TWO AND THREE PHASE PARTITIONING FOR PURIFICATION AND CONCENTRATION OF SELECTED BIOMOLECULES AND FOOD COLORANTS

A thesis submitted to the

University of Mysore

for the award of the degree of

DOCTOR OF PHILOSOPHY

in

Biotechnology

by

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DEDICATED TO MY PARENTS ……

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DECLARATION

I hereby declare that the thesis entitled **"Two and three phase partitioning for purification and concentration of selected biomolecules and food colorants"** which is submitted herewith for the degree of **Doctor of philosophy** in **Biotechnology** of the University of Mysore, is the result of the research work carried out by me in the Department of Food Engineering, Central Food Technological Research Institute, Mysore, India, under the guidance of **Dr. KSMS Raghavarao**, during the period 2002 to 2006.

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

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CERTIFICATE

I hereby certify that this Ph.D. thesis entitled **"Two and three phase partitioning for purification and concentration of selected biomolecules and food colorants"**, submitted by **Mr. A.V.Narayan** for the degree of **Doctor of Philosophy in Biotechnology** of the 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 September 2002 to December 2006.

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ACKNOWLEDGEMENT

I express my deep sense of gratitude to the Council of Scientific and Industrial Research (CSIR), New Delhi for the award of a Senior Research Fellowship, which provided an opportunity to work at this prestigious institute, Central Food Technological Research Institute, Mysore.

I would like to express my sincere gratitude to my guide **Dr. KSMS Raghavarao** for his thought provoking guidance, encouragement, suggestions and support during the course of the work. He has been a great source of inspiration to me.

I wish to express my deep sense of gratitude to Dr. V. Prakash, Director-General, CSIR, New Delhi and former Director, CFTRI, for permitting and providing all the facilities to carry out my research work.

I would like to express my sincere gratefulness to Ms. T.N.Indira, Scientist, Protein Chemistry and Technology for her invaluable suggestions, encouragement and providing all the facilities to carry out my work.

I would like to express my sincere gratefulness to Dr. G. A. Ravishankar, Head, Department of Plant Cell Biotechnology, CFTRI, Mysore, for providing all the facilities during the course of my work. I would also express my sincere gratefulness to Dr.M.Mahadevaswamy, former scientist, Department of Plant Cell Biotechnology, CFTRI, Mysore for all the help rendered.

I gratefully acknowledge the help received from the staff of Department of Food Engineering, Central Instrumentation and Facility Services, library (Fostis), Human Resource and Development and of various other divisions such as the Pilot Plant, Workshop, Glass blowing, Electrical & Maintenance, Administration, Stores and Purchase, IFTTC Hostel and Mess, etc.

I thank my present colleagues in the Food Engineering Department Mr. E. Nakeeran, Ms. B.S. Roopa, Ms. S. Manjula, Mrs. Hemavathi Satish, Mrs V. Sumana, Mr. Pankaj Sharma and Ms. Shashikala for their affectionate support. I wish to give special thanks to Mr. Chetan. A. Nayak, Mr. S.N. Raghavendra, Mr. B. Ravindrababu and Mr. M.C. Madhusudhan, for all the help they had given through out my work. I also wish to give special thanks to my former colleague Dr. S.Chethana and present colleague Mr. Ganapathi Patil, for all the help they had given through out my work.

I would like to thank Mr.C.Anandharamakrishnan, Scientist, HRD, CFTRI, for all the help given by him. I would also thank my former colleagues, Dr. Naveen Nagaraj, Dr. Rajendrakumar Barhate, Mr. U. Hanumesh, Ms. Rajashri, Mr. Premsingh Samuel, Dr. G. K. Nagesh, Dr. T.V. Ranganathan, Dr. P.Krishnamurthy, Mr. C. Shankar, Ms. M.B. Shubhashini and Mrs. Brinda. I also thank my colleagues Ms. Vidyavathi, Sandesh Kamath and Shaktivelu, Plant cell Biotechnology, CFTRI and former colleague Dr. K.S. Venkatesh, Food Microbiology, CFTRI for all the help and support rendered by them.

I wish to thank the staffs of Food Engineering Mr. A.Chakkaravarthi, Dr. Suvendu Bhattacharya and Dr.K.Udayasankar for providing all the help to carry out my work. I also thank Mr. A. Appukuttan, Steno, Food Engineering Department who helped me in all my official works.

I express my indebtedness to my beloved parents, brother, friends and teachers, who have supported me and helped me to come up to this level.

A.V. Narayan

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LIST OF ABBREVIATIONS

LLE	Liquid-liquid extraction
ATPE	Aqueous two phase extraction
ATPSs	Aqueous two phase systems
ATPS	Aqueous two phase system
TPP	Three phase partitioning
MLFTPP	Macroligand facilitated three phase partitioning
TLL	Tie line length
PEG	Polyethylene glycol
TMA-PEG	Trimethyl amino- Polyethylene glycol
S-PEG	Sulfonate- Polyethylene glycol
P-PEG	Pamitate- Polyethylene glycol
IDA-PEG	Iminodiacetic acid- Polyethylene glycol
Met-PEG	Methionine- Polyethylene glycol
TED	Triscarboxymethylated ethylxene diamine
Cm	carboxymethylated
LDH	Lactate dehydrogenase
EO	Ethylene oxide
PO	Propylene oxide
EG	endoglucanase
HFB	Hydrophobin
HRP	Horse radish peroxidase
GFP	Green fluorescent protein
Vr	Volume ratio
K	Partition coefficient
BSA	Bovine serum albumin
C_T	Concentration of protein in top phase
C_B	Concentration of protein in bottom phase
V_f	Relative free volume
ΔV_f	Delta free volume
ρ_T	Density of top phase
RBO	Rice bran oil
FFA	Free fatty acid
PC	Phosphatidylcholine
PA	Phosphatidic acid
PE	Phosphatidylethanolamine

Contents

Synopsis

Recent developments in biotechnology have opened up new avenues towards the production of many biomolecules important in research, pharmaceutical/clinical and industrial applications. In view of the recognized fact that product recovery costs become critical in the overall economics of modern biotechnological processes, there has been an increased interest in the development of efficient downstream processing methods for separation, concentration and purification of biomolecules from fermentation and cell culture media. For the downstream processing, in recent years liquid-liquid extraction (LLE) using aqueous-two phase systems is gaining attention for purification and concentration of biomolecules. Aqueous two phase system is formed by dissolving a polymer and a salt or a pair of polymers in water above a certain critical concentration. Major advantages of aqueous two phase extraction (ATPE) include high extraction capacity, biocompatible environment, low interfacial tension, high throughput, low process time as well as low energy requirements, easy scale-up and continuous operation. However, the major hindrances for the practical applicability of aqueous two-phase extractions for industrial practice are high cost of the phase forming polymers and slow rate of phase demixing. The former aspect has been addressed to a great extent by temperature induced phase separation for recovery and recycling of the polymers. The latter aspect has been addressed by the application of electric, magnetic, acoustic and microwave fields to enhance the phase demixing rate. The slow demixing in aqueous two phase systems (ATPSs) is due to a low difference in density between the phases,

high viscosity of the individual phases and low interfacial tension. The difficulty of recovery and recycling and disposal of spent phases after extraction is yet another major drawback of ATPS. Therefore, there exists a need for the development of an alternate method that facilitates easy recovery of product and recycling of phase components.

Keeping this in view, the present study explores the possibility of combining the concept of traditional aqueous-organic extraction and modern aqueous two-phase extraction. This enables to arrive at a biocompatible two-phase system (for a given solute) which results in three phase partitioning (TPP) as an alternative to aqueous two phase systems. TPP utilizes the phase components like t-butanol and inorganic salts like $(NH_4)_2SO_4$ to precipitate proteins from aqueous solution, at the interface of two-phase system. This method was first developed as an upstream method for precipitation of crude cellulases and subsequently as downstream processing method.

Anticipated advantages of employing TPP for purification and concentration of proteins are lower cost of phase components, easy recovery and recycling of phase components, high selectivity in removal of contaminants (protein complexing agents such as phenolics and tannins). Besides, the other advantages include removal of enzyme inhibitors, polar/non-polar lipids that are difficult to remove otherwise and easy product recovery from phase components in concentrated form.

The contents of the thesis are divided into four chapters.

Chapter 1. Review of literature

This chapter comprises literature review regarding the downstream processing of biomolecules employing aqueous two phase extraction and three phase partitioning. Application of these methods and advantages they have over conventional purification techniques are presented. Further, the parameters needed to optimize the partitioning of a biomolecule employing aqueous two phase extraction and three phase partitioning are discussed. This chapter is divided into two sections, namely, General Introduction and Aim and Scope of Present Investigation.

Chapter 2. Three phase partitioning and aqueous two phase extraction

This chapter is divided into two sections.

Section A- Physiochemical characterization and demixing kinetics of phases

This section comprises of the physicochemical characterization of aqueous two phase systems (PEG 4000 and potassium phosphate) and aqueous/organic phase system (t-butanol and ammonium sulfate) with respect to density and viscosity at different tie line lengths (composition). Viscosity of the individual phases indicates whether the total demixing time required for separation is high or low while the kinetics of demixing depicts the entire behaviour of the system during equilibration and demixing after the extraction. The kinetics of phase demixing at the low, intermediate and high tie line lengths for different phase volume ratios of aqueous two phase system (for H/D ratios of 0.75 and 2.85) and aqueous/organic phase system (three phase partitioning) are determined.

Section B- Three phase partitioning of a plant peroxidase

This section deals with the extraction and purification of peroxidase from *Ipomoea palmata* leaves. The parameters employed for optimizing extraction such as pH of the protein, concentrations of ammonium sulfate and t-butanol and effect of temperature and concentration of the crude are discussed. The enzyme purification achieved is 18-fold with a 123% activity recovery. Comparison of TPP with conventional ammonium sulfate precipitation for the extraction of peroxidase is also presented.

Chapter 3. Comparative studies of three phase partitioning and aqueous two phase extraction

This chapter is divided into two sections.

Section A- Standardization of process parameters for BSA

In this section a comparative study has been undertaken between three phase partitioning and aqueous two phase extraction, employing a model solute (BSA) and a real solute C-phycocyanin, a natural colorant. Volume ratio and partition coefficient are important factors which determine the effectiveness of extraction. One sided partition coefficient with extreme volume ratio both in favor of extracting phase reduces the volume to be handled in subsequent purification steps thereby aiding process integration. In aqueous two phase extraction, PEG 6000/ ammonium sulfate system is employed to find the effect of volume ratio (70/30, 50/50 and 30/70) on the partitioning of BSA at different concentrations. In three phase partitioning, the ratio of volume of crude extract to the co-solvent employed is necessary for

optimization of the biomolecule recovery. In three phase partitioning, a study is carried out to find the effect of volume ratio as in the conventional aqueous-organic system for different concentrations of BSA at phase volume ratios of 70/30, 50/50 and 30/70. A yield of 96%, each for ATPE and TPP is obtained.

Section B- Standardization of process parameters for C-phycocyanin

In this section a comparative study with respect to extraction and purification of C-phycocyanin from *Spirulina platensis* employing aqueous two phase extraction and three phase partitioning is undertaken. In aqueous two phase extraction, the parameters to optimize studied are tie line length, volume ratio and neutral salt. Phycocyanin of 0.73 purity and 90% yield is achieved. In three phase partitioning, C-phycocyanin irreversibly denatured and precipitated at the interphase due to its strong hydrophobic interaction with alcohols. However, byproducts such as carotenoids are extracted to alcohol phase. Different alcohols studied were ethanol, isopropanol and t-butanol with ammonium sulfate. Aqueous two phase extraction is found better than three phase partitioning for extraction and purification of C-phycocyanin from *Spirulina platensis*.

Chapter 4. Extraction and purification of oryzanol from rice bran oil soapstock

This chapter deals with extraction and purification of oryzanol from rice bran oil soapstock. This chapter is divided into three sections.

Section A- Extraction based processes

A.1- Solid-liquid extraction (leaching)

This section consists of extraction based process, namely, leaching (solid-liquid extraction) for the recovery of oryzanol from the raw material dried soapstock. The solvent mixtures used for leaching are different proportions of ethyl acetate, acetone and water. 26-36% purity of oryzanol with a recovery of 44-59% is obtained. Since the purity and recovery of oryzanol are low, ethyl acetate and acetone, each alone, are attempted as leaching solvents. The purity and recovery of oryzanol with ethyl acetate obtained are 34% and ~100% while that with acetone are 33% and 58%, respectively.

Section A.2- Liquid-liquid extraction

This section consists of extraction based process namely liquid-liquid extraction (LLE) for the recovery of oryzanol from dried soapstock. The solvents used are mixtures of hexane and alcohol (in presence of alkali) in different proportions. A purity of 23-38% with recovery of 28-35% is achieved.

Section B- Enzymatic degumming

This section deals with enzymatic degumming studies with unsaponified fraction (obtained after leaching of dried soapstock) as the starting material. Degumming of rice bran oil with enzyme lecitase to convert the non-hydratable phospholipids to hydratable is a conventional way. In the present study two protocols are adopted. In the first one, the unsaponified fraction is treated with lecitase under different processing conditions followed by drying, saponification, leaching and crystallization. Oryzanol of 34% purity

with 80% yield is obtained. In the second protocol, the unsaponified fraction after treatment with lecitase is saponified directly without drying followed by crystallization. Oryzanol of 30-40% purity with a recovery of 70-80% is obtained.

Section C- Crystallization

In this section a combination of leaching and crystallization is described. The product obtained after leaching (i.e. the unsaponified matter) is subjected to crystallization with solvents such as acetone and methanol in suitable proportions. A purity of 60-65% with a recovery of 70-75% is achieved.

Finally, references and suggestions for future work are presented.

Chapter- 1

REVIEW OF LITERATURE

Section- A

GENERAL INTRODUCTION

1A. General Introduction

1A.1. Preamble

Studies on protein solubility in organic solvents started in 1950's (Morton, 1950, Rees and Singer, 1956, Singer 1960). However, due to limitations of enzyme solubility and functionality, biocatalysis did not take off at a higher pace. Non-aqueous enzymology gained attention and grew exponentially in the last two decades (Castro et al., 2003) by the pioneering work of Klibanov (1977) and Halling (1984). The enzyme function and stability in organic solvents has emerged out as a potential area of research (Gupta et al. 1997). There are two reasons for it. Firstly, such studies give the contribution of different molecular forces to maintain the native structure of protein. Secondly, a number of enzyme catalyzed processes, such as the synthesis of peptides and esters, transformation of some hormones, fats and steroids, etc. need to be performed at low water levels (Mozhaev et al., 1989).

1A.2. Three phase partitioning

n-butanol as an extracting solvent was first used by Morton (1950) for several enzymes. In this study, extraction of lipids and esters was found to be effective thus aiding protein purification. Later, Tan and Lovrien (1972) found that a number of enzymes maintain their activities in t-butanol-water cosolvent mixtures, some even have enhanced activity while some other denatured. Further, Lovrien and coworkers (1987) extended their work by a technique called "Three phase partitioning" (TPP). Initially, TPP was first developed as an upstream method for precipitation of crude cellulases (Odegaard et al., 1984). TPP is a process by which target enzymes or proteins are precipitated

to interface from crude extracts by employing suitable compositions of an organic solvent such as alcohol (ex: t-butanol) and a inorganic salt (ex: ammonium sulfate) (Lovrien et al., 1987).

t-butanol, unlike other solvents, stabilizes the protein structure for most of the enzymes or proteins since it is a powerful structure promoter. Because of its size and bushy structure, t-butanol, unlike ethanol and methanol, does not easily penetrate folded and partly folded proteins. Hence t-butanol acts as a differentiating solvent and co-solvent whereas the C1 and C2 alcohols enter into the tertiary structure of proteins denaturing many of them (Dennison and Lovrien, 1997). It also inhibits enzyme activity at a concentration greater than 30% (v/v). The presence of 30% (v/v) t-butanol reduces the generation of artifacts either by proteolysis or by protein-protein interactions (Dennison et al., 2000). The salting out of a protein by sulfate ion can be viewed in six ways. Ionic strength effect, kosmotropy, cavity surface enhancement osmotic stress (dehydration), sulfate anion binding to cationic protein molecules thus promoting macromolecular contraction and conformational shrinkage and as an exclusion crowding agent (Dennison and Lovrien, 1997).

There are possibly two advantages of TPP over conventional salting out. In conventional salting out, the solution becomes denser with an increase in ammonium sulfate concentration until a point is reached where it is difficult to sediment the precipitated protein. In TPP, the precipitated enzyme/protein floats on the aqueous layer and with an increase in concentration of ammonium sulfate, more and more protein partitions to the interface.

Secondly, in conventional salting out, the precipitated protein needs to be desalted since the salt content is high, while in TPP, the precipitated protein has low salt content which can be easily separated by ion exchange chromatography (Dennison and Lovrien, 1997). Hence, TPP has emerged as a potential technique for the biomolecule purification.

Currently, three groups have been actively working on TPP (Dennison et al., 2000, Sharma et al., 2000, and Szamos and Kiss, 1995). Dennison and coworkers and Sharma and coworkers are working on the purification of enzymes and proteins by TPP, the group of Szamos and Kiss are more interested in the rheological properties of the recovered proteins at the interface after TPP. Table 1 shows the list of biomolecules extracted and purified by TPP.

The overexpression of protein in *E.coli* results in inactive aggregates called inclusion bodies and it is desirable to recover the active protein from them. One way of recovering the active protein is by TPP. An interesting aspect of TPP found by Roy et al. (2003, 2005) and Sharma et al. (2004) is its ability to renature the structure of enzymes such as xylanase, pectinase, cellulose, cellobiase, β-glucosidase from inclusion bodies which are initially denatured by urea. The authors concluded that the renaturation of the structure of protein may be due to the presence of sulfate and t-butanol which act as structure promoting agents thus aiding in correct refolding. The exact mechanism by which TPP takes place is yet to be known completely.

1A.2.1. Systematic approach for the recovery of biomolecules by TPP

The parameters required to be optimized for the extraction and purification of a biomolecule in TPP are isoelectric point (pI) of the protein, concentrations of alcohol (ex: t-butanol) and salt (ex: ammonium sulfate), temperature at which TPP is to be carried out and different concentrations of the crude extract. The procedure usually adopted is as follows:

a) pI of the protein is determined experimentally by isoelectric focusing or by cross-partitioning method of aqueous two phase extraction and the values need to be cross checked with literature values.

b) On fixing the concentration of t-butanol (say 20-30% v/v) the concentration of ammonium sulfate is varied (say 20-60% w/v). The concentration of ammonium sulfate that gives the highest purity and yield for the fixed concentration of t-butanol is selected for further experiments.

c) After knowing the optimum concentration of ammonium sulfate, the ratio of concentrations of crude extract to t-butanol is varied (1:0.3, 1:0.5, 1:0.75, 1:1). The concentration of t-butanol which gives the highest purity and yield for the fixed concentration of ammonium sulfate is selected for further experiments.

d) If, most of the enzymes or proteins are found in the aqueous phase even after step (c), a second cycle of TPP is carried out.

e) After knowing the optimum concentrations of t-butanol and ammonium sulfate, the temperature at which TPP can be carried out is determined (say 25-60°C), depending on the temperature tolerance of a given

enzyme. Temperatures below 25°C are not recommended since t-butanol solidifies below it.

f) Finally optimum enzyme or protein concentration (low, intermediate or high) of the crude at which TPP needs to be performed is determined.

1A.3. Aqueous two phase extraction

One drawback of TPP is that some biomolecules such as hemoglobin get denatured irreversibly due to the organic solvent, t-butanol (Pike and Dennison, 1989). Hence, in such cases, liquid-liquid extraction using aqueous two phase systems (ATPS) can be a better alternative. Aqueous two phase extraction (ATPE) has been recognized as potential purification step for the downstream processing of biomolecules ever since Albertson's discovery (Albertsson, 1986). The systems employed in ATPE are of two types namely, polymer-polymer or polymer-salt. Polymer-salt systems are generally preferred over polymer-polymer ones because of low cost and low viscosity. Protein purification from cells (or cell debris) of the intracellular proteins and enzymes at a large scale encounters the problems of small size of particle (such as bacteria), viscosity of slurries, separation of nucleic acids accompanied by high loss in activity of product (Husted et al., 1985). Conventional unit operations such as filtration and centrifugation can solve these problems to some extent but cannot represent a total solution for it. The problems associated with handling of solids in filtration are compressibility of biological materials leading to formation of compact cakes.

ATPE can be employed as an alternative to solve the problems encountered in filtration and centrifugation for the isolation of target biomolecule and removal of insoluble contaminants such as cell debris as well as soluble contaminants. ATPE has been successfully employed for the purification of biomolecules in large scale (Husted 1978, Kula, 1981, 1982a, 1982b; Kroner 1978, 1982; Hart, 1994).

The applications of ATPE are listed in the monographs (Albertsson, 1986; Walter, 1985 and Walter and Johansson, 1994) as well as in the reviews (Albertsson, 1970; Kula et al., 1982a; Diamond and Hsu, 1992; Raghavarao et al., 1995; Kula and Selber, 1999).

1A.3.1. Systematic approach for the recovery of biomolecules by ATPE

Optimization of the partitioning of target biomolecule and its purification by ATPE depends on systematic variation of system parameters (Albertsson, 1986) as well as the solute parameters. The system parameters are choice of phase components, system pH, PEG molecular weight, tie line length (phase composition), volume ratio, and type and concentration of neutral salt while the solute parameters are hydrophobicity, charge, size, molecular weight of biomolecule (Albertsson, 1986; Cascone, 1991). The flow chart for the recovery of biomolecules by ATPE is shown in Figure 1A.

a) The polymer to be used in ATPE is first fixed. To select the salt of PEG/salt system, ATPE is carried out using different PEG/salt systems at low molecular weight PEG (1000 or 1500), low TLL, at constant volume ratio of one, and at a pH 7 (or the pH at which the biomolecule

is stable). Usually low molecular weight of polymer is preferred since majority of contaminants, cell debris and nucleic acids can be partitioned to bottom (salt rich) phase.

b) The phase diagram is constructed for the selected polymer-salt system or obtained from literature if available.

c) The molecular weight of the polymer is chosen by carrying out ATPE (at pH 7 or optimum pH of biomolecule) at low, medium and high molecular weights (say 1500, 4000, 6000 and 8000). The experiments are executed at a constant pH and volume ratio of one. The molecular weight which gives the optimum purity, partition coefficient and yield for the biomolecule is selected for further experiments.

d) The molecular weight chosen from step (c) is employed for selecting the volume ratio. In this case, TLL and pH are kept constant. The optimum volume ratio is decided based on the purity, partition coefficient and yield for the biomolecule obtained in these ATPE experiments.

e) Finally, pH of the phase system is selected from ATPE experiments by keeping the TLL and volume ratio constant.

1A.4. Recent developments in ATPE

1A.4.1. Affinity partitioning

The term “affinity partitioning” was coined by Flanagan and Barondes (1981) who used affinity ligand bound polymer in the form of dinitrophenyl-PEG in PEG-dextran system for the purification of S-23 myeloma protein (Diamond and Hsu, 1992). In affinity partitioning a ligand is covalently attached to one of the polymers, and this macroligand partition selectively to

one of the phases similar to underivatized polymer while the contaminants partition to opposite phase (Carlson, 1988, Diamond and Hsu, 1992).

1A.4.2. Use of charged PEG

When a protein is added to the ATPS, a non-covalent protein-complex is formed, that partitions to one of the phases. If the macroligand is a third polymer, such as the derivatized PEG, it partitions in the similar manner as that of PEG at low concentration. If the concentration of the derivatized PEG is increased then the partition coefficient significantly increases even 10 times higher that the normal PEG (Johansson and Shanbhag, 1984; Johansson et al., 1983). Thus a charged PEG makes the partitioning of the protein highly pH dependent which aids in adjustment of their partitioning in a most favorable way (Johansson, 2000). Charged PEG derivatives such as trimethyl amino-PEG (TMA-PEG) and PEG-sulfonate (S-PEG) gives information about the net charges and isoelectric points of proteins as well as particles. TMA-PEG enhances the affinity of the particles to interface and top phase if the particles in the original system are partitioning to bottom phase. On the other hand, if the particles are partitioning to the top phase, the selectivity to interface and bottom phase can be increased by the addition of S-PEG.

