of lipase from Pseudomonal cepacia. They obtained about 76% of enzyme recovery with 4-fold concentration and 24-fold purification. Cole (1991) partitioned nucleic acids selectively to the salt phase in a PEG/salt type ATPS containing Chaotropic agents and detergents. Proteins and cellular components partitioned to top phase or precipitated at the interface.

1. 6. PRESENT STUDY

The present study is divided mainly into four chapters. In the first chapter, a general introduction about the ATPSs has been documented, covering fundamental aspects, highlighting various applications and emphasizing recent developments in this field. In the second chapter, extraction and purification studies of a plant peroxidase extracted from the leaves of Ipomoea palmetta (a new source of the enzyme) has been carried out using various ATPSs. Laboratory scale purification of the enzyme has been attempted using ATPE coupled with gel filtration and relatively larger-scale purification of the enzyme has been investigated using ATPE in combination with ultrafiltration. In addition to this enzyme another fungal enzyme, amyloglucosidase produced from solid state fermentation is also subjected to ATPE to study its extraction and purification behavior.

Phase demixing is a slow process in ATPSs. Existing methods to enhance the phase demixing rate have many drawbacks making them unsuitable to implement at large-scale operations. Hence, in the third chapter, an attempt has been made to enhance the demixing rates in ATPSs by employing external fields such as acoustic and microwave. A detailed study on the kinetics of phase demixing has also been reported in this chapter.

Fourth chapter mainly deals with the mass transfer aspects and drop dynamics in ATPSs. Mass transfer aspects of the enzyme horseradish peroxidase using spray extraction column has been investigated in a PEG/salt system and a model has been developed to predict the drop sizes in ATPSs at low flow rates and its accuracy is tested with the experimental values.

At the end, suggestions for future work has been presented followed by references.

Table 1. 1. Examples of polymer/polymer aqueous two-phase systems

Polymer 1	Polymer 2
Polyethylene glycol	Dextran
	Ficoll
	Pullulan
	Polyvinyl alcohol
	Maltodextrin
	Polyvinyl pyrrolidone
	Hydroxypropyl starch
Polypropylene glycol	Dextran
	Hydroxypropyl dextran
	Polyvinyl pyrrolidone
	Polyvinyl alcohol
	Polyethylene glycol
	Methoxy polyethylene glycol
	Hydroxypropyl starch
Polyvinyl alcohol	Dextran
	Hydroxypropyl dextran
	Methyl cellulose
	Acrylic/methacrylic acid copolymers
Polyvinyl pyrrolidone	Dextran
	Hydroxypropyl dextran
	Methyl cellulose
Methyl cellulose	Dextran
	Hydroxypropyl dextran
Ethylhydroxyethyl cellulose	Dextran
Hydroxypropyl dextran	Dextran
Ficoll	Dextran
Dextran	Ethylhydroxyethyl cellulose
	Hydroxypropyl dextran
	Ficoll

Table 1. 2. Examples of polymer-salt aqueous two-phase systems

Polymer	Salt
Polyethylene glycol	Potassium phosphate
	Sodium sulfate
	Ammonium sulfate
.	Sodium formate
	Magnesium sulfate
	Sodium citrate
	Sodium potassium tartarate
	Sodium succinate
	Aluminium sulfate
	Calcium carbonate
	Ferrous sulfate
	Alum
	Copper sulfate
	Sodium formate
Polypropylene glycol	Potassium phosphate
	Ammonium sulfate
Methoxypolyethylene glycol	Potassium phosphate
Polyvinyl pyrrolidone	Potassium phosphate

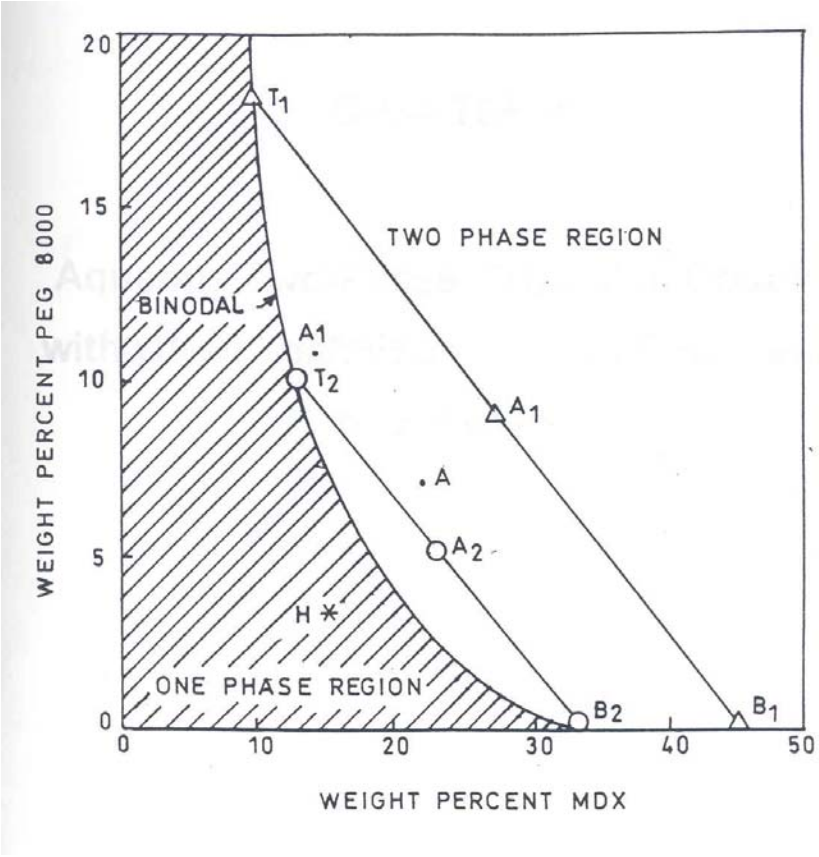

Figure.1.1 Phase diagram of a PEG/MDX system

CHAPTER 2

Aqueous Two-Phase Extraction Coupled with Other Techniques for the Purification of Enzymes

SECTION - A

Partition Behaviour and purification of a Plant Peroxidase

2A. 1. INTRODUCTION

Peroxidases (PODs, E.C. 1.11.1.7) belong to the group of oxidoreductases are widespread in nature. They have the ability to catalyze the oxidation of large varieties of substrates through the reaction with hydrogen peroxide. They are widely used in clinical biochemistry and enzyme immunoassay (V amos- Vigyazo, 1981). Some novel applications of PODs suggested include treatment of wastewater containing phenolic compounds, synthesis of various aromatic chemicals and removal of peroxide from materials such as foodstuffs and industrial wastes (Agastini et al., 1997). Horseradish root tubers are commonly employed as a commercial source for POD production (Yamada et aI., 1987; Saitou et aI., 1991; Kim and Y 00, 1996). However other cultivated species may provide PODs exhibiting similar or better properties, especially the recombinant species (Alexy et aI., 1995).

A major limitation for the wide spread use of POD is the current high cost of production of the enzyme (Kim and Y 00, 1996). The enzyme cost can be reduced either by reducing the production cost or by reducing its recovery and purification cost or preferably both. The present work aims at the latter and therefore is confined to the downstream processing of the enzyme. Here we selected leaves of Ipomoea palmetta as a source for POD production, which has not been attempted before. It could be an alternative commercial source for high activity POD.

The problems encountered during the isolation, extraction and purification of plant enzymes were extensively studied (Vilter and Kula, 1989; Vilter and Jordan, 1989; Vilter, 1990). Some of the problems could be due to the rigid cell walls, high viscosity of the extract, presence of phycocolloids, phenolic compounds etc. Recent studies have demonstrated the suitability of ATPSs for the successful extraction of enzymes from plant sources (Vilter and Kula, 1989;Vilter and Jordan, 1989). A detailed study on large-scale extraction and purification of algal peroxidase from Ascophyllum nodosum (300 liter scale) and vanadate-dependant peroxidase from Laminaria digitata (1 liter scale) were carried out by using ATPSs followed by chromatographic techniques (Vilter, 1990). Horseradish peroxidase (HRP) was partitioned and purified by two-stage ATPE containing polyvinylpyrrolidone (Miranda and Cascone, 1997). Further, two-phase extraction and purification methods for large-scale operations of HRP have also been established (Miranda and Cascone, 1994; Miranda et aI., 1995). However, large-scale extraction and purification of the Ipomoea peroxidase is not yet attempted. Since it was reported that these enzymes could be used as commercial sources of high activity peroxidases (Lin et al., 1996), a simple and cost effective method for large-scale purification of these enzymes would be highly beneficial. Hence, in the present study, apart from studying the various factors that affect the partition behavior of the enzyme we investigated the feasibility of utilizing ATPE followed by gel filtration for the purification of the enzyme at lab scale (100 ml). In addition, we also attempted for the large-scale (I-liter) purification of the enzyme by A TPE coupled with ultrafiltration.

2A. 2. MATERIALS AND METHODS

2A. 2. 1. Chemicals

Polyethylene· glycols (MWs 4000 and 6000) were purchased from Sisco Research Laboratory (Mumbai, India). Polyethylene glycol (MW 1550) was procured from Sigma Chemicals (St. Louis, MO). Ammonium sulfate, sodium sulfate, di-potassium hydrogen phosphate and potassium di-hydrogen phosphate were from Ranbaxy Chemicals (Punjab, India). Maltodextrin was from Laxmi Starch Pvt, Ltd. (Mumbai, Inida) and comassie brilliant blue G-250, guaiacol, and H_202 were from Fluka Chemie AG (Buchs, Switzerland). All other chemicals used were of analytical grade.

2A. 2. 2. Crude enzyme extract preparation

Leaves of Ipomoea palmetta were collected from the plant nursery of Department of Applied Botany and Biotechnology, University of Mysore (Mysore, India) and were immediately processed. Fresh leaves were washed thoroughly with distilled water at room temperature for 5-10 minutes. Washed leaves were cut into pieces and homogenized with distilled water for 10 minutes at room temperature. The extract was filtered with cheese-cloth (four-fold) and used for the partition behavior and lab scale (100 ml) purification studies of the enzyme. Larger scale (1 liter) extraction and purification studies were carried out with unfiltered extract only. This unfiltered extract contained cell debris and some uncrushed small leaf particles. 2A. 2. 3. Two-phase preparation

Phase systems were prepared by mixing required quantities of phase forming solutes in water (dilute enzyme extract). The phases were mixed thoroughly for sufficient time and were allowed to separate in a separating funnel. Top and bottom phases were separated and volumes measured. Aliquots of the phases were taken for enzyme assay and protein concentration determination. Aliquots of the pure systems were used as blanks for protein concentration determination. pH of the phase systems wherever required was adjusted using either 2N NaOH or 2N HCl.

2A. 2. 4. Determination of partition coefficient

Partition coefficient (m) of the enzyme was calculated as the ratio of the equilibrium concentration of the enzyme in the top phase to that in the bottom phase. That is, $m= C_T /C_B$, where C_T and C_B are equilibrium concentrations of enzyme in the top phase and bottom phase, respectively.

2A. 2. 5. Enzyme assay and protein concentration determination

Peroxidase activity was determined at room temperature with a spectrophotometer following the formation of tetraguaiacol (A max = 470 nm $\varepsilon = 26.6$ $mr^{\cdot 1}$ $cm^{\cdot 1}$) in a 3 ml reaction mixture containing 1 ml of 0.1 M phosphate

buffer, pH 6; 1 ml of 15 mM 2-methoxyphenol (guaiacol); 1 ml of 3 mM H202; and 50 III of enzyme extract. One unit of peroxidase activity (U) represents the amount of enzyme catalyzing the oxidation of 1 μ mole of guaiacol in 1 minute (Agostini et al., 1997). Protein concentration was determined by comassie brilliant blue G-250 method, using bovine serum albumin as standard (Bradford, 1976).

2A. 2. 6. Purification of the enzyme by ATPE coupled with gel filtration

Hundred milliliters of ATPS comprising of PEG (24%, w/v), ammonium sulfate (7.5%, w/v) and NaCI (2 %, w/v) along with 80 ml of crude extract was prepared. Sixty-six milliliters of the top phase and 34 ml of bottom phase were separated. Enzyme activity and protein concentration of both phases were measured. Partition coefficient and specific activity of the enzyme were calculated. The salt rich bottom phase was completely dialyzed against distilled water to remove salt and further concentrated to 5.5 ml by antidialysis against 25% PEG (6000).

2A. 2.7. Gel filtration on Sephadex G-IOO column

Five milliliters of the concentrated enzyme sample from the previous step was loaded onto a Sephadex G-I00 column. The column (2 x 90 cm) was equilibrated and eluted with 10 mM sodium phosphate buffer, pH 6.0, at a flow rate of 15 ml/h. Three milliliter fractions were collected throughout the elution and absorbance at 280 nm and peroxidase activity were monitored.

2A. 2. 8. Ultrafiltration

Ultrafiltration was carried out in a Millipore Minaton ultrafiltration unit (Millipore, USA) with a membrane of molecular weight cut off 30,000.

2A. 3. RESULTS AND DISCUSSION

Partitioning behavior of biomolecules in ATPSs is an important criterion for the separation, characterization and purification of biomolecules. A large number of interdependent parameters, which include, type of phase forming components, system pH, molecular weight of phase forming polymers, phase composition and additives like neutral salts are responsible for governing partitioning behavior of the enzyme (Kula, 1985). In order to understand the partitioning behavior of Ipomoea peroxidase, influence of above-mentioned parameters was studied in a number of experiments.

2A. 3. **1. Influence of pH**

Variation of partition coefficient (m) of the enzyme with pH studied in the three systems is shown in Figure 2A.l. PEG/potassium phosphate system did not show phase separation below pH 7 at the studied phase composition. Similar trend of partitioning was observed in all the systems with an increase in pH. That is 'm' increased with an increase in pH up to around neutrality and then it decreased with any further increase in pH. An interesting

observation may be made at neutral pH where 'm' remained practically same (=0.2) in all the systems. In all the studies, yield of the enzyme in the bottom phase was found to be greater than 80 % around neutral pH.

Influence of pH on the partition behavior of the enzyme could be explained by two major physico-chemical factors in a given A TPS. One is the changes in the properties of the aqueous media in the phases induced by a given pH change. That is the changes in the solution pH that results in an electrical potential difference between the phases, which causes a change in the partitioning behavior of the protein except at the isoelectric pH (Abbot and Hatton, 1988). Other is the pH-induced change in the solute resulting in the change of interactions of solute with an aqueous medium (Zaslavski, 1995). That is, the biopolymers eg, proteins, peptides etc., contain large variety of acidic and basic groups with different pKa values, differently charged at different pH values. The pH changes not only alter the solute net charge, however, they may also induce conformational changes in the structure of a given biopolymer as well as their association and dissociation into subunits. It has been suggested that in PEG/salt systems the partition coefficient of protein is strongly influenced by pH, and it changes over 2-3 orders of magnitude depending on the protein, at certain small pH intervals (Kula, 1985).

2A. 3. 2. Influence of phase composition and polymer molecular weight

Influence of phase composition on the partition coefficient of the enzyme studied in ATPSs was depicted in Table 2A.l. It was observed that as the phase composition increased the partition coefficient as well as phase volume ratio increased gradually in PEG/salt systems. The Increase In phase volume ratio indicates an increase in the concentration of salt in the salt rich bottom phase and availability of water in the polymer rich top phase. Hence, the solubility of the enzyme in the salt rich bottom phase decreases, exhibiting partitioning as a salting out type (Zaslavski, 1995). However, in PEG/MDX system 'm' did not show much variation as phase composition varied.

Figure 2A.2 shows the influence of PEG molecular weight on partition coefficient of the enzyme in different ATPSs. PEG(4000)/potassium phosphate system did not show phase separation for the attempted composition (10/11), hence is not represented in the figure. It was observed that partition coefficient decreased with an increase in PEG molecular weight (Fig. 2A.2). The phase polymer molecular weight influences the solute partitioning both by altering the phase diagram and by changing the number of polymer enzyme interactions. An increase in molecular weight decreases the amount of water in the polymer rich phase available to solubilize the enzyme and the salt. The effective enzyme and salt concentrations in the free solvent would therefore increase with increase in molecular weight of the polymer causing the enzyme to partition more strongly into the salt rich bottom phase (Hustedt et aI., 1985; Albertsson, 1986).

2A. 3. 3. Influence of neutral salt

Influence of NaCI concentration on partition coefficient of the enzyme in four ATPSs studied is depicted in Figure 2A.3. An increase in salt concentration (up to 2%) has resulted in a considerable decrease in partition coefficient. However, any further increase has resulted in an increase in the partition coefficient. Thus the lowest partition coefficient was observed with NaCl concentration of 2%.

Generally, addition of a neutral salt (additive) to an ATPS alters the phase diagram of the system and also the properties of the partitioning solute (Abbot and Hatton, 1988). A slight variation in the volume ratio with the addition of NaCI observed in our studies has indicated that the phase diagram is not influenced much by the presence of NaCl. It should be noted that the extent of variation in the partition coefficient is much higher when

compared to the previous parameters such as pH, phase composition and polymer molecular weight, which basically influence the phase diagram. Hence it may be inferred that the latter mechanism is responsible for the observed results. For example, when a protein being partitioned changes its size depending on the ionic strength of the medium, undergoes conformational changes in the presence of a particular additive, or binds to the additive. In all these cases the nature or relative amount of particular group of the protein macromolecule exposed to the solvent is changed. This means that the solute-solvent interaction of this changed solute differs from those of the same solute in the absence of a given additive (Potschker, 1988). In the same manner, addition of NaCl appears to have changed the interaction of POD with the system, resulting in a significant decrease in the partition coefficient.

The differential response of individual proteins in PEG/salt systems in the presence of added salt has been used for the selective partitioning of a number of enzymes viz, 1,4 a-glucon phosphorylase (Hustedt et aI., 1978), formate dehydrogenase (Kroner et aI., 1982), etc. At very high salt concentration, solubility limits of proteins may be reached resulting in high apparent partition coefficients (Menge et aI., 1983), a phenomenon observed in our case also.

2A. 2. 4. Purification of the enzyme by ATPE coupled with gel filtration

The purification protocols of the I. palmetta peroxidase are summarized in Table 2A.2. Aqueous two-phase extraction employed here as a primary purification step, has achieved its main purpose of improving the degree of enzyme purification (purification factor = 2.18) while considerably reducing the volume of the crude sample (57.5%) that has to be further processed by chromatography step. The extraction conditions employed has resulted in the enrichment of enzyme specific activity (15.7), which is due to the differential partitioning of the desired enzyme and contaminating enzymes/proteins to the opposite phases. G-I00 column chromatography of the antidialysate resulting from salt-rich bottom phase of the ATPE has markedly increased the degree of enzyme purification (about 49 fold). Figure 2A.4 shows the elution profile of the enzyme in sephadex G-l 00 column.

By the combination of methods the enzyme was purified about 49 times with a recovery of 75.3%. The specific activity was 331.4 u/mg, which is similar to those for plant or fungal PODs reported in the literature (Alexey et al., 1995). Similar work reported elsewhere (Lin et al., 1996) wherein the POD extracted from leaves of Ipomoea cairica, purified by conventional methods has produced only 16.7 fold pure enzyme after G-I00 column chromatography. Thus it could be reiterated that ATPE could be an efficient purification step in the purification chain of certain biomolecules and the present case of plant peroxidase is a shining example.

2A. 3. 5. Parameters optimization for Larger-scale extraction and purification

In large-scale operations for enzyme purification by ATPE, clarification of unfiltered extract by filtration or centrifugation, prior to ATPE becomes prohibitively expensive and highly unpredictable (Hart et al., 1994). Therefore integration of clarification with ATPE in the form of differential partitioning of cell debris and desired product to the opposite phases will be economically highly beneficial on large-scale operations. Therefore we attempted to achieve differential partitioning of the enzyme and cell debris of the unfiltered extract in ATPSs at 100 ml scale, which can be easily adapted for large-scale operations.

Partitioning experiments carried out with unfiltered extract in ATPSs with PEG- 6000 and/or 4000 showed that along with the enzyme, cell debris and solid particles also partitioned to the bottom phase. However, when PEG-1550 was replaced with PEG-4000 or 6000, differential partitioning of the enzyme and cell debris was observed. Enzyme partitioned to the bottom phase and cell debris partitioned to the top phase. Therefore partitioning experiments were carried out in a number of other ATPSs with PEG-1550 as one of the phase forming component, in order to obtain optimum differential partitioning of the enzyme and cell debris and the results are summarized in Table 2A.3. PEG (1550) /K2HP04 system showed the optimum value for differential partitioning of the enzyme and cell debris. In this system, top PEG rich phase was found enriched with cell debris along with the residual leaf particles and enzyme was enriched in the bottom salt rich phase. Addition of NaCI (2%) further enabled the enzyme to partition more selectively to the bottom phase and also improved enzyme purity.

2A. 3. 6. Larger-scale (1 liter) extraction in combination with ultrafiltration

In a typical experiment, 600ml of the unfiltered crude extract prepared from 100 g of ipomoea leaves was subjected to ATPE in PEG (1550) / K_2HP04 / NaCI system. Total volume of the system was 1000 ml with net enzyme activity of 996 units and specific activity of 3.3 u/mg protein. After mixing thoroughly, 334 ml of the top phase and 668 ml of the bottom phase were separated and the partition coefficient of the enzyme was found to be 0.1. The bottom phase contained enzyme activity of 912 units with a specific activity of 10.5 u/mg protein. Therefore, the yield and purity in the bottom phase was calculated to be 91.6% and 3.26 fold respectively.

Six sixty milliliters of bottom phase (901 units) was subjected to ultrafiltration with a membrane of molecular weight cut off 30,000. Volume of the concentrate was found to be 47 ml with total enzyme activity of 684.8 units and specific activity of 19.4 u/mg protein. Thus, A TPE followed by ultrafiltration has resulted in a total enzyme recovery of 76% with 6.0 fold purification, and 8.8 fold activity concentration.

2A. 3.7. Enzyme stability

The enzyme (49 fold pure obtained from 0-100 column) showed optimum activity at 50°C as indicated in Figure 2A.5 under the assay conditions employed. The thermal stability was rather high. The enzyme was stable at 60°C for 5 hours (Fig. 2A.6) and drastic loss in activity (around 80%) was observed when the enzyme was incubated at 80°C for 1 hour. Optimum pH of the enzyme was found to be 6.0 (Fig. 2A.7) and was stable in the pH range 5-11 as shown in Figure 2A.8.

2A. 3. 8. Kinetic studies

Kinetic studies of the enzyme (49- fold) carried out using guaiacol as substrate are depicted in Figure 2A.9. Inset is Linweaver-Burk plot for Km and V_{max} estimation. The enzyme showed Michaelis-Menten kinetics, with the kinetic constant, Km being 18.15 mM and Vmax 27.1 μ moles/min.

2A. 4. CONCLUSIONS

Polyethylene glycol/salt aqueous two-phase systems consisting of 2% NaCl were successfully employed for extraction and purification of the enzyme Ipomoea peroxidase. By coupling gel filtration with A TPE the enzyme was purified to 49 fold with 75.3% recovery and by coupling ultrafiltration with ATPE the enzyme was purified to 6 fold with 76% recovery. The enzyme preparation with 6 fold purity is pure enough for waste water treatment with added amount of polyvinylpyrrolidone, which is a good additive for enhancing the enzyme performance (Arseguel

and Baboulene, 1994). The enzyme preparation with 49 fold purity may be used in more sophisticated applications such as enzyme immunoassay and histochemistry (Miranda and Cascone, 1997). The variation of partition coefficient with system pH in ipomoea POD indicates that the structural properties of this enzyme may be differ from that of the HRP, since it is reported in latter case that partition coefficient remains practically unchanged with the variation in system pH (Miranda and Cascone, 1997). Furthermore, the preferential partitioning of the enzyme to the bottom phase in the PEG/di-potassium phosphate, PEG/tri-potassium citrate and PEG/potassium carbonate systems (Table 2A.3) reveals that the structural properties of the enzyme may be different from that of the other plant peroxidases, which have preferred top phase under similar conditions (Vilter, 1990).

Table 2A. 1. Influence of phase composition on partition behavior of ipomoea peroxidase in ATPSs

System composition	Volume ratio (top / bottom)	Partition coefficient (m)	% Yield (bottom phase)
PEG/sodium sulfate			
9/8.5	38/62	0.118	80.0
10.5/8.5	41/59	0.123	86.3
12.5/8.5	45/55	0.185	79.9
10.5/12	30/70	0.267	83.2
PEG/ammonium sulfate			
8/12.5	25/75	0.212	80.0
11/12.5	33/67	0.208	83.8
13/12.5	35/65	0.214	87.9
15/12.5	39/61	0.248	81.3
PEG/maltodextrin			
10/30	69/31	0.135	71.2
10/40	55/45	0.133	79.1
15/30	70/30	0.138	71.0
15/40	65/35	0.124	74.0

Table 2A. 2. Purification of peroxidase from Ipomoea palmetta leaves

Fraction	Partition coefficient (m)	Total enzyme (u)	Total protein (mg)	Specific Activity (u/mg)	Purification Factor	% Recovery
Crude extract	----	576.0	80.0	7.2	1.00	----
ATPE	0.042					
Top Phase		42.5	36.4	1.2	0.17	7.37
Bottom Phase		527.0	40.3	15.7	2.18	91.50
G-100 column Chromatography	----	433.7	1.24	349.8	48.6	75.30

Table 2A. 3. Extraction and purification of ipomoea peroxidase in ATPSs having PEG of molecular weight 1550 in presence of NaCl (2%)

System	Composition (%, w/w)	Partition coefficient	% Yield (bottom phase)	Purification Factor
PEG/sodium Sulfate	10/15	0.08	91	2.73
PEG/potassium	10/15	0.67	75	2.74

Carbonate				
PEG/potassium Citrate	10/29	1.67	44	3.60
PEG/di-potassium Phosphate	10/17.5	0.097	93	3.40

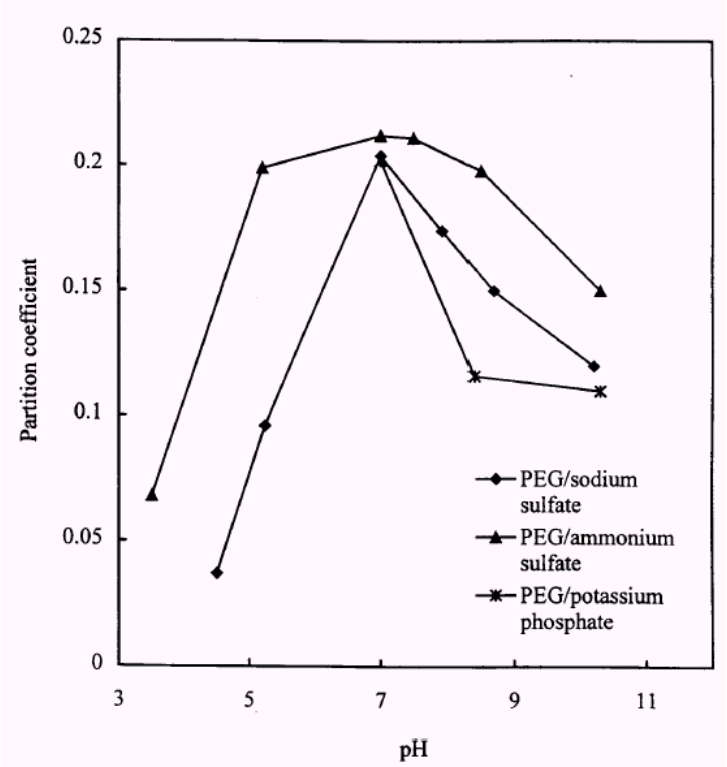

Figure 2A. 1. Influence of pH on partition coefficient of plant peroxidase in A TPSs

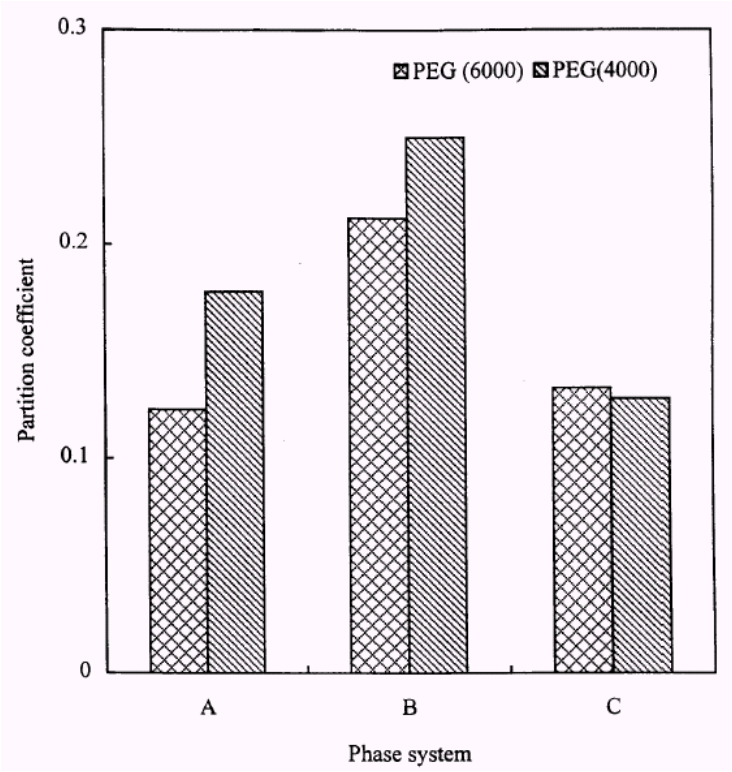

Figure 2A. 2. Influence of PEG molecular weight on partition coefficient of plant peroxidase in A TPSs. jt; t
A = PEG/sodium sulfate, B = PEG/ammonium sulfate and C = PEG/maltodextrin

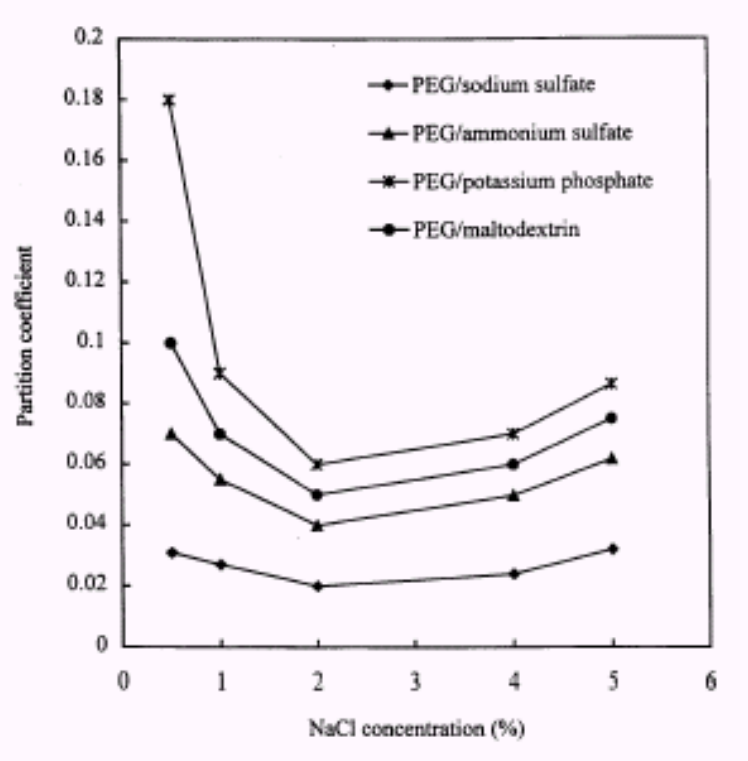

Figure 2A. 3. Influence ofNaCI concentration on partition coefficient of plant peroxidase in A TPSs

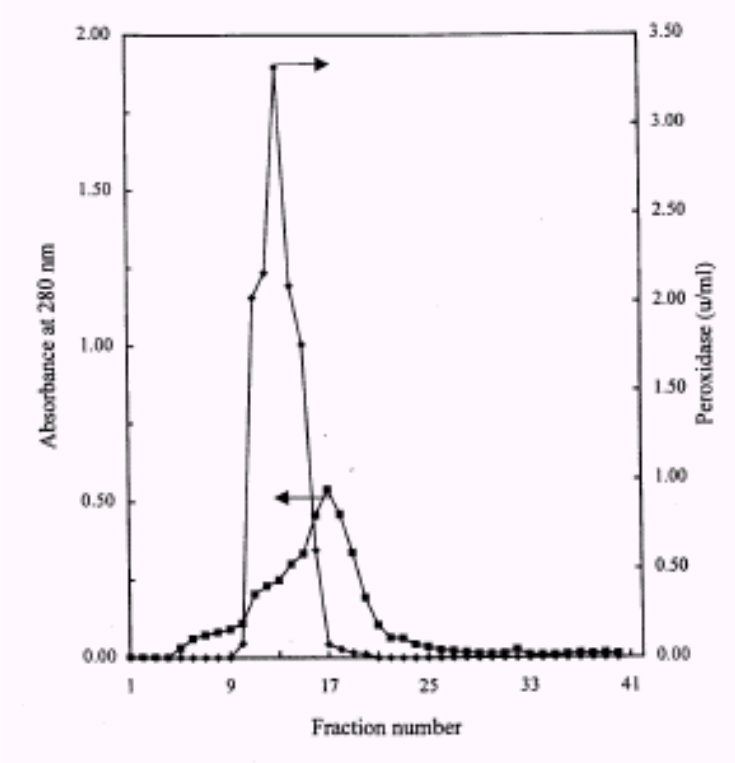

Figure 2A. 4. Gel filtration of plant peroxidase on Sephadex G-100 column (2 X 90 cm)