1A.4.3. Use of hydrophobic polymer derivatives

In PEG/dextran system, if a fraction of PEG is replaced by PEG to which hydrophobic groups are covalently coupled, it dramatically enhances the partitioning of the biomolecules of interest (Shanbhag and Jensen, 2000). PEG-palmitate was used for the affinity partitioning of human serum albumin in PEG-dextran system wherein the partition coefficient was found to increase 3 times when the concentration of P-PEG was in creased to 1-10% (Shanbhag and Johansson, 1974). Partition coefficient of proteins (BSA, β-lactoglobulin, hemoglobulin and cytochrome-c) increased when PEG bound fatty acids were introduced in PEG/dextran systems (Shanbhag and Axelsson, 1975).

1A.4.4. Dyes as affinity ligands

Reactive dyes can be directly coupled to PEG, dextran, ficoll, polyvinyl alcohol and hydroxypropyl starch (Kopperschlager and Kirchberger, 2000). The reactive dyes are used as ligands because of high specificity, low cost, ease of coupling and high chemical as well as biological stability. Triazine dyes such as Cibacron-blue were used for the purification of phosphofructokinase from baker's yeast to give 58 fold purification (Kopperschlager et al., 1982). Procion Red dyes was used as an affinity ligand for the large scale extractions such as formate dehydrogenase (FDH) from *Candida boidinii* employing PEG 8000-dextran system and Procion Red yellow (a triazine dye) was used in lactate dehydrogenase (LDH) from pig muscle using hydroxylpropyl-starch two phase system (Cordes and Kula, 1986; Joelsson and Tjerneld, 1994).

1A.4.5. Metal ions as affinity ligands

Metal affinity partitioning increases the selectivity of separating a biomolecue by incorporating chelated transition metal ions covalently bound to PEG as affinity ligands (Wuenschell, 1990). When metal-chelated PEG is added to ATPS containing the protein, proteins having affinity (surface accessible amino acids particularly histidine residues) towards the metal ion, partitions preferentially to PEG phase. Some of the chelating-PEG derivatives used are iminodiacetic acid (IDA)-PEG, L- and D- isomers of methionine(Met)-PEG, as well derivatives of tris-carboxymethylated ethylxene diamine (TED) agents and carboxymethylated (Cm-TREN). The most commonly used is IDA-PEG complexed with metal ions such as Cu, Co, Zn, Ni and Fe. Proteins such as human hemoglobin, bovine hemoglobin, whale and horse myoglobins were purified using metal affinity two phase extraction (Wuenschell, 1990, Chung and Arnold, 1991, Birkinmeier et al., 1991).

1A.4.6. Smart and thermoseparating polymers

There is an increasing interest among researchers to use smart polymers because of two reasons. Firstly, easy separation of target molecule from polymer and thereby recovering the polymer. Secondly, due to various possibilities to modulate the interaction between polymer and the target molecule by changing environmental variables such as temperature, pH and ionic strength and specific affinity (Johansson, 2002). The polymers such as chitosan, eudragit, alginate and hydrogels have been used as bio-ligands. Chitosan has been used to purify chitinases from neurospora crassa, cabbage and puffballs in PEG 6000-K_2HPO_4 system (Teotia et al., 2004). Eudragit S-

100 has shown its selectivity towards recombinant protein A in PEG 8000-Reppal PES200 system from *E.coli* cell homogenate. Alginate has been used in PEG 6000-K_2HPO_4 system to purify α and β amylase by 42 and 43 fold with activity recoveries of 92% and 90% respectively.

Thermoseparating polymers separate from water above a certain temperature, either cloud point temperature or the lower critical solution temperature. The thermoseparting polymers offer an attractive alternative to PEG/polymer or PEG/salt systems since system involves only one phase forming component, that is, the polymer (and no salt) in water solution. The random copolymer of ethylene oxide (EO) and propylene oxide (PO) is one such example that has lower cloud point and finds its application in protein purification (Harris et al. 1991; Alred et al., 1994). In the first stage, the target protein partitions to the EOPO-rich phase in a two-phase system (polymer-polymer). In the second stage, the EOPO-rich phase is removed and thermoseparated by increasing the temperature to its cloud point. In this water- polymer two-phase system the proteins are partitioned predominantly to top phase (containing water) leaving the copolymers in bottom phase that can be recycled (Johansson, 1999).

The use of smart hydrogels has attracted attention in biotechnology. Hydrogels is a network of hydrophilic polymers that hold a large amount of water. The key property of hydrogels is its swelling/deswelling property in aqueous solutions (Kim and Park, 2002). Hydrogels which swell or deswell upon changes in environmental conditions (pH, temperature, solvent, salt

type) are called smart hydrogels. Ucon a random copolymer of 50%ethylene oxide (EO) and 50% propylene oxide (PO) is used along with Repal PES to form ATPS. Ucon rich phase on separation and subjecting to temperature induced phase separation facilitated in extraction and purification of ecdysteroids from spinach leaves and apolipoproteins from *E.coli* (Modlin, 1994; Johansson, 1999).

1A.4.7. Detergent based aqueous two phase systems

The detergent based systems or cloud point extraction systems form two-phases in solutions of non-ionic detergents above a certain temperature called the cloud point temperature (Selber, 2004). A single isotropic micellar phase separates into two isotropic phases, one of the phases called coazervate or detergent rich phase and the other a detergent depleted phase (Minuth, 2000). The detergent forms micelles in the detergent-depleted phase and exists in the form of lamellar stacks in detergent-rich phase. This kind of ATPE was used to extract amphiphilic or hydrophobic biomolecules (usually from the membrane bound proteins). This also is a good alternative to polymer-polymer or polymer-salt aqueous systems since this system uses only a single phase forming component, that is, the surfactant in aqueous solution. Bordier (1981) first demonstrated it using nonionic polyoxyethylene detergents. Ramelmier et al. (1991) and Terstappen et al. (1992) demonstrated successfully the extraction of water-soluble extracellular proteins such as cholesterol oxidase and lipase. Cutinase was extracted from *S.cerevisiae* expressing cutinase variants carrying a tryptophan-rich peptide tag by using Agrimul NRE1205 (Rodenbrock et al., 2001). A

genetically engineered protein, endoglucanase I (EGI) and the hydrophobin I (HFBI) protein was extracted from *T.reesei* using the detergent Agrimul NRE 1205 in large scale (1200 L) with partition coefficient of 3.8 and yield of 59% (Selber et al., 2004).

1A.4.8. Application of Nanotechnology in ATPE

Nanotechnology, the science and technology that focuses on special properties of material which emerges from nanometer size is becoming the most promising scientific fields of research (Moraru et al., 2003). ATPE was used in the extraction and purification of bioparticulate products such as viral or plasmid gene therapy vectors and particulate protein vaccines (Walker and Lyddiatt, 1998). ATPE has been successfully employed for the recovery of inclusion bodies (100-200 nm) from *E.coli* in PEG 8000-potasium phosphate system. The inclusion bodies particulates partitioned to the lower phase while the cell debris to the interface (Walker and Lyddiatt, 1998). Recently, ATPE has been explored for the biorecognition-driven nanoparticle assembly which finds application in biosensing (Helfrich, 2005). DNA-derived gold nanowires were partitioned to interface in a PEG 8000-dextran10000 system which retain their ability to selectively bind to complementary DNA. ATPE has also been explored in diagnostics and material application. Gold-horse radish peroxidase (HRP) conjugate when partitioned in a PEG 8000-dextran 500 000 system resulted in a increase in partition coefficient of 150:1 in favor of PEG-phase and 2000:1 for the contaminants to dextran phase as compared to without conjugation (Long and Keating, 2006).

Table 1A. Biomolecules purified by TPP

AC-affinity chromatography, IEC-ion exchange chromatography

Enzyme/inhibitor	Source	process	Degree of purification (fold)	Activity yield (%)
1. Protease	T.brucei brucei	TPP	15	63
2. Cathepsin D	Bovine spleen	TPP+ AC	83.3	7.7
3. Catalase	Human erthrocytes	TPP+ IEC	640	5
4. Peroxidase	Horse radish	TPP+ IEC	23.6	29.5
5. Peroxidase	turnip	TPP+ MAC	80.3	20.3
6. Lipase	F.odoratum	TPP+ IEC+HC	14	18.4
7. Oligopeptidase	T.congolense	TPP	1269	34
8. M.dehydrogenase	A.parasiticus	TPP+ GFC	110	-
9. Alkaline phosphatase	Chicken intestine	TPP	23	88

MAC- metal affinity chromatography, HC-hydrophobic interaction chromatography, GF-gel filtration chromatography

Continued….

Enzyme/inhibitor	Source	process	Degree of purification (fold)	Activity yield (%)
10. Amylase inhibitor	Wheat germ	25	25	85
11. Phospholipase D	Dacus carota	TPP	13	72
12. Pectinase	A.niger tomato	TPP	10 9	76 183
13. Xylanases	A.niger	MLFTPP	95	60
14. Trypsin	soybean	TPP	13	72
15. Pectinase Cellulase	commercial	MLFTPP	13	96 92
16. Denatured xylanase	A.niger	TPP	21	93
17. Glucoamylase Pullanase	A.niger B.acidopullulyticus	MLFTPP	20 38	83 89

TPP- Three phase partitioning

MLFTPP-Macroligand facilitated three phase partitioning

Continued…

Enzyme/inhibitor	Source	process	Degree of purification (fold)	Activity yield (%)
18. Green fluorescent protein(GFP)	*E.coli*	TPP	20	78

19. Denatured pectinase	commercial	MLFTPP	4	100
20. Urea denatured cellulase Cellobiase β-glucosidase	commercial	TPP	73 65 101	94 98 90
21. α-amylase	*B. myloliquefaciens* Wheat germ Porcine pancreas	MLFTPP	74 77 92	5.5 55 10

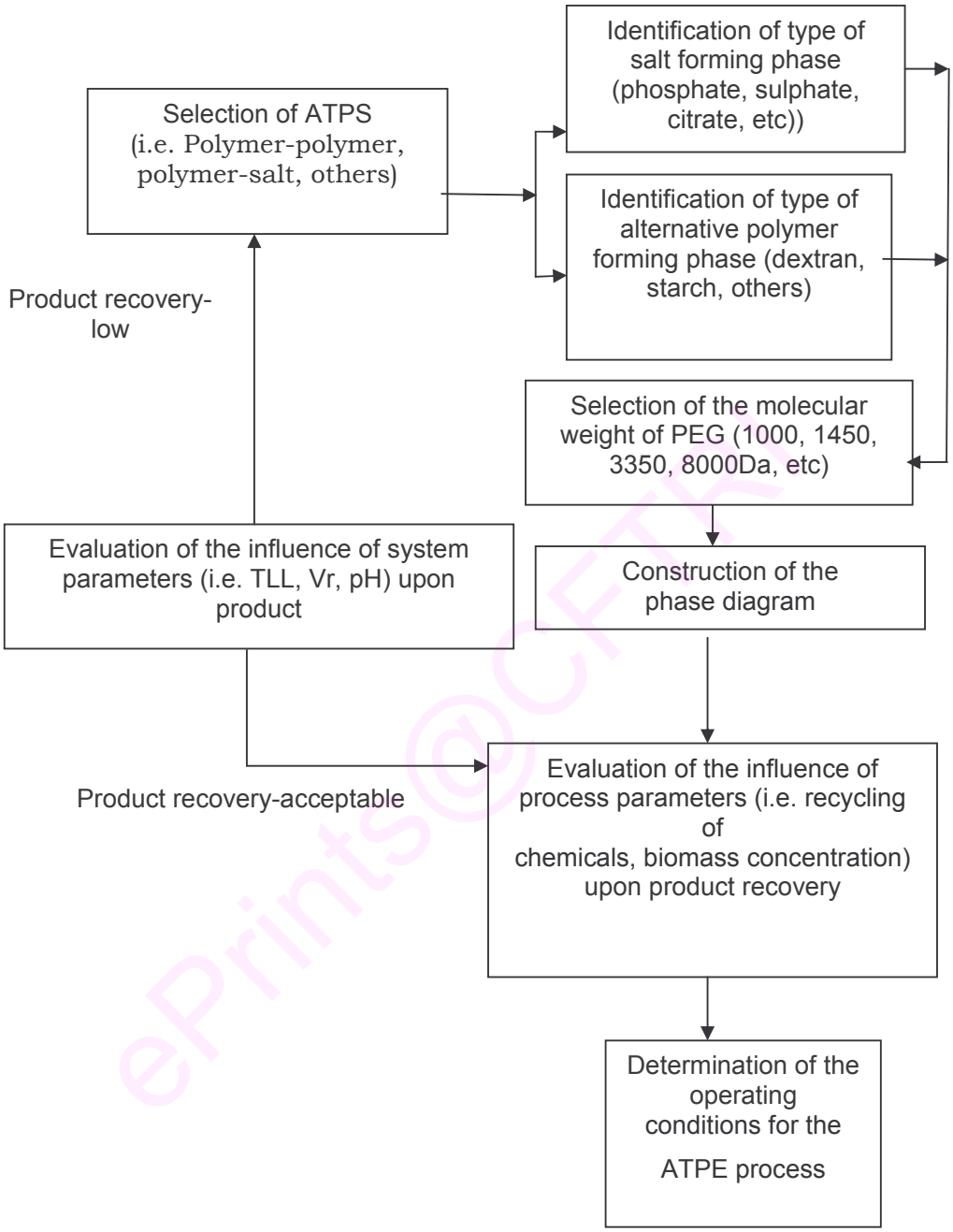

Figure 1A. Approach for the optimization of process parameters in ATPE[*]

[*d]Palomares (2004)

Section- B

AIM AND SCOPE OF PRESENT INVESTIGATION

Downstream processing of biological products using liquid-liquid extraction has been known from a long time. Since most of the enzymes and proteins are stable and function effectively in the water-rich medium, the potential of aqueous-organic system was not recognized in the past. In the last two decades with the growth of biocatalysis, aqueous/organic systems have been gaining enormous attention. In view of this, a process called "Three phase partitioning" consisting of t-butanol/ammonium sulfate system has been explored for its potential for the extraction and purification of biomolecules and comparison has been made with aqueous two phase extraction.

In Chapter 1, The downstream processes such as Three phase partitioning (TPP) and Aqueous two phase extraction (ATPE) are reviewed, highlighting their applications.

In Chapter 2, The phase systems of TPP and ATPE (without protein) were characterized with respect to density and viscosity and the kinetics of phase demixing are evaluated. Further the optimization of the process parameters for the extraction and purification of peroxidase from *Ipomoea palmetta* leaves employing TPP is presented.

In Chapter 3, a comparative study between TPP and ATPE is presented for the model protein, BSA and also for a real system, C-phycocyanin a natural colorant from *Spirulina platensis*. Effect of volume ratio in TPP and ATPE for the extraction of BSA and the evaluation of the process

(ATPE/TPP), with respect to purity and yield, for the extraction and purification of C-phycocyanin are discussed.

In Chapter 4, liquid-liquid extraction explored for the extraction and purification of the value added product-oryzanol from rice bran oil soapstock is described. The waxes and gums which are the major impurities that hinder in the extraction processes (leaching, liquid-liquid extraction) and other processes (enzymatic degumming and crystallization) explored for their efficiency in removing these impurities are discussed.

Chapter- 2

THREE PHASE PARTITIONING AND AQUEOUS TWO PHASE EXTRACTION

Section- A

Physicochemical characterization and Kinetics of demixing of phases

2A.1. Introduction

In LLE, physicochemical characterization of phases with respect to density and viscosity enables to know the phase system having high or low demixing times. The interfacial tension data in LLE is important for the optimization of partitioning of product and impurities. Generally low values of interfacial tension are preferred in ATPE and TPP in order to facilitate the mass transfer. The values of interfacial tension of different systems are in the order of t-butanol/salt system> polymer/salt system> polymer/polymer system.

The kinetics of phase demixing depicts the entire behavior of the phase system during and after extraction. Kinetics of phase demixing was well documented in literature for aqueous/organic and aqueous two phase systems [Backes et al., 1990, Kaul et al., 1995 and Srinivas et al., 2000]. Merchuk et al. [1980] studied the extraction of copper solutions by LIX64N in aqueous/organic systems with various types of motionless mixers. It was found that for the same amount of copper recovered, the motionless mixer required half the volume of the equipment used as compared to a stirred vessel. Tidhar et al. [1986] found the phase inversion points comprising of water/kerosene, water/CCl_4 and mixture of water/CCl_4/kerosene.The experimental values were in good agreement with the proposed model.

In ATPE, Asenjo and coworkers [Kaul et al., 1995, Salamanca et al.,1998, Merchuk et al.,1998] studied the kinetics of demixing by gravity separation and found the phase inversion points in PEG 4000 and potassium phosphate system. They concluded that by representing the dispersion height

as a function of initial height, a profile can be obtained, independent of geometrical dimension of the separator, which is of prime importance during design of large scale separators. The experimental values agreed well with their predicted model [Mistry et al., 1996 and Asenjo et al., 2002].

The present study deals with the kinetics of demixing in aqueous/organic system comprising of t-butanol/ammonium sulfate system at H/D ratio of 5.89 employing different volume ratios (0.43-2.33) and TLL's (52%-71%). In case of ATPS, the kinetics of demixing of PEG 4000/potassium phosphate system was studied at H/D ratios of 0.75 and 2.85, volume ratios (0.43-2.33) and TLL's (13.12-44.65%).

2A.2. Materials and Methods

t-butanol and ammonium sulfate were purchased from Merck, Germany and Rankem Mumbai, India, respectively. PEG 4000, potassium di-hydrogen phosphate (KH_2PO_4) were procured from SRL, Mumbai, India and di-pottasium hydrogen phosphate (K_2HPO_4) from Ranbaxy, New Delhi, India. All chemicals purchased were LR grade. The photograph of the spinning drop tensiometer instrument is shown in Fig 2A.1.

2 A.2.1 Phase system preparation

In aqueous/organic system, predetermined quantities of ammonium sulfate, and distilled water were mixed thoroughly followed by addition of t – butanol and stirred for 30 min in a magnetic stirrer to prepare 100 %w/w of the system based on the phase diagram (Kiss et al., 1998). The mixture was left for complete phase separation in a separating funnel and the individual phases separated.

In aqueous two phase system, predetermined quantities of PEG 4000, water and potassium phosphate were weighed based on the phase diagram (Albertsson, 1986) and mixed thoroughly for equilibration. The mixture was left for complete phase separation in a separating funnel and the individual phases separated.

2A.2.2. Measurements of density, viscosity and interfacial tension

Density and viscosity measurements were carried out using specific gravity bottle of 10 ml capacity and Ostwald-U-tube viscometer of 25 ml capacity, respectively. All measurements were carried out in duplicates at 25±1°C.

Interfacial tension measurements were performed by spinning drop tensiometer as shown in Figure 2A.1 (SITE100 No. 20002806, Kruss, Germany). Interfacial tension was calculated as mean of rotation for 2500-7500 rpm for 15 counts at 25±1°C using the drop shape analysis software (DSA 2).

2A.2.3. Kinetics of demixing

The desired volume ratios were obtained by taking the required volumes of top and bottom phases from the equilibrated and separated phases of the phase system. For all the experiments in aqueous/organic system, the demixing studies were carried out in a 100 ml measuring cylinder (height to diameter ratio, 5.89). The kinetics of demixing was studied by measuring the height of dispersion as a function of time. In aqueous two phase system, PEG 4000 and potassium phosphate were used (K_2HPO_4:KH_2PO_4=1.82:1, pH=7) and the demixing studies were carried out similar to aqueous/organic system. In aqueous two phase system, demixing studies were carried out in a 100 ml beaker filled to half the height (height to diameter ratio 0.75) and 100 ml measuring cylinder filled to half the height (height to diameter ratio 2.85).

2A.3. Results and Discussion

2A.3.1. Aqueous/organic system (t-butanol/ammonium sulfate system)

2A.3.1.1. Effect of TLL on density of phases

With an increase in TLL, the density of the top phase practically remained constant while that of the bottom phase showed an increase by 14% (1045 to 1190 kg/m^3) (Figure 2A.2). With an increase in TLL, the density difference between top and bottom phase increased. For example, at 71% TLL, difference in density 108% higher than that at 52%TLL.

2A.3.1.2. Effect of TLL on viscosity of phases

With an increase in TLL, the viscosity of top and bottom phases were unaffected because of less variation in composition of phases (Figure 2.A.3). The viscosity of top and bottom phases (and hence difference in viscosities between top and bottom phases) from 52% to 71% TLL remained practically constant.

2A.3.1.3. Effect of TLL on interfacial tension of the phases

With an increase in TLL, interfacial tension increased and it ranges from 0.26-11.3 mn/M for low (52%) to high (71%) TLL (Figure 2A.4). With an increase in TLL, the composition of both phase increases, the surface tension increases and thus results in increase in interfacial tension.

For the partitioning of biomolecules and to preserve its native structure, low TLL (≤ 52 %) are preferred in t-butanol/ammonium sulfate system because of the low interfacial tension of the phases.

2A.3.1.4. Kinetics of phase demixing

The kinetics of demixing are shown for low (52%), medium (63.5%) and high (71%) TLL's in Figures 2A.5, 2A.6 and 2A.7. All the plots show a sigmoidal curve. In organic/aqueous systems two types of separation profiles are generally observed. When the rate of drop sedimentation is greater than rate of drop coalescence, then a sigmoidal plot is observed. If the rate of drop coalescence is greater, an exponential curve is observed (Kaul, 1995).

At low TLL (52%), the volume ratios 0.43-2 showed almost similar demixing times (Figure 2A.5). At volume ratio of 2.33, the demixing time reduced to nearly half as that compared to volume ratios of 0.43-2. At low volume ratio (0.43 and 0.66), it was expected that the rate of demixing to be higher (low demixing time) than at 1-2.33. This was because at volume ratios of 0.43 and 0.66, the less viscous aqueous (bottom) phase forms the continuous phase and more viscous t-butanol (top) phase forms the dispersed phase. However, the observed trend is in fact the opposite (Figure 2A.5). The decrease in demixing rate at volume ratios of 0.43 and 0.66 was because of low interfacial tension of the system (0.26 mN/m). The low interfacial tension leads to slow rate of coalescence and in turn slow rate of demixing (Srinivas et al, 2000). At medium TLL (63.5%), all the volume ratios showed similar demixing rates (Figure 2A.6). The demixing rate at volume ratio of 2.33 decreased as compared to its demixing rate at low TLL (52%) (Figure 2A.5 and 2A.6). This was due to the high volume of continuous (top) phase which was more viscous as result of increase in phase composition at 63.5% TLL. At high TLL (71%), the demixing rates at volume ratios of 0.43-2 show similar trends while the demixing rate at 2.33 increased by 43% as compared to low TLL (52%) (Figure 2A.7). This may be attributed to the high interfacial tension of the system (11.3 mN/m) which results in high demixing rate due to faster drop coalescence of the dispersion and thus faster separation of the phases.

2A.3.2. Aqueous two phase systems (PEG 4000/K_2HPO_4)

2A.3.2.1 Effect of TLL on density of phases

As TLL increased, the density of the top phase remained practically constant while that of bottom phase increased (Figure 2A.8). With an increase in phase composition, the density difference between top and bottom phases increased and at high TLL (44.65%), it was about 73% higher than that of low TLL (13.12%). As the TLL increased the phase composition at higher TLL's contained very high PEG content (about 28-40 %w/w) in top phase and similarly very high salt content (20-27 %w/w) in bottom phase. Similar observation was reported previously (Asenjo, 2002). The phosphate concentration had a significant effect on density than PEG concentration.

2A.3.2.2. Effect of TLL on viscosity of phases

With an increase in TLL, the viscosity of top phase increased at higher TLL (44.65%) significantly, while that of bottom phase remained constant (Figure 2A.9). This is because with an increase in TLL, the concentration of PEG and phosphate in top and bottom phases increased. The increase in concentration of PEG increased the viscosity of top phase and at higher TLL (>31 %) it increased dramatically (about 93%) while the increase in salt concentration did not effect the viscosity of bottom phase.

2A.3.2.3. Effect of TLL on interfacial tension of the phases

With an increase in TLL, interfacial tension of the individual phases increased significantly. At 44.65% TLL, the interfacial tension of the phases was 100% higher than that at 13.12% (Figure 2A.10). As the TLL increased

the difference between the top and bottom phase composition increased, the surface tension increased and thus the interfacial tension also increased.