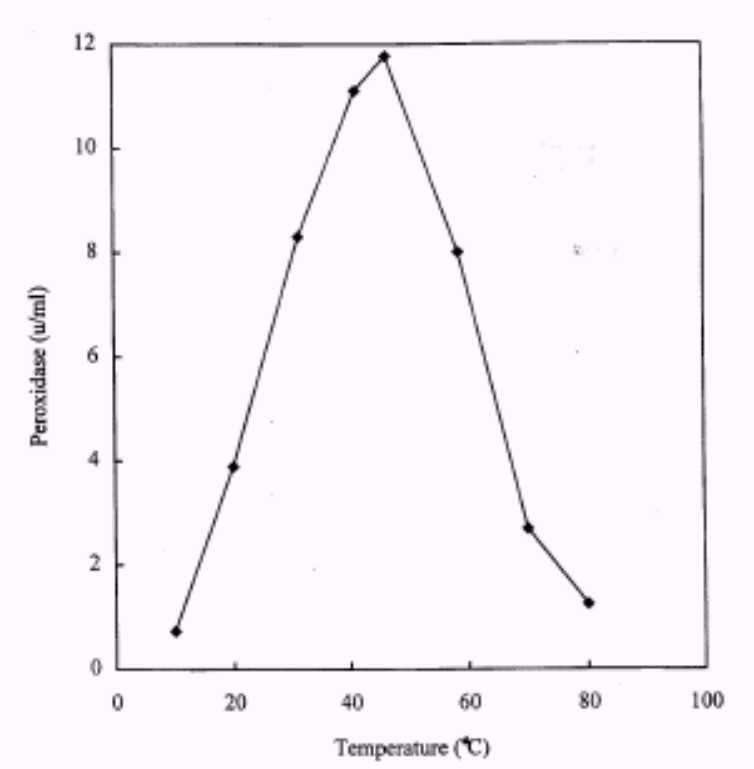

Figure 2A. 5. Optimum temperature of plant peroxidase

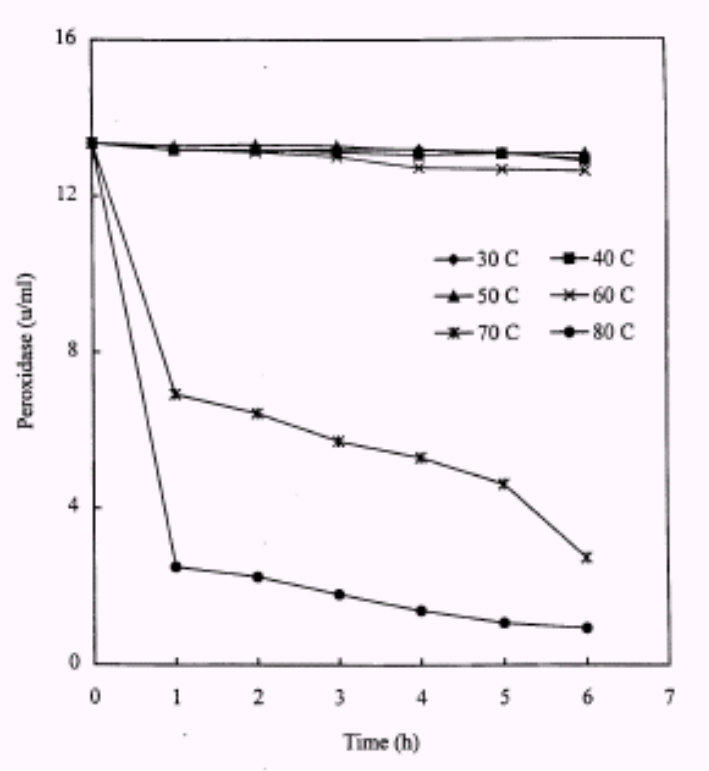

Figure 2A. 6. Effect of temperature on stability of plant peroxidase

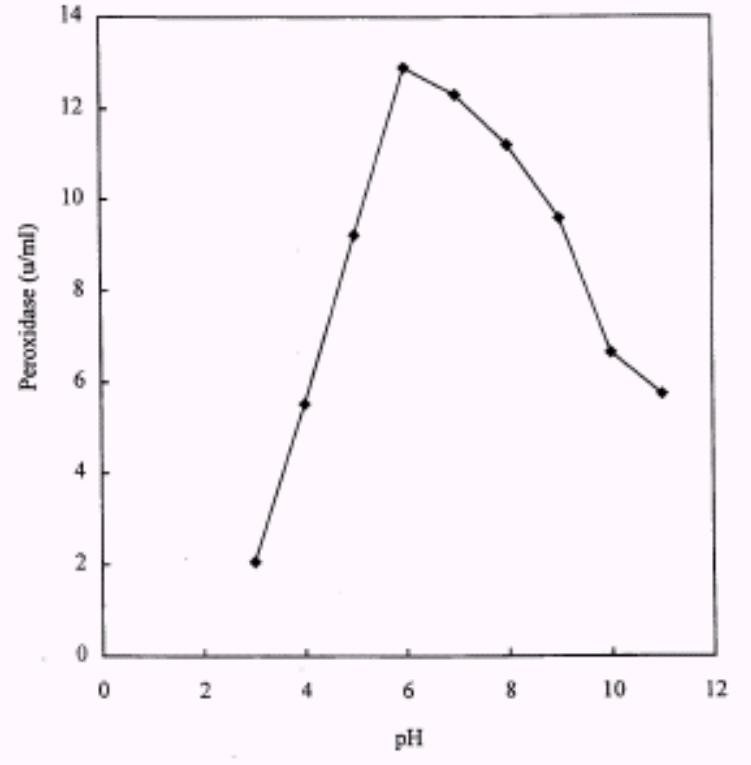

Figure 2A. 7. Optimum pH of plant peroxidase

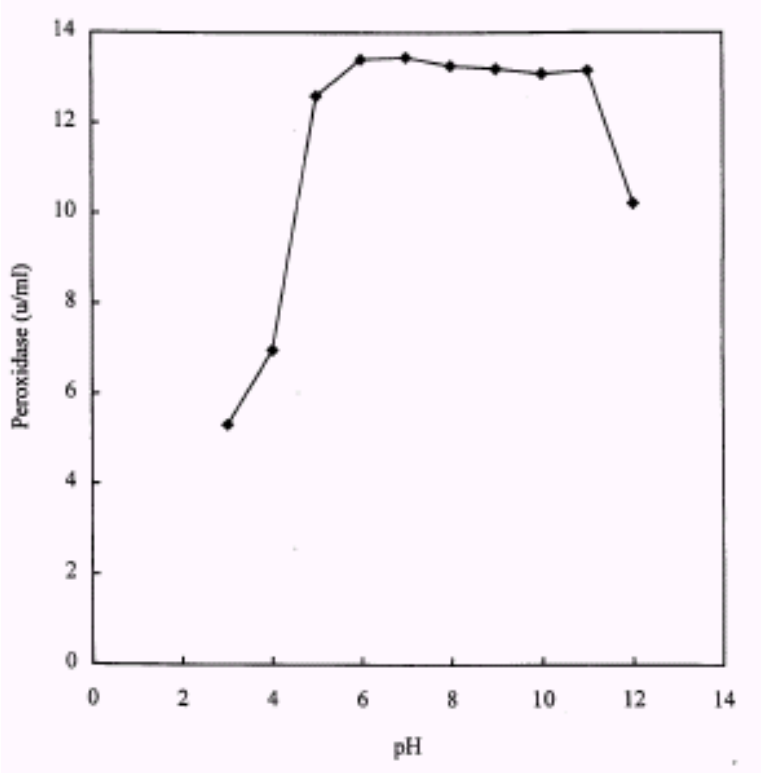

Figure 2A. 8. Effect of pH on the stability of plant peroxidase

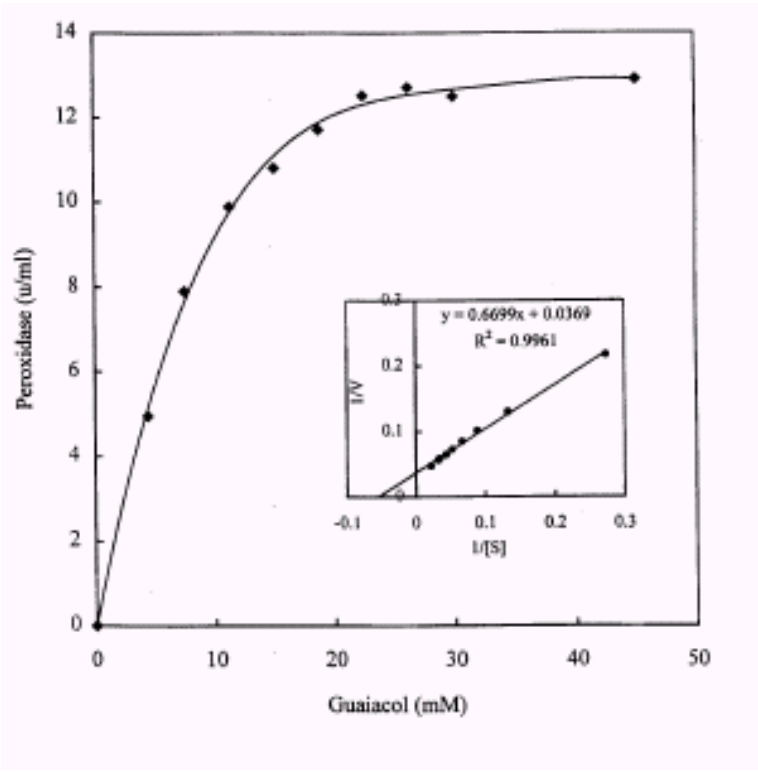

Figure 2A. 9. Guaiacol saturation curve for plant peroxidase: Inset is Lineweaver Burk plot

SECTION -B

Partition Behaviour and Purification of an Amyloglucosidase

2B. 1. INTRODUCTION

Amyloglucosidases (Glucoamylase E.C. 3.2.1.3) that hydrolyses α-l,4 and α-l,6 linkages of starch molecules to produce glucose are known to occur almost exclusively in fungi and microbial kingdom (Mahajan et aI., 1983). The most important application of this enzyme is in the production of high glucose syrup, which is used to produce crystalline glucose and high fructose syrup (Mase et al., 1996). Its other commercial applications include production of dextrose for confectionery and pharmaceuticals and liberation of glucose from different xylans (Ghosh et al., 1995).

Although several successful applications of ATPE on large-scale have been demonstrated (Walter et al., 1985; Cordos and Kula, 1986; Boland et al., 1991; Hart et al., 1994), very few reports are available in the literature on A TPE coupled with ultrafiltration for the recovery and purification of enzymes (Walter et aI., 1985; Veide et al., 1989) and none for the enzymes produced by solid state fermentation (SSF). It is known that SSF results in moldy bran (unlike the fermentation broth from submerged fermentation), leaching of which result in a dilute extract of enzyme that needs to be purified and concentrated. It may be noted that SSF offers economical and technical advantages over submerged fermentation (Ramana Murthy et aI., 1993).

The scope of this work is to investigate the performance of an ATPE with an integrated ultrafiltration step for downstream processing of the enzyme amyloglucosidase produced by solid state fermentation. ATPE coupled with ultrafiltration could be used for the final purification and concentration of this enzyme or could be utilized as a primary purification protocol in the overall purification train, depending on the application of the enzyme and the degree of enzyme purity required in that particular application.

In the present work, the partition behavior of amyloglucosidase in different ATPSs such as PEG/MDX, PEG/Na_2HPO_4, PEG/KH_2PO_4 and PEG/Na_2S0_4 was evaluated. Binodals for PEG (4000, 6000)/MDX systems, not reported earlier were developed. The effect of buffering salts such as NaCI, Na_2HPO_4, KH_2PO_4 and Na_2S0_4 on the partition coefficient of the enzyme in PEG/MDX system was studied. The enzyme extract was concentrated in a PEG/MDX system in the presence of KH_2PO_4 (1%), and further extracted from the PEG rich top phase back into a Na_2HPO_4 rich bottom phase and finally concentrated by ultrafiltration.

2B. 2. MATERIALS AND METHODS

2B.2. 1. Chemicals

Polyethylene glycols (MW 4000 and 6000) were purchased from Sisco Research Laboratories (Mumbai, India). Sodium chloride, sodium sulfate, potassium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Ranbaxy chemicals (Punjab, India). Maltodextrin was from Laxmi Starch Pvt., Ltd. (Mumbai, India). Dinitro salicylic acid and soluble starch were procured from Loba Chemie (Mumbai, India) and all other chemicals used were of analytical grade.

2B. 2. 2. Enzyme preparation

Amyloglucosidase enzyme produced using Aspergillus niger CFTRI 1105 by solid state fermentation was extracted (with distilled water) from dry moldy bran in order to obtain the dilute enzyme extract (Ramakrishna et al., 1982). A Millipore Minitan model ultrafiltration system (Millipore, USA) with a membrane of molecular weight cut off 30,000 was used for the concentration of enzyme.

2B. 2. 3. Construction of phase diagram

Phase diagrams for PEG (4000, 6000)/MDX were constructed at room temperature based on the cloud point determination method (Bamberger et al., 1985). Binodal compositions of PEG and MDX were calculated from the estimation of mal to dextrin by polarimetry method. Water content was obtained by drying and concentration of PEG was then obtained by subtraction (Walter et al., 1985). Ostwald U-tube viscometer and specific gravity bottles were used for the measurement of viscosity and density, respectively.

2B. 2. 4. Partition studies

Predetermined quantities of phase forming polymers in % w/w [PEG(4000)/MDX, (12/40); PEG(6000)/MDX, (10/40); PEG(4000, 6000)/Na_2HPO_4, (9/10); PEG(4000, 6000)/KH_2PO_4, (10/11) and PEG(4000, 6000)/Na_2S0_4, (10.5/8.5) were accurately weighed and to this the enzyme extract of desired quantity (decided by the total composition of the phase system, in other words water is replaced by the enzyme extract) was added. The entire mixture was stirred in a mechanically agitated contactor for 25 minutes. Then the phases were allowed to settle for sufficient time, which led to a distinct phase separation. The samples were removed from both the phases and

analyzed for enzyme activity. The partition coefficient was calculated by the equation, m = CT/CB where CT and CB are the equilibrium concentrations of the enzyme in the top and bottom phases, respectively.

Influence of buffering salts was studied in the following manner. To the predetermined quantities of PEG (6000)/MDX phase forming polymers, 1% (w/w) buffering salts like NaCI, Na_2HPO_4, Na_2S0_4 and KH_2PO_4 were added and to this the enzyme extract of desired quantity was added. The partition coefficient (m) of the enzyme was calculated for each type of salt as described above.

2B. 2. 5. Enzyme assay and protein concentration determination

Five milliliters of buffered 4% starch solution (acetate buffer, 0.2 M, pH 4.2) was taken in a test tube and placed in a water bath (60 DC) for 10 minutes for equilibration. The enzyme solution was added to the tube at the desired level (0.2-0.5 ml) and the reaction mixture was incubated at 60 °C for 60 minutes and the reaction was arrested by adding 0.8 ml of 4N NaOH solution. Aliquots of the hydrolysate in the range of 0.2-1.0 ml were used for estimation of glucose by the dinitrosalicylic acid method (Miller, 1959). The activity is expressed in international units (IV); one IV is equal to the ~M of glucose released per minute per ml or g under the defined conditions, i.e., temperature 60°C, pH 4.2 and 4% starch solution (Ramakrishna et al., 1982). Protein concentration was determined by comassie brilliant blue G-250 method, using bovine serum albumin as standard (Bradford, 1976).

2B. 3. RESULTS AND DISCUSSION

2B. 3. 1. Phase diagrams

For the first time, phase diagrams for PEG (4000)/MDX and PEG (6000)/MDX, were developed on the basis of the distribution of MDX and PEG between the two coexisting phases at 293 K and are shown in Figures 2B.I and 2B.2, respectively. Phase diagrams for PEG (8000)/MDX were reported in the literature (Szlag and Guliano, 1988). Phase systems were prepared at the points A, B and C for PEG (4000)/MDX and at a, b, c and d for PEG (6000)/MDX phase diagrams, respectively. Tie-lines were drawn (Figs 2B.I and 2B.2) and phase composition, viscosity and density of top and bottom phases were determined at all these points and summarized in Tables 2B.I and 2B.2. Based on the physical properties and the phase volume ratios, phase compositions at point' A' for PEG (4000)/MDX and at point 'c' for PEG (6000)/MDX systems were selected for the partitioning studies of the enzyme, amyloglucosidase.

2B. 3. 2. Extraction of amyloglucosidase

Amyloglucosidase enzyme produced by Aspergillus niger by SSP was extracted with distilled water from dry moldy bran in order to obtain the dilute enzyme extract. The ratio of bran to water in the range of 1:10 was employed to standardize the extraction of the enzyme from dry moldy bran. Appreciable increase in enzyme activity (730 u/ml) was observed when the dry moldy bran was equilibrated with water before subjecting to ATPE when compared to the situation where bran was equilibrated with ATPS (336 u/ml). This clearly indicates that thorough prior mixing enables efficient mass transfer/extraction of the enzyme into the water phase.

2B. 3. 3. Partition studies

Partition behavior of the enzyme was examined in PEG (4000, 6000)/Na_2HPO_4, PEG (4000, 6000)/Na_2S0_4, PEG (4000, 6000)/KH_2PO_4, and PEG (4000, 6000)/MDX systems and represented in Figure 2B.3. Optimal partitioning conditions were seen in PEG (6000)/MDX system at a composition of PEG 7.5% (w/w) and MDX 40% (w/w) with amyloglucosidase selectively partitioning to the top phase (m=4.8). In this PEG / MDX system, the effect of buffering salts on partition coefficient was studied by the addition of NaCI, KH_2PO_4, Na_2HPO_4 and Na_2S0_4 (Fig. 2B.4). Maximum effect on the partitioning behavior of amyloglucosidase was found with KH_2PO_4 giving the highest partition coefficient of m=18.1. This PEG/MDX system buffered with KH_2PO_4 was employed for the larger scale experiments for separation and purification of amyloglucosidase.

Salts are added to aqueous solution of enzymes to help buffer the solution and stabilize the biomolecule. The variation of the salt type and concentration in two-phase aqueous polymer system provide one of the most versatile means by which the selectivity and yield of the extraction may be manipulated. Changes in the salt type often produce an electrical potential difference between the two phases caused by the preference of one of the ions for one phase relative to other (Johansson and Hartman, 1974).

Johansson, (1974) has correlated the effect of salt on protein partition behavior with partition coefficients of the salt itself. Monobasic phosphate has a smaller partition into the lower phase than the dibasic, and in fact the potential was found to be smaller for the monobasic salt. Larger ions such as phosphate, citrate and sulfate partition more unequally than monoatomic ions such as chloride and so will give a large potential (Johansson, 1970; Zaslavski et al., 1983). It has been suggested that the partitioning of the salts is caused not by their affinity for the

dextran phase, but by their rejection from the PEG phase because of water structuring about the polymer which inhibits the hydration of the phosphate ion (Bamberger et al., 1984). Kula, (1990) has suggested that these changes in the partition coefficient are due to steric exclusion, the phosphate ion possibly bridging the hydroxyl groups of the dextran molecule. This increases the excluded volume above that of the free chains, thus forcing the proteins to partition away from the dextran phase. In our experiments, for a given tie line length, partition coefficient of the amyloglucosidase decreased in the following order of the salt; NaCl < Na2S04 < Na2HPO4 < KH2PO4. This result is consistent with literature reports (Hartounian et al., 1994). This trend reflects the partitioning behavior of these salts, i.e., monobasic potassium dihydrogen phosphate partitions to the bottom phase more strongly than does disodium hydrogen phosphate, sodium sulphate and sodium chloride.

2B. 3. 4. Ultrafiltration

The dilute enzyme extract of volume 500 ml was concentrated by ultrafiltration in an experiment to a final volume of 50 ml. The initial activity of the extract was 730 u/ml with a specific activity of 256 u/mg of protein. After concentration by ultrafiltration, amyloglucosidase activity was found to be 6278 u/ml with a specific activity of 353.3 u/mg of protein. The total activity before and after ultrafiltration was 3,65,000 units and 3,13,900 units, respectively. Thus the total recovery of the enzyme after ultrafiltration was 86% with 1.38 fold purification.

2B. 3. 5. Enzyme extraction coupled with ultrafiltration

In a typical experiment, as summarized in Table 2B.3, the dilute enzyme extract of volume 515 ml was mixed with PEG (6000) / MDX to form ATPS having the total composition, in % w/w basis as PEG-7.5, MDX-40, KH_2PO4-1 and enzyme extract-51.5. After equilibration the enzyme was partitioned into top phase of volume 320 mI. The top phase containing enzyme was subjected to one more ATPE (back extraction) by diluting the total volume to 512 ml, and then adding required amount of Na_2HPO4 in order to obtain the desired phase composition that gave the lowest partition coefficient (Fig. 2B.3) that makes the extraction most efficient. After equilibration the enzyme partitioned to the Na_2HPO_4 rich bottom phase (332 ml), which was further concentrated to 43 ml by ultrafiltration. The enzyme recovered by this method (78.4%) was comparable to enzyme recovered by direct ultrafiltration (86%). The back extraction of the enzyme from PEG phase into salt phase has facilitated a simple way to remove most of the PEG from the enzyme solution and enables the recycling of the PEG phase. This removal of

the polymer is required in later purification procedures for the enzyme. In addition, employing ATPE prior to ultrafiltration has considerably reduced the bulk volume by half that is to be processed by ultrafiltration. This becomes much more significant on large-scale. More importantly by partitioning the contaminants into the opposite phases, relatively higher degree of purification (3.1 fold) is achieved by A TPE followed by ultrafiltration than direct ultrafiltration alone (1.38 fold). Thus, a two stage ATPE followed by ultrafiltration yielded an overall recovery of 78.4% with 12 fold concentration and 3.1 fold purification of the enzyme, amyloglucosidase produced by SSF.

2B. 3. 6. Economical aspects

One of the important factors that is critical in the industrial purification of enzymes by ATPE is the selection of the appropriate system. Most of the enzyme purification methods employing aqueous two-phase extraction reported in the literature use PEG/dextran system. However, the cost of fractionated dextran is very high (approximately $ 500/Kg). The PEG/MDX system appears to be the most cost effective polymer-polymer type ATPS (cost of MDX is $ 1.0 Kg). Further, recycling of PEG rich phase improves the economics of aqueous two-phase extraction.

2B. 4. CONCLUSIONS

Partitioning behavior of an enzyme (amyloglucosidase) produced by SSF was studied for the first time by employing ATPS. Phase diagrams were developed for PEG (4000, 6000)/MDX two-phase systems. A purification approach by using two stage ATPE coupled with ultra-filtration was shown to be more efficient (with higher purification factor) and economical alternative for extraction, purification and concentration of amyloglucosidase produced by SSF on large-scale.

Table 2B. 1. Physical properties of PEG (4000) / MDX system.

System	Composition (% w/w)			Viscosity (mPa s)		Density (kg m-'»		L1 density
	MDX	PEG	H_2O	Top	Bottom	Top	Bottom	(kg m-^{3})
A I	30	12	58	15.2	64.3	1032	1162	I 30

B	I	32	13	55	16.7	66.8	1036	1164	I 28
C	I	32	14	54	17.1	67.5	1038	1165	I 27

Table 2B. 2. Physical properties of PEG (6000) / MDX system.

System		Composition (% w/w)			Viscosity (mPa s)		Density (kg m -.>)		~ density (kg m -3)
		MDX	PEG	H_2O	Top	Bottom	Top	Bottom	
a	I	40	7.5	52.5	14.3	62.6	1037	1079	42
b	I	40	8.7	51.3	15.8	65.7	1038	1080	42
c	I	40	10.0	50.0	16.8	66.2	1039	1082	43
d	I	40	11.2	48.0	17.2	66.8	1041	1083	I 42

Table 2B. 3. Purification and concentration of amyloglucosidase using two-stage

A TPE coupled with ultrafiltration

Fraction	Enzyme activity (ulml)	Total activity (u)	Total protein (mg)	Specific activity (ulmg protein)	Purification factor	% Recovery
Crude extract	730.0	75950	1468.8	256.0	1.00	
I-ATPE (top Phase)	1047.0	35040	472.2	709.6	2.77	89.12
II-ATPE (bot. Phase)	941.6	12611	375.2	833.3	3.26	83.15
Ultrafiltration	6856.3	94821	371.7	793.2	3.10	78.42

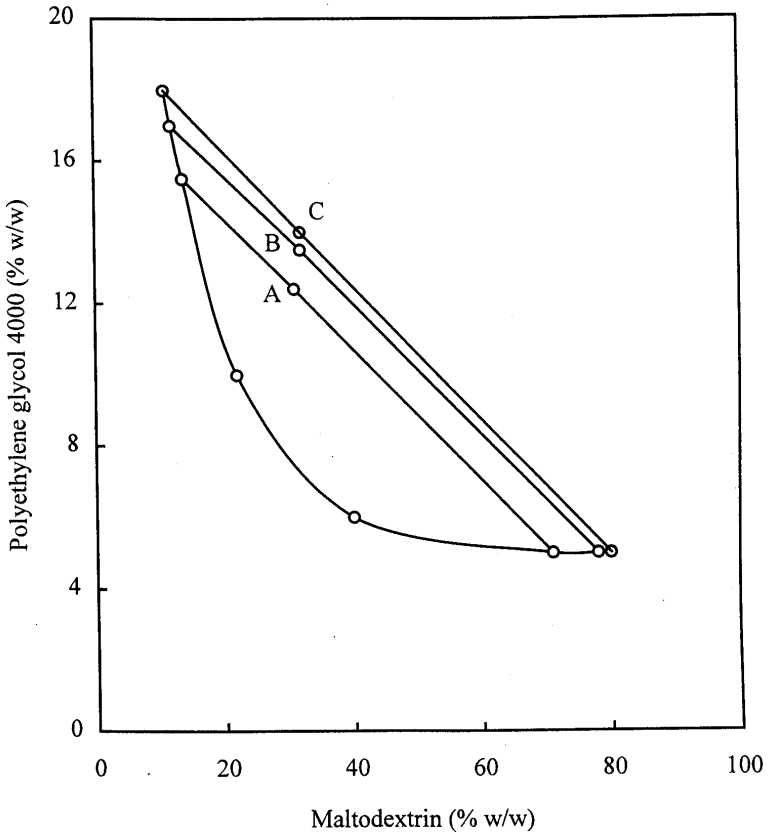

Figure 2B. 1. Phase diagram for PEG (4000) / maltodextrin aqueous two-phase system

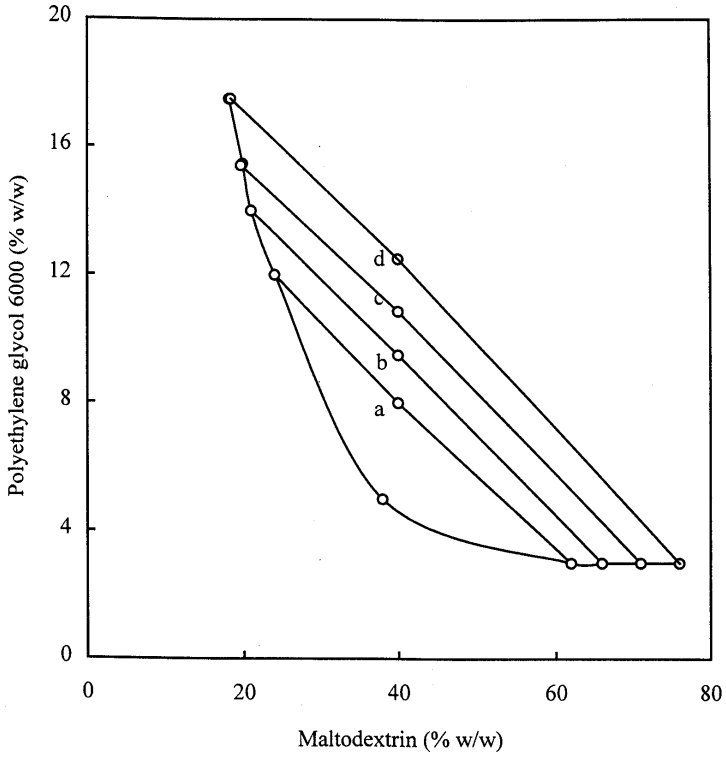

Figure 2B. 2. Phase diagram for PEG (6000) / maltodextrin aqueous two-phase system

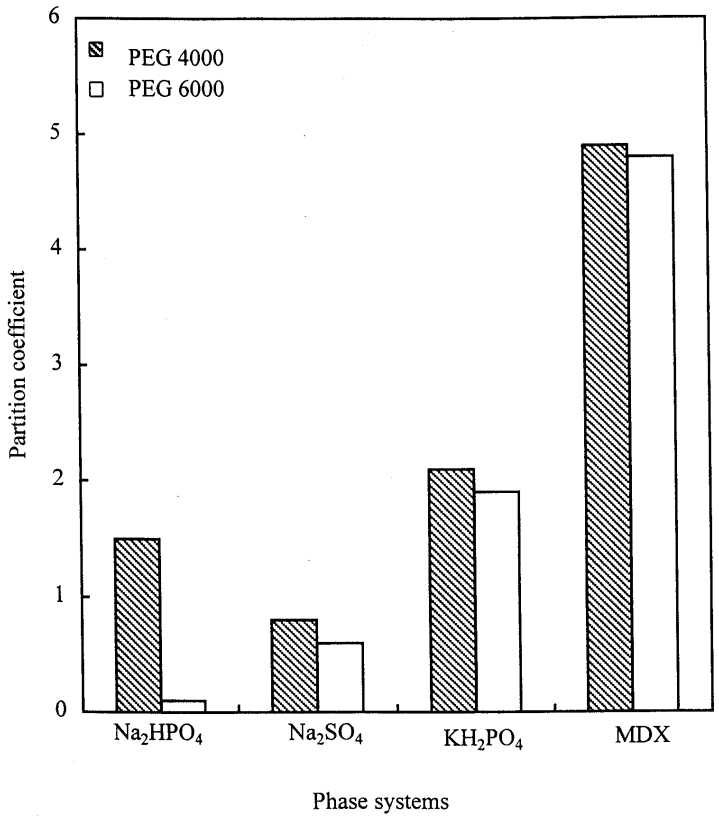

Figure 2B. 3. Partition behavior of amyloglucosidase in different phase systems containing PEG (4000/6000) as one of the phase forming polymers

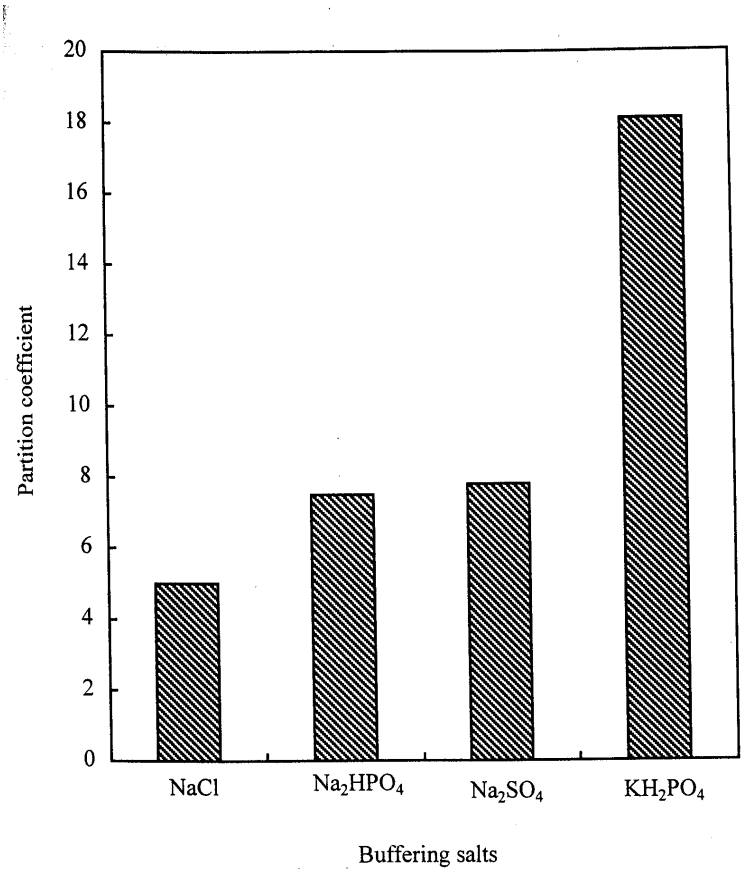

Figure 2B. 4. Effect of different buffering salts on partition behavior of amyloglucosidase in PEG (6000)/maltodextrin two phase system

CHAPTER 3

Field Assisted Enhanced Demixing of Aqueous Two-Phase Systems

SECTION - A

Acoustic Field Assisted Enhanced Demixing of Polymer/Salt Aqueous Two Phase Systems

3A. 1. INTRODUCTION

Major hindrances for application of aqueous two-phase extraction (ATPE) technique in bio-industries are; high cost of the phase forming polymers and slow demixing rates (Raghavarao et aI., 1995, 1998). The former aspect is solved to a great extent by adapting temperature induced phase separation for recovery and recycling of the polymers (Galaev and Mattiasson, 1993; Johansson et aI., 1997), while, the latter aspect is not addressed to the same extent. The slow demixing of the thoroughly mixed phases is due to a small difference in densities between them, high viscosity of the individual phases and low interfacial tension (Hustedt et aI., 1985; Albertsson, 1986). In polymer/salt systems where the desired molecule has a favorable partition coefficient, a single partition step may suffice and phase demixing by gravity may be satisfactory. However, in systems, where multistep partitioning is required gravitational separation of the phases becomes very time consuming and faster demixing is highly desirable (Vikrostrom et aI., 1987).

Asenjo and coworkers (Kaul et aI., 1995) have studied in detail phase separation kinetics of polyethylene glycol (PEG, MW 4000)/potassium phosphate system under gravity. Their investigations were found important for the design of large-scale aqueous two-phase separators. They observed a characteristic change in phase separation time at the phase inversion point and this point at each tie-line length corresponds to a fixed phosphate concentration. Albertsson (1986) has described separation times for various systems at different volume ratios and observed a sudden decrease in time of phase separation as the volume ratio (top:bottom) was increased above a certain point.

Efforts have been directed to speeding up phase separation by methods alternative to the conventional gravity settling (Hustedt et aI., 1985) and centrifugation (Hustedt et aI., 1989) such as electrokinetic demixing (Brooks et aI., 1984; Raghavarao et aI., 1990, 1991, 1998) and magnetic field assisted demixing (Vikrostrom et aI., 1987; Larsson 1994)-each having its own drawbacks (Sikdar et aI., 1991). From the economic point of view, centrifugation becomes prohibitively expensive on large scale. Electrokinetic demixing requires fabrication of special equipment and chemical additions such as salts to the polymer/polymer systems (Raghavarao et aI., 1990, 1991) and this method is not applicable to PEG/salt systems. Magnetic field assisted process requires addition of

micron sized iron particles or ferro-fluids to the system. The technique was not found useful when the PEG phase was dispersed in a PEG/dextran system (Vikrostrom et aI., 1987). Further micron-sized particles may aggregate in a strong magnetic field, which may cause the procedure to become inoperative, since the magnetic field would force the particles out of the two-phase system. Ferro-fluids may also affect the activity of the enzymes when they are present at low concentrations owing to poorly coated iron oxide particles, which will segregate into larger entities (Larsson, 1994).

All these observations indicate that there is a need for newer methods to enhance the demixing rates in ATPSs in a simple and cost effective manner. Application of acoustic field is one such potential method. Conventional wisdom indicates that application of acoustic field causes mixing rather than demixing of the systems, since it imparts energy to the system in order to achieve dynamic agitation, shear, cavitation, heating etc. This can be easily seen by the application of acoustics for cleaning of surfaces and disruption of microbial cell walls. However, we found that this is not true in case of acoustic field of higher frequency (MHz range). Our preliminary experiments showed that application of acoustic field has decreased the demixing time in PEG/salt systems by about 50%. Since the method was found simple, easy to scale-up and economical (the acoustic transducer being high voltage and low current devices), we carried out a detailed study of this method in PEG/potassium phosphate ATPS.

It is known that kinetics of phase demixing strongly depends on the physical properties such as density, viscosity and interfacial tension of the system. Changing the composition of the system can largely vary these properties. Hence, we studied the effect of phase composition on demixing times in PEG/phosphate system both under gravity and in the presence of acoustic field. From the practical point of view it is desirable to concentrate the target biomolecules/cells into small volumes of the phases. Hence, the effect of volume ratio was investigated. Large scale processing involves large amounts of cells in the phase system. Presence of cells and cellular components in the system significantly increases the demixing time due to the formation of stabilized emulsion (Larsson, 1994). Therefore, we also investigated the effect of microbial cells on the demixing times in PEG/phosphate system in settling under gravity alone and under the influence of acoustic field.

3A. 1. 1. Theoretical aspects of phase demixing

Some of the researchers have assumed that approximately the Stoke's law (which was developed for a rigid sphere)

can describe phase de mixing under unit gravity;

$$V = \frac{D^2 \Delta \rho g}{18 \mu_C} \qquad (1)$$

where D is the droplet diameter, Δp is the density difference between the phases, μc is the dynamic viscosity of the continuous phase, g is the acceleration due to gravity and V is the droplet rise/fall velocity.

Asenjo and coworkers (1995) have rightly indicated that for a swarm of droplets considerable deviations from the Stoke's law can be expected. As the droplets are not rigid the circulation inside them (induced by the drag of the continuous phase) has to be taken into account as given by the Hadamard Rybzcynski equation

$$V = \frac{D^2 \Delta \rho g}{18 \mu_C} \left(\frac{3\mu_D + 3\mu_C}{3\mu_D + 2\mu_C} \right) \qquad (2)$$

where μc and μD are the viscosities of the continuous and dispersed phases, respectively.

Phase demixing can be seen as a combined effect of droplet rise/fall and droplet coalescence. If a single droplet is considered then the two steps are clearly in series. The droplet has to rise/fall to the interface and there it coalesces with the interface (Kaul et aI., 1995). In this situation, droplet migration will be the controlling step in the overall demixing process. In ATPSs this situation can be seen when the phase volume ratios are either very high or very low. The time required for the separation of the two phases at this situation can be represented by equation (2).

However, this may not be the case generally. The presence of multiple droplets leads to considerable droplet-droplet interaction, which leads to coalescence as they rise/fall. This will increase the droplet size and in turn alters their rise/fall velocities (proportional to the square of the droplet diameter). Hence, coalescence will be the controlling step, which is also the observation made by us in the present study.

3A. 2. MATERIALS AND METHODS

3A. 2. 1. Chemicals

Polyethylene glycol (Batch No. T/823081) with a molecular weight of 6000 daltons was obtained from Sisco Research Laboratories (Mumbai, India). Potassium dihydrogen phosphate and di-potassium hydrogen phosphates were obtained from Ranbaxy Chemicals (Punjab, India). Glucose and yeast extract were obtained from Difco Research Laboratories (Delhi, India). All other chemicals used were of analytical grade.

Yeast cells (Saccharomyces cerevisiae) in the form of cakes were purchased from local market. Cell mass of Lactobacillus casei was obtained by growing them in a 2 liter bioreactor at 37°C with the medium composition explained elsewhere (Demirci et aI., 1998). Prior to use, both yeast cells and 1. casei cells were thoroughly washed with distilled water and centrifuged at 5,000 rpm for 30 minutes. The supernatants containing some of the soluble contaminants were discarded. The pellets were dried at 50°C for 2 hours and then used for the experimentation.

3A. 2. 2. Phase systems preparation

Predetermined, weighed quantities of polyethylene glycol and potassium phosphates (K_2HPO_4:KH_2P0_4=1.82:1) were added to a known quantity of distilled water, so as to make the total weight of the system as 100% on w/w basis. After dissolving the components, the system was mixed thoroughly for 1 hour and allowed to separate in a separating funnel for overnight. The equilibrated and separated phases were then collected and used as stock solutions for further experiments. In this way 300g of systems were prepared at each phase composition. The phase compositions of the different systems employed in the present work are given in Table 3A.l.

3A. 2. 3. Phase demixing experiments

All the demixing experiments were carried out in a 100 ml capacity-measuring cylinder with a height to diameter ratio of 8.75. All the demixing studies were carried out at 3 volume ratios of the top to bottom phase, viz. 30/70, 50/50 and 70/30. The column was filled with a 100 ml freshly prepared thoroughly mixed (for 10 minutes) phase dispersion. The dispersion was subjected to ultrasonication at a frequency of 1.2 MHz and at a power level of 1.2 W/cm^2 using an ultrasonic transducer (model # HM-460, Holmer Products Corp, MA). The dispersion height was defined as the height of the un-separated cloudy/turbid region of dispersion. The dispersion height was recorded as a function of time. For all the experiments, sonication was given from the bottom and with a travelling wave mode with the transducer at the bottom of the dispersion. Schematic diagram of acoustically assisted process is shown in Figure· 3 A.I. Similar demixing experiments were carried out under gravity alone in the absence of ultrasonic at ion. For all the experiments, the time for complete phase separation was taken as the time required for a clear horizontal interface to be formed. However, in the experiments with the cells, one or two droplets were found to present at the interface even after the formation of the interface. For the experiments with the cells, predetermined, weighed quantities of cells were added to a 100 ml of dispersion.

All the demixing experiments were carried out in triplicate and mean values reported. Accuracy of the measurements was found well within ± 5%.

3A. 2. 4. Phase density and viscosity measurements

Density and viscosity of the individual phases were measured by usmg specific gravity bottle and Ostwald- U -tube viscometer of 10 ml capacity, respectively. All the measurements were carried out in duplicate at 27 ± 0.5 °C and average values reported.

3A. 3. RESULTS AND DISCUSSION

3A. 3. 1. Demixing behavior with respect to phase composition

Systems of various phase compositions (Table 3A.l) were selected to examine the effect of phase composition on demixing in PEG/phosphate system. Figures 3A.2 - 3A.4 depict the kinetics of phase demixing (dispersion height as a function of time) in some of the selected phase compositions both in the presence and absence of acoustic field. Demixing times for remaining phase compositions were given in Table 3A.2.

Droplet coalescence is the critical factor for demixing in ATPSs. Ultrasonication has caused a mild circulation currents in the phase dispersion thereby increasing the droplet-droplet interaction (collision), as the binary collision frequency is proportional to the shear (Arp and Mason, 1976). Increase in collision frequency enhances the probability of coalescence as the droplets rise/fall, which in turn enhanced the demixing rates due to increased migration velocity of the larger droplets. This could be observed by the enhanced slopes in Figures 3A.2 - 3A.4.

In order to bring out the positive effects of acoustics more clearly, demixing time was plotted against phase volume ratio with phase composition as a parameter in Figure 3A.5. It can be seen that as phase composition increased demixing time decreased gradually under gravity as well as in acoustically assisted process. The acoustically assisted process has resulted in an about 14, 43 and 55 % reduction in demixing times at high (35/11), intermediate (15/11) and low phase compositions (7/11), respectively as shown in Figure 3A.6. This decrease in the efficacy of acoustics with an increase in phase composition is due to attenuation of ultrasound intensity by increase in phase viscosity. Attenuation of ultrasound intensity was also observed in a given phase composition when the top viscous phase was continuous (Fig. 3A.6).

Kula et al. (1982) have suggested that in PEG/salt systems, the fastest separation could be expected at intermediate compositions especially when the volume of the higher viscous phase is smaller than that of the lower viscous phase. However, we did not observe the fastest separation at the intermediate phase composition. Instead, as phase composition increased, de mixing time kept on decreasing (Figs. 3A.2 - 3A.4). Further, at all the studied phase compositions, the demixing time was higher when the volume of the higher viscous (PEG rich) phase was smaller than that of the lower viscous (salt rich) phase (30/70 volume ratio) as shown in Table 3A.2 and Figure 3A.5. The

observed decrease in demixing time with an increase in phase composition mentioned above (Fig. 3A.5) is mainly due to the increase in droplet size of the dispersed phase with an increase in phase composition.

Increase in phase composition increases the interfacial tension, density difference and phase viscosities (Table 3A.1). The combined effect of these properties is that the average drop size and the drop velocity increase with an increase in the system composition (Jafarabad et aI., 1992). Visual observations indicated that as phase composition increases, average droplet size increases at all volume ratios. Higher the droplet size, it rises/falls faster before it coalesces with the next droplet (Kaul et aI., 1995) thereby decreasing the demixing time. As a result the demixing time decreased with an increase in phase composition. In polymer/polymer type aqueous two-phase systems, it was reported that at high phase compositions demixing rate becomes very slow owing to a very high viscosity of the phases (Alberts son, 1986), which drastically reduces the migration of the phase droplets. However, such effect of viscosity in reducing the migration of droplets was not observed in our studies at the examined phase volume ratios.