2A.3.2.4. Kinetics of demixing

At low TLL (13.12%) and H/D=0.75 high demixing rates are observed at volume ratios of 2 and 2.33 as compared to 0.43-1.5 (Figure 2A.11). At volume ratios of 1.5-2.33, the continuous and dispersed phases were top (PEG) and bottom (salt) phases, respectively. It was expected that high volume ratios (2 and 2.33) would result in low demixing rates (high demixing times), since the top phase was continuous and viscous, however, the trend observed is the opposite (Figure 2A.11). This was because at these volume ratios, the droplet size of the dispersed phase was high, resulting in faster rate of coalescence and hence high demixing rates (low demixing time). At medium TLL (25.52%), and volume ratios of 0.43-1.5, increase in demixing rate by 31-40% is observed as compared to that at low TLL (13.12%), while, at high volume ratios (2 and 2.33) any significant effect is not observed (Figure 2A.11 and Fig.2A.12). The increase in demixing rate at volume ratios of 0.43-1 (25.52% TLL) was due to increase in phase composition as well as high volume of continuous (less viscous) bottom phase. At a volume ratio of 1.5, though the continuous phase was the top phase, the higher phase composition at medium TLL (higher density differences between the phases) leads to higher demixing rates than low TLL (13.12%). The demixing rate at medium TLL (25.52%) and high volume ratios (2 and 2.33) were found to be similar to low TLL (13.12%). This was because the viscosity of the continuous (top phase) at 25.52% and 13.12% TLL's were almost similar due to less

differences in phase compositions. At high TLL (44.65%) and volume ratios 0.43-1.5 the increase in demixing rate was about 42-66% as compared to low TLL (13.12%) (Figure 2A.13). The increase in demixing rate for volume ratios 0.43-1 was due to the synergistic effects of high phase composition and large volume of the continuous (bottom) phase. At a volume ratio of 1.5, though the more viscous (top) phase was continuous, the density difference between top and bottom phases at 44.65% TLL was about 278% higher than 13.12% TLL which leads to faster demixing rate. The high volume ratios (2 and 2.33) showed similar demixing rates at medium (25.52%) and low (13.12%) TLL's as compared to other volume ratios (0.43-1.5). This was due to the larger droplet size of the dispersed phase that in turn leads to faster coalescence rates and thus higher demixing rates (due to the higher interfacial tension of the system).

At low TLL (13.12%), H/D=0.75, the trend followed by the volume ratios 0.43-2.33 show that demixing rate increased by about 13-35% as compared to low TLL (13.12%), H/D=2.85, which was expected because of high H/D ratio (reduced cross sectional area) (Figure 2A.14). Similar results were obtained for medium TLL (25.52%), H/D=0.75 and volume ratios 0.43-2.33, where increase in demixing rate by about 19-50% is observed as compared to medium TLL (25.52%), H/D=2.85 (Fig.2A.15 and Fig. 2A.12). At high TLL (44.65%), H/D=0.75, the demixing rate increased for the volume ratios 0.43-2.33 by about 8-60% as compared to high TLL (44.65%), H/D=2.85 (Figure 2A.16and Figure 2A.13).

2A.3.2.5. Kinetics of phase demixing in presence of cells

Geometry of the separation equipment has a significant effect on the separation time (Solano-Castillo, 2000). For the recovery of the biomolecules from crude homogenate, where the cell debris partition to interface rather than either of the phases, H/D<1 are preferred as compared to H/D>1.This is because for H/D<1, the cell debris distribute across the interface, thereby improving the separation efficiency (expressed as relative volume ratio, that is, the volume ratio of the phase system in presence of the cells to that without cells [blank system])of the desired product in top or bottom phase (Solano-Castillo and Rito-Palomares, 2000).

H/D ratios of <1 (0.17 and 0.85) has been employed for the study of kinetics of demixing of *Spirulina platenisis* homogenate (2 L scale). The trend shows that for H/D=0.17, the dispersion heights (after 60 min and 840 min) are constant while that for H/D=0.85, even after 720 min (12 hrs) the separation is incomplete (Figure 2A.17). Hence, low H/D ratio showed high demixing rate as compared to higher ones for the cell debris partitioning at the interface.

2A.4.Conclusions

The density, viscosity and interfacial tension increased with an increase in TLL in t-butanol/ammonium sulfate system and PEG 4000/potassium phosphate system. The increase in density, viscosity and interfacial tension was more prominent in PEG 4000/potassium phosphate system than in t-butanol/ammonium sulfate system. This is because the

density of top phases (PEG 4000) in PEG 4000/potassium phosphate system is higher than that of top phase (t-butanol) in t-butanol/ammonium sulfate system.

The kinetics of demixing in t-butanol/ammonium sulfate and PEG 4000/potassium phosphate systems showed a sigmoidal plot. Low H/D ratios showed higher demixing rates than high H/D. In t-butanol/ammonium sulfate and PEG 4000/potassium phosphate systems, high volume ratios showed higher demixing rates as compared to low volume ratio.

The kinetics of demixing employing cell homogenate in aqueous two phase system showed higher demixing rates for the low H/D values than high H/D for the cell partitioning at interface.

Figure 2A.1. Photograph of Spinning drop tensiometer

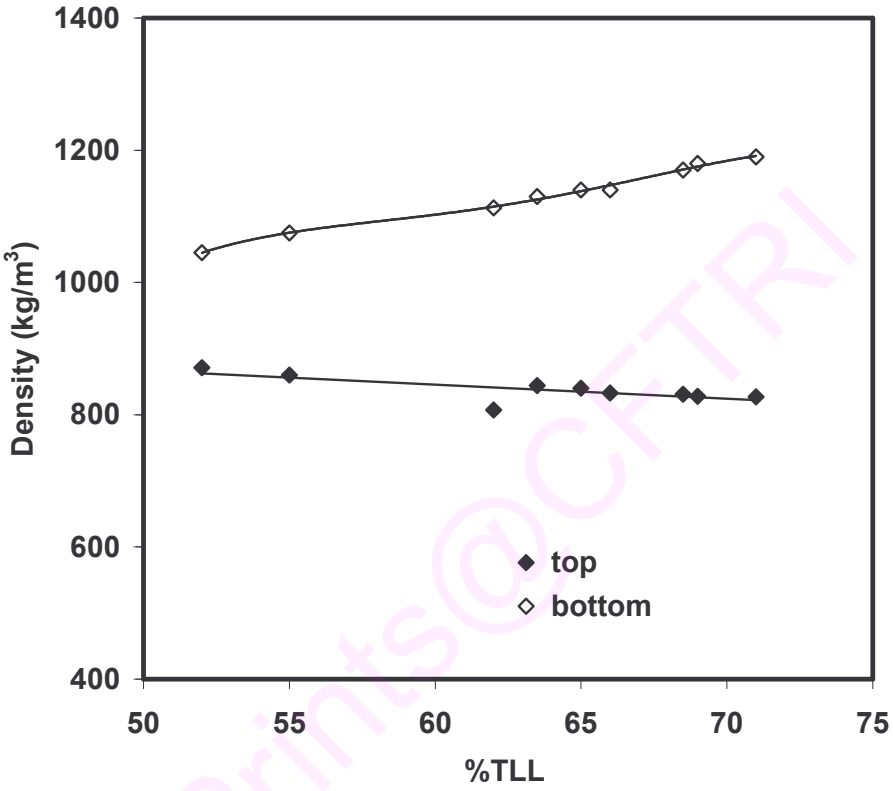

Figure 2A.2. Effect of TLL on phase density of t-butanol/ammonium sulfate system

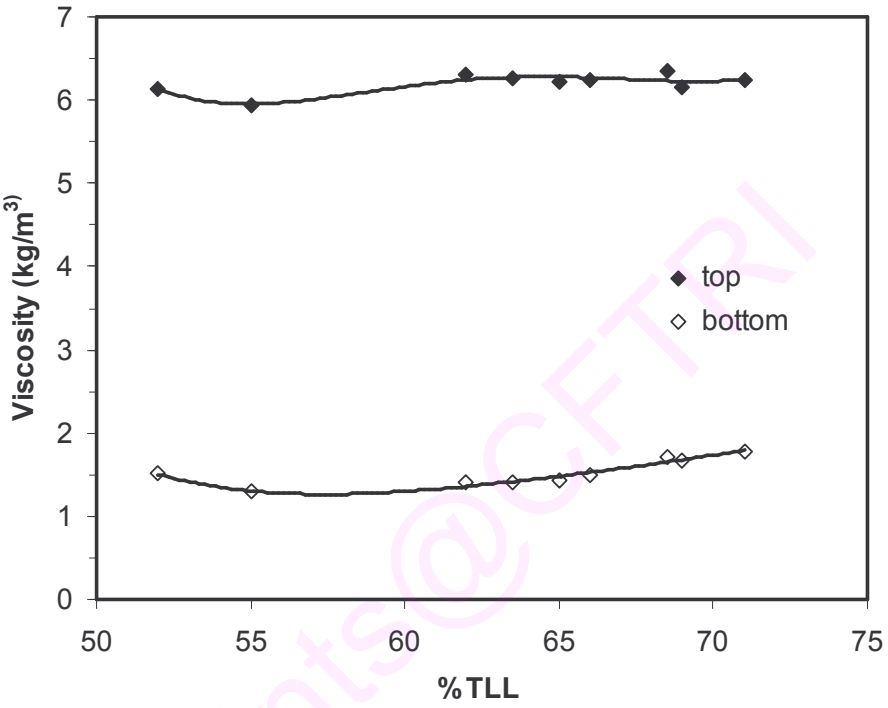

Figure 2A.3. Effect of TLL on phase viscosity of t-butanol/ammonium sulfate system

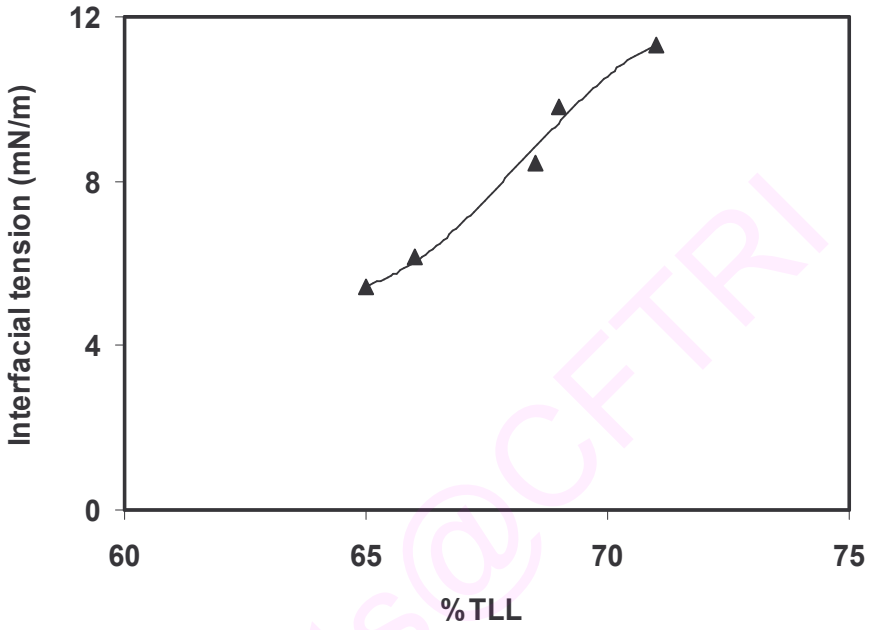

Figure 2A.4 Effect of phase composition on the interfacial tension of t-butanol and ammonium sulfate system

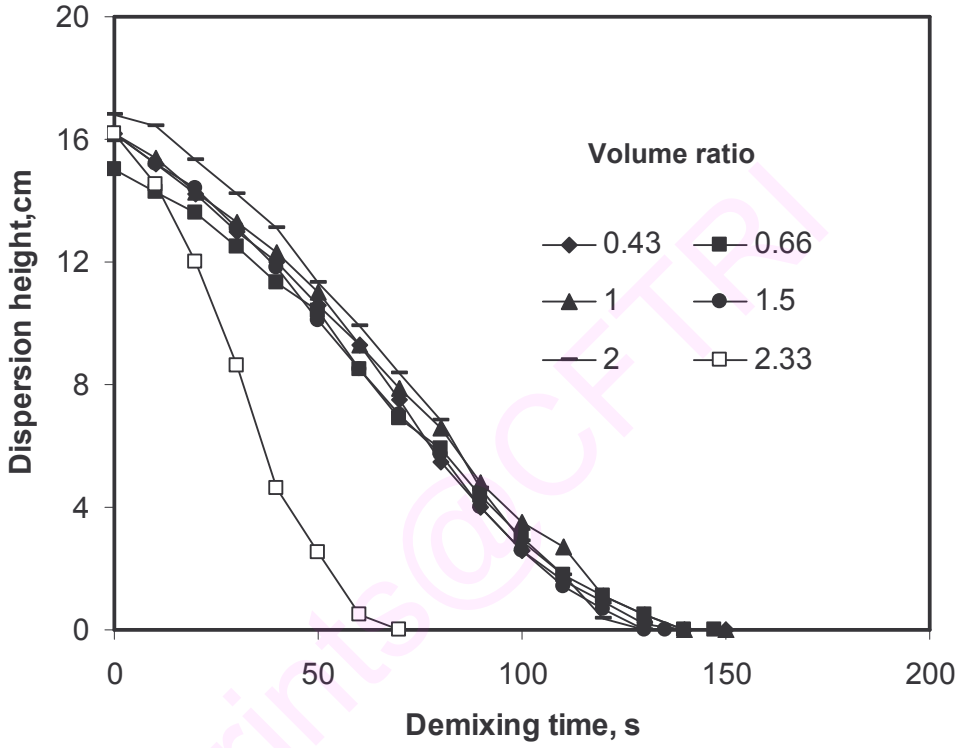

Figure 2A.5. Kinetics of phase demixing of t-butanol and ammonium sulfate at low TLL (52%)

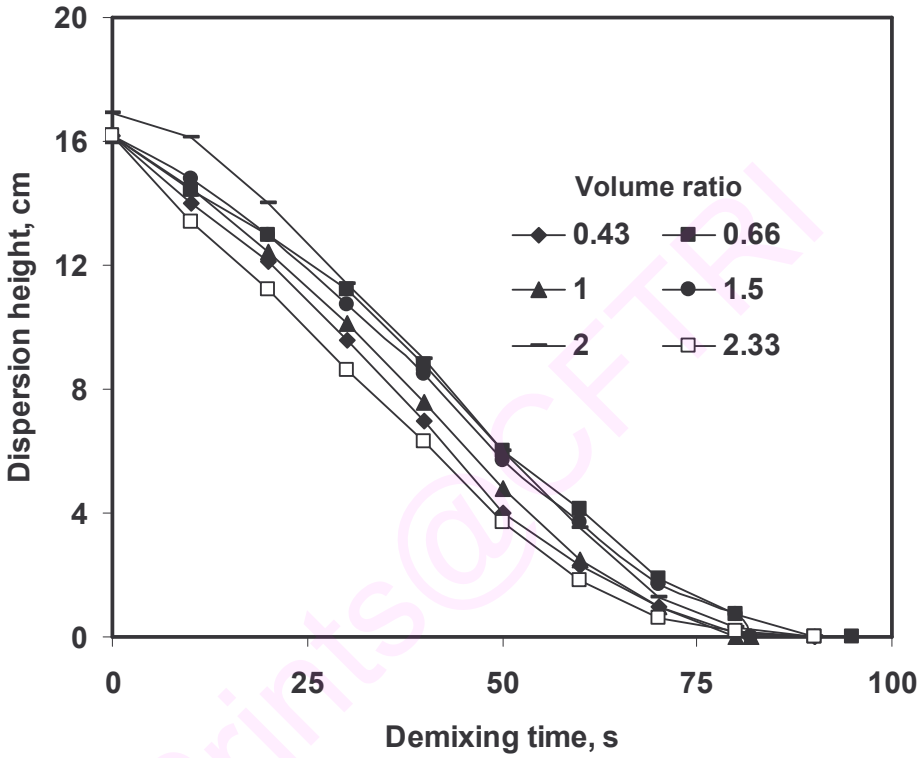

Figure 2A.6. Kinetics of phase demixing of t-butanol and ammonium sulfate at medium TLL (63.5%)

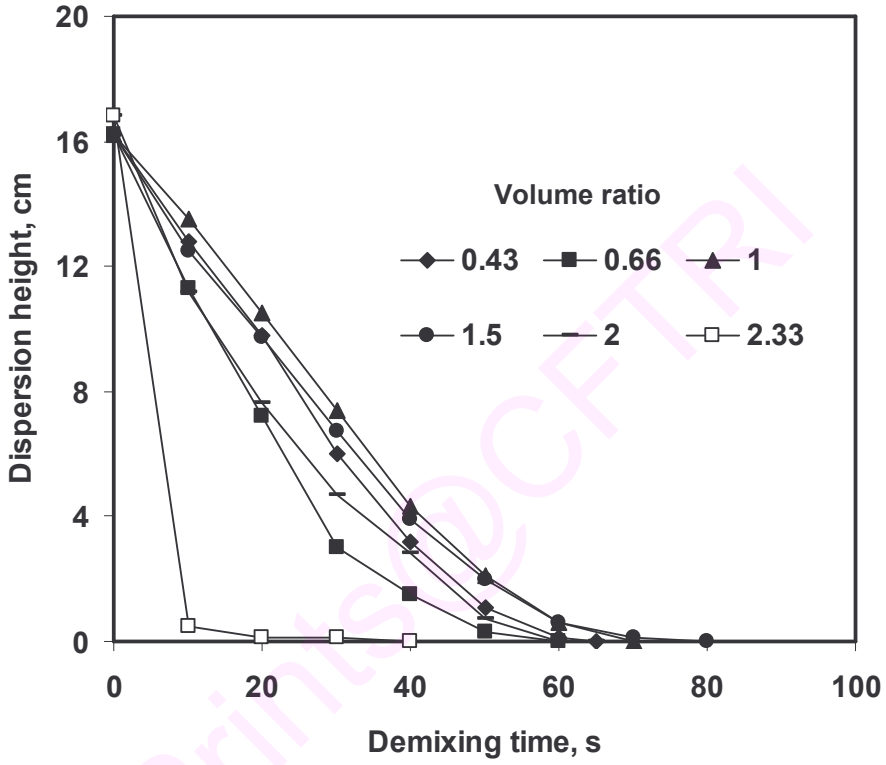

Figure 2A.7. Kinetics of phase demixing of t-butanol and ammonium sulfate at high TLL (71%)

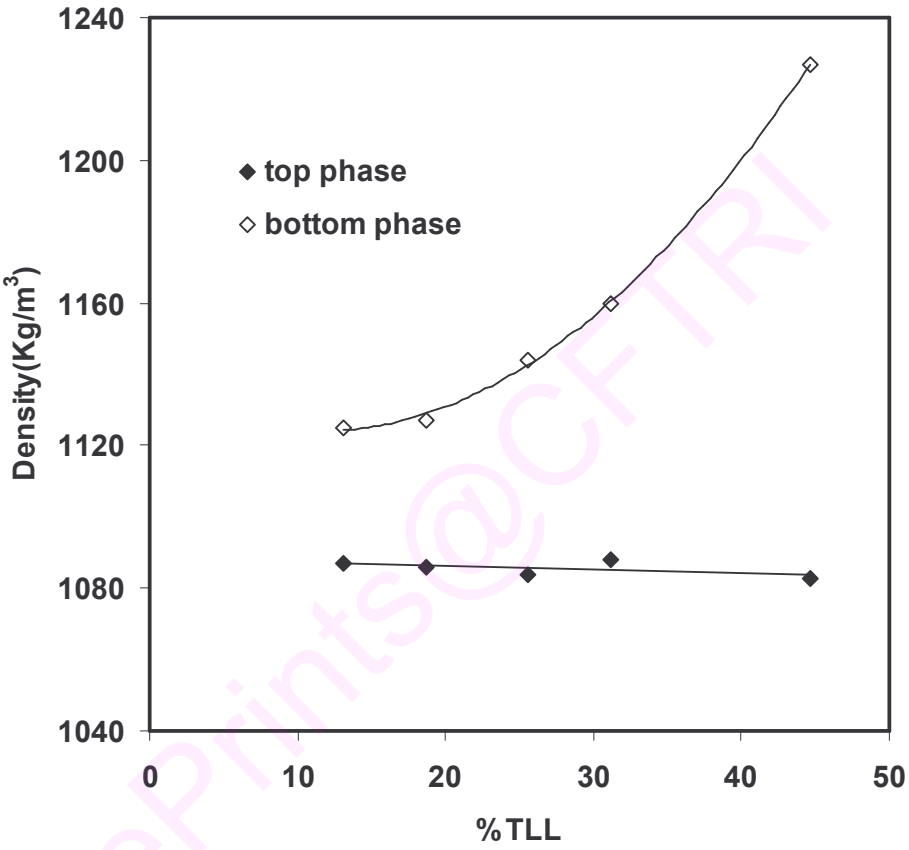

Figure 2A.8. Effect of TLL on phase density of top and bottom phases in PEG 4000 and potassium phosphate system

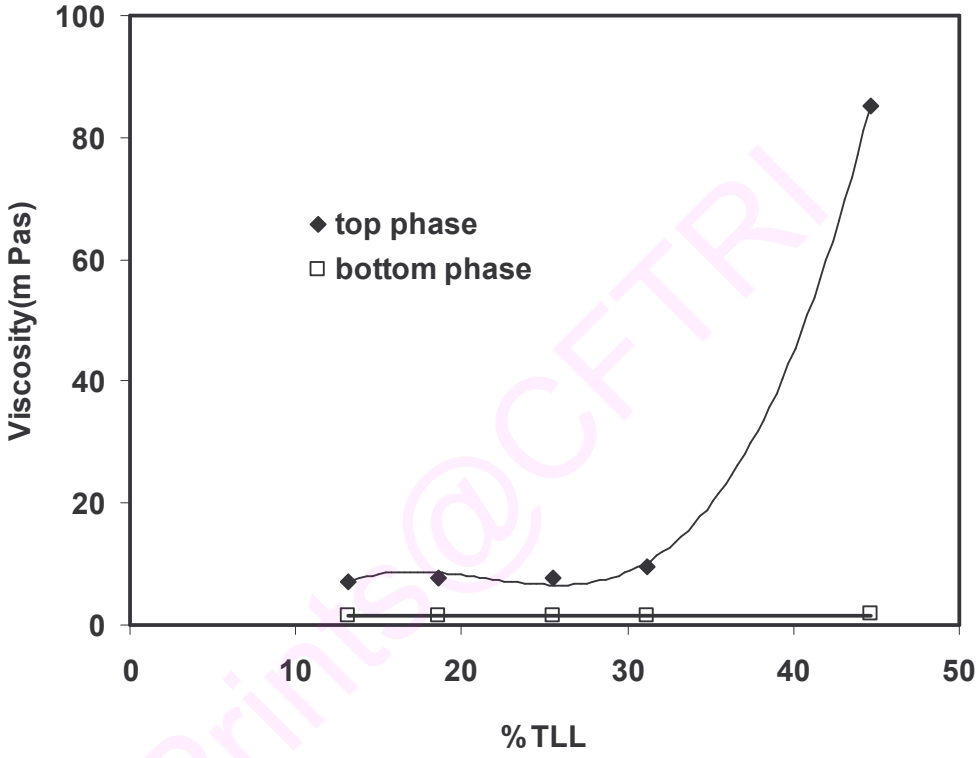

Figure 2A.9. Effect of TLL on the phase viscosity of the phases in PEG 4000 and potassium phosphate system