3A. 3. 2. Demixing behavior with respect to phase volume ratio

It may be noted from Figure 3A.5 that volume ratio has a significant effect on demixing time at low phase composition and the effect decreased gradually as phase composition increases. As a general trend, demixing time decreased with an increase in volume ratio. An exception for this trend is at 50/50 volume ratio of low (7/11) phase composition where demixing time was higher than 30/70 volume ratio. Visual observations indicated that at a given phase composition, droplet size increased with an increase in phase volume ratio, resulting in a decrease in demixing time with an increase in phase volume ratio. At 70/30 volume ratio salt is the dispersed phase and more viscous PEG phase forms the continuous phase. Owing to higher viscosity of the continuous phase, relatively less energy out of the energy given to form the dispersion, will be transferred to the dispersed phase when compared to the situation where PEG phase is dispersed and salt phase of much lesser viscosity forms the continuous phase (50/50 and 30/70 volume ratios) Hence, the droplet size at 70/30 is larger than that at 50/50 or 30/70 volume ratio, resulting in lower demixing time at this volume ratio at any given composition.

At 50/50 and 30/70 volume ratios, dispersed phase being the same (PEG phase), the lower demixing time at 50/50 volume ratio than that at 30/70 volume ratio at intermediate and high phase compositions (Fig 3A. 5) is mainly due to the higher droplet size at 50/50 volume ratio (visual observations). This higher droplet size at 50/50 is the result of relatively lower energy availability (energy/unit volume) to form the dispersion owing to higher volume of the dispersed phase when compared to that of 30/70 volume ratio. However, as an exception to this trend, at 50/50 volume ratio of low phase composition, although the droplet size was observed to be higher, demixing time was higher than that at 30/70 volume ratio as shown in Figure 3A.5. Visual observations indicated that at this volume ratio the droplets after reaching the interface, assembled near the interface forming a densely packed zone and took longer time to coalesce with the already formed PEG layer. The formation of dense packed zone was due to the lower rate of droplet coalescence with the interface when compared to the rate of migration of the droplets. The droplet packing was also observed in 30/70 volume ratio of low phase composition. However, due to lower

concentration of the dispersed phase droplets the density of packing was lower than that at 50/50 volume ratio. This kind of accumulation was observed only at this phase composition.

It can be noted that, the extent of decrease in demixing time when the volume ratio was increased from 30/70 to 70/30 reduced with an increase in phase composition (Figs. 3A.5 and 3A.6). For instance in gravity demixing, the extent of decrease in demixing was about 51, 18 and 8 % at 7/11, 15/11 and 35/11 phase compositions, respectively (Fig. 3A.5). This is due to the synergistic effect of increase in droplet size with an increase in phase composition as well as phase volume ratio.

3A. 3. 3. Phase demixing in presence of microbial cells

Addition of microbial cells to PEG/phosphate system significantly increased the demixing time. Presence of the cell surface associated biomolecules and their interaction with the phase system will affect the charge, size and hydrophobicity of the dispersed phase droplet and thus alters its surface properties, which in turn discourages droplet coalescence. This could be responsible for increase in the de mixing time. It was reported that presence of cellular components and other biomolecules in ATPSs can form stabilized emulsions that will considerably increase the phase separation time (Larsson, 1994). Table 3A.3 shows the experimental results of effect of yeast and L. easei cells on the demixing behavior in PEG/phosphate system at 1 and 8 % cell concentration. Experiments were carried out at an intermediate composition of PEG 15% and potassium phosphate 11 % because of suitability of this phase composition for industrial practice. In both the cases, almost all cells partitioned to bottom phase forming a band between the interface and bottom phase. Demixing time in the case of yeast cells was found to be much higher when compared to 1. easei cells. It was observed that in both the cases volume ratio had a little effect on demixing time, like that of the pure system at intermediate phase composition. Hence, effect of cell concentration (2-7%) was studied at 70/30 volume ratio only.

As cell concentration was increased, at a constant volume ratio, demixing time increased significantly in case of yeast cells. However, a very marginal increase in demixing time was observed in case of 1.easei cells as indicated in Figure 3A.7. It is known that yeast cell wall contains manno-proteins and lipo-polysaccharides (Valetin et aI., 1984), which stabilizes the dispersion by adsorbing at the interface. As the extent of this stabilization increases with an increase in yeast cell concentration, demixing time increased consistently with an increase in cell concentration (Fig. 3A.7). However, 1.easei cell wall is not known to contain any such materials having emulsifying properties, which appears to be the reason for insignificant increase in demixing time with an increase in cell concentration.

The acoustically assisted process was found to decrease the demixing time significantly in these systems having cells. In case of L. easei cells up to 40% and in case of yeast cells up to 60% decrease in demixing times were observed (Table 3A.3 and Fig. 3A.7). In addition to the advantages already mentioned, another attractive feature of this acoustic method is it provides gentle treatment to the system, thereby not causing any damage to the cells. This is quite in contrast to the classical application of acoustic field of lower frequency (kHz range) for disruption of cells

to release intracellular compounds. Further, the rise in temperature of the system due to acoustic field application was found to be very less when compared to that of electrokinetic demixing process. This is more true in case of PEG/salt systems wherein application period of acoustic field is short.

Addition of microbial cells did not affect the physical properties such as density and viscosity of the systems (Tables 3A.4 and 3A.5). However, it may be noted that as the cell concentration increased a slight decrease in the viscosity of the top phase in case of L. casei cells (Table 3A.4) and a slight increase in case of yeast cells were observed (Table 3A.5). This slight variation in viscosity may be due to adsorption of polymeric components at the cell surface and release of surface associated cellular components to the aqueous medium. The adsorption at the cell surface appears to be predominant in case of L. casei cells, hence a slight decrease in viscosity was observed. On the other hand, release of cell associated components appears to be predominant in case of yeast cells, therefore a slight increase in viscosity was seen.

3A. 4. CONCLUSIONS

Acoustic demixing, a new method for enhancing the demixing rates of PEG/salt ATPSs is reported. The effect of phase composition and volume ratio on demixing behavior was explained mainly based on the dispersed phase droplet size, which is the resultant effect' of physical properties of the system. The present work suggests that the acoustic demixing of two-phase systems would be useful in enhancing the demixing rates of the phases especially at low and intermediate phase compositions irrespective of phase volume ratio and even at high microbial cell concentration. The process is simple, economical, easy to scale up and readily available ultrasonic transducers could be employed.

Table 3A. 1. Viscosity and density at various phase compositions of PEG (6000) 1 potassium phosphate two phase system

Sl. #	Phase composition (PEG/salt,% w/w)	Viscosity Top phase (mPa.s)	Viscosity Bot. phase (mPa.s)	Density Top phase (kg m-3)	Density Bot. phase (kg m-3)
1	7/11	11.68	1.21	1121.9	1143.0
2	10/11	12.29	1.23	1130.9	1152.9
3	15/11	24.47	1.41	1135.2	1190.7

4	10/16	36.77	1.40	1138.2	1209.0
5	25/11	56.79	ISI	1142.6	1253.7
6	20/16	71.99	1.68	1144.4	1276.3
7	35/11	97.75	1.83	1152.0	1330.0

Table 3A.2.Demixing times at different volume ratios of various phase compositions in PEG(6000) / potassium phosphate two phase system

Phase	70/30		50/50		30/70	
Composition	volume ratio		volume ratio		volume ratio	
PEG/salt, %w/w)	Gravity	Acoustics	Gravity	Acoustics	Gravity	Acoustics
10/11	5.80	3.35	11.96	5.83	10.92	4.80
10/16	4.83	3.14	5.28	3.35	6.16	3.82
25/11	4.10	2.82	4.20	2.86	4.40	2.94
20/16	3.5	2.5	3.70	2.6	3.87	2.65

Table 3A. 3. Demixing times at 15% PEG and 11 % potassium phosphate two-phase system in presence of yeast and L.casei cells

Cell	V olume ratio	L.casei cells		Yeast cells	
Concentration	(top/bot.)	demixing time (min)		Demixing time (min)	
(% w/v)		Gravity	Acoustics	Gravity	Acoustics
	70/30	12.00	8.00	18.00	10.00
1	50/50	12.20	8.10	19.00	10.30
	30/70	12.10	8.00	18.40	10.00

	70/30	13.20	8.10	89.00	37.00
8	50/50	13 .40	8.20	94.00	40.00
	30/70	13.20	8.10	92.00	40.00

Table 3A. 4. Viscosity and density of 15 % PEG and 11 % potassium phosphate two phase system in presence of L. casei cells

Cell concentration (% w/v)	Volume ratio (top/bot.)	Viscosity (mPa.s)		Density (kg m -3)	
		Top phase	Bot. phase	Top phase	Bot. phase
0	70/30	17.01	1.46	1134.1	1166.9
	70/30	17.23	1.46	1134.2	1166.4
1	50/50	17.21	1.46	1132.1	1167.3
	30170	17.19	1.46	1131.0	1171.3
2	70/30	16.45	1.46	1134.5	1163.8
4	70/30	15.75	1.46	1133.3	1165.8
6	70/30	15.42	1.46	1133.8	1167.5
8	70/30	14.98	1.46	1134.6 .	1168.3

Table 3A. 5. Viscosity and density of 15 % PEG and 11 % potassium phosphate two phase system in presence of yeast cells

Cell concentration (% w/v)	Volume ratio (top/bot.)	Viscosity (mPa.s)		Density (kg m-3)	
		Top phase	Bot. phase	Top phase	Bot. phase
0	70/30	17.01	1.46	1134.1	1166.9
	70/30	17.51	1.46	1137.1	1166.8
1	50/50	17.19	1.46	1138.1	1166.7
	30/70	17.33	1.46	1140.0	1165.8
2	70/30	18.21	1.46	1138.0	. 1166.2
4	70/30	19.98	1.46	1141.6	1166.0
6	70/30	21.24	1.46	1142.0	1166.2
8	70/30	22.64	1.46	1143.3	. 1166.3

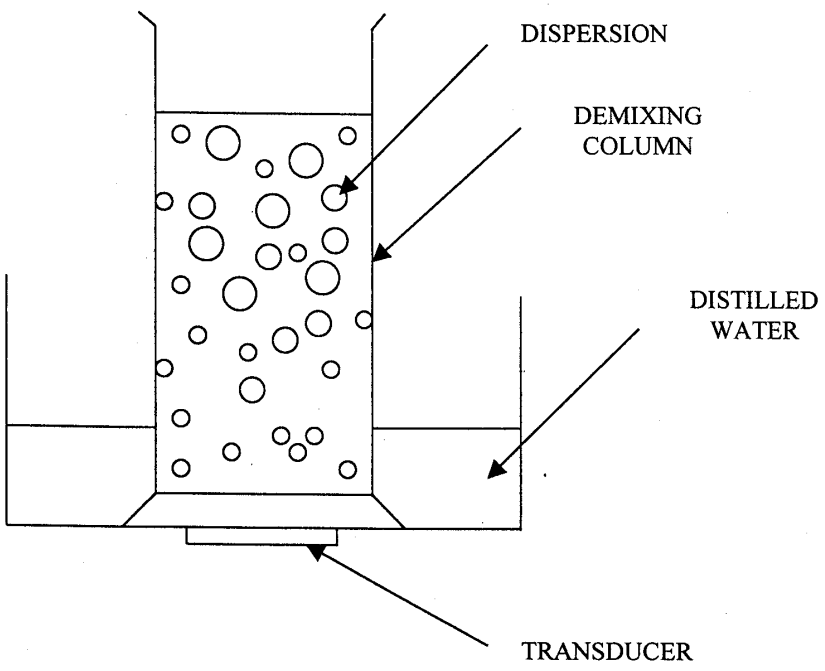

Figure 3A. 1. Schematic diagram of acoustically assisted demixing process

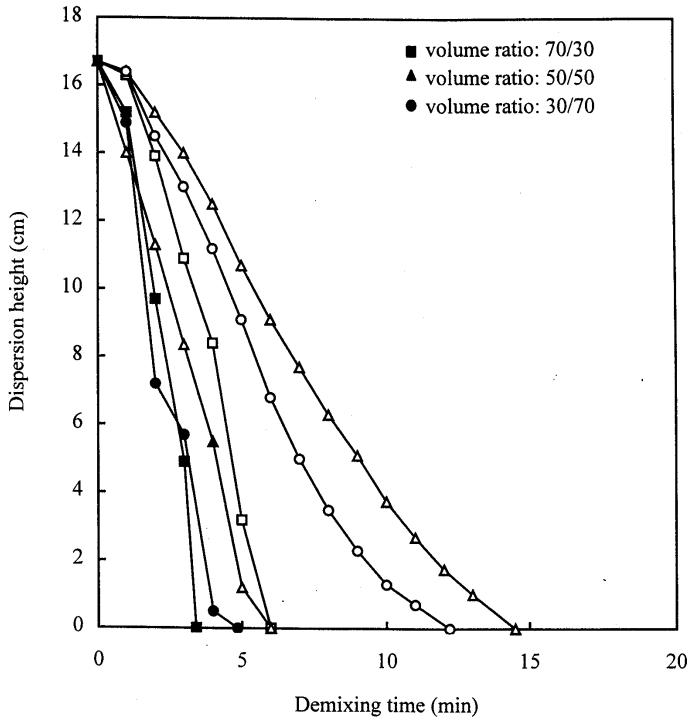

Figure 3A. 2. Kinetics of phase de mixing at 7 % PEG and 11 % potassium phosphate two phase system (dispersion height as a function of time)
open symbols: demixing under gravity alone
closed symbols:demixing under ultrasoncation

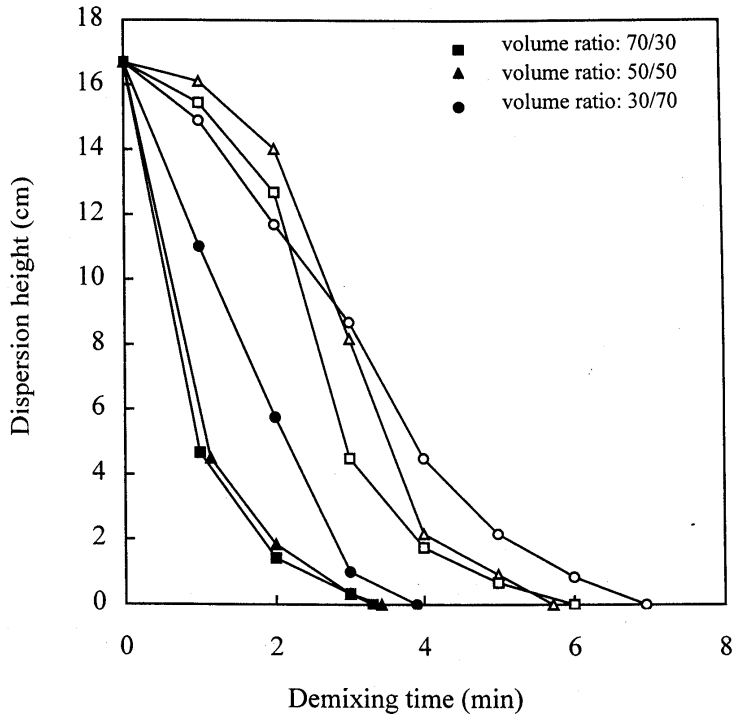

Figure 3A. 3. Kinetics of phase de mixing at 15 % PEG and 11 % potassium phosphate two phase system

(dispersion height as a function of time)

open symbols: demixing under gravity alone

closed symbols: demixing under ultrasoncation

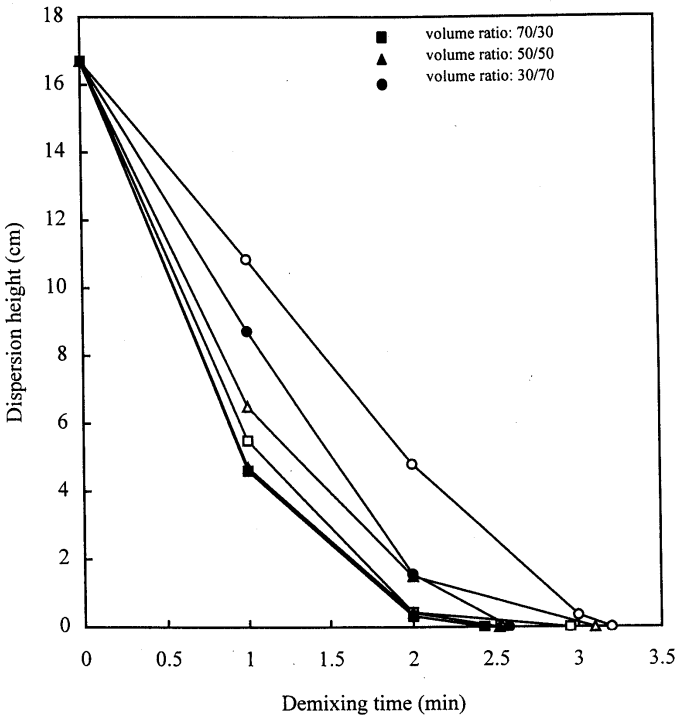

Figure 3A. 4. Kinetics of phase demixing at 35 % PEG and 11 % potassium phosphate two phase system (dispersion height as a function of time

open symbols: demixing under gravity alone

closed symbols: demixing under ultrasoncation

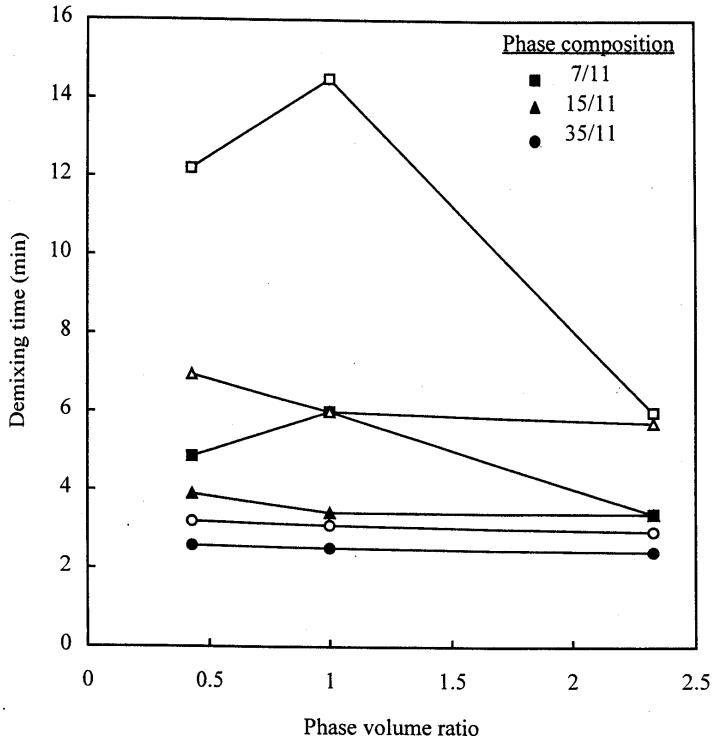

Figure 3A. 5. Effect of phase volume ratio on demixing time in PEG/potassium phosphate two phase system

open symbols: demixing under gravity alone

closed symbols: demixing under ultrasonication

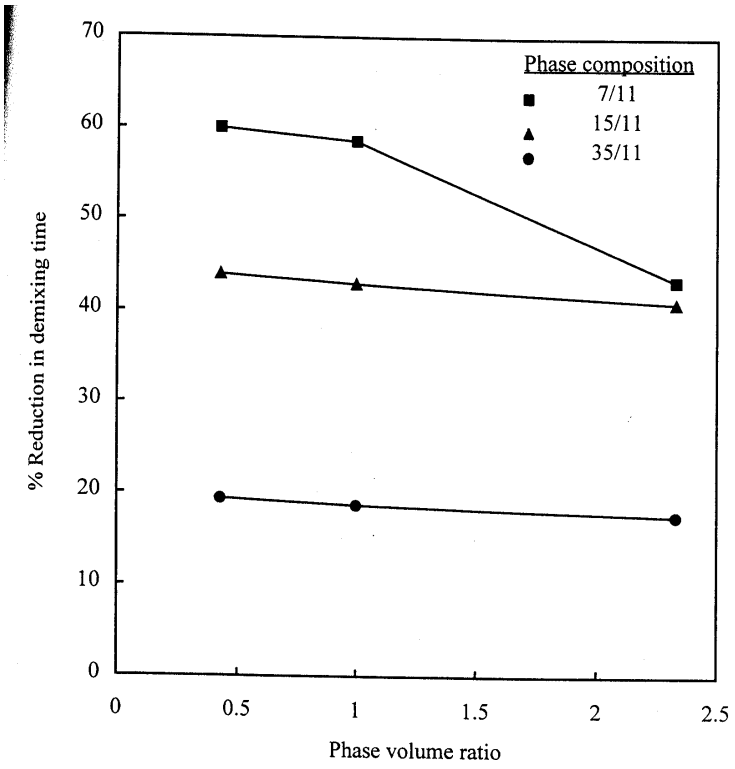

Figure 3A. 6. Efficacy of acoustics on de mixing behavior in PEG/potassium phosphate two phase system