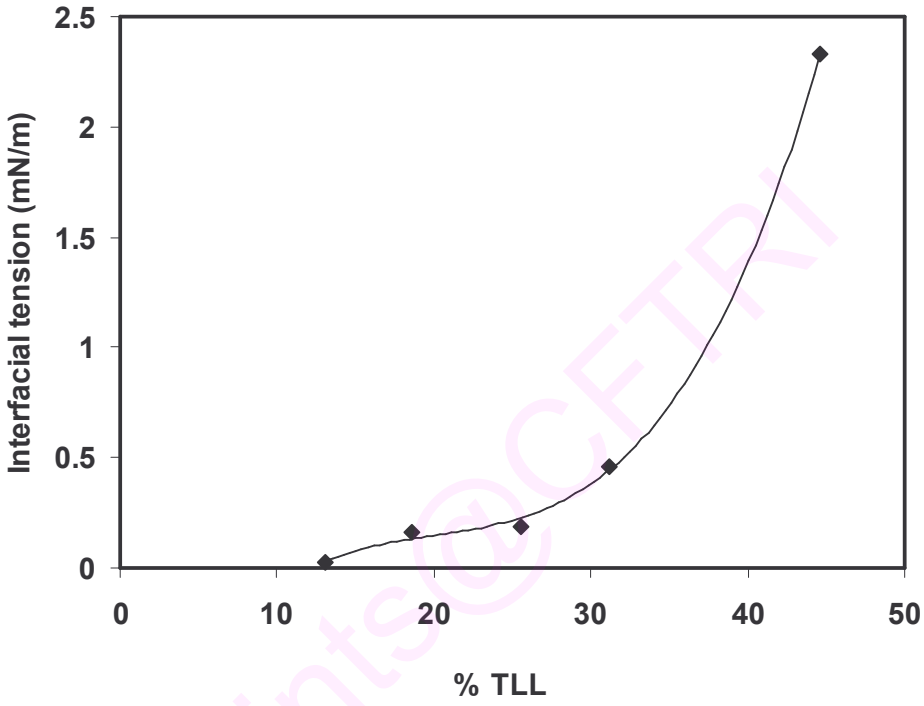

Figure 2A.10. Effect of % TLL on the interfacial tension of PEG 4000 and potassium phosphate system

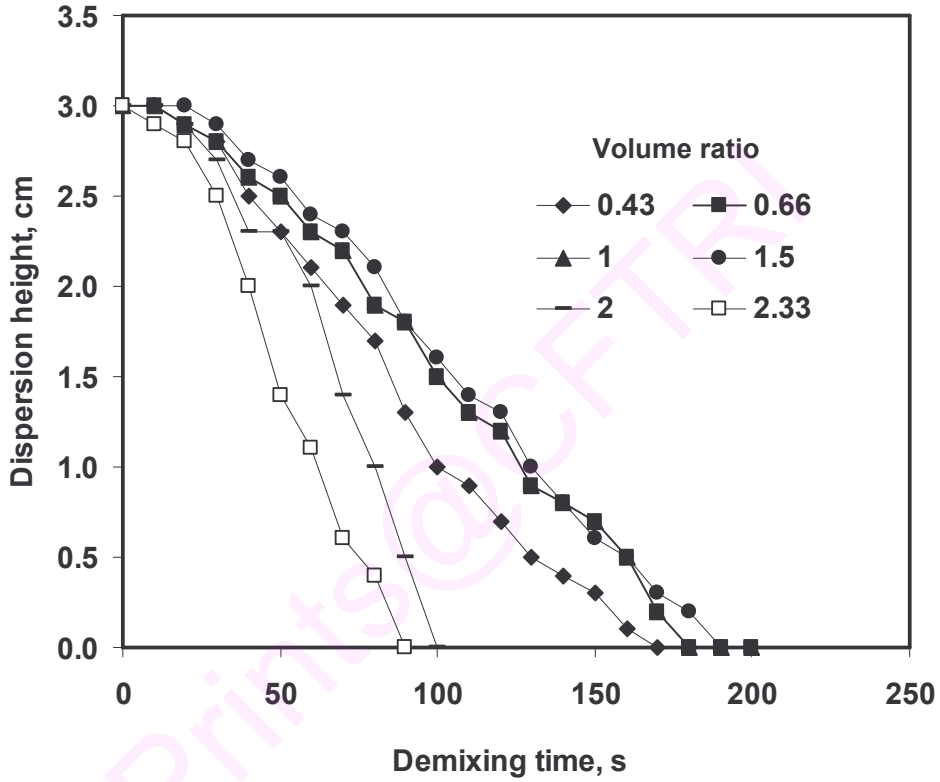

Figure 2A.11. Kinetics of phase demixing in PEG 4000 and potassium phosphate systems at low TLL (13.12% w/w) and H/D=0.75

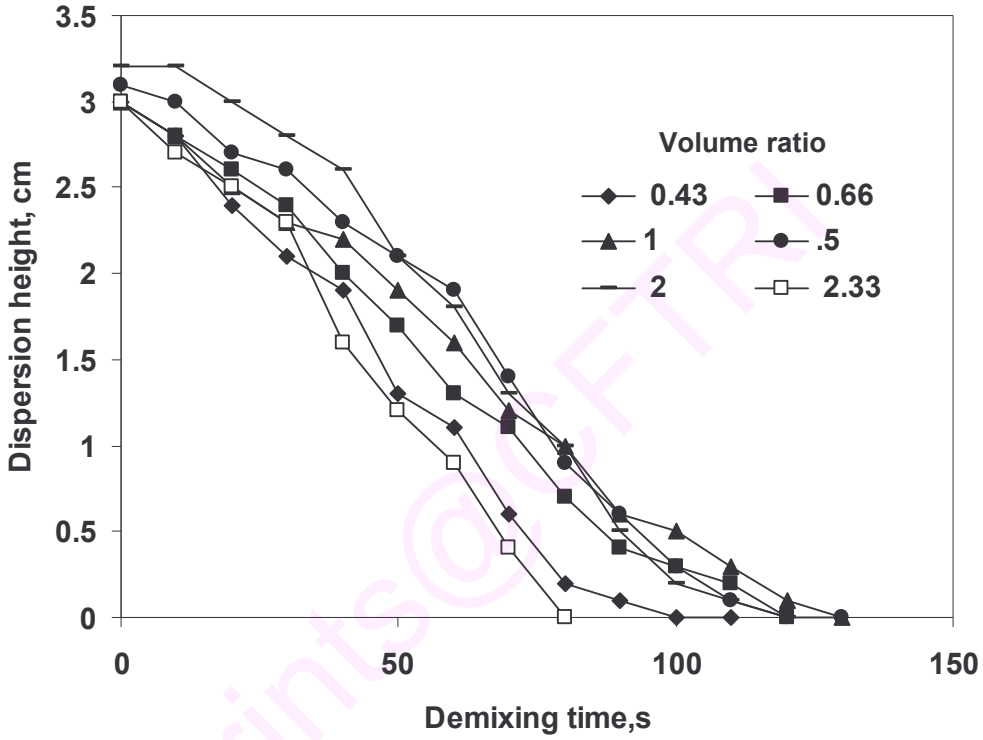

Figure 2A.12. Kinetics of phase demixing in PEG 4000 and potassium phosphate systems at medium TLL (25.52% w/w) for H/D =0.75

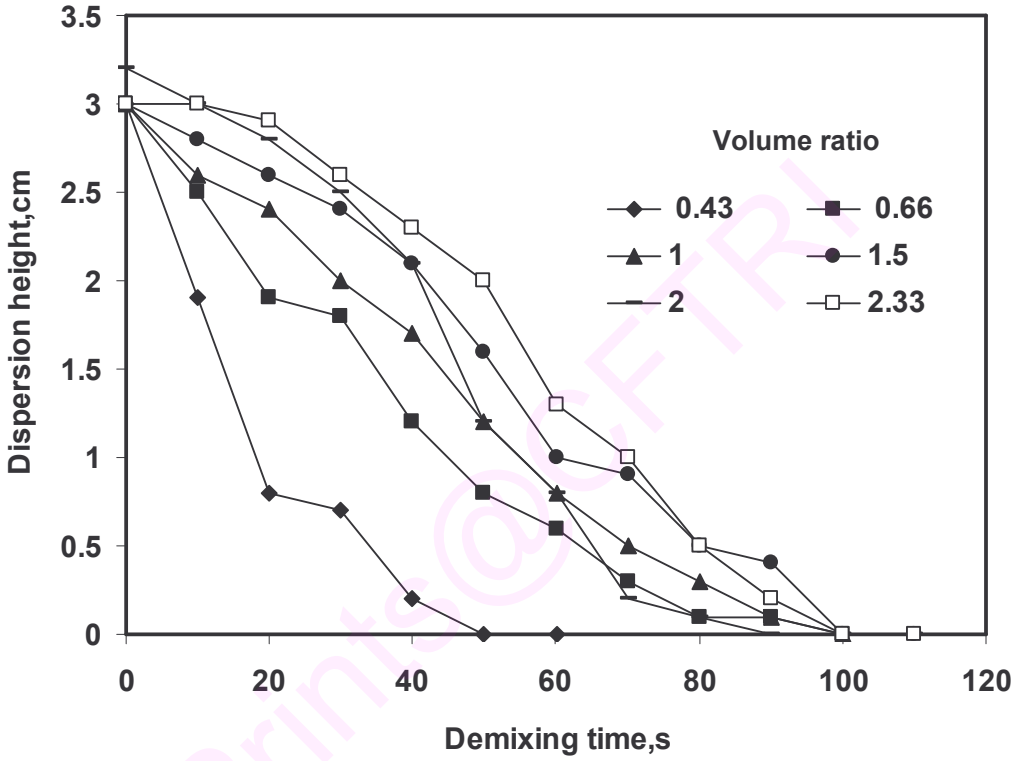

Figure 2A.13. Kinetics of phase demixing in PEG 4000 and potassium phosphate systems at high TLL (44.65% w/w) for H/D=0.75

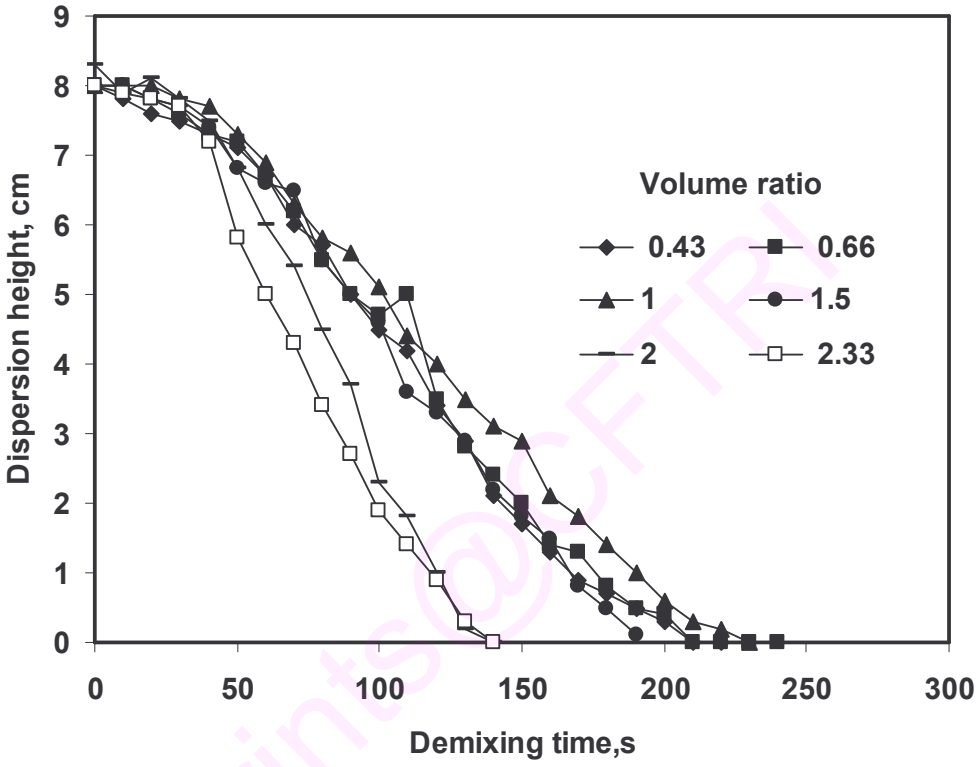

Figure 2A.14. Kinetics of phase demixing in PEG 4000 and potassium phosphate systems at low TLL (13.12% w/w) for H/D =2.85

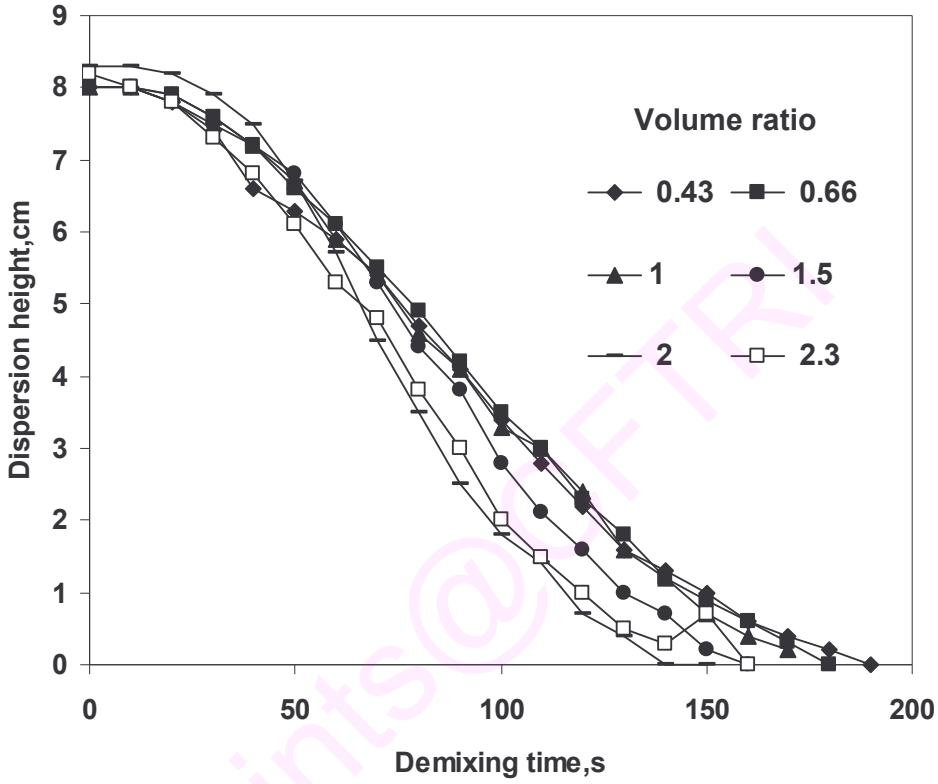

Figure 2A.15. Kinetics of phase demixing in PEG 4000 and potassium phosphate systems at medium TLL (25.52% w/w) for H/D =2.85

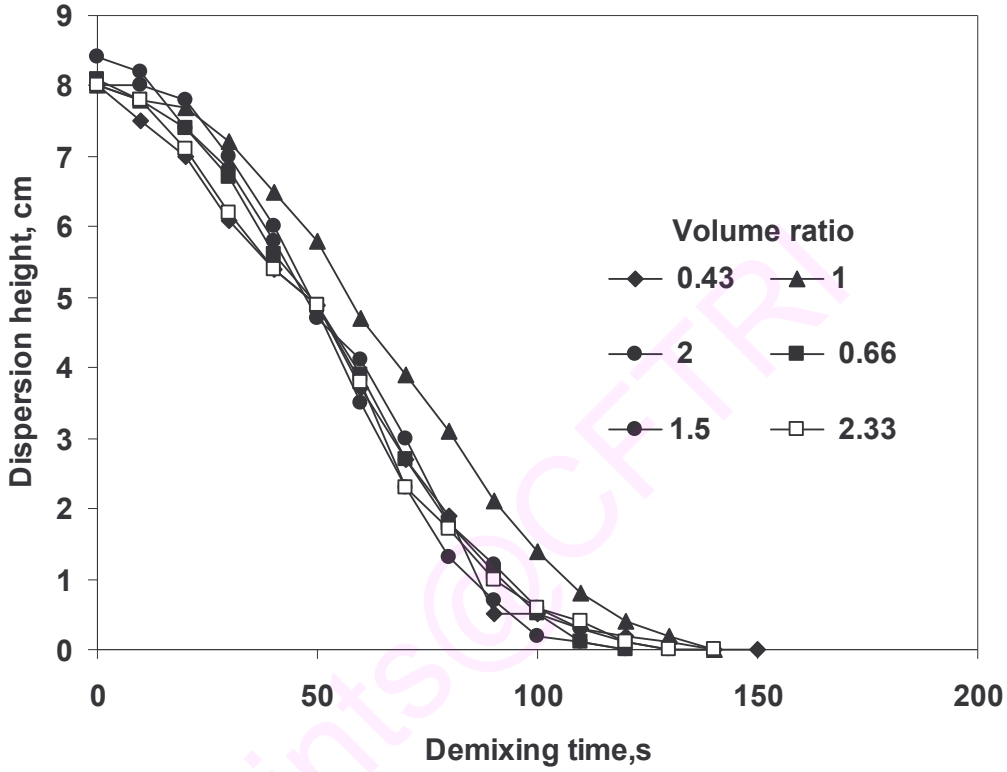

Figure 2A.16. Kinetics of phase demixing in PEG 4000 and potassium phosphate systems at high TLL (44.65% w/w) for H/D =2.85

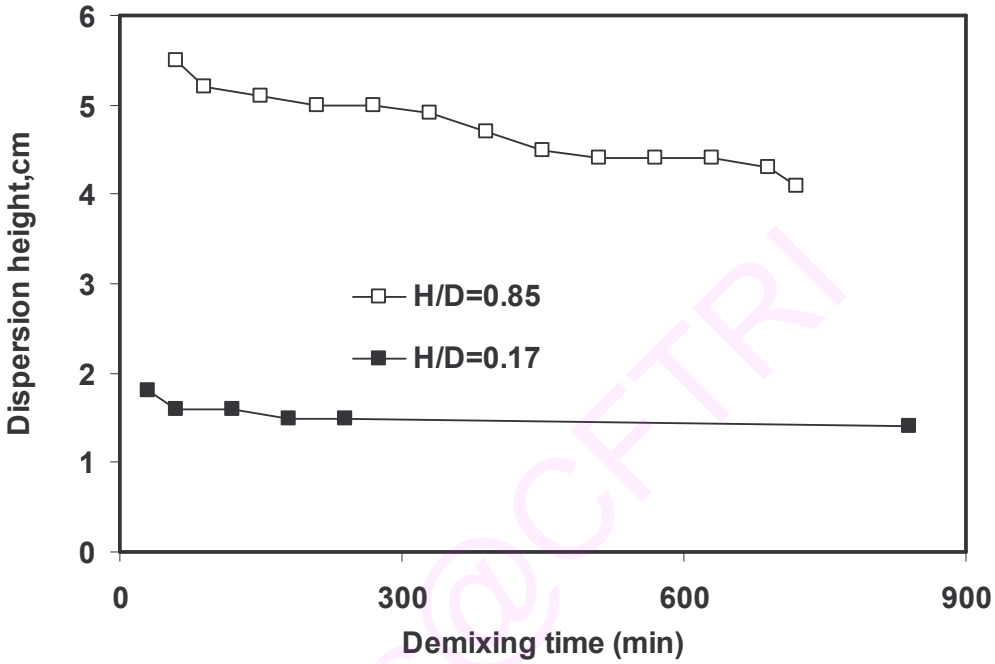

Figure 2A.17. Kinetics of phase demixing in PEG 4000 and potassium phosphate system (with cell homogenate) at 13.12% TLL

Section- B

Three phase partitioning of a plant peroxidase

2B.1. Introduction

Peroxidases [E.C.1.11.1.7] are haemoproteins widely distributed in nature which are found in plant cells, animal organs and tissues. Plant peroxidases are found mainly in cell wall, vacuoles and transport organelles and also on rough endoplasmic reticulum [Azevedo et al., 2003]. They play an important role in plant physiological responses including auxin catabolism, modification of the cell wall, lignification, pathogen defence and wound healing. Peroxidases are widely used in enzyme immunoassays, clinical biochemistry, treatment of waste water containing phenolic compounds, decolorization of dyes, catalyst in organic synthesis [Vamos-Vigazo, 1981, Azevedo et al., 2003, Regalado et al., 2004]. They have been divided into three superfamilies according to their source and mode of action as plant peroxidase, animal peroxidase and catalases [Azevedo et al., 2003].

The separation and purification accounts for a major fraction (70-80%) of the total production cost [Sadana and Beelaram, 1994]. In view of this, the present study has been undertaken for the extraction and purification of peroxidases from *Ipomoea palmata* leaves by using a process called “Three Phase Partitioning” (TPP). TPP is a novel separation technique in which organic/aqueous system such as t-butanol and ammonium sulfate are used to selectively extract the target biomolecules to the interface (in the form of precipitate) partitioning the contaminants in the alcohol and aqueous phases [Dennison and Lovrien, 1997]. Plant peroxidase from *Ipomoea* is a good alternative source to the conventional horseradish peroxidase (HRP).

Earlier work on the purification of peroxidase from *Ipomoea cairica* include ammonium sulfate precipitation, acetone fractionation and gel filtration to result in 51 fold purification with 84% activity recovery [Lin et al., 1996]. Srinivas and coworkers [1999] reported extraction and purification of peroxidase from *Ipomoea palmata* leaves achieving 49 fold purification and 75% activity recovery while Shaffiqu and coworkers [2002] used ammonium sulfate precipitation, ion exchange chromatography and gel filtration chromatography to obtain 78% purification.

Two groups used combination of TPP and chromatographic techniques for the extraction and purification of peroxidase from horseradish and turnip, respectively [Szamos and Hoschke (1992), Singh and Singh (2003)]. The former group reported 24 fold purification with 30% activity recovery by the integration of TPP and ion exchange chromatography. The latter achieved 80.3 fold purification with 20% activity recovery by a combination of TPP and metal affinity chromatography.

In all these studies, the aims of the authors were to recover the protein in highly purified form by a primary purification process followed by a chromatography step. Though the use of chromatography often shows encouraging results on a small-scale, it is difficult to scale up to a large-scale owing to the problems of column clogging and cost of gel used. Hence, in the present study, the potential of TPP alone was explored for the extraction and purification of peroxidase by optimizing the parameters such as pI of the

protein, concentrations of t-butanol, ammonium sulfate and crude extract followed by the temperature of the extraction.

2B.2. Materials and Methods

Ipomoea palmata leaves were procured from the campus of CFTRI, Mysore, Karnataka, India. t-butanol and ammonium sulfate were LR grade and procured from Merck, Germany and Rankem, Mumbai, respectively.

2B.2.1. Preparation of crude extract

Fresh leaves (*Ipomoea palmata*) were washed thoroughly with distilled water and cut into pieces. The leaves were then homogenized with 10mM phosphate buffer (pH 6). The ratio of amount of leaves to that of buffer was maintained at 1:1(w/v). The crude extract was filtered through cheese cloth and centrifuged to remove traces of fibrous particles and cell debris. The supernatant was stored at 4°C and used as stock solution for all the experiments. A plot of pH versus K for two salts gives two different curves intersecting or crossing at a point called cross point [Walter and Johansson, 1994]. The pH corresponding to the cross point gives the isoelectric point of the enzyme.

2B.2.2. Cross partitioning using PEG/salt system

Predetermined quantities of PEG 1500, potassium phosphate (pH- 5-11), and distilled water/crude extract were mixed separately with NaCl and Na_2SO_4 (two sets of experiments) and equilibrated using a magnetic stirrer for

30 min and kept for phase separation. After complete phase separation, the top and bottom phases were separated and analyzed.

2B.2.3. Three phase partitioning

Predetermined quantities of ammonium sulfate and distilled water/crude extract were mixed thoroughly followed by addition of t-butanol and stirred for 30 min using a magnetic stirrer while maintaining the system pH to 9 (pI of the protein). After mixing, the phase system was equilibrated in a water bath at 25°C for 30 min for three phase formation. After complete phase separation, the top, bottom and interfaces were separated. The bottom phase and interface were analyzed for enzyme activity and total protein content. After the first cycle of TPP it was found that most of the enzyme partitioned to bottom phase, hence, a second cycle of TPP similar to first cycle was carried out in order to recover the peroxidase at the interface.

2B.2.4. Estimation of enzyme and total protein content

The activity of the peroxidase was determined at 25°C by spectrophotometer following the formation of tetraguaiacol [Kim, Kim and Yoo, 1997]. A 3 ml reaction mixture containing 1 ml of 15 mM 2-methoxyphenol (guaiacol), 1ml of 3mM H_2O_2 and 50 µl enzyme extract were mixed and the absorbance at 470 nm taken at intervals of 1 min for total of 3-5 min [ε (extinction coefficient) = 26.6 $mM^{-1}cm^{-1}$]. One unit of peroxidase activity represents the amount of enzyme catalyzing the oxidation of 1 µmol of guaiacol in 1 min. The protein assay was carried out by coomasie brilliant blue G-250 method, using BSA as standard (Bradford, 1976).

2B.2.5. Measurement of interfacial tension of the system

Interfacial tensions were measured by spinning drop tensiometer (SITE100 No. 20002806, Kruss, Germany) according to the method described by Vonnegut (1942). More denser phase (ammonium sulfate) was filled in a glass tube and a small drop of less dense phase (t-butanol) injected and rotated about its axis of symmetry. This caused the drop to migrate to the centre of the tube. At higher rotational speed the drop becomes elongated. The interfacial tension was calculated as the mean of rotation at 5000-10000 rpm for 10 counts at 25±2°C using the drop shape analysis software (DSA 2).

2B.3. Results and discussion

2B.3.1. Determination of pI of the protein by cross partitioning

Cross-partitioning is a method employing aqueous two phase extraction by which the isoelectric point of a protein similar to that by electrophoretic means (isoelectric focusing) can be obtained [Walter and Forciniti, 1994]. In the present study, PEG 1500 and potassium phosphate were used in which the effect of salts (NaCl and Na_2SO_4 , each 1%) on the partitioning of peroxidase was evaluated by a plot of K vs pH (Figure 2B.1). The pH corresponding to the point where the two curves cross or intersect (pI of the protein) was found to be 9. Further experiments for the optimization of other parameters were carried at pH 9 since pI is the optimum pH for TPP [Dennison and Lovrien, 1997].

2B.3.2. Effect of t-butanol concentration

The amount of ammonium sulfate was fixed to 30% (w/w) and the ratio of crude to t-butanol was varied from 1:0.3 to 1:1 (Figure 2B.2). It was found that during the first cycle of TPP most of the enzymes partitioned in aqueous phase (low activity yield of 12-47%), hence a second cycle of TPP was carried out similar to first cycle to recover the protein at interface. In the second cycle of TPP, for 1:0.75 and 1:1 crude to t-butanol ratios, the content of ammonium sulfate was decreased to 20% and 15%, respectively. The decrease in ammonium sulfate content was because at concentrations higher than 15 and 20% most of the ammonium sulfate remained insoluble indicating that the solubility limit of ammonium sulfate has been crossed. After second cycle of TPP also activity yield and extent of purification was found high in supernatant than in the precipitate. Equal volumes of crude to t-butanol showed 22 fold purification with 52% activity recovery [Figure 2B.2].

The observed optimum ratio of crude to t-butanol of 1:1 may be because of two factors. If t-butanol content is less, the synergistic effect of ammonium sulfate required to recover the protein at interface is less [Sharma and Gupta, 2001a]. If t-butanol content is high (>1:1) the denaturation of the protein is more likely, in addition to an increase in the viscosity of top phase.

2B.3.3. Effect of ammonium sulfate concentration

The effect of ammonium sulfate was studied by maintaining the ratio of crude: t-butanol constant (1:1) and varying the concentration of ammonium sulfate from 30-60% w/w. The second cycle of TPP was carried out similar to

first cycle as explained in Section 2.B.3.2 (Effect of t-butanol concentration). With an increase in ammonium sulfate concentration, the degree of purification decreased significantly above 30% w/w and remained practically constant [Figure 2B.3]. The activity recovery initially increased to 60% (at 40% w/w) and then decreased to 38% (at 60% w/w) with an increase in concentration of ammonium sulfate.

Sulfate ion and t-butanol are known to be excellent protein structure makers (kosmotropes) [Dennison and Lovrien, 1997]. The principle of sulfate ion for salting out protein has been viewed in five different ways namely ionic strength effects, kosmotropy, cavity surface tension enhancement osmotic stressor (dehydration), exclusion crowding agent, binding of SO_4^{2-} to cationic sites of protein [Dennison and Lovrien, 1997]. TPP depends upon the concentration of ammonium sulfate and the pI of the protein. It may be noted that all the experiments were carried out at the pI (9) of the protein. At 40% w/w and higher concentrations of ammonium sulfate, 'salting out' of protein was observed and at very high salt concentrations (>40% w/w) due to the inactivation of the protein, the activity yield decreased dramatically [Figure 2B.3]. This result was confirmed from the experiments of ammonium sulfate precipitation [Figure 2B.4]. In addition, the high concentration of ammonium sulfate reduces the selectivity of extraction thus reducing the degree of purification.

2B.3.4. Effect of the phase composition

With an increase in phase composition (t-butanol and ammonium sulfate), the difference between the top and bottom phase composition increased, the surface tension increased and thus the interfacial tension also increased [Figures 2B.5 and 2B.6]. The increase in interfacial tension in turn increased the partitioning of peroxidase at the interface [Figure 2B.7]. Higher interfacial tension values signify higher density differences and also higher polarity differences between the phases (Szamos and Kiss, 1995).

2B.3.5. Effect of temperature

The thermal stability was studied by incubating the peroxidase subjected to TPP at 25°, 37° and 50°C for 1h in a water bath. At higher temperature (50°C) the degree of purification decreased (by 50%) but dramatic increase in the activity yield was observed (4 fold increase at 50°C as compared to 25°C) [Figure 2B.8]. This may be due to the synergistic effects of the phase components along with the effect of temperature itself coming into play which activates the enzyme molecule thus increasing the activity yield but decreasing the selectivity of extraction thus reducing the degree of purification. The thermal stability of present study is in good agreement with earlier reports which have shown that peroxidase to be stable at 60°C for 5-6 h [Lin et al., 1996, Srinivas et al., 1999].

2B.3.6. Effect of initial activity of crude extract

Effect of initial activity on the degree of purification and activity yield in TPP was evaluated. It is observed that low activity (0.018 U/ml) of the crude

extract showed highest degree of purification (purification factor) (about 30 fold) than the high activity (3.357U/ml) after TPP [Figure 2B.9]. With an increase in initial activity of the crude, both the degree of purification and the activity yield have decreased markedly (over 100 times). The reason may be that the removal of the contaminants at low enzyme activity is more effective than at high activities. The system parameters standardized for one protein concentration (say low) generally do not show similar results for other protein concentration (say high). This is because at high protein concentration, the system parameters such as concentration of t-butanol and ammonium sulfate needs to be decreased suitably (or else it leads to irreversible protein denaturation) and an optimum concentration required to be found. Also, at low enzyme activity the partitioning of protein becomes independent of initial protein analogous to that observed in aqueous two phase extraction [Schmidt, 1996].

2B.3.7. Ammonium sulfate precipitation

The concentration of $(NH_4)_2$ SO_4 was varied from 30-90 % in increments of 5% and the degree of purification for the precipitate as well as the supernatant were determined. The specific activity of the enzyme was observed to be higher in the supernatant (1.5-5.5 U/mg) than in the precipitate (<1 U/mg). The 35-45% ammonium sulfate cut showed maximum degree of purification and activity recovery in the supernatant [Figure 2B.4]. The degree of purification of peroxidase was found to be high in TPP however, the activity yield was found to be lower than in ammonium sulfate precipitation.

2B.3.8. Comparison with literature

The results of the present study were compared with the existing literature values [Table 2B.1]. The literature values show that in all the processes a minimum of two steps are used with the second step invariably being chromatography. The results of present study showed that high degree of purification and activity yields could be obtained in a single step of TPP.

2B.4. Conclusions

TPP employed for the extraction and purification of peroxidase from *Ipomoea palmata*, has shown potential to be an attractive process as the primary purification step. Present study has shown high values of degree of purification and activity yield in a single step as compared to the existing literature methods. However, the mechanism of TPP is not yet fully understood due to the complexity of the process involved and hence the scale-up still remains a challenge.

Table 2B.1. Comparison of the process from literature values for peroxidase purification

Sl No	Process	Source	In Primary Purification method		After Chromatography		Reference
			Initial purification factor	Activity yield (%)	Overall purification factor	Activity yield (%)	
1	Ammonium sulfate precipitation + acetone fractionation+gel chromatography	*I.cairica*	1.25	77.7	51	84	Lin et al.,1996
2	Aqueous two-phase extraction+ gel chromatography	*I.palmata*	2.18	91.5	48.6	75.3	Srinivas et al., 1999
3	Ammonium sulfate precipitation+ ion exchange chromatography+ gel chromatography	*I.palmata*	-	-	77.8	-	Shaffiqu et al.,2003
4	TPP +ion exchange chromatography	Horseradish	5.7	65.6	23.6	29.5	Szamos and Hoschke, 1992
5	TPP + immobilized metal affinity chromatography	Turnip	6.8	36	80.3	20.3	Singh and Singh, 2003
6	TPP	*I.palmata*	18	123	Not employed		Present study

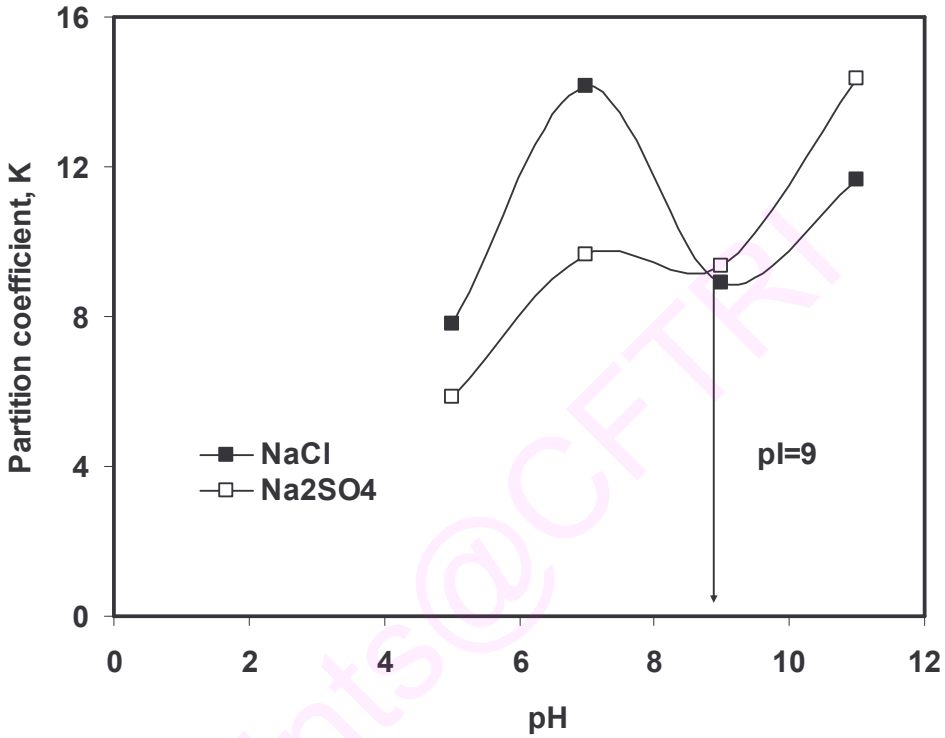

Figure 2B.1. Cross partitioning of peroxidase from the leaves of *Ipomoea palmata* employing PEG 1500/pottasium phosphate system

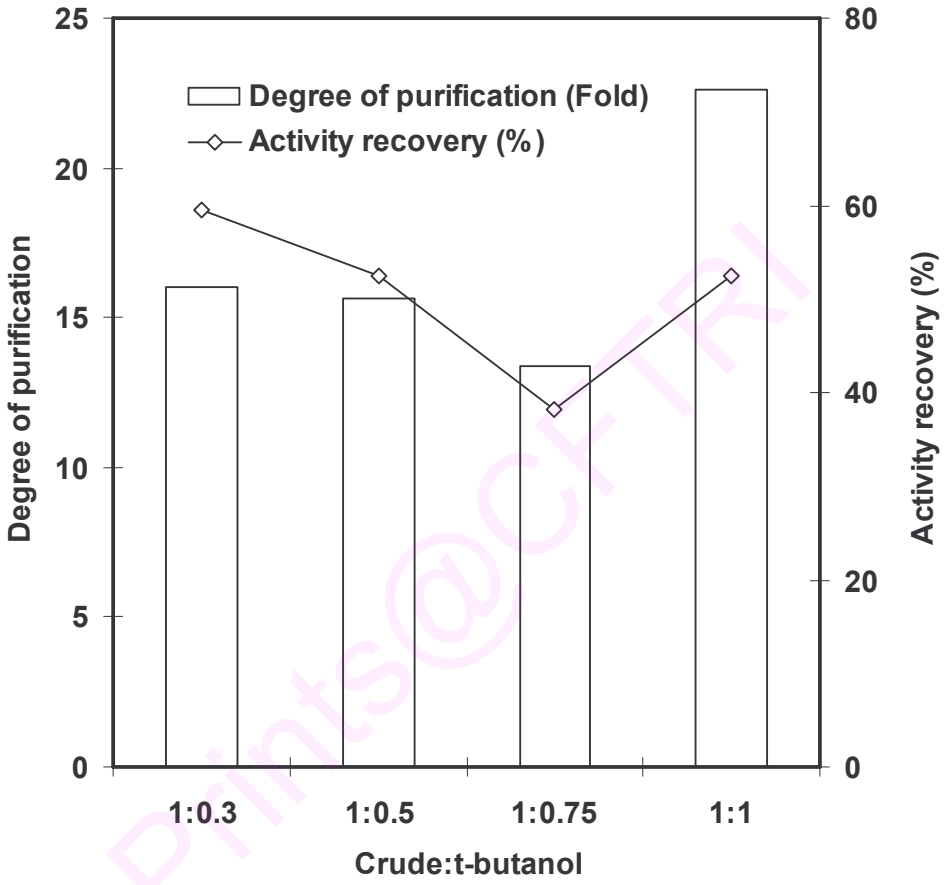

Figure 2B.2. Effect of t-butanol concentration on the degree of purification and activity recovery of peroxidase in the supernatant

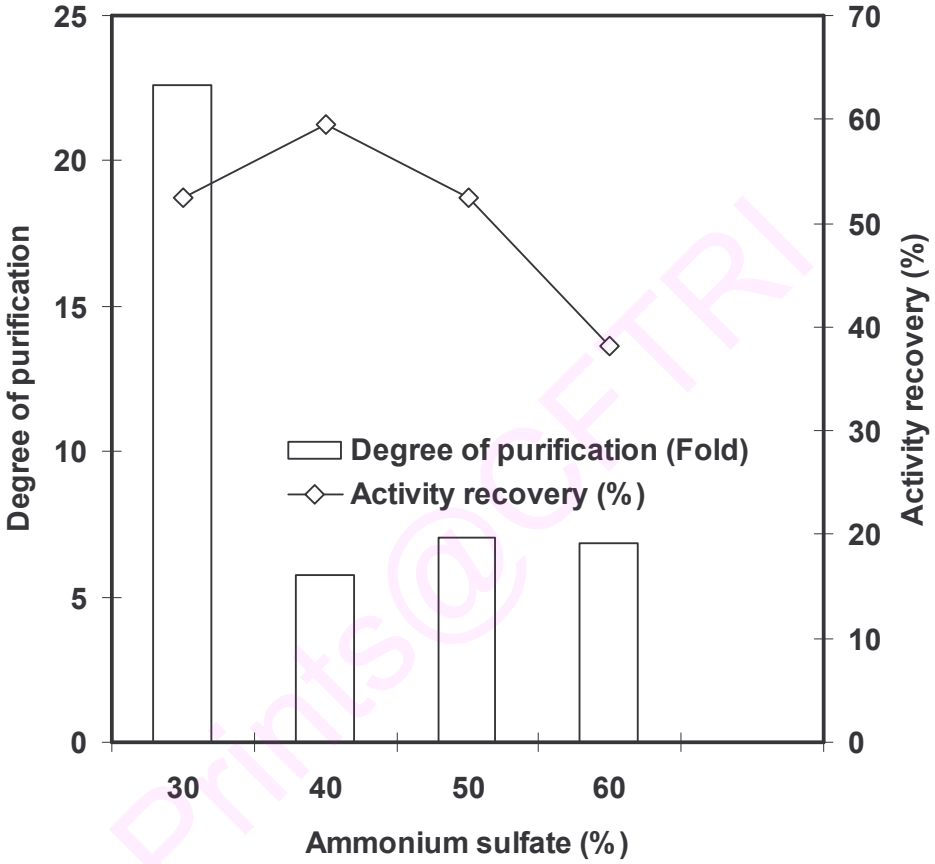

Figure 2B.3. Effect of ammonium sulfate concentration on the degree of purification and activity recovery of peroxidase in the supernatant

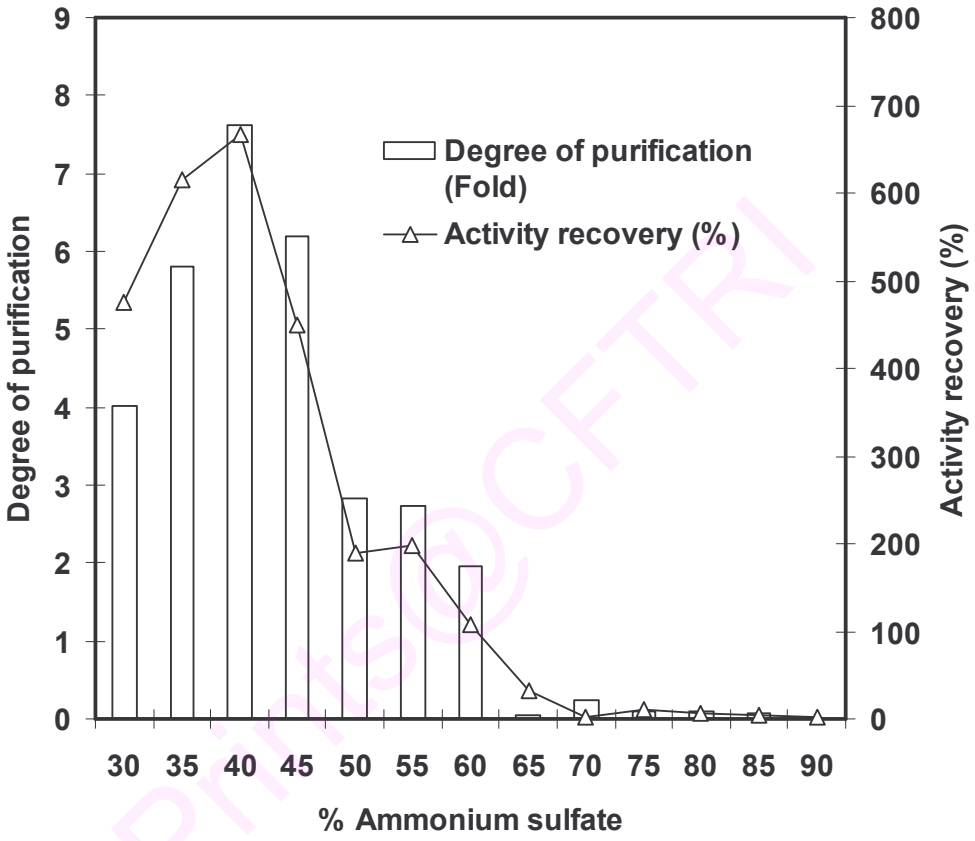

Figure 2B.4. Effect of different concentrations of ammonium sulfate (ammonium sulfate precipitation) on the degree of purification and activity recovery of peroxidase

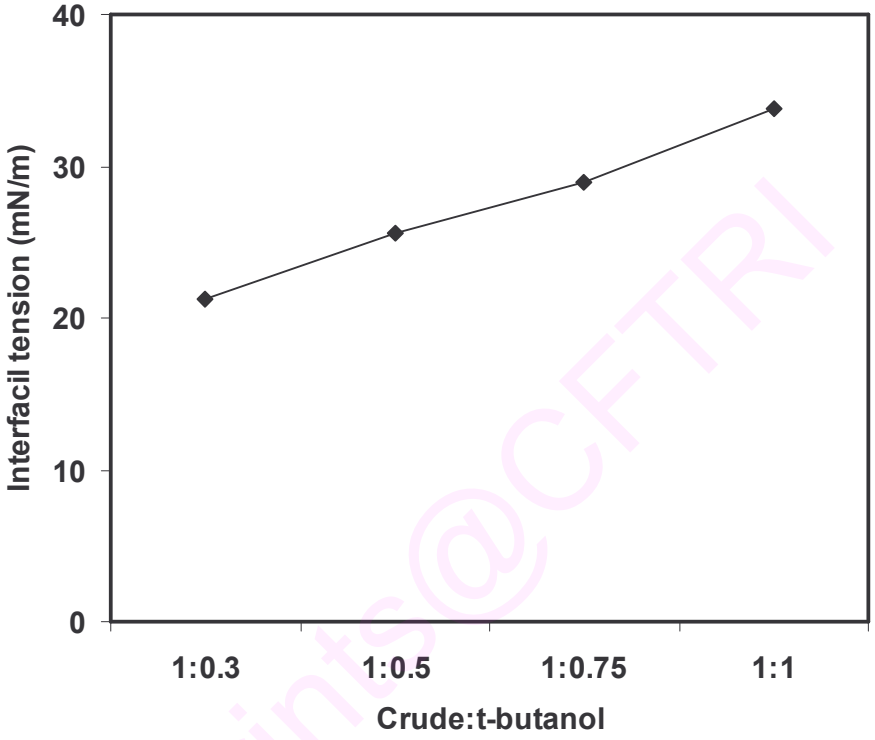

Figure 2B.5. Effect of concentration of t-butanol on the interfacial tension of the system

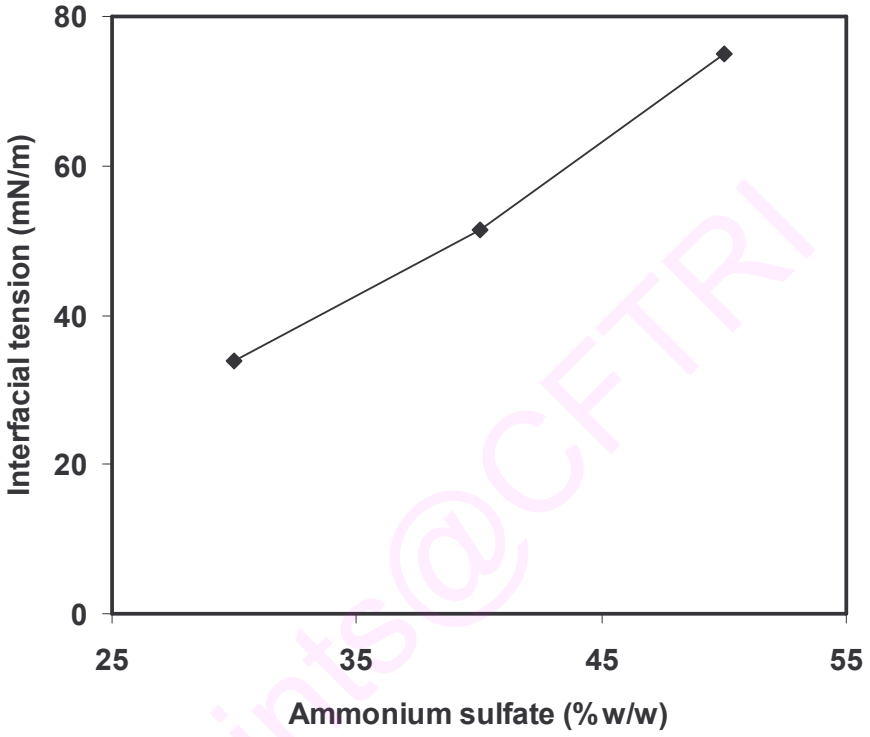

Figure 2B.6. Effect of concentration of ammonium sulfate on the interfacial tension of the system

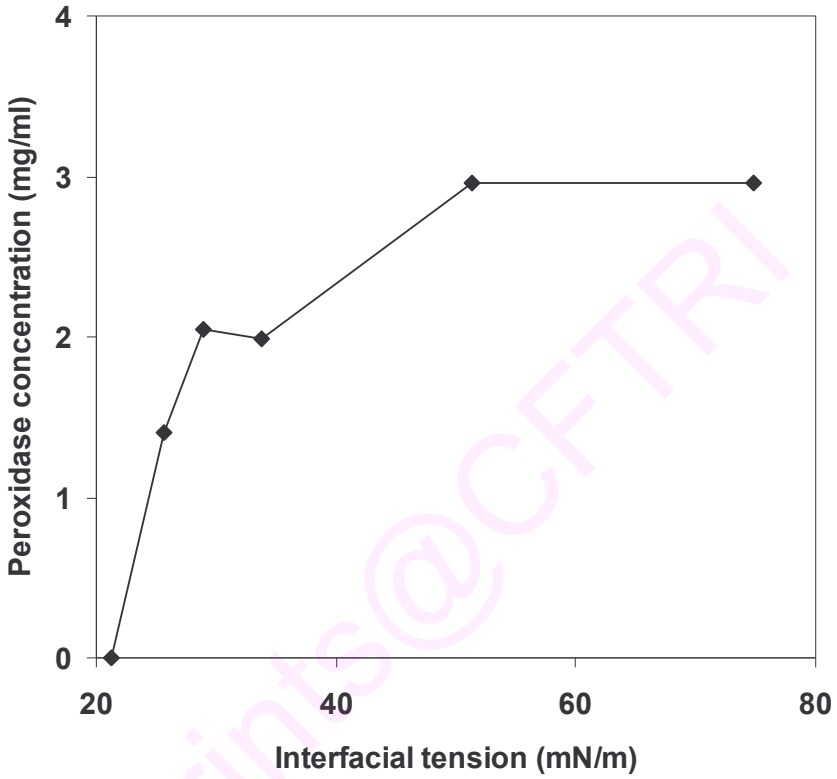

Figure 2B.7. Effect of the interfacial tension of the system on the partitioning of peroxidase at the interface