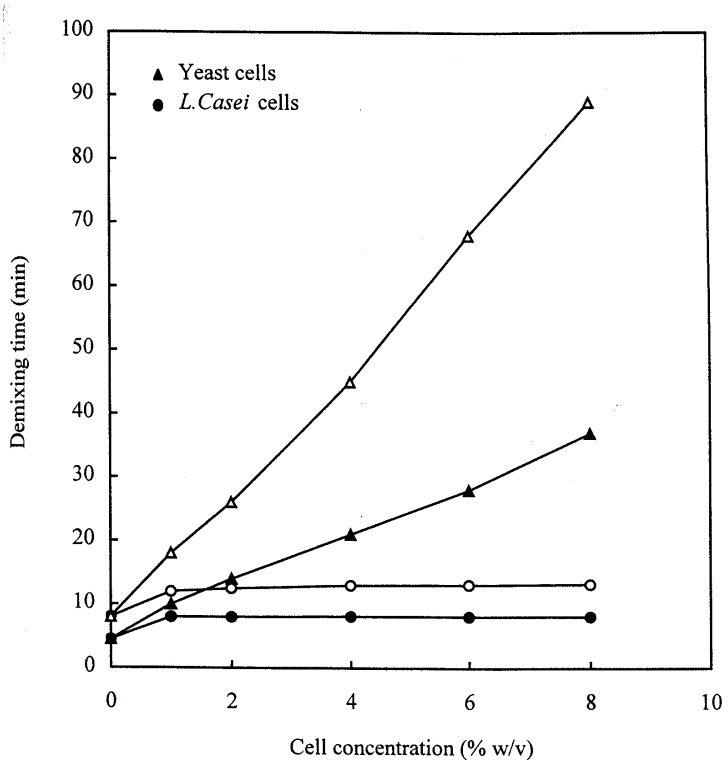

Figure 3A. 7. Effect of microbial cell concentration (1 to 8 %) on demixing time at 70/30 volume ratio of 15 % PEG and 11 % potassium phosphate two phase system.

open symbols: demixing under gravity alone

closed symbols: demixing under ultrasoncation

SECTION - B

Acoustic Field Assisted Enhanced Demixing of Polymer / Polymer Aqueous Two-Phase Systems

3B. 1. INTRODUCTION

In the previous section (3A) of this chapter, the problems encountered due to slow rate of phase demixing in ATPSs, different approaches available in the literature to enhance this slow demixing rate, the draw backs of these processes, which necessitates the need for newer methods have been discussed in detail. For the first time, it was shown that acoustically assisted process could be conveniently employed to enhance the demixing rates in polymer/salt ATPSs. Furthermore, the process was demonstrated to be simple, easy to scale up and cost effective, since available transducers could be used.

Although polymer/salt systems are highly suitable for industrial extraction of proteins, polymer/polymer systems are required in affinity partitioning. This is due to the interference caused by the high salt concentration of polymer/salt systems in majority of the protein-ligand affinity interactions, which are of electrostatic in nature (Walter et aI., 1991). Demixing rates of polymer/polymer systems are much slower than polymer/salt systems, due to high phase viscosities, much lower interfacial tension and similar phase densities. Hence, there is certainly a need to enhance the demixing rates in polymer/polymer systems and this has been attempted in the present study by employing acoustic field.

The most popular polymer/polymer system studied for research purpose is PEG/dextran. Purified dextran being very expensive ($ 500 per kg) severely limits its use on large-scale. In order to improve the economics, crude dextran and partially hydrolyzed dextran were explored as phase forming polymers along with PEG and detailed partition studies were conducted for many enzymes/proteins to demonstrate their suitability (Kroner et aI., 1982). PEG/hydroxypropyl starch system (cost of hydroxypropyl starch $20 per kg), which showed similar characteristics as that of PEG/dextran system was also successfully employed for the large-scale extraction and purification of enzymes from pig muscle (Tjemeld et aI., 1987). On similar lines, an inexpensive polyethylene glycol/maltodextrin (PEG/MDX) system (cost of MDX $ 1.0 per kg) developed by Slag and Guilano (1988) appears to have a good scope on industrial scale. Hence, in the present work we selected PEG/MDX system to study the influence of acoustic field on phase demixing. The study in detail include, the effect of various parameters such as phase composition, salt concentration, duration of time of applying acoustic field and microbial cell concentration on demixing time in this system.

3B. 2. MATERIALS AND METHODS

3B. 2. 1. Chemicals

Polyethylene Glycol (PEG; MW 6000, Batch # T/820326) was purchased from Sisco Research Laboratories, Mumbai, India and mal to dextrin (MDX; MW 105000) was procured from Laxmi Starch Private Limited, Mumbai, India and sodium chloride was purchased from Qualigens Fine Chemicals, Mumbai, India.

Escherichia coli D21 obtained through the courtesy of Dr. M.A Linggood, Uniliver Research, United Kingdom, which is maintained as stock culture in the Department of Food Microbiology, CFTRI, Mysore was used for the study. The culture was maintained at 4 °C on a brain heart infusion (BHI) agar slants and subcultured at 30 day interval. Prior to use in experimental trials, the culture was propagated twice in BHI broth at 37°C. BHI broth in aliquots of 3 liters were sterilized, inoculated with active culture of E.coli, mixed well and incubated at 370C for 48 hours. Cells were harvested by centrifuging the medium for 30 minutes at 5000 rpm. The pellet obtained was washed with distilled water thoroughly and again centrifuged for 30 minutes at 5000 rpm. The washed pellet was dried at 50°C for 2 hours and then used for experimentation.

3B. 2. 2. Phase system preparation, demixing experiments and, density and viscosity measurements

Preparation of phase systems, phase demixing experiments and phase density and viscosity measurements were all carried out as explained in the section 3A. 2. However, in the case of phase demixing experiments, ultrasonication was provided with repeated cycles of sonication for a known period and followed by gravity alone for a known period. This was done to avoid excess heat generation in the system due to continuous application of acoustic field.

All the demixing experiments, and viscosity and density measurements were repeated thrice and average value was reported. Accuracy of the measurements were found well within ± 5%.

3B. 3. RESULTS AND DISCUSSION

3B. 3. 1. Effect of phase composition

Variation in phase composition changes the physical properties such as viscosity, density and interfacial tension of the system. As kinetics of phase demixing is greatly dependent on these physical properties, we studied the effect of phase composition on demixing. Various compositions selected are given in Table 3B.1 along with their viscosity and density values. Kinetics of phueeomilUng (dispersion height versus time) for a composition is shown in Figure 3B.1. Demixing times for various phase compositions are represented in Figure 3B.2. At each phase composition the demixing studies were conducted at 30170, 50/50 and 70/30 volume ratios. This study was conducted by realizing the fact that, from the process point of view, it is highly desirable to concentrate the product by reducing the volume of the phase to which it preferentially partitions.

Application of acoustic field has resulted in a notable decrease in demixing time in these systems. At low phase composition the intensity of decrease was insignificant. However, at intermediate and high phase compositions it has resulted in significant decrease in demixing time, particularly at 30/70 volume ratio where bottom phase is continuous. In these systems up to 2-fold decrease in demixing time was observed as indicated in Figure 3B.2. Although application of acoustic field has decreased the demixing time, the demixing behavior (total demixing time and demixing rate) was found to be same as that of gravity demixing.

In general, it was observed that in a given phase composition, demixing time remained practically similar or slightly varied at 70/30 and 50/50 volume ratios where top PEG light phase is continuous while, it increased dramatically at 30170 volume ratio where bottom MDX heavy phase is continuous (Fig. 3B.2). This indicates that demixing time depends largely on which of the phase is continuous, precisely on the viscosity of the continuous phase. Interfacial tension being very low, even lower than PEG/salt systems, its contribution to demixing can be neglected in polymer/polymer systems. Density difference remains same at any volume ratio of a given phase composition. Hence, it is only the viscosity, which largely determines the demixing rate in polymer/polymer systems. At 30/70 volume ratio, high viscosity of the continuous bottom phase retards the migration (rise/fall) velocity of the dispersed phase droplets, which eventually results in lengthening the demixing time as can be seen from the Hadamard-Rybzcynski equation given in section 3A of this chapter.

Albertson (1986) has reported that near the critical point the density difference is small and therefore the demixing time will be high. For way from the critical point polymer concentration and the viscosity are high, hence the demixing time will be high. Thus, at intermediate compositions demixing time is the shortest. Similar behavior was observed in our studies with an increase in phase composition only at volume ratios where top phase was continuous (70/30 and 50/50), but not at volume ratio where bottom phase was continuous (30/70). At this volume ratio (30/70), demixing time increased significantly with an increase in phase composition (Fig. 3B.2). The reason is that the high viscosity of the continuous bottom phase, which further increases with an increase in phase composition, dominates over the density effect causing significant retardation in migration velocity of the droplets and hence, results in higher demixing time.

Another noteworthy observation from Figure 3B.2 was that at any given phase composition, demixing time was shortest at 50/50 volume ratio. Continuous phase being same at 70/30 and 50/50 volume ratios, the lower demixing time in the latter case is due to higher volume of the dispersed phase, which provides higher concentration of droplets. The higher droplet concentration has lead to higher droplet-droplet interaction. Increased droplet interaction hastened the rate of droplet coalescence, resulting in an increase in droplet size. Bigger droplets traveled faster to the interface, as V is proportional to D^2 (Eqs. 1 and 2 of section 3A), eventually resulting in lower demixing time.

3B. 3. 2. Effect time duration of applying acoustic field

It is known that continuous application of ultrasonication increases the temperature of the system. Hence, to avoid this it is prudent to apply the acoustic field to the system discontinuously. That is in repeated intervals of a known period of acoustic field followed by gravity demixing. However, this problem does not arise in case of polymer/salt system, as the overall time required for phase demixing is very less. To find out the optimum condition we carried out the demixing experiments at various modes of repeated intervals of acoustic field followed by gravity field as listed below.

Mode (1) : 5 minutes acoustics field followed by 5 minutes gravity
Mode (2) : 10 minutes acoustics field followed by 10 minutes gravity
Mode (3) : 10 minutes acoustics field followed by 20 minutes gravity and
Mode (4) : 20 minutes acoustics field followed by 10 minutes gravity

Experiments were carried out at 30170 volume ratio of low and intermediate phase compositions and the results were tabulated in Table 3B.2. In view of the fact that acoustic effect was prominent only at 30/70 volume ratio (Fig. 3B.2), we selected this volume ratio for this aspect of study. In both phase compositions mode (4) type produced best results in terms of fold decrease in demixing time (about 1.7 to 2.2). However, the rise in temperature measured in the dispersion immediately after 20 minutes exposure to acoustic field was found to be around 10°C. From the practical point of view, this may not be suitable for many of the biomolecules especially for enzymes/proteins, which are heat sensitive. Hence, mode (1) type with practically similar results (about 1.5 to 2. I-fold decrease in demixing time), but with only around 1.5-2.0 °C rise in temperature, was found to be suitable.

3B. 3. 3. Effect of NaCI concentration on demixing

Addition of a salt to ATPSs can be used rather effectively to alter the phase diagram, partition coefficient of biomolecules and even the mass transfer of biomolecules as reported in the next chapter (section A). The effect of salt type and concentration can be dramatic when the proteins are far away from their isoelectric points. The dependence of the partition coefficient at high salt concentration in the PEG/DX aqueous system has been explored by Albertson (1986) and results for protein partitioning in the PEG/DX system with increasing NaCI concentration was studied. It was found that at high NaCI concentration proteins tend to favor the upper PEG rich phase. This could possibly be due to hydrophobic interactions with PEG or salting out. We have shown in chapter-2 that addition of salts to ATPSs has dramatically improved the partition behavior of a plant peroxidase and amyloglucosidase. Many researchers have achieved optimum extraction conditions with respect to purity and recovery of biomolecules in ATPSs by addition of neutral salts (Hustedt et aI., 1978; Kroner et aI., 1982; Menge et aI., 1983). Thus it is evident that addition of natural salt is highly required to achieve selective partitioning in ATPSs. Hence, we thought that it is worthwhile to study the effect of NaCI on phase de mixing as well. As very low demixing times were observed at 50/50 volume ratio of any studied phase composition, we restricted ourselves to study the effect ofNaCl concentration on phase demixing only at this volume ratio in an intermediate composition. Results obtained are shown in Figure 3B.3 and density and viscosity values of these systems are tabulated in Table 3B.3. It was observed that demixing time increased gradually with an increase in NaCI concentration of up to 4%. However, any further increase in salt concentration (4 to 6%) has resulted in a dramatic increase in demixing time (Fig. 3B.3). The gradual increase in demixing time up to 4% salt concentration· is mainly due to a decrease in density difference between the individual phases with an increase in salt concentration (Table 3B.3). Lower the density difference lower is the buoyant motion of dispersed phase droplets, eventually resulting in increased demixing time as can be seen from equation (1) of section 3A. However, the dramatic increase at 6% is mainly due to decrease in volume ratio of the system with an addition of salt (Table 3B.4), which has changed the lower viscous

dispersed phase to continuous phase. As a result of very high viscosity of the continuous phase, demixing time increased significantly. Nevertheless, acoustically assisted process has showed about 1.25 to 1.5 fold decrease in demixing time at all the salt concentrations studied.

3D. 3. 4. Effect of microbial cell concentration

It was reported that addition of microbial cells to the system increases the demixing time due to the formation of stabilized emulsions (Larsson, 1994). Another reason for the same could be due to the alteration in charge and hydrophobicity of the droplets by cell wall associated compounds. However, in our experiments we observed that addition of E. coli cells to the system at 50/50 volume ratio slightly increased the demixing time up to certain concentration (3 % w/v) and there after (46 % w/v) it decreased slightly to values lower than that of the pure system (Fig. 3B.4). E.coli cells were selected for the study as opposed to the yeast and L.casei cells, which were studied in PEG/salt system (section 3A), because, the latter two were found to act on MDX releasing carbon dioxide. The slight decrease in density difference with the addition of cells up to 3% w/v and an increase in density difference after 3% as indicated in Table 3B.4 are the probable reasons for an increase and a decrease in demixing times, respectively. Density difference being directly related to droplet migration velocity (Eqs. 1 and 2 of section 3A), higher the density difference lower will be the demixing time and vise versa.

In all these systems application of acoustic field has decreased the demixing time approximately about 1.4-fold. Visual observations of the column after demixing showed a clear top phase and somewhat a yellowish turbid bottom phase indicating that E.coli cells predominantly partitioned to the bottom phase. In addition, a slight increase in the volume of the bottom phase after demixing also supported the statement that cells predominantly partitioned to the bottom phase.

38. 4. CONCLUSIONS

Acoustic demixing a method for decreasing phase demixing time in polymer/polymer (PEG/MDX) system is reported for the first time. In these systems phase demixing depends greatly on which of the phases is continuous and viscosity of the continuous phase strongly influences the movement of the droplets and hence the phase demixing. Addition of NaCl (> 4% w/v) at 50/50 volume ratio, has changed the bottom dispersed phase to continuous phase thereby increasing the demixing time.

Acoustically assisted process employed here significantly decreased the demixing time. Ultrasonication has induced mild circulation currents in the phase dispersion, which has enhanced the rate of droplet coalescence, eventually resulting in decreased demixing time. The processes is simple, economical (low current and high voltage device), easy to scale-up and readily available ultrasonic transducers could be employed.

Table 3B. 1. Viscosity and density values at various phase compositions of PEG/MDX system

Phase composition (PEG/MDX % w/w)	Viscosity(mPa. s) Top	Bot.	Density (kg m -3) Top	Bot.	Δ Density (kg m-^{3})
7/30	10.4	33.5	1106.3	1187.3	81.0
10/30	13.5	85.1	1107.0	1226.4	119.4
10/40	33.4	202.5	1123.0	1326.0	203.0

Table 3B. 2. Demixing times at 30/70 volume ratio of two phase compositions at various duration of application of acoustic field/gravity in cycles

Phase composition (PEG/MDX, % w/w)	Gravity demixing (min)	Acoustics/gravity (min) 5/5	10/10	20/10	10/20
7/30	178	120	150	110	170
10/30	250	140	160	115	200
Rise in Temp. (OC)	-	1.5-2	3.5-4	8-9	3.5-4

Table 3B. 3. Viscosity and density values in presence of NaCl at 50/50 volume ratio of 10% PEG and 30% MDX phase composition

NaCl (% w/v)	Change in volume ratio	Viscosity (mPa. s) Top	Bot.	Density (kg m-3) Top	Bot.	Density difference (kg m-^{3})
0	50/50	1107.0	1226.4	13.5	85.1	119.4
1	49/51	1114.8	1227.4	14.0	85.8	112.6
2	48/52	1121.2	1230.5	14.2	86.4	1 09.3
4	46/54	1133.8	1237.8	16.0	87.2	104.0
5	45/55	1137.6	1239.9	17.2	87.5	101.2

6	43/57	1142.9	1242.5	18.9	87.7	99.6

Table 3B. 4. Viscosity and density values in presence of E. coli at 50/50 volume ratio of 10% PEG and 30% MDX phase composition

E. coli (% w/v)	Change in volume ratio	Viscosity (mPa. s) Top	Bot.	Density (kg m -3) Top	Bot.	Density difference (kg m-^{3})
0	50.0/50.0	1107.0	1226.4	13.5	85.1	119.4
1	49.5/50.5	1112.2	1229.8	14.4	89.1	117.6
2	49.0/51.0	1113.4	1231.5	15.3	97.2	118.1
3	48.5/51.5	1113.9	1232.7	16.2	105.9	118.8
4	48.0/52.0	1114.5	1240.0	17.8	113.4	125.5
6	47.0/53.0	1116.1	1260.7	19.7	121.9	144.6

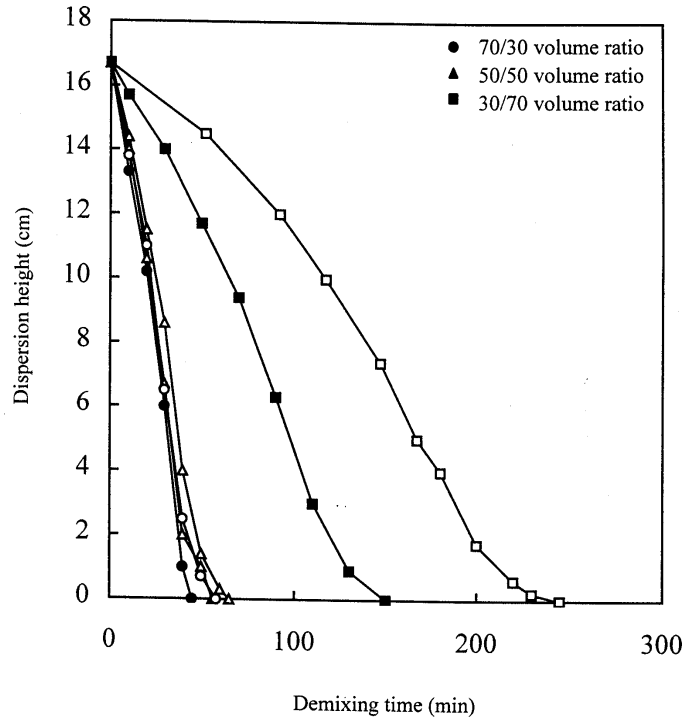

Figure 3B. 1. Kinetics of Phase demixing in PEG 10% and MDX 30% two phase system

open symbols: demixing under gravity alone closed symbols: de mixing under ultrasonication