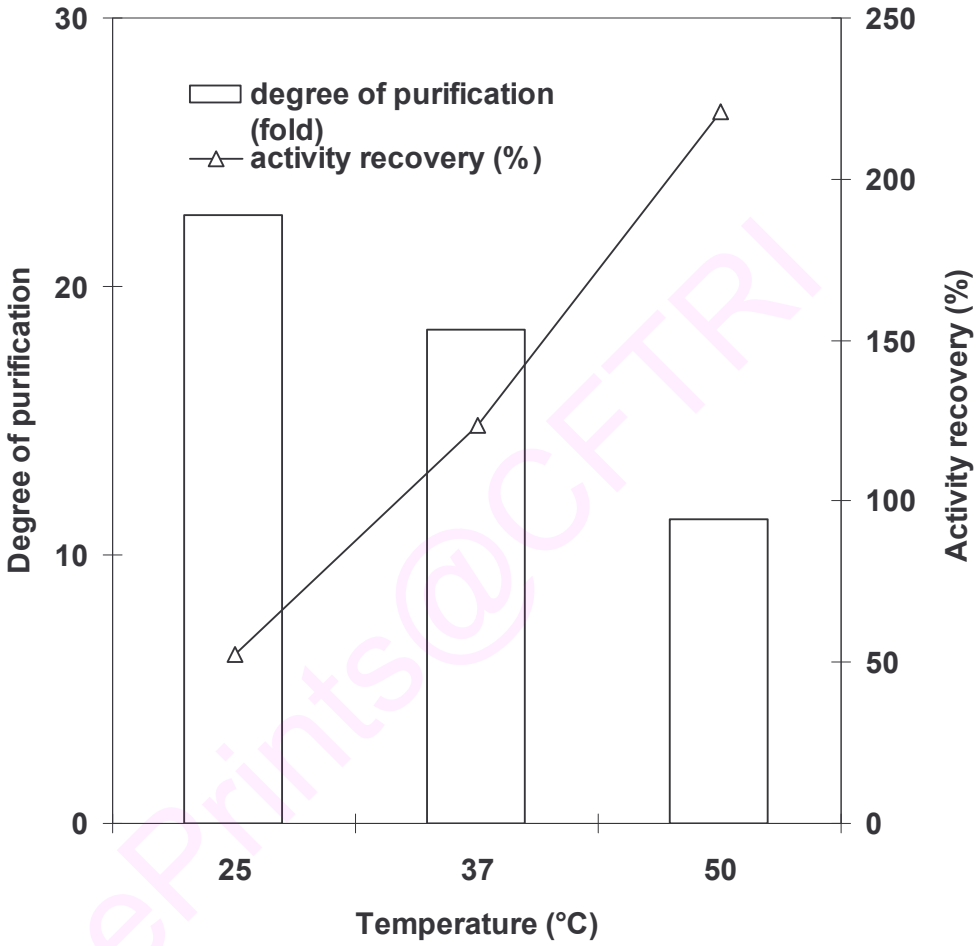

Figure 2B.8. Effect of temperature on the degree of purification and activity recovery of peroxidase

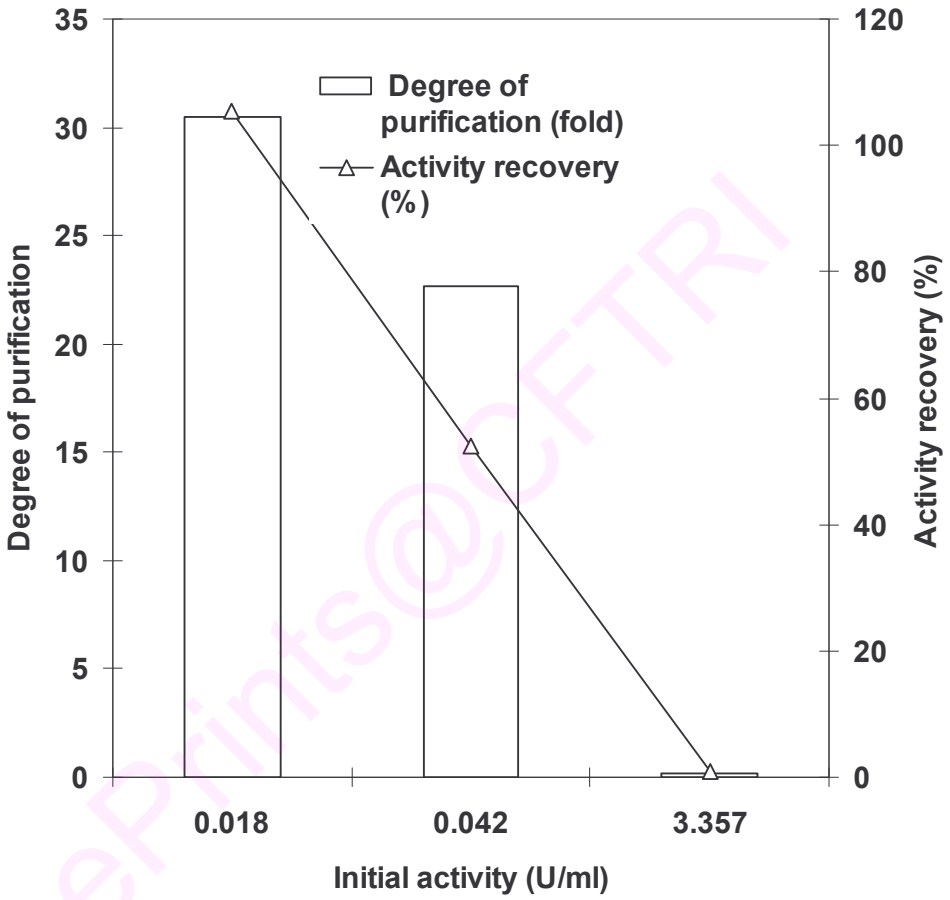

Figure 2B.9. Effect of initial activity on the degree of purification and activity recovery of peroxidase

Chapter- 3

COMPARATIVE STUDIES OF THREE PHASE PARTITIONING AND AQUEOUS TWO PHASE EXTRACTION

Section- A

Standardization of process parameters for BSA

3A.1. Introduction

The partitioning of BSA in Aqueous two phase extraction (ATPE) has been reported in both polymer/salt and polymer/polymer systems [Huddleston et al. (1994), Gunduz and Korkmaz (2000)]. Huddleston and coworkers (1994) studied the partitioning of BSA in PEG 1450/potassium phosphate system at different TLL's (13.2, 27.5 and 38.4%) and volume ratios (0.2-5). The authors reported that partition coefficient of protein (BSA) varied with phase volume ratio along a fixed TLL (27.5 and 38.4 %) and remained constant only at low TLL (near to the binodial). The authors explained their results on the basis of salting out of proteins and observed that at high TLL, precipitation of protein occurs at interface. Gunduz and Korkmaz (2000) have studied the partitioning of BSA in PEG 3350/dextran 37500 system at different pH (4.2-9.8) and different NaCl concentration (0.06-0.34 M). It was found that BSA partition coefficient decreases below 0.2 M at pH (4.2-5). Marcos and coworkers, (1998) studied the partitioning of penicillin acylase in PEG 1000/sodium citrate and PEG 3350/ sodium citrate systems. They reported that in PEG 1000/sodium citrate system, decrease in volume ratio increases the partition coefficients of penicillin acylase and total protein. However in PEG 3350/ sodium citrate system, the partition coefficient of penicillin acylase decreased while total protein remained constant. The authors concluded that generally, it is not possible to increase the purification factor with decrease in phase volume ratio due to large variation of protein partition coefficient along a given TLL. Kim and Rha [1996] used a combination of the theories of salting out of protein and excluded volume of PEG to explain the partitioning of BSA and lysozyme in ATPE involving PEG 8000 and potassium phosphate system.

In Three phase partitioning (TPP), the extraction of model proteins such as BSA, cytochrome, γ-globulin, hemoglobin, lysozyme, myoglobin and ovalbumin was studied by optimizing the parameters such as pI of the protein, concentrations of t-butanol, ammonium sulfate and crude, temperature [Pike and Dennison, 1989]. The ratio of crude to t-butanol of 1:0.3 with 30% ammonium sulfate at pI of the proteins resulted in recovery higher than 95%. Gupta and coworkers [2000, 2001a, 2001b, 2001c, 2003] found the ratio of crude extract to t-butanol of 1:1 with 30% ammonium sulfate was optimum for the extraction of enzymes such as alkaline phosphatase, phospholipase, protease inhibitor, glucoamylase, pectinase and cellulase.

In the present study, the effect of volume ratio (0.43-2.33) on the partitioning of a model protein BSA (0.5-2 mg/ml) was studied comprising of PEG 6000/ammonium sulfate at low (14% w/w) and medium (31% w/w) TLLs. In aqueous/organic systems, effect of volume ratio (0.43-2.33) on t-butanol/ammonium sulfate was studied for recovering BSA (0.5-2 mg/ml) at interface. The objective of the study is a comparative study between ATPE and TPP for the partitioning of BSA.

3A.2. Materials and Methods

PEG 6000 and ammonium sulfate were obtained from Rankem, Mumbai and SRL, Mumbai, India, respectively. BSA was obtained from Loba Chemie, Mumbai. t-butanol and ammonium sulfate were procured from Merck, Germany and Rankem, Mumbai, respectively.

3A.2.1. Aqueous two phase extraction

Predetermined quantities of PEG 6000, ammonium sulfate and distilled water were added to prepare 100 %w/w of total phase system [Zaslavsky, 1995]. The contents were mixed thoroughly for equilibration using a magnetic stirrer for 30 min and then transferred to a separating funnel for complete separation of phases. The desired volume ratio was obtained by taking required volumes of top and bottom phases, respectively and then a predetermined quantity of BSA was added (in order to obtain the final concentration of BSA 0.5-2 mg/ml). ATPE was performed by mixing the contents for 30 min and left for complete phase separation (about 12 hrs). After phase separation aliquots of top and bottom phases were analyzed for total protein content. K and yield were determined and volume ratio vs K and yield plotted.

In TPP, predetermined quantities of ammonium sulfate and distilled water were mixed thoroughly followed by the addition of t-butanol and stirred for 30 min in a magnetic stirrer to prepare 100 %w/w of the system. The mixture was left for complete phase separation (about 6 hr) in a separating funnel and the individual phases separated. The rest procedure (partitioning of BSA in TPP) remained similar to aqueous two phase extraction. The protein partitioned at top, bottom and interfaces were analyzed.

3A.2.2. Analytical procedure

3A.2.2.1. Protein determination

Total protein content was determined by measuring the absorbance at 280 nm.

3A.2.2.2. Partition coefficient

Partition coefficient (K) was calculated as the ratio of the equilibrium concentration of the protein (C_T) in top phase to that of bottom phase (C_B).

$$K = \frac{C_T}{C_B} \qquad \text{..... (1)}$$

3A.2.2.3. Yield of BSA

Yield of BSA was calculated using the following equation

$$Y = \frac{C_B V_B}{C_{Tot} V_{Tot}} x100 \qquad \text{..... (2)}$$

where, C_B and C_{Tot} are concentration of bottom phase and total concentration of the phase system. V_B and V_{Tot} are volume of bottom phase and total volume of the phase system.

3A.3. Results and Discussion

3A.3.1. Aqueous two phase extraction (ATPE)

In ATPE, at low TLL (14% w/w), with an increase in volume ratio from 0.43-1, at different BSA concentrations (0.5- 1.5 mg/ml) decrease in K was observed [Figure 3A.1]. A significant decrease in K (about >70%) for 0.5 and 1.5 mg/ml was observed, while that for 1 mg/ml the decrease was not significant (practically constant). The decrease in K was because with an increase in volume ratio, the volume of the bottom phase decreased and thus the concentration of BSA partitioned increased. However at higher concentrations of 2 mg/ml, K remained constant which was due to the reason that the change in volume ratio (0.43-1) did not alter the partitioning of BSA.

At volume ratios of 0.43-1, yields for initial BSA concentration 0.5- 2 mg/ml is practically constant because of the less difference in values [Figure 3A.2]. Highest yield of 96% was obtained for 0.5 mg/ml while 1-2 mg/ml showed yield 80-90%.

With an increase in volume ratio from 1 to 2.33, at initial BSA concentrations of 0.5 and 1 mg/ml, K remained constant [Figure 3A.1]. At 1.5 mg/ml, K increased by about 50%. This was because with increase in volume ratio, the protein partitioning in top and bottom phases increased. However, the increase in concentration of protein partitioned in bottom phase was higher than that in top phase (about 10%) as a result, K increased. At higher concentration of 2 mg/ml and volume ratio of 2.33, K decreased significantly by about 58%. At a volume ratio of 2.33, the volume of the extracting (bottom) phase reduced to a minimum, thus the concentration of BSA partitioned increased to a very high value (on substituting the values in equation 1) thereby decreasing K.

As the volume ratio increased from 1 to 2.33, the yield at 0.5,1 and 2 mg/ml was practically constant since the change in volume ratio (1-2.33) did not alter the partitioning of BSA. The trend followed by the yield is similar to that of K for 0.5-1.5 mg/ml [Figure 3A.1 and Figure 3A.2]. At 1.5 mg/ml and volume ratio 2.33, an increase in yield by about 9% was observed. With an increase in volume ratio, the volume of extracting phase (bottom) decreased and the concentration of BSA at volume ratio of 2.33 increased by about 43% as compared to that at 1 mg/ml at the same volume ratio. This significant

increase in BSA concentration is the cause of increase in yield for 1.5 mg/ml though the volume of bottom phase was least among all volume ratios (0.43, 1, 2.33).

At medium TLL (31% w/w), with increase in volume ratio from 0.43-1, K for 0.5 mg/ml showed increase by about 78% while that for 1 mg/ml remained practically constant [Figure 3A.3]. The increase in K for 0.5 mg/ml can be explained as follows. With increase in volume ratio, concentrations of both top and bottom phases increased. At a volume ratio of 1, the increase in concentration of top phase was more significant (about 175%) than that of bottom phase (about 48%), resulting in an increase in K [Figure 3A.4]. With an increase in volume ratio from 0.43-1, for 1 mg/ml, K remained constant [Figure 3A.3]. This was because with an increase in volume ratio, the increase in concentration of top phase was counterbalanced by a corresponding increase in bottom phase [Figure 3A.5]. At 1.5 and 2 mg/ml, a drop in K by about 20% and a significant decrease (by about 60%) respectively, are observed [Figure 3A.3]. At 1.5 mg/ml, with increase in volume ratio from 0.43-1, the bottom phase concentration increased (about 35%) but that of top phase remained practically constant which resulted in a drop of K [Figure 3A.6]. As the volume ratio increased from 0.43-1, at 2 mg/ml the concentration of BSA in top phase decreased (by about 48%) while that at bottom phase increased (by about 38%) with the net result being decrease in K [Figure 3A.7].

The yields for BSA concentration 0.5-2 mg/ml at volume ratios 0.43-1 show similar trend as K which is expected [Fig.3.A.3 and Fig.3.A.8]. The yield for 0.5- 2 mg/ml was about 81-93%.

As volume ratio further increased from 1 to 2.33, at BSA concentrations of 0.5 and 1 mg/ml, K decreased significantly by about 47-80% [Figure 3A.3]. A similar trend for K of 1.5 and 2.33 mg/ml are observed [Figure 3A.8]. The decrease was most prominent at low BSA concentration (0.5 mg/ml) and least prominent at high BSA concentration (2 mg/ml). As the volume ratio further increased from 1-2.33 for initial BSA concentrations of 0.5-2 mg/ml, the concentration of protein partitioned to bottom phase reached a maximum and thus K decreased [Figure 3A.3].

With increase in volume ratio from 1 to 2.33, the yield at 0.5 mg/ml showed an increase by about 11% [Figure 3A.8]. With increase in volume ratio, the protein partitioning in top and bottom phases increased and the volume of extracting (bottom) phase reduced to a minimum. At 0.5 mg/ml, it was expected the yield to decrease since K decreased but the trend observed was the opposite [Figure 3A.3 and Figure 3A.8]. The concentration of protein partitioned in bottom phase for 0.5 mg/ml was higher (about 65%) than that of top phase (13%). This significant increase in concentration of bottom phase resulted in an increase in yield at 0.5 mg/ml, though the volume of bottom phase was less [Figure 3A.8]. The yields for 1-2 mg/ml were practically constant because an increase in volume ratio from 1 to 2.33 did not cause any significant effect in partitioning.

3A.3.2. Three Phase Partitioning (TPP)

The effect of the volume ratio at a given % TLL was explored similar to that in conventional aqueous/organic system. In aqueous/organic system comprising t-butanol/ammonium sulfate, BSA partitioning was studied similar to that in ATPE at 0.5-2 mg/ml and at low, medium and high TLL's respectively. With an increase in TLL, the protein recovery at interface increased. At low TLL (52%) and low BSA concentration (0.5 mg/ml), it was observed that protein did not partition at interface (visual observation) rather to top (t-butanol) and bottom (ammonium sulfate) phases similar to the partitioning in conventional aqueous/organic system [Figure 3A.9]. At medium (63.5%) and high (71%) TLL's, BSA partitioned at interface. At medium TLL (63.5%), with change in volume ratio from 0.43-2.3, the yield remained practically constant. At 63.5% TLL, yield of about 20% (at interface) is achieved with the remaining protein partitioning to bottom (aqueous) phase for all the volume ratios studied. At high TLL (71%) with an increase in volume ratio from 0.43-1, the yield increased by 21%. The increase in yield at a volume ratio of 1 is because of the high interfacial tension of the system (11.3 mn/M). The value of interfacial tension implies higher density differences and greater polarity between the phases [Szamos and Kiss, 1995]. As the volume ratio further increased from 1-2.33, the yield decreased by about 8% [Figure 3A.9].

Since the highest yield of BSA was found at 0.5 mg/ml and high TLL (71%), further experiments with higher concentration of BSA (1-2 mg/ml) were carried out at this TLL and the trend shown in Figure 3A.10. As the volume

ratio increased from 0.43-1, the yield for 1 and 2 mg/ml practically remained constant while that for 1.5 mg/ml showed a decrease by about 9%. At volume ratio greater than 1 (that is from 1-2.33), the yield decreased significantly. With increase in concentration of BSA the decrease in yield was also significant. For example, at a volume ratio of 2.33, 2 mg/ml showed a significant reduction in yield by about 44% as compared to 1 and 1.5 mg/ml [Figure 3A.10].

It was observed that high volume ratio (2.33) and high TLL (71%) had a significant effect on the partitioning of BSA at interface. On analysis of the top and bottom phases it was found that about 0.2-6% BSA partitioned at the bottom phase for all the volume ratios studied and a negligible quantity partitioned at top phase. Hence the loss of yield at volume ratio of 2.33 cannot be attributed to quantity of BSA partitioning to either or both phases. TPP process is complicated by a number of biological phenomenon occurring at a time such as ionic strength effects, kosmotropy, cavity surface tension enhancement osmotic stressor (dehydration), exclusion crowding agent, binding of SO_4^{2-} to cationic sites of protein [Dennison and Lovrein, 1997]. Hence, the reason for significant decrease in yield at high TLL (71%) at volume ratio of 2.33 is because of the high concentration and volume of top (t-butanol) phase which may possibly affect the tertiary structure of BSA by t-butanol (denaturation). However, the mechanism of the effect of higher concentrations and volumes of t-butanol on BSA requires further understanding of the various phenomena taking place.

3A.4. Conclusions

Partitioning of BSA was studied in PEG 6000/ammonium sulfate system at low, medium TLL's and at different volume ratios. At high TLL, it was found that the protein precipitates at interface indicating the solubility limit of BSA. At 14% and 31% TLL's and volume ratios of low (0.43) and unity the partitioning did not show any particular trend. However, at high volume ratio, (> 1) the yield of BSA either decreased or remained constant. The yield of BSA was in the range 81-96% for both the TLL's and at all volume ratios studied. Both the TLL's show similar yields because of the less differences in the composition of phase forming components. It was found in earlier reports that the partition coefficient is independent of volume ratio at low TLL (close to binodial) and at low concentration of protein [Huddleston, 1994, Asenjo, 1996]. However, in the present study, the partition coefficient was not found independent of volume ratio (true partitioning) even at low concentration of BSA and at low TLL. This may be due to the molecular weight of PEG (6000), which was slightly higher than the medium (4000) and low molecular weights (1500), that has higher excluded volume effects. Also, the salt chosen in the present study is ammonium sulfate that is conventionally used as precipitating agent because of its highest salting out ability, being ranked first in Hofmeister series. Possibly, the net result (even at low TLL), because of the nature of phase forming components would be particulate formation of protein at bottom phase and precipitation at interface which was difficult to be detected by visual observations at low and medium TLL's. Hence, due to these reasons, the true partitioning of BSA was not observed.

In TPP, high yield (about 96%) were obtained at high TLL and at high BSA concentration. Volume ratio has a prominent effect (significant reduction in yield) only at high values and at high BSA concentration (greater than 1).

A comparative yield of about 96% was obtained for both ATPE and TPP at different experimental conditions. Both ATPE and TPP require additional steps of removing the phase forming components such as ultrafiltration and dialysis respectively, to obtain BSA in pure form. However, the economics suggests that the cost of phase forming components in TPP (such as t-butanol) is nearly half of ATPS (PEG) [Dennison and Lovrein, 1997]. Hence, TPP is a suitable process than ATPE for the extraction of BSA.