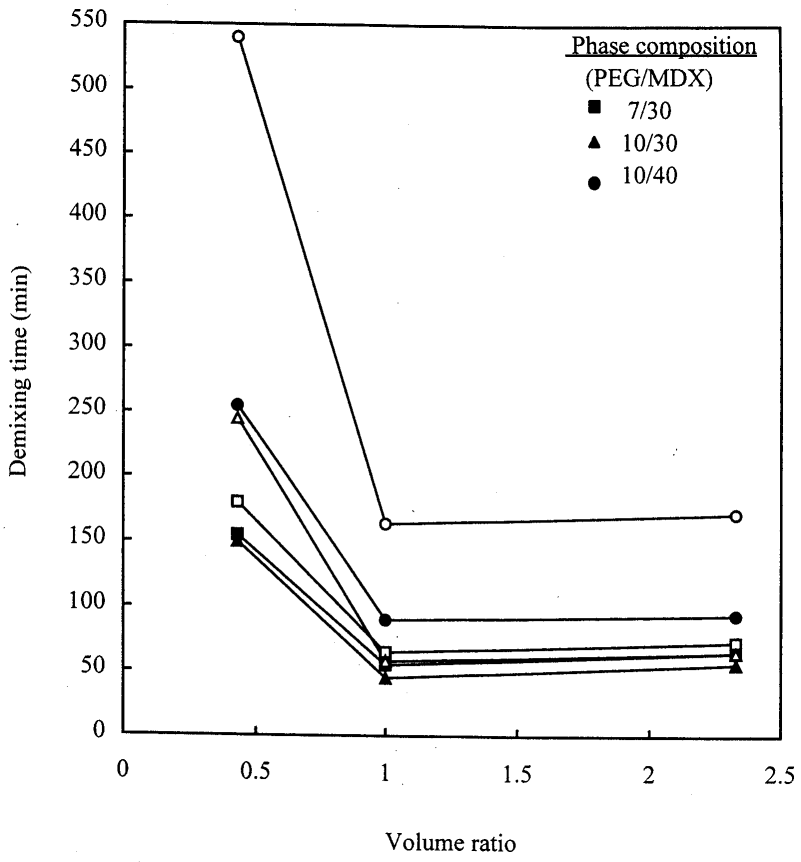

Figure 3B. 2. Effect of phase volume ratio on demixing time in various PEG/MDX two phase systems open symbols: demixing under gravity alone closed symbols: demixing under ultrasonication

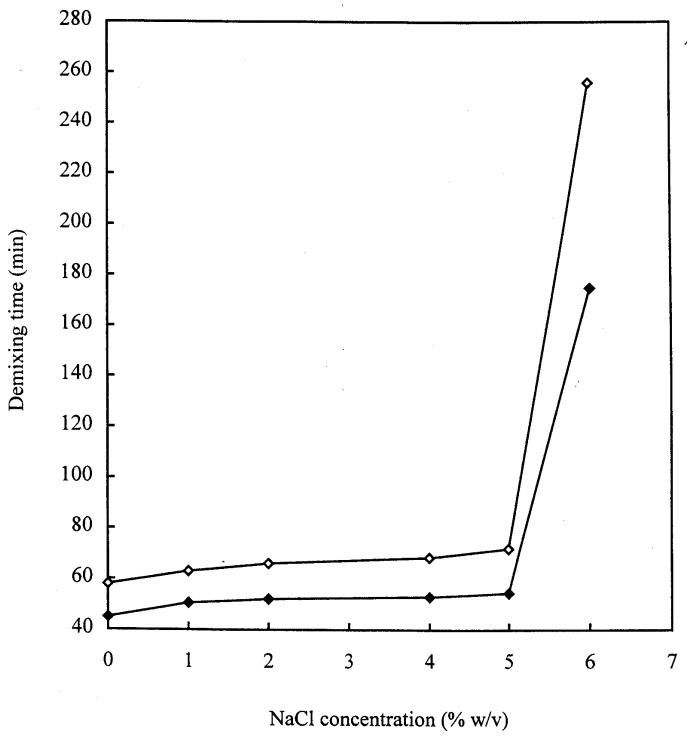

Figure 3B. 3. Effect of NaCI concentration on demixing time in PEG 10 % and MDX 30 % two phase system at 50/50 volume ratio

open symbols: demixing under gravity alone closed symbols: demixing under ultrasonication

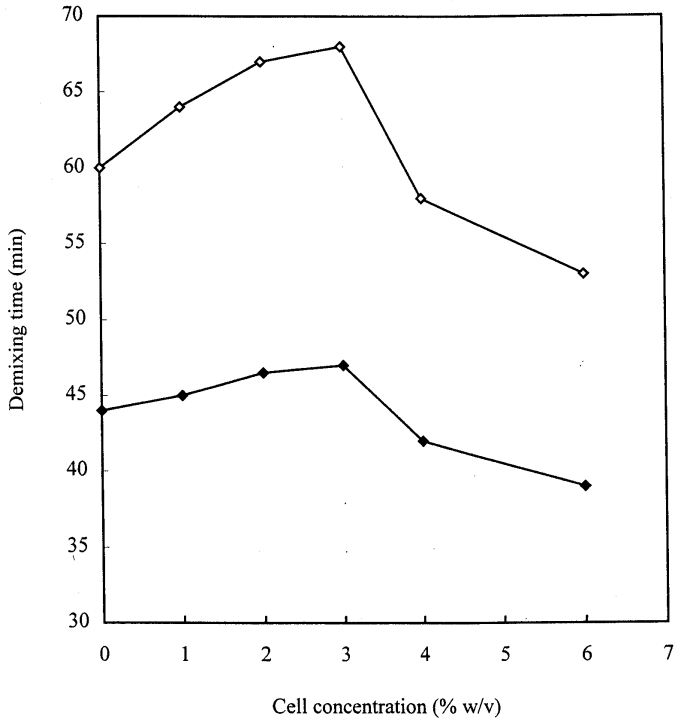

Figure 3B. 4. Effect of E. coli cell concentration on demixing time at 50/50 volume ratio of PEG 10 % and MDX 30 % two phase system

Open symbols: demixing under gravity alone closed sysmbols: demixing under ultrasonication

SECTION - C

Microwave Field Assisted Enhanced Demixing of *Aqueous Two-Phase Systems*

3C. 1. INTRODUCTION

In this section, for the first time microwave field has been explored as an external field to enhance the demixing rates of aqueous two-phase systems. Experiments were carried out using both PEG/potassium phosphate and PEG/MDX two-phase systems.

3C. 2. MATERIALS AND METHODS

Chemicals used such as polyethylene glycol (PEG, MW 6000), potassium phosphates and maltodextrin were same as listed in section 3A.2 and 3B.2 of this chapter and phase systems used in case of PEG/potassium phosphate (10/15, % w/w) and PEG/MDX (10/30 % w/w) were also same as that used in sections 3A.2 and 3B.2 of this chapter, respectively.

3C. 2. 1. Phase demixing experiments

Experiments were carried out in a microwave oven (Kelvinator Magic Cook Model T23) at a frequency of 2450 ± 50 MHz. A 100 ml capacity column with height to diameter ratio of 5 was used for the demixing experiments. Experiments were carried out at three phase volume ratios of top to bottom phase viz. 70/30, 50/50 and *30/70.* For the demixing experiments of PEG/MDX phase system, 100 ml of dispersion at each volume ratio (premixed for 10 minutes) was allowed to demix in the presence of a microwave field (inside a microwave oven) for two minutes and then taken out and allowed to separate under gravity alone for five minutes. In this way the systems were subjected to repeated cycles of exposure to microwave field for two minutes and non-exposure for five minutes until complete demixing was achieved. However, for PEG/phosphate demixing experiments the dispersion (premixed for 10 minutes) was subjected to microwave field for only one minute and then taken out and allowed to separate completely under gravity. In this case repeated cycles of exposure and non-exposure to microwave field was not necessary because of the very less over all demixing time (<4 minutes). For all the volume ratios, demixing time under gravity alone was also noted down.

3C. 3. RESULTS AND DISCUSSION

The demixing behavior of ATPSs studied in this section both in the presence and absence of microwave field has been summarized in Table 3C.1. The present method enabled to enhance the demixing rate by 1.5-2 fold over the demixing rate under gravity demixing alone. Application of microwave field energy increased the temperature of the system, thereby decreasing the viscosity of the continuous phase. This lower viscosity of the continuous phase offers lesser resistance to migration velocity of the dispersed phase droplets (since it is evident from the Stoke's law that migration velocity of the droplets is inversely proportional to viscosity of the continuous phase), leading to enhanced demixing rates of the phases. This process has some similarities to acoustic demixing of ATPSs. Like the earlier one this process is also simple, easy to scale-up and easy to adopt as continuous process.

The major application of this microwave field assisted de mixing process would be in the following aspects of bioprocessing: (1) In downstream processing of biomolecules (proteins, enzymes etc), especially in the case of thermostable biomolecules (2) In the recovery of thermally separated phase forming polymers (Galaev and Mattiasson, 1993; Johansson et aI., 1997) where after extraction, the phase polymer will be separated by increasing the temperature to certain value and then reuse of this separated polymer and (3) In the design of a new bioreactor for extractive fermentation.

3C. 4. CONCLUSIONS

For the first time microwave field was explored to enhance the demixing rates in ATPSs. About 1.5-2 fold decrease in demixing times were observed in A TPSs. The process was found to be simple, easily scalable and easily adaptable.

Table 3C. 1. Demixing times (minutes) for ATPSs both in the presence and absence of microwave field

Phase volume Ratio	PEG/MDX system (10/30, % w/w)		PEG/phosphate system (l0/15, % w/w)	
	gravity	microwave	Gravity	microwave
70/30	66	41	6.10	3.0
50/50	44	32	6.20	3.0
30170	150	106	6.32	3.0

CHAPTER 4

Mass Transfer and Drop Dynamics in Aqueous Two-Phase Systems

SECTION - A

Mass Transfer

4A. 1. INTRODUCTION

In a batch mode practice, protein extraction using aqueous two-phase system is performed by mixing the protein solution with the phase forming polymers and subsequently, allowing the dispersion to separate. When the dispersion separates back into two equilibrated phases, the desired protein concentrates in one of the phases facilitating its isolation. However, the dispersion produced in the agitated vessel is relatively stable making the rate of phase separation very slow under gravity necessitating the use of centrifuge. The agitated vessel-centrifuge assembly provides one theoretical stage and is expensive on large-scale. In many ATPE more than one theoretical stage is often required to achieve desired level of purity. Column type of extractors can be employed as alternative extraction equipment as they provide more than one theoretical stage and faster phase demixing is achieved by gravity eliminating the need for centrifugation.

Spray columns, the simplest of column contactors can be conveniently employed for the protein extraction using A TPSs. One of the phases is made continuous and the other is dispersed in the form of droplets. Spray columns are easy to operate, simple in construction and economical. More over, the continuous phase residence time can be easily varied. Major draw back with these columns is the back mixing which limits the use of these columns only to the systems requiring less than five theoretical stages and fortunately is the case with A TPE (Sawant et aI., 1990).

Jafarabad et al. (1990) reviewed the literature on spray columns. At that time there was practically no information regarding the mass transfer aspects in spray columns using ATPS, except that of Sawant et al. (1990), who have reported the mass transfer coefficient for bovine serum albumin (BSA) in a 9.7 mm internal diameter spray column. Authors observed that the dispersed phase hold up (ED) and mass transfer coefficient (KLa) increased with an increase in dispersed phase velocity (V d) and true mass transfer coefficient (K_L) was independent of V d. Later on Jafarabad et al. (1992) and Pawar et al. (1993) have extended the work using different ranges of column diameters with BSA and amyloglucosidase as solutes. A systematic study for the measurement of ED and KLa were performed by studying the effect of various parameters such as distributor design, column height and phase composition in a PEG/salt system. KLa and ED were found to decrease with an increase in phase composition. Prafulla et al. (1994) investigated the effects of various parameters on ED and KLa in a sieve plate column of 60 mm internal diameter using a PEG/salt ATPS with amyloglucosidase as solute. They observed that both Kla and ED increased with an increase in V d, number and size of orifice and decreased with an increase in phase composition. Authors calculated the contributions to mass transfer during the formation, rise and coalescence of droplets. Recently Pawar et al. (1997) measured the mass transfer coefficients of amyloglucosidase and galactosidase in a modified spray column both in the co-current and counter current mode using a PEG/sodium sulfate ATPS. The performance of the modified column was compared with that of the conventional one, with the former one showing about 10-fold enhancement in the throughput.

The present study is aimed at investigating the extraction capacity of a simple spray column for a new solute,

horseradish peroxidase (HRP) using a PEG/potassium phosphate ATPS. The effects of dispersed phase superficial velocity, orifice size and NaCI concentration on KLa and ED were studied. Effect of NaCI was studied for the first time considering the fact that addition of NaCI has a significant effect on partition coefficient of an enzyme in A TPS (chapter 2).

4A. 2. **MATERIALS AND METHODS**

Polyethylene glycol (MW 6000), K_2HP0_4, KH_2P0_4, NaCl, guaiacol, and H_20_2 were all same as listed in section 2A. Horseradish peroxidase (HRP) was purchased from Sigma Chemicals (USA).

4A. 2. 1. Phase system preparation and partition coefficient measurements

Three thousand gram of phase system comprising of PEG 15% and potassium phosphate 11 % was prepared as explained in the section 3A.2. The individual phases were separated and used as stock for further experiments. Similarly to study the effect of NaCI, 3000 g of phase system comprising of PEG 15%, potassium phosphate 11 % and NaCI 2% was prepared, individual phases were separated and used as stock for further experiments. Partition coefficient of HRP was calculated as explained in the section 2A.2.

4A. 2. 2. Mass transfer experiments

The schematic diagram of the Experimental set up for mass transfer is shown in Figure 4A.I. The internal diameter of the column was 18 mm. Experiments involving the HRP transfer from dispersed phase to continuous phase were carried out with salt-rich (heavy) phase as continuous phase and PEG-rich (light) phase as dispersed phase. The droplets of the dispersed phase coalesced at the top of the continuous phase and the coalesced layer was collected through the overflow outlets.

In all the experiments, solute HRP was dissolved in PEG-rich phase at a predetermined concentration (7.5 u/ml) and the stock solution was stored in a glass tank. The extent of deactivation of the enzyme was found to be negligible during the time of experimentation (-3 hours). The flow of the PEG-rich phase was monitored using a peristaltic pump (Pharmacia, Sweden). The PEG phase was removed from the top through the overflow line and the flow rate was calculated by measuring the amount of PEG phase collected for a known period of time. In all the experiments 110 ml of the continuous phase was added into the column and the flow of the dispersed phase continued for a period which was always 10 times greater than the residence time of PEG droplets. The duration was provided to ensure a steady state with respect to dispersion characteristics.

The fractional dispersed phase hold up (ED) was calculated by using the formula,

$$\varepsilon_D = \frac{H_D - H}{H_D} \qquad (1)$$

where Ho is the dispersion height and H is the continuous phase height.

The overall mass transfer coefficient (KLa) was calculated by using the expression,

$$K_L a = \frac{L}{V_D} \ln\left[\frac{(C_i - mC_s)}{(C_o - mC_s)}\right] \qquad (2)$$

where L is the flow rate of the dispersed phase, V_D is the dispersion volume (calculated from H_D value), Cj and Co are the inlet and outlet concentration of HRP in the PEG-rich dispersed phase, C_s is the concentration of HRP in the salt-rich continuous phase after extraction and m is the partition coefficient of HRP.

All the experiments were carried out in triplicates and average values represented. All the experiments were performed at 27 ± 0.5 °C.

4A. 3. RESULTS AND DISCUSSION

4A. 3. 1. Effect of superficial dispersed phase velocity

Effect of superficial dispersed phase velocity on KLa and ED was studied at various nozzle diameters in a given phase system. In all the cases the values of KLa and ED increased with an increase in V s as shown in Figures 4A.2 and 4A.3, respectively. Kumar and Hartland (1982) indicated that the drop size decreases with an increase in V s. Visual observations also indicated a decrease in drop size as well as an increase in drop frequency (number of drops/minute) with an increase in V_d• This decrease in drop size decreases the drop rise velocity and increases the drop residence time in the column and hence ED as well as KLa increases. In addition, the increase in drop frequency and a decrease in drop size, increase the interfacial area available for protein transfer, resulting in an increase in KLa. Similar results were reported in the previous works (Sawant et aI., 1990; Jafarabad et aI., 1990; Jafarabad et aI., 1992; Pawar et aI., 1993; Prafulla et aI., 1994; Pawar et aI., 1997).

4A. 3. 2. Effect of orifice diameter

Effect of orifice diameter on KLa and ED was studied at 3 different orifice diameters viz. (i) 0.504 mm (ii) 0.924 mm

and (iii) 1.326 mm and the results were depicted in Figures 4A.2 and 4A.3, respectively. It can be seen that the values of KLa and ED increased with an increase in orifice diameter. This is explained based on the visual observations made on the drop size, which indicated that at a given dispersed phase velocity, drop size decreased and drop frequency increased with an increase in orifice diameter. This decrease in drop size and an increase in drop frequency are eventually responsible for an increase in the values of KLa and ED as explained earlier.

4A. 3. 3. True mass transfer coefficient

As the behavior of KLa and ED were similar with respect to V d (Figs. 4A.2 and 4A.3), a plot of KLa / ED (HRP transfer per unit hold up) versus V d was made as shown in Figure *4AA*. The ratio was found to be independent of V d which indicates that true mass transfer coefficient was independent of superficial velocity. This result is consistent with that of Sawant et al. (1990) and is important in designing scale-up aspects of spray column extraction.

4A. 3. 4. Effect of NaCI

As it is evident from chapter 2 that addition of NaCl to the system has a profound effect on partition coefficient of enzymes, we thought it is worthwhile to study its effect on mass transfer. Experiments on HRP partition coefficient studies at various NaCI concentration indicated that lowest partition coefficient could be obtained at 2% NaCI (Fig. 4A.4). Hence, we selected only this concentration to study its effect on mass transfer. It was observed that KLa increased in the presence of 2% NaCI up to about 2 fold (Fig. 4A.5). However, ED remained practically same (data not shown). Although exact explanation is not known at this stage, it is thought that attractive electrostatic interactions between NaCI and HRP may be responsible for this increase in KLa. However, detailed investigations on the effect of NaCI on interfacial tension of the system and on the internal circulation of the drops have to be performed to provide a proper explanation for the observed enhancement of KLa in presence of NaCl.

4A. 4. CONCLUSIONS

This chapter deals with the extraction of HRP in a simple spray column of 18 mm internal diameter using a PEG/salt ATPS. The main advantage of the spray column extraction method is that the phases separate easily and quickly without the need of a centrifuge. In addition, the process is simple and easy to operate at continuous mode. KLa and ED were found to increase with an increase in V d as well as orifice size. As expected, true mass transfer coefficient was found to be independent of V d. Preliminary experiments on the effect of NaCI showed that KLa increases by the presence of NaCI in the system. Detailed investigations on the effect of NaCI on interfacial tension and internal circulation of the drops are required to provide an appropriate interpretation for the observed increase in KLa.