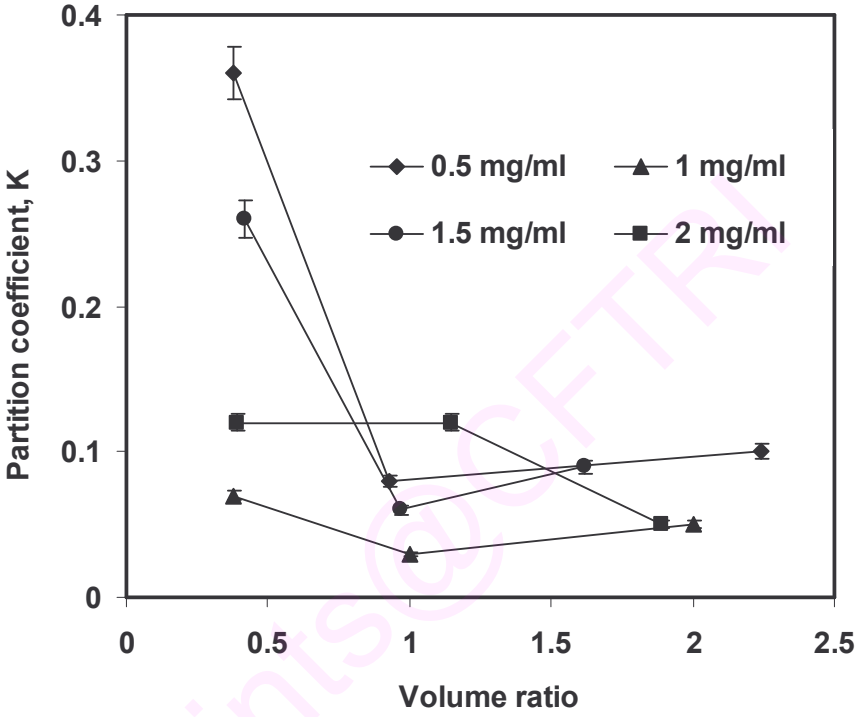

Figure 3A.1. K of BSA (0.5-2 mg/ml) using PEG 6000/$(NH_4)_2SO_4$ at low TLL(14%)

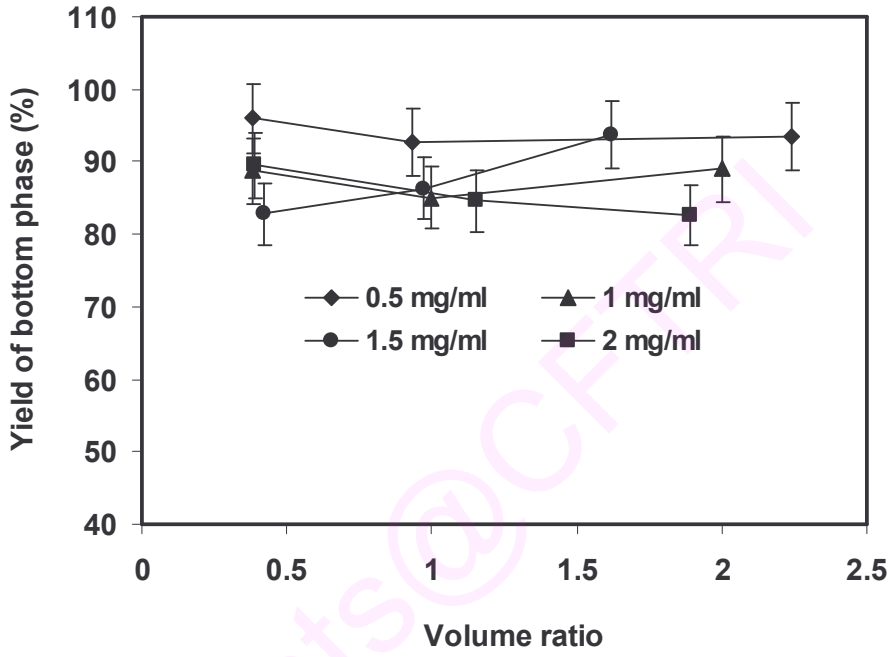

Figure 3A.2. Yield of BSA (0.5-2 mg/ml) using PEG 6000/$(NH_4)_2SO_4$ at low TLL(14%)

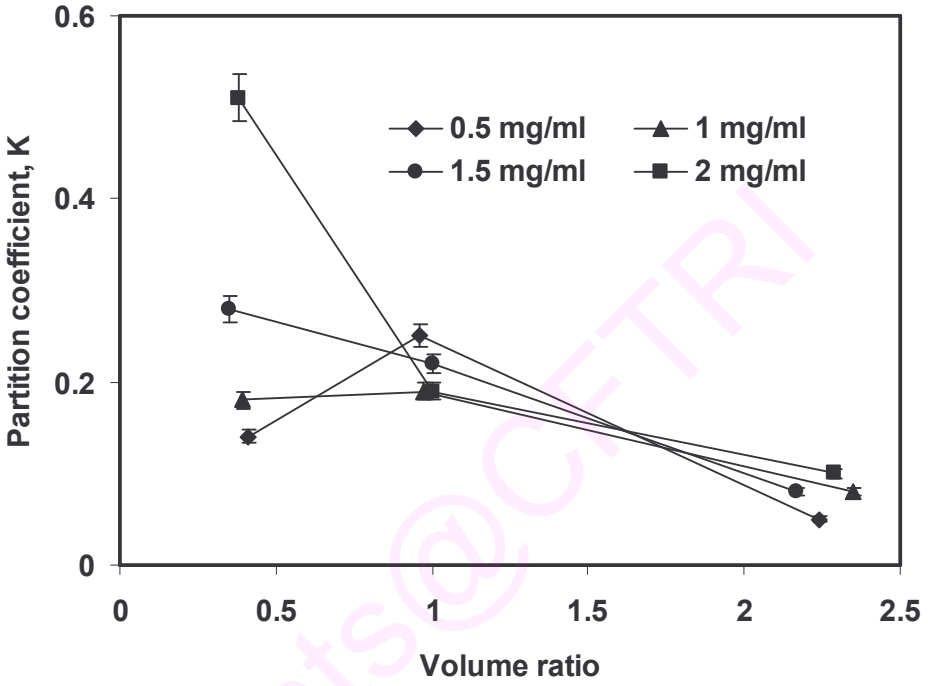

Figure 3A.3. K for BSA (0.5-2 mg/ml) using PEG 6000/$(NH_4)_2SO_4$ at intermediate TLL(31%)

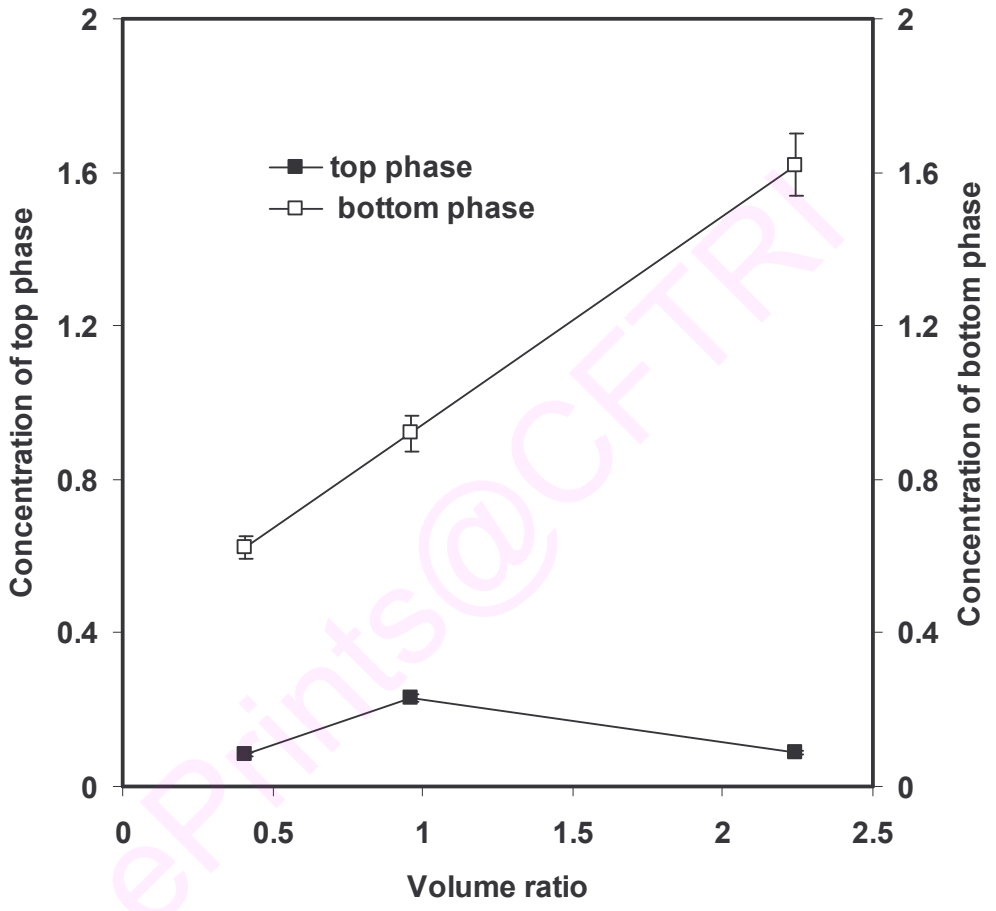

Figure 3A.4. Effect of volume ratio on concentrations of top and bottom phases at medium TLL (31w/w %) for 0.5 mg/ml

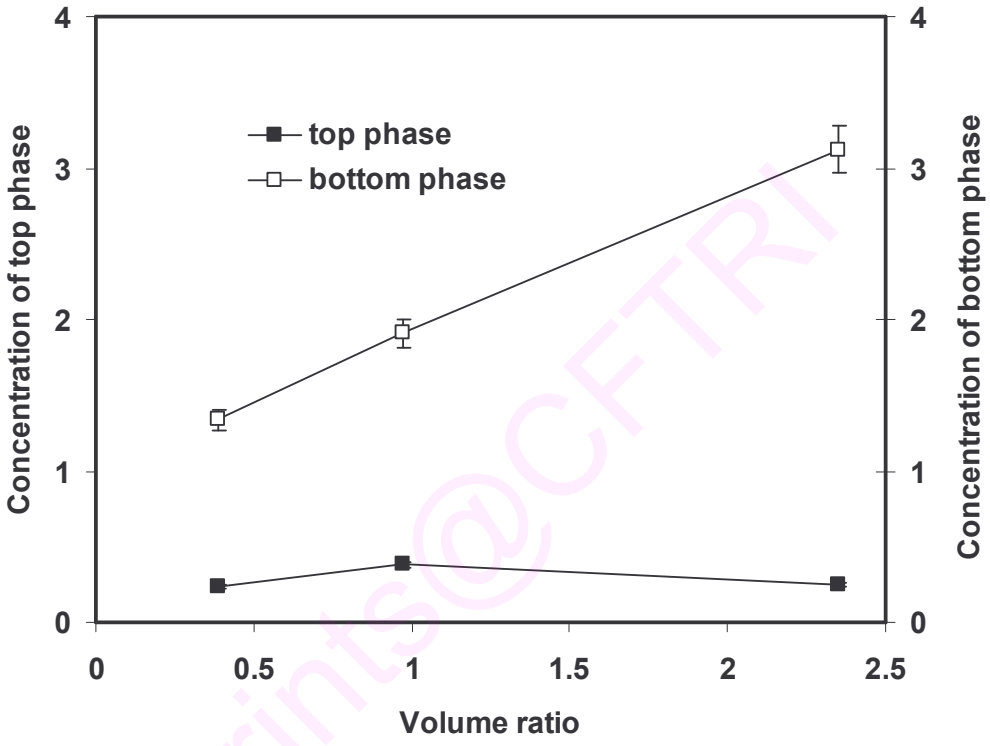

Figure 3A.5. Effect of volume ratio on concentrations of top and bottom phases at medium TLL (31w/w %) at 1 mg/ml

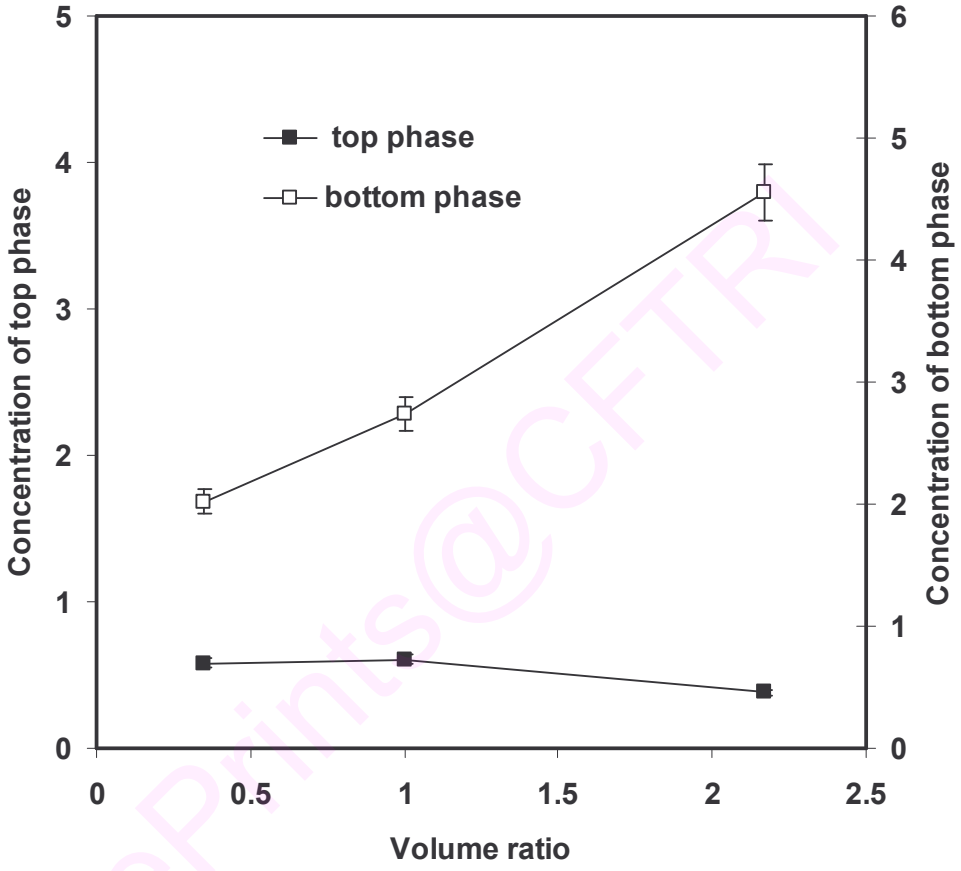

Figure 3A.6. Effect of volume ratio on concentrations of top and bottom phases at medium TLL (31w/w %) at 1.5 mg/ml

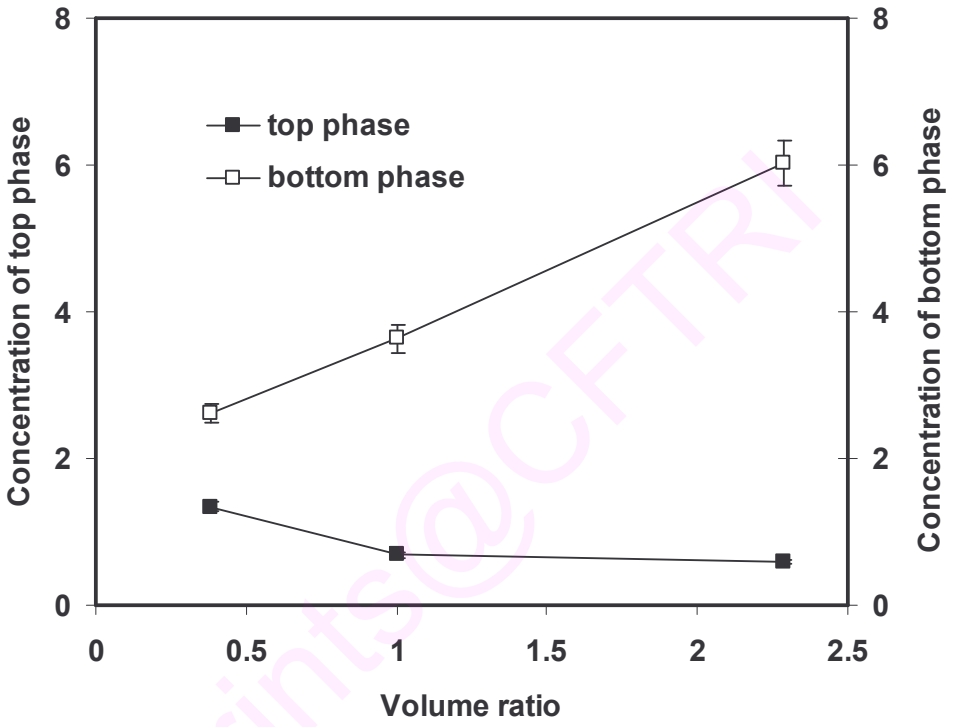

Figure 3A.7. Effect of volume ratio on concentrations of top and bottom phases at medium TLL (31w/w %) at 2 mg/ml

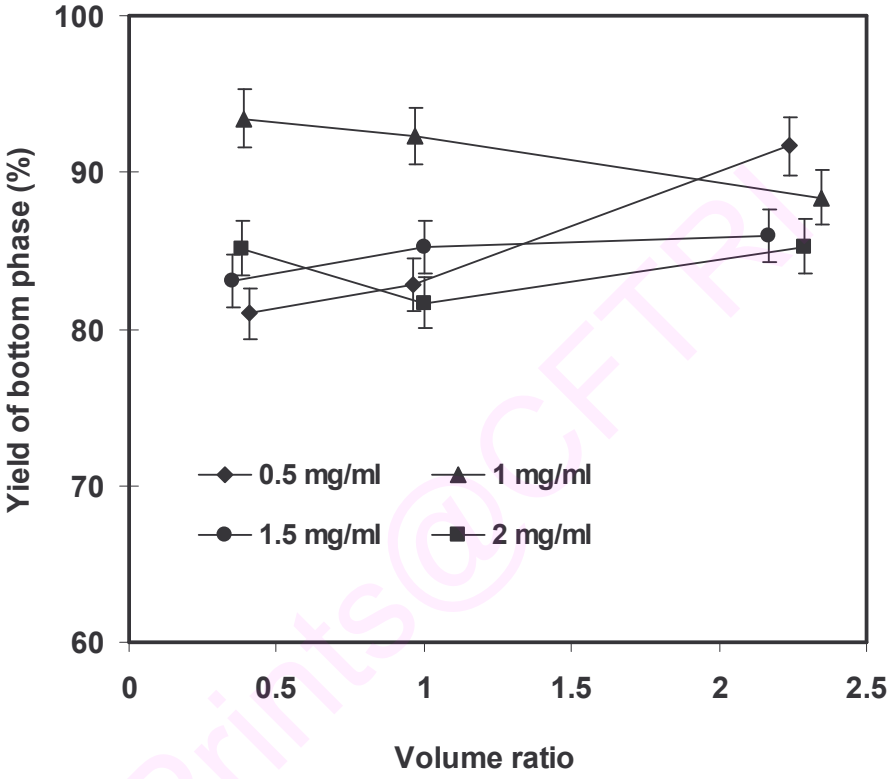

Figure 3A.8. Yield for BSA (0.5-2 mg/ml) using PEG 6000/$(NH_4)_2SO_4$ at intermediate TLL(31%)

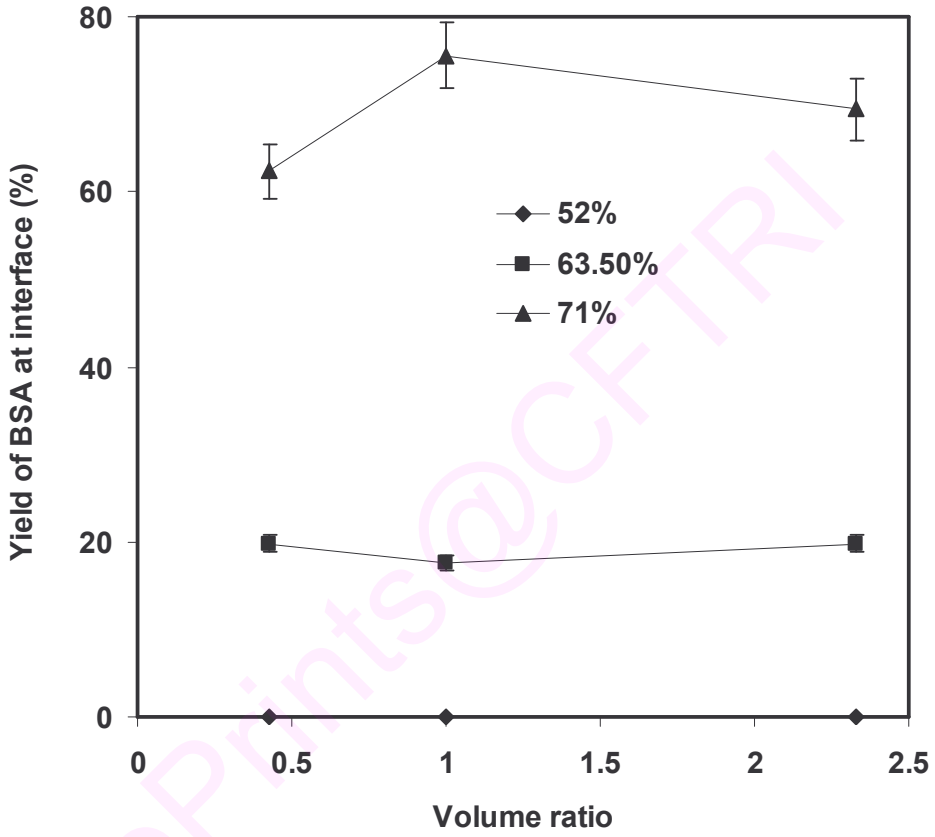

Figure 3A.9. Effect of volume ratio on yield of BSA at interface for 0.5 mg/ml in t-butanol/ammonium sulfate system

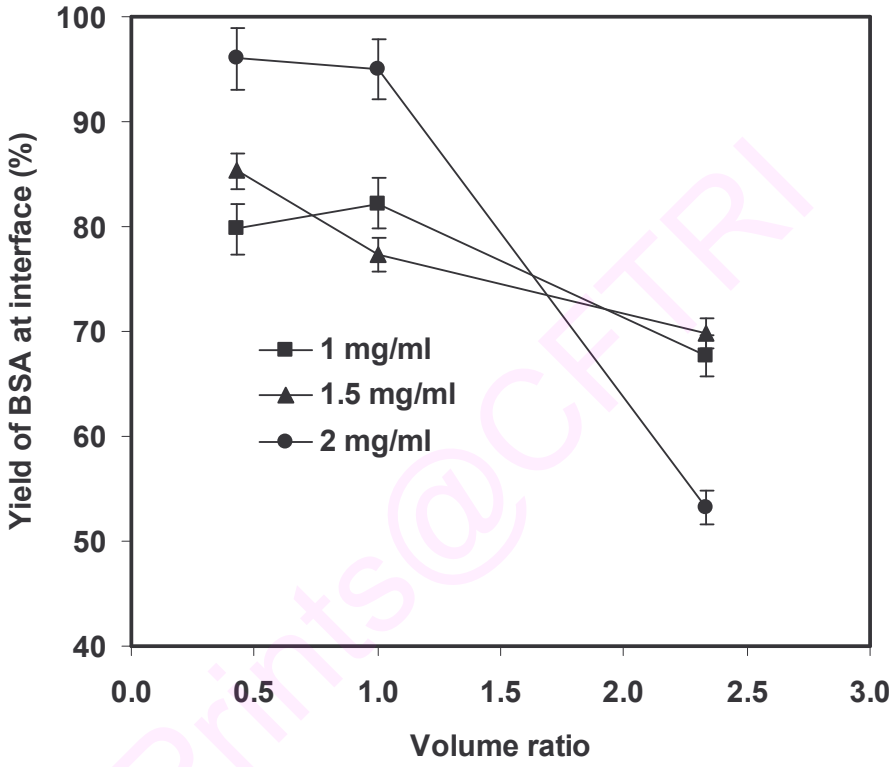

Figure 3A.10. Effect of volume ratio on yield of BSA at highest TLL (71%) with varying concentration (0.5-2mg/ml) in t-butanol/ammonium sulfate system

Section- B

Standardization of process parameters for C-phycocyanin

3B.1. Introduction

Natural colors in food, cosmetics and molecular genetics are of commercial significance because of lack of toxicity as compared to synthetic colors. Phycocyanin, a natural blue colorant has a high commercial value because of its various applications in food, cosmetics and biomedicine [Arad and Yaron, 1992]. In downstream processing, the methods conventionally used to recover the intracellular biomolecules are isolation or extraction step followed by a purification step. The isolation procedures commonly used for the release of phycocyanin from the algal cells are cell disintegration by a homogenizer or freezing and thawing or cell lysis by lysozyme [Berns, 1963]. The extraction and purification process used are initial steps for primary purification (such as ammonium sulfate precipitation) followed by a high resolution chromatographic step for final purification [Teale and Dale, 1970, Boussiba and Richmond, 1979, Glazer 1988, Bermejo, 1997, Abalde, 1998, Reis, 1998, Zhang and Chen, 1999]. Further, the chromatographic process requires an additional concentration step, since the product after purification gets diluted to a high level. Also the chromatographic processes have scale up problem making them very expensive at large scale.

Three phase partitioning (TPP) and Aqueous two phase extraction (ATPE) are the two potential non-chromatographic processes, known for a long time, which are used as alternative methods for the extraction and purification of biomolecules. In the present work, for the first time, the feasibility of TPP for the extraction and purification of C-phycocyanin from *Spirulina platensis* has been explored.

The previous work of aqueous two phase extraction for the extraction of C-phycocyanin include preparation of stabilized pure phycocyanin from *Spirulina platensis* using polymer-polymer and polymer-salt systems [Raghavarao et al., 1996, Chethana et al., 2003, Rito-Palomares et al., 2001]. In polymer-polymer system, PEG and maltodextrin were used to extract phycocyanin in maltodextrin rich phase [Raghavarao et al., 1996]. In polymer-salt system, PEG-sodium sulfate and PEG-potassium phosphate were used to extract the phycocyanin in PEG rich phase [Rito-Palomares et al. 2001, Chethana et al., 2003].