Figure 4A. 1. Schematic diagram of experimental set up for HRP transfer studies

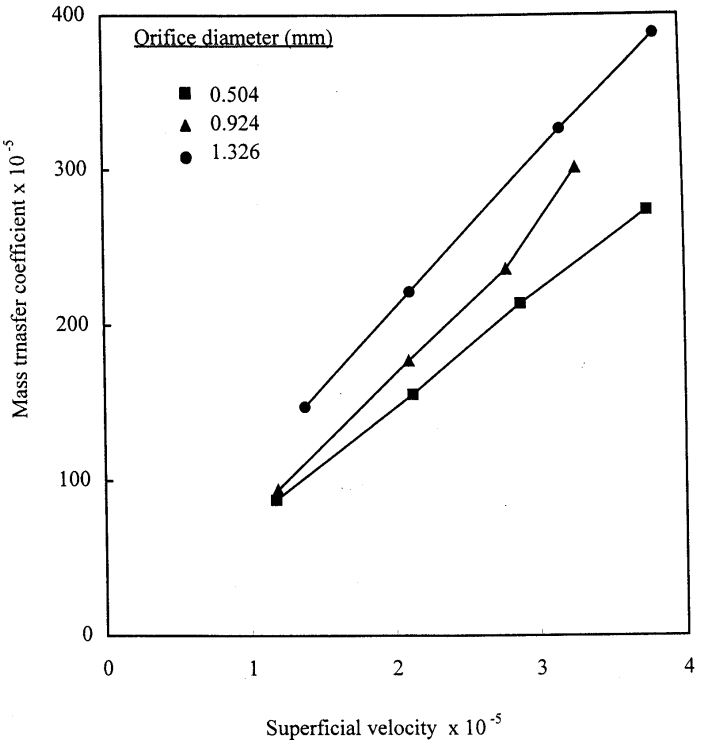

Figure 4A. 2. Effects of dispersed phase superficial velocity and orifice diameter on the mass transfer coefficient of HRP in a PEG/potassium phosphate ATPS.

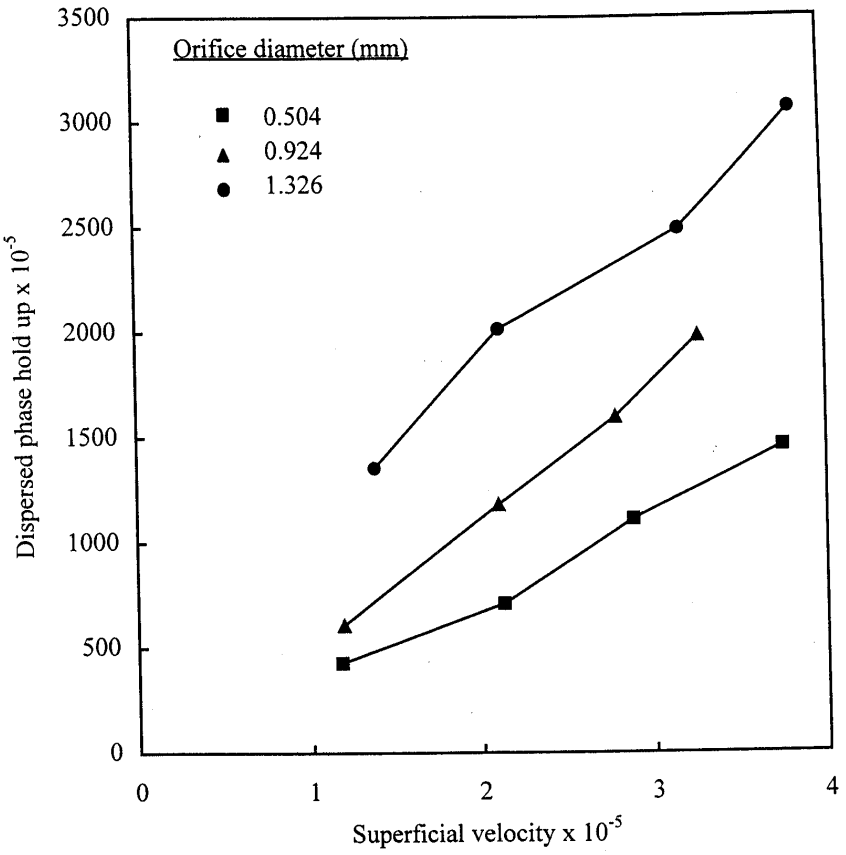

Figure 4A. 3. Effects of dispersed phase superficial velocity and orifice diameter on the fractional dispersed phase hold up in a PEG/potassium phosphate ATPS.

Figure 4A. 5. Effect of NaCl concentration on partition coefficient of HRP in a PEG/potassium phosphate ATPS.

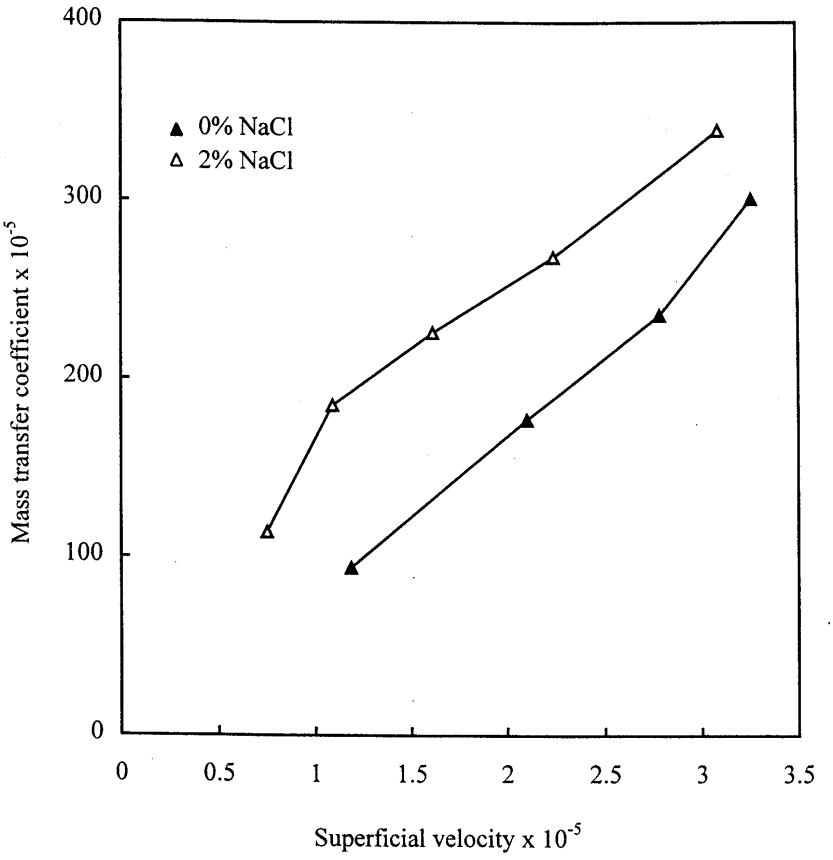

Figure 4A. 6. Effect of NaCl (2%) on mass transfer coefficient of HRP in a PEG/potassium phosphate ATPS (Orifice diameter: 0.924 mm)

SECTION - B

Drop Dynamics

4B. 1. INTRODUCTION

Drop dynamics drop size and migration (rise/fall) velocity] plays a crucial role for efficient mass transfer during extraction of biomolecules in spray columns. Mass transfer studies of an enzyme, horseradish peroxidase using a simple spray extraction column has been demonstrated in section 4A of this chapter. Existing models available in the literature for prediction of drop volume during formation are all pertain to organic-aqueous systems (Hayworth and Treybal, 1950; Null and Johnson, 1958; Narasingarao et aI., 1966; Scheele and Meister 1968). ATPSs differ from organic-aqueous two-phase systems in their physical properties. That is, they have similar densities and high viscosity of the individual phases (especially in polymer/polymer systems) and low interfacial tension. A review of published information (Prafulla et aI., 1994) indicates that the dispersed phase viscosity in the A TPS is more than 14 mPa s. Similarly, in conventional organic-aqueous extraction, the difference in density is at least twice that in the case of A TPS. In addition the interfacial tension is more than 13 mNm^{-1} in organic-aqueous two phase systems whereas in the ATPS it is in the range of 10-4 to 10^{-1} mNm^{-1} . Hence, we attempted to model the drop dynamics in A TPSs by modifying the force balance involved in organic-aqueous two-phase systems. In this study, we have considered only low volumetric flow rates of the dispersed phase to model the drop dynamics in ATPSs. Experimental data have been presented to test the model using polyethylene glycol/potassium phosphate two phase system.

4B. 1. 1 Theory

At low flow rates, static drops forms at the nozzle at a very slow rate. During the process of this drop formation, two major forces will act on it. 1) The buoyancy force due to the density difference between the two fluids and 2) The interfacial tension force at the nozzle tip. The drag force exerted by the continuous phase and the inertial or kinetic force associated with the fluid flowing out of the nozzle are too low to be considered in this case.

The buoyancy force is

$$F_B = V_d \Delta \rho g \qquad (1)$$

The interfacial tension force is

$$F_S = \pi D_N \gamma \qquad (2)$$

At static condition,

$$F_B = F_S$$

That is

$$V_d \Delta \rho g = \pi D_N \gamma \qquad (3)$$

However, the Harkins-Brown factor which corrects for the fraction of the liquid volume that remains attached to the nozzle after drop breaks off has to be taken into account.

Therefore

$$V_F = \frac{\pi D_N \gamma}{\Delta \rho g} \phi \left(\frac{D_N}{V_d} \right) \qquad (4)$$

where, V_d is the static drop volume, y is the interfacial tension, D_N is the nozzle diameter, Δp is the density difference between the continuous phase and dispersed phase and V_F is the drop volume .

48. 2. MATERIALS AND METHODS

Polyethylene glycol (MW 6000), K_2HP0_4 and K_2HP0_4 were all same as listed in section 2A. A PEG (6000)/potassium phosphate two phase system with a composition of PEG 10% and potassium phosphate 11 % (K_2HP0_4 : KH_2P0_4 :: 1.82 : 1, pH 7.0) whose interfacial tension is already reported in the literature (Jafarabad et aI., 1992) is selected for the study. Preparation of this system at 3000 g scale and separation of the equilibrated individual phases were done as explained in the section 3A.2,

4B. 2. 2. Experimental set up for drop formation studies

Figure 4B.l presents a schematic diagram for the drop formation studies. The glass column with 950 mm length and 340 mm diameter was filled with 600 ml of continuous phase (salt-rich). Prior to filling, the column was packed from below with a rubber cork containing a capillary tube of known diameter. Dispersed phase (PEG rich) stored in a constant level reservoir was sparged into the column through the capillary. Flow rate of the dispersed phase was varied using a peristaltic pump (Pharmacia, Upsala, Sweden). The drops rise and collect at the top of the column. The following items are noted down during the experimentation for the computation of the drop volume: 1) flow rate of the dispersed phase 2) frequency of drop

formation (number of drops/min) and 3) Volume of the dispersed phase collected for a known period of time.

4B. 2. 3. Phase density and orifice diameter measurements

Density and viscosities of the individual phases were measured by using Specific gravity bottle and Ostwald-U-tube viscometer of 10 ml capacity, respectively and the values are shown below Table 4B.l as footnotes. Orifice diameters were measured using a microscope (Leitz LABORLUX S model of Leica, Germany).

4B. 3. RESULTS AND DISCUSSION

Drop volumes were calculated using equation (3) as well as experimentally (Table 4B.l). The model calculations were performed at this stage using equation (3) neglecting the Harkins-Brown correction factor. This is because of the complications and lack of facilities in deducing the Harkins-Brown correction factor. A detailed study on this factor revealed that the values available in the literature are valid only to various organic-aqueous two-phase systems (Harkins and Brown, 1919, Scheele and Meister, 1968) and not applicable to ATPSs. Furthermore, it depends on various variables such as orifice diameter, flow rate, physico-chemical characteristics and nature of the system and is not a single constant value for a particular system.

It may be noted from the Table 4B.l that, at a given orifice diameter, droplet size increased with an increase in flow rate. This is contradictory to the observations made by various researchers in their mass transfer studies using spray columns (Sawant et al., 1990; Jafarabad et al., 1990; Jafarabad et al., 1992; Pawar et al., 1993; Prafulla et al., 1994; Pawar et aI., 1997) as well as by us in the previous section of this chapter where in droplet size decreased with an increase in dispersed phase flow rate. The major difference between the previous mass transfer studies and the present drop dynamic studies is that the mass transfer studies were performed at relatively higher flow rates where as the drop dynamic studies are performed at very low flow rates. Narasingarao et ai. (1966) in their drop dynamic studies using various organic aqueous two phase systems have observed a gradual increase in drop volume with an increase in volumetric flow rates of the highly viscous dispersed phase. This observation is consistent with our observation. The increase in drop volume with an increase in volumetric flow rate could be attributed to the change in the time of detachment of the drop from the orifice tip. As it was observed that the time of detachment of the drop remained practically same with an increase in volumetric flow rate, the drop size increased steadily with an increase in volumetric flow rate.

A good agreement was seen between the theoretical and experimental calculations of drop volume, especially at very low flow rates in the lowest studied orifice diameter (Table 4B.l). Therefore it can be said that the model is applicable for predicting the drop volumes in ATPSs at very low flow rates. At low flow rates, though the model is similar to that of organic -aqueous phase systems, a considerable difference is expected at high flow rates, due to consideration of drag and kinetic forces, as these forces vary largely from organic-aqueous to aqueous two phase systems.

Table 4B. 1. Comparison of predicted and experimental drop volumes at low flow rates of three different orifice diameters

Orifice diameter (m) X 10^{-3}	Flow rate ($m^3 S^{-1}$) X 10^{-10}	Drop volume (m^J) X 10^{-9} Experimental	Predicted	% Error
0.504	6.60	1.03	1.048	1.38
0.504	6.78	1.04	1.048	1.50
0.504	6.81	1.06	1.048	-0.70
0.504	7.06	1.07	1.048	-2.25
0.504	10.2	1.23	1.048	-14.96
0.924	3.12	1.56	1.92	23.07
0.924	3.15	1.61	1.92	19.2
0.924	3.18	2.05	1.92	-6.34
1.326	3.08	0.99	2.756	175.9
1.326	3.27	1.13	2.756	143.9

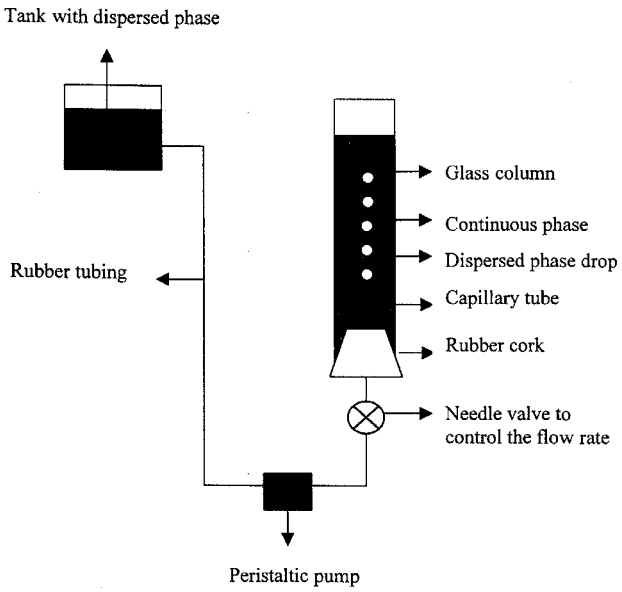

Figure 4B. 1. Schematic diagram of experimental set up for drop dynamics studies

SUGGESTIONS FOR FUTURE WORK

The two major deterrents for the industrial adaptation of aqueous two-phase systems are high cost of the phase forming polymers and slow rate of phase demixing. Extensive work on thermo separating aqueous two-phase extraction for the recovery and recycling of phase forming polymers by the swedish group (Alred et al, 1993; Berggren et al., 1999) appears to have solved the problem of high cost of the phase forming polymers to a great extent. However, PEG being the most extensively studied polymer in ATPS, efficient, simple and cost effective methods for its recovery and recycling have not been discovered. Ultrafiltration, the existing method is not simple and prohibitively expensive on large-scale. Hence, there is a need to find out efficient, simple and cost effect methods for the recovery and recycling of PEG in ATPSs. We started working in this direction and preliminary experiments have shown promising and encouraging results. Detailed investigations should be carried out in future.

In the second chapter (section A) extraction and purification of a plant peroxidase from the leaves of *Ipomoea palmetta* has been discussed. This enzyme from a plant source is having high activity and could be used as an alternative for the currently available commercial peroxidase from the roots of horseradish (HRP), which is expensive owing to its high cost of production. Keeping in view of this point, there is a need to continue to investigate on various aspects of *Ipomoea* peroxidase such as purification to homogeneity, characterization, etc.

Acoustic demixing was shown to be a promising technique for enhancing the demixing rates of ATPSs in chapter 3. Although detailed studies have been conducted both in polymer/salt and polymer/polymer ATPSs, some other major parameters, viz. effects of intensity and frequency of acoustic field as well as axial location from transducer on phase demixing was not studied due to the limitations of the equipment. Hence, this study needs to be performed in the near future. From the industrial point of view, scale up studies have to be performed in this field and new and efficient acoustic bioreactors has to be designed. Further, keeping in view the encouraging results obtained from the preliminary studies on microwave field assisted enhanced phase de mixing in ATPSs (chapter 3, section C), it would be a worthy exercise to go into details of this study, especially in case of thermo separating polymers. In addition, visualizing the popularity gained by the A TPE using thermo separating polymers in recent years, it is desirable to study the demixing kinetics of these systems in detail especially in the presence of external fields such acoustic, electric and microwave.

For the first time we have shown that addition of NaCI has increased the overall mass transfer coefficient in ATPSs using simple spray columns with HRP as solute (chapter 4, section A). However, we could not provide a complete explanation for this observed enhancement in overall mass transfer coefficient. For the same a detailed study has to be performed to elucidate the effect of NaCI on the internal circulation of the droplets, droplet size and interfacial tension. These studies are under progress in our laboratory at present.

A good agreement has been observed between the theoretical and experimental values of droplet volumes in the fourth chapter (section B). These studies have been conducted only at very low flow rates of the dispersed phase. The

encouraging results have prompted us to continue to work on the drop dynamics of A TPSs, a relatively new area and we are the first to explore it. As a result we have developed the models to predict the drop volumes at higher flow rates of the dispersed phase and presently our group is engaged in calculating the drop volumes experimentally, which will enable us to check the validity of the model.

The encouraging results of electrokinetic demixing opens the scope for a systematic study on electroextraction for achieving the selective partitioning of biomolecules.

LLE using reverse micellar extraction (RME) is another attractive and well established method for the extraction and purification of biomolecules (Luisi and Magid, 1986). Hence, comparison studies for the extraction and purification of biomolecules (especially for *Ipomoea* peroxidase) using ATPE as well as RME would be interesting. Further, kinetic studies and field assisted methods for the enhanced demixing of reverse micellar systems needs to be performed for the industrial suitability of this technique.

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1. Harikrishna S, Srinivas ND, Raghavarao KSMS & Karanth NG Reverse micellar extraction for downstream processing of proteins/enzymes Advances in Biochemical Engineering/Biotechnology -Accepted

PATENTS

1. Raghavarao KSMS & **Srinivas ND** An improved process for separation of biomolecules using demixing technique of aqueous phases generated in bioreactors (NF 94/2000)

INTERNATIONAL CONFERENCES / SYMPOSIA ATTENDED

1. **Srinivas ND** & Raghavarao KSMS Drop formation in aqueous two-phase systems

International Conference on Processed Foods for the 21 st Century, Calcutta, India (Jan 14-16, 2000) - **Poster presented.**

2. **Srinivas ND,** Raghavarao KSMS & Todd P Acoustically enhanced drop coalescence in aqueous two phase systems with and without cells. Eighth World Filtration Congress (WFC 8), Brighton, UK (April 2000) - **Accepted for Oral presentation.**