In the present study, a comparative study between TPP and ATPE for the extraction and purification of C-phycocyanin from *Spirulina platensis* has been undertaken. In TPP, clarified phycocyanin (re of cell debris) was used as the stock solution for further experiments. ATPE was employed directly to the cell homogenate of *Spirulina platensis* for the downstream processing of C-phycocyanin. This enables to integrate the process steps of cell removal, extraction and concentration of C-phycocyanin into a single unit operation of aqueous two phase extraction. The aim of the study is to determine the suitable system with optimal processing conditions for the downstream processing of C-phycocyanin.

3B.2. Materials and Methods

t-butanol and ammonium sulfate were of LR grade and procured from Merck, Germany and Rankem, Mumbai, respectively. Polyethylene glycol 1000 was procured from Merck, Germany while PEG 4000 and 6000 were procured from SRL, Mumbai, India. Potassium di-hydrogen phosphate (KH_2PO_4) and di-potassium hydrogen phosphate (K_2HPO_4) were procured from Ranbaxy, New Delhi, India. Sodium sulfate (Na_2SO_4) and BSA were procured from Rankem, Mumbai and Loba Chemie, India respectively.

3B.2.1. Preparation of cell homogenate

The blue green algae Spirulina platensis was obtained from Department of Plant Cell Biotechnology, CFTRI, Mysore, India. The biomass was washed thoroughly for the removal of media components. The wet biomass was weighed, an equal quantity of water added and homogenized at a pressure of 100-400 kg/cm^2 to release the phycocyanin [Manoj et al., 1996]. The homogenate was stored at 4□C for further use.

3B.2.2. Three phase partitioning

The predetermined quantities of ammonium sulfate and phycocyanin were mixed followed by the addition of t-butanol to constitute 100% w/v and stirred using a magnetic stirrer for 30 min at 25°C. The individual phases after complete phase separation (about 30min) were separated and the interface (precipitate of phycocyanin) collected. The interface was dialyzed overnight (about 12 hrs) in distilled water. The interface was analyzed for phycocyanin and total protein content.

3B.2.3. Aqueous two phase extraction

Predetermined quantities of PEG, potassium phosphate, and sodium sulfate selected from the phase diagram reported in literature were weighed and known quantity of cell homogenate (3% w/w) was added to prepare a total of 100% w/w of phase system [Albertson, 1986; Snyder et al., 1992; Haghtalab et al., 2004]. In PEG 4000/pottasium phosphate the pH of the system was maintained neutral by varying the ratio of salts (K_2HPO_4:KH_2PO_4 = 1.82:1) while in PEG 1000/sodium sulfate the pH of both phases varied in the range 5.5-6.0 (the system pH resulted by the addition of sodium sulfate was taken as the final pH of the system). After mixing the contents thoroughly for 30 min at 25ºC, the mixture was centrifuged at 8000 rpm for the separation of individual phases. After complete phase separation, the volumes of top and bottom phases were measured and the volume ratio calculated. The aliquots from both the phases were analyzed for total protein and phycocyanin content. The analysis was performed in duplicates with standard deviation of 0.001-8.8%. The densities of both phases were determined by using a specific gravity bottle.

3B.2.4. Analytical Procedures

3B.2.4.1. Determination of total protein

The total protein content in the cell homogenate was determined by measuring the absorbance at 280 nm using a spectrophotometer (Shimadzu UV 1601).

3B.2.4.2. Determination of C- phycocyanin concentration

The phycocyanin concentration was measured using a spectrophotometer (Shimadzu UV 1601) and determined by Beer Lambert's law, taking the extinction coefficient of phycocyanin as 70 [MacColl and Guard-Friar, 1987]. The final equation used was:

PC concentration (mg/ml) = Optical Density at 620 nm x 0.1429 ……. (1)

3B.2.4.3. Determination of C-phycocyanin purity

The purity of phycocyanin was calculated as the ratio of absorbance at 620nm to 280 nm. 620 nm and 280 nm are the absorbance maximas of C-phycocyanin and total protein, respectively that was measured using a spectrophotometer (Shimadzu UV 1601).

3B.2.4.4. Calculation of partition coefficient

The partition coefficient (K) was calculated as the ratio of the equilibrium concentration of the phycocyanin/total protein in top phase (C_T) to that in respective bottom phase (C_B).

$$K = \frac{C_T}{C_B} \qquad \text{…….. (2)}$$

3. B.2.4.5. Calculation of C-phycocyanin yield

Yield (%) of phycocyanin in top phase was calculated by using the following equation [Kula et al., 1982]:

$$\text{yield} = \frac{kV_r}{1 + kV_r} \times 100 \qquad \text{..... (3)}$$

where,

V_r = volume ratio (volume of top phase to that of bottom phase)

K = partition coefficient of C-phycocyanin

3B.2.4.6. Calculation of relative free volume

Relative free volume was calculated using the following equation [Eiteman and Gainer, 1989]:

$$V_f = \frac{1}{\rho} - \frac{1}{\rho_o} \qquad \text{..... (4)}$$

where, ρ and ρ_0 are densities of top or bottom phase and reference solution (pure water) respectively.

3B.2.4.7. Change in free volume

Change in free volume was calculated using the equation:

$$\Delta V_f = \left(\frac{1}{\rho_{top}} - \frac{1}{\rho_{bottom}}\right) \qquad \text{..... (5)}$$

3. B.2.4.8. High Performance Liquid Chromatography (HPLC)

The separation of components of carotenoids were carried out in C_{18} reverse phase column (5 µm spherisorb ODS, Shimadzu, Japan). The solvents used and their ratios were acetonitrile: dichloromethane: methanol = 70:20:10 v/v at a flow rate of 1ml/min (De Leehheer and Nelis, 1992).

3B.3. Results and Discussion

3B.3.1. Three phase partitioning

In TPP, different alcohols were tested by maintaining the concentration of ammonium sulfate constant (30%) to find the suitability in the extraction of C-phycocyanin [Table 3B.1]. It was found that methanol and ethylene glycol formed a homogeneous phase while n-butanol, ethanol, isopropanol and t-butanol formed three phases while phycocyanin partitioned at interface. Irreversible denaturation of phycocyanin was observed for all the four alcohols used. In further studies, t-butanol and ammonium sulfate were selected as the phase forming components at low compositions [Table 3B.2]. Phycocyanin recovered at interface was dialyzed and analyzed. It was observed that the purity of phycocyanin decreased from 0.54 (crude) to 0.3 (after TPP). Also, the interface did not dissolve completely at pH 6 and 7 buffers, indicating the partial denaturation. The yield was not determined owing to very low content of the sample recovered after dialysis. It was observed that carotenoid a byproduct, partitioned to top (alcohol rich) phase. The extraction of carotenoids to alcohol rich phase was observed in ethanol, isopropanol and t-butanol (except methanol and ethylene glycol). The HPLC analysis showed lutene and β-carotene as the major components of carotenoid both having absorbance maxima (λ_{max}) of about 440-450 nm after comparing with a known standard [Figure 3.B.1].

The fluorescence spectrum was recorded in order to know the concentration of t-butanol at which the phycocyanin fluorescence decreased. The fluorescence spectrum of different proportions of t-butanol and

phycocyanin is shown in Figure 3.B.2. The fluorescence intensity was measured after 2 hrs of exposure of phycocyanin to t-butanol. Increase in concentration of t-butanol (10-70%) resulted in the decrease of fluorescence intensity significantly (about 90%). OhEocha [1963] observed about 1000 fold decrease in fluorescence intensity of phycocyanin when subjected to 50% ethanol. Phycocyanin is enclosed in the hydrophobic region of protein [O hEocha, 1963]. t-butanol has stronger tendency for pairwise interaction between its molecules which in turn leads to greater ability for solute-solute hydrophobic interaction with protein [Chen, 1980]. t-butanol thus binds to hydrophobic part of the protein and weakens the trimer-trimer interaction that is necessary in the formation (aggregation) of hexamer [Chen, 1980]. Thus, confirmation of the protein is lost that result in irreversible denaturation of protein.

3B.3.2. Aqueous two phase extraction

The phycocyanin and total protein content in the cell homogenate (crude) were 0.14-0.26 mg/ml and 0.34-0.38 mg/ml, respectively. The parameters studied for selecting the suitable system were molecular weight of polymer, phase composition, volume ratio and neutral salt.

3B.3.2.1. PEG 4000/Potassium Phosphate System

The previous studies [Barhate, 2004] showed differential partitioning (partitioning of target protein or enzyme to one phase and the contaminants to opposite phase) of C-phycocyanin in PEG 4000/potassium phosphate system

and hence this system was employed for partitioning studies of cells and C-phycocyanin.

3B.3.2.1.2. Effect of Tie line length (TLL)

To study the effect of TLL, the volume ratio was maintained constant. As the %TLL increased from 13.12 to 25.52, K, yield and the purity of C-phycocyanin remained practically constant (Table 3B.3). However, further increase of TLL to 44.65%, resulted in decrease of K and yield of phycocyanin due its solubility limits. With increase in TLL, the concentrations of both PEG and salt increases in top and bottom phases. The increase in salt concentration of bottom phase reaches a high value which “salts out” the proteins as their solubility limit is exceeded. The salting out drives the proteins to top phase but the excluded volume effects of PEG tend to push it out of the top (PEG rich) phase as well [Huddleston et al., 1991]. The net result is that protein precipitates at the interface which was confirmed by visual observation.

As the %TLL increased, the relative free volume of top phase remained practically constant but that of bottom phase decreased (Table 3B.4). Relative free volume is the difference in the specific volume of the phase and that of the reference solution (pure water) (equation 4). It is an indication of the extent of the rearrangement of water upon phase splitting (Grossman and Gainer, 1989). The reduction in free volume of bottom phase resulted in partitioning of the contaminating proteins to top phase thus decreasing the purity of C-phycocyanin. At 44.65% TLL, the purity decreased

to a minimum of 0.44 (Table 3B.3). The lowest TLL (13.12%) showed the highest purity and yield of phycocyanin (0.73 and 90.34%, respectively) hence further studies to find the effect of the volume ratio was employed at this TLL.

3B.3.2.1.3. Effect of volume ratio

With an increase in volume ratio from 0.32 to 1.33, the yield of C-phycocyanin increased by 10% while the purity decreased by about 35% (from 0.69 to 0.45) (Table 3B.5). The observed increase in yield is because the volume of extracting phase was high and hence phycocyanin partitioned to top phase. The decrease in purity is due to the reason that the contaminating proteins ($K_{total\ protein}$) also partitioned to top phase with phycocyanin (Table 3B.5). At low volume ratios purity is high with low yield while at high volume ratio converse holds good as expected (Table 3B.5). However, improvement in purification can be achieved without loss of yield if both partition coefficients of the target protein/enzyme and total protein remain constant with the variation of volume ratio [Walter and Johansson, 1994]. Table 3B.6 shows that K of total protein varied with an increase in volume ratio, and hence at low volume ratio, high yield was not obtained. The optimum values of purity and yield were 0.73 and 90.1%, respectively. The absorption spectrum of the crude and purified phycocyanin is shown in Figure 3B.3.

3B.3.2.1.4. Effect of neutral salt

The addition of neutral salt (NaCl) alters the partition coefficient of the target protein [Albertson, 1986] or that of contaminants [Cascone et al., 1991;

Schmidt et al., 1994]. The first extraction was carried out at 13.12% TLL without adding NaCl. The effect of NaCl during second extraction was studied at volume ratio of 0.32 and K total protein 2.89. The objective of NaCl addition during second extraction was to find whether the partition coefficient of contaminating protein could be reduced or partitioning of C-phycocyanin could be altered in favor of bottom phase. During the second extraction, equal volume of the bottom phase (without phycocyanin) of same composition was added to the existing top phase obtained after first extraction (C-phycocyanin rich phase) followed by the addition of NaCl (1-5%) and the contents were mixed thoroughly. A control without the addition of NaCl (i.e. only second extraction) was carried out for comparison purpose. As the NaCl concentration increased from 0 to 5%, the yield slightly decreased but K decreased significantly by 73% (Figure 3B.4). Phycocyanin has an isoelectric point (pI) of 5.8, above which it is negatively charged and below which it is positively charged. It is reported that addition of NaCl decreases the K of negatively charged protein [Albertson, 1986]. The experiments were carried out at neutral pH and thus, decrease in K of phycocyanin from 42.8 to 11.7 is observed (Figure 3B.4). Among the two cases studied (with and without NaCl), the control (second ATPE at 0% NaCl) was found better than with addition of NaCl. The control resulted in K and yield of 42.8 and 92% respectively (Figure 3B.4).

3B.3.2.2. PEG 1000/Sodium Sulfate System

3B.3.2.2.1. Effect of molecular weight

PEG/sodium sulfate systems with different molecular weights of PEG (1000, 4000, 6000) were employed for the selection of suitable molecular weight for partitioning studies of C-phycocyanin. The composition chosen were near to the binodial and the volume ratios were maintained constant. It was observed that with an increase in molecular weights of PEG, K, yield and purity of phycocyanin in top phase decreased (Table 3.B.6). With an increase in PEG molecular weight, the relative free volumes in both top and bottom phases increased (Table 3B.7). The relative free volume available in bottom phase of PEG 4000 and 6000 is high as compared to that of respective top phases resulting in the partitioning of C-phycocyanin from top to bottom phase (as seen by K values of C-phycocyanin in Table 3.B.6). PEG 1000/sodium sulfate system showed high yield, K and purity of phycocyanin as compared to PEG 4000 and 6000 and hence PEG 1000/ sodium sulfate system was selected for further studies (Table 3B.6).

3B.3.2.2.2. Effect of TLL

The effect of TLL was studied by maintaining the volume ratio constant. As the %TLL increased, the yield initially increased to 97% at 34.98% TLL and then decreased to 93% at 45.43% TLL, while the purity of phycocyanin decreased from 0.41 to 0.29 (Table 3B.8). The decrease in yield can be explained similar to PEG 4000 and potassium phosphate system. As the phase composition increased, the concentrations of PEG and salt increased

in both phases, and at 45.43% TLL, precipitation of phycocyanin was observed at interphase, thus resulting in decrease in the yield.

With an increase in %TLL, the free volume of top phase increased from 23.29 to 34.98% and remained constant while that of bottom phase decreased (Table 3B.9). As a result, the contaminating proteins also partitioned to top phase, thus decreasing the purity of C-phycocyanin (Table 3B.8).

3. B.3.2.2.3. Effect of volume ratio

The effect of volume ratio was studied at 34.98% TLL. It is observed that with an increase in volume ratio the purity slightly decreased from 0.52 to 0.32, while the yield increased from 84.8% to 96.52% (Table 3B.10). The increase in yield is due to increase in volume of extracting (top) phase and the decrease in purity is since the contaminating proteins also partition to top phase with phycocyanin (Table 3B.10). It may be noted that in Tables 3B.6, 3B.8 and 3B.10 the standard deviation for K are as high as 4-8%. This is due to the contaminants such as cell debris, nucleic acids etc. present in cell homogenate effect the partitioning when subjected to ATPE without prior purification. Similar observation was reported previously [Kepka et al. 2005].

3B.3.2.2.4. Effect of neutral salt

The effect of neutral salt was studied during second extraction in similar manner as in PEG 4000/potassium phosphate system at a volume ratio and K total protein of 0.21 and 3.23, respectively. With an increase in

concentration of NaCl from 1-4%, the yield of C-phycocyanin remained constant however K was found to decrease significantly (Figure 3B.5). The pH of the top phase (without NaCl) was above the isoelectric point of phycocyanin (5.8) and addition of NaCl did not alter the pH of top phase significantly (Figure 3B.6). The K of C-phycocyanin was found to decrease since C-phycocyanin is negatively charged. The second extraction without NaCl (control) was found better than with addition of NaCl wherein K of C-phycocyanin as high as 720 with 99% yield was achieved (Figure 3B.5).

3B.4. Conclusions

ATPE was found better than TPP for the extraction and purification of C-phycocyanin from *Spirulina platensis*. TPP caused irreversible denaturation of C-phycocyanin, due of the strong hydrophobic interaction of t-butanol and C-phycocyanin. However, byproducts such as carotenoid were extracted to t-butanol phase.

Application of ATPE directly to cell homogenate was carried out which enabled the integration of cell separation, extraction and concentration into single step of ATPE. PEG 4000 and potassium phosphate system was found better than PEG 1000 and sodium sulfate system for the extraction and purification of C-phycocyanin. This is due to the phosphate salt and pH (neutral) at which extraction of phycocyanin was carried out cause minimal adverse effect to phycocyanin. Addition of NaCl did not increase the K like in many systems previously studied and in fact the opposite trend was observed.

Table 3B.1. Different alcohols used for the extraction of phycocyanin

Sl no.	Phase system		Interphase
1.	0%w/v $(NH_4)_2SO_4$ + 30%v/v methanol	homogeneous	-
2.	30%w/v $(NH_4)_2SO_4$ + 30%v/v ethanol	Three phase	denaturation
3.	30%w/v $(NH_4)_2SO_4$ 30%v/v isopropanol	Three phase	denaturation
4.	30%w/v $(NH_4)_2SO_4$ + 30%v/v t-butanol	Three phase	denaturation
5.	30%w/v $(NH_4)_2SO_4$ + 30%v/v ethylene glycol	homogeneous	-

Table 3B.2. Different compositions of ammonium sulfate and t-butanol used or the extraction of phycocyanin

Phase system	pH	Purity of C-phycocyanin
1. 20%w/v $(NH_4)_2SO_4$ / 20%v/v t-butanol	7	0.31
2. 20%w/v $(NH_4)_2SO_4$ / 15%v/v t-butanol	7	0.32
3. 20%w/v $(NH_4)_2SO_4$ / 20%v/v t-butanol	8	0.29
purity of crude phycocyanin		0.54

Table 3B.3. Effect of TLL on K and yield of phycocyanin in PEG 4000 and potassium phosphate system

% TLL	$K_{C\text{-}phycocyanin}$*	$K_{total\ protein}$*	purity of phycocyanin	% yield of phycocyanin
13.12	20.33 ± 0.18	1.72±0.07	0.73 ± 0.01	90.34
18.64	18.5 ± 0.71	1.58±0.05	0.70 ± 0.01	88.35
25.52	18 ± 0.71	1.93 ± 0.21	0.65 ± 0.03	89.01
44.65	12.25 ± 1.06	2.19 ± 0.17	0.44 ± 0.01	86.43

* values represent mean± standard deviation

Table 3B.4. Effect of TLL on free volume changes in PEG 4000 and potassium phosphate system

% TLL	Density		Relative Free Volume		Change in free volume (cc/g)
	Top phase (g/cc)	Bottom phase (g/cc)	Top phase (cc/g)	Bottom phase (cc/g)	
13.12	1.080	1.125	-0.070	-0.107	0.037
18.64	1.079	1.133	-0.069	-0.113	0.044
25.52	1.078	1.143	-0.068	-0.121	0.053
44.65	1.088	1.231	-0.077	-0.184	0.107

Table 3B.5. Effect of volume ratio on k, yield and purity of phycocyanin in PEG 4000 and potassium phosphate system at 13.12% TLL

volume ratio	$K_{C\text{-}phycocyanin}$	$K_{total\ protein}$	purity	Yield (top)
0.32	19.38 ± 0.03	1.50 ± 0.29	0.69 ± 0.01	86.11
0.45	20.25 ± 0.01	1.72*	0.73 ± 0.01	90.11
1.33	20.00 ± 0.02	1.06 ± 0.46	0.45*	96.38

*represents standard deviation is nil

Table 3B.6. Effect of molecular weight of PEG on K and yield of phycocyanin for PEG and sodium sulfate system

PEG mol. wt	$K_{C\text{-}phycocyanin}$	$K_{total\ protein}$	Purity C-Phycocyanin (top phase)	% Yield of C-Phycocyanin (top phase)
1000	17.07 ± 4.14	1.55 ± 0.31	0.46	88.92
4000	1.07 ± 0.04	1.14 ± 0.28	0.24	28.37
6000	0.45	1.50 ± 0.31	0.08	13.04

Table 3B.7. Effect of molecular weight of PEG on change in free volume in PEG and sodium sulfate system

PEG mol.wt	Density		Relative free volume (ml/g)		Change in free volume (cc/g)
	Top phase (g/cc)	Bottom phase (g/cc)	Top phase (cc/g)	Bottom phase (cc/g)	
1000	1.093	1.141	-0.082	-0.121	0.038
4000	1.080	1.123	-0.071	-0.107	0.035
6000	1.070	1.120	-0.062	-0.104	0.042

Table 3B.8. Effect of TLL on K and yield of phycocyanin in PEG 1000 and sodium sulfate system

% TLL	$K_{C\text{-}phycocyanin}$	$K_{total\ protein}$	Purity of phycocyanin	% Yield of phycocyanin
23.29	9.63 ± 0.18	1.53 ± 0.01	0.48	88.51
34.98	38 ± 1.41	2.15 ± 0.1	0.41	97.62
40.52	27.5 ± 0.71	2.64± 0.01	0.29 ± 0.05	92.42
45.43	14.67± 8.84	1.98 ± 0.2	0.29	93.96

Table 3B.9. Effect of %TLL on free volume changes in PEG 1000 and sodium sulfate system

% TLL	Density		Relative Free volume		Change in free volume (cc/g)
	Top phase (g/cc)	Bottom phase (g/cc)	Top phase (cc/g)	Bottom phase (cc/g)	
23.29	1.100	1.121	-0.087	-0.104	0.017
34.98	1.084	1.163	-0.073	-0.136	0.063
40.52	1.081	1.201	-0.071	-0.163	0.092
45.43	1.081	1.220	-0.071	-0.176	0.105

Table 3B.10. Effect of volume ratio on k, yield and purity of phycocyanin in PEG 1000 and sodium sulfate system at 34.98% TLL

Volume ratio	$K_{C\text{-}phycocyanin}$	$K_{total\ protein}$	purity	% Yield of Phycocyanin
0.21	26.57 ± 8.07	2.87 ± 0.11	0.52	84.80
1.08	30.12 ± 6.4	2.72 ± 0.14	0.41	97.02
1.5	18.46 ± 2.33	2.27 ± 0.07	0.32	96.52

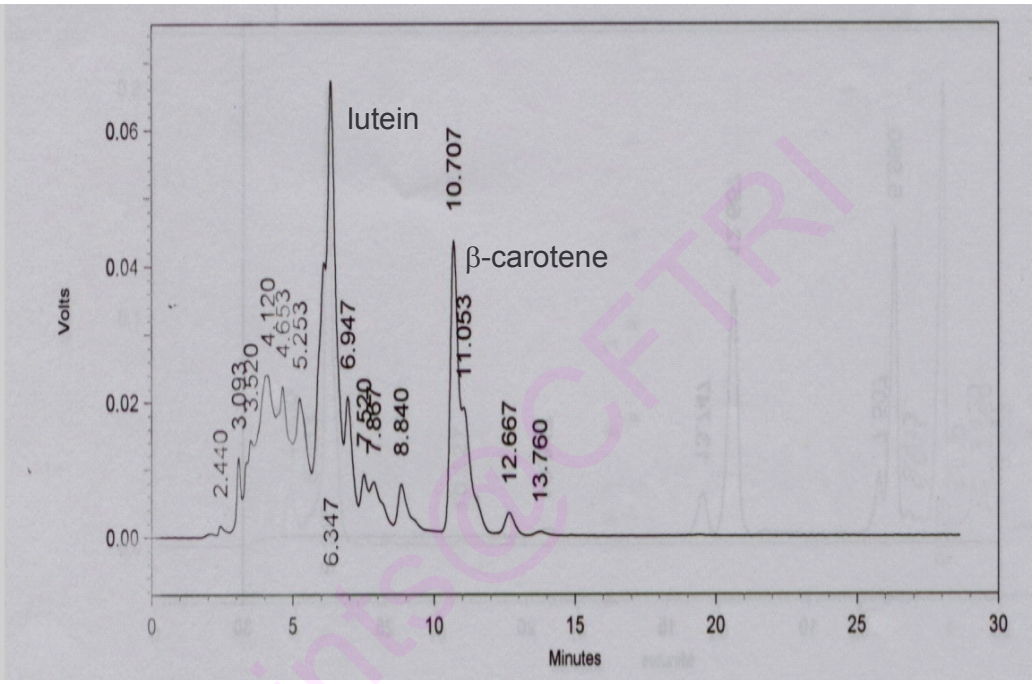

Figure 3B.1. Carotenoid components detected by HPLC (composition - 30% t - butanol +30% $(NH_4)_2SO_4$)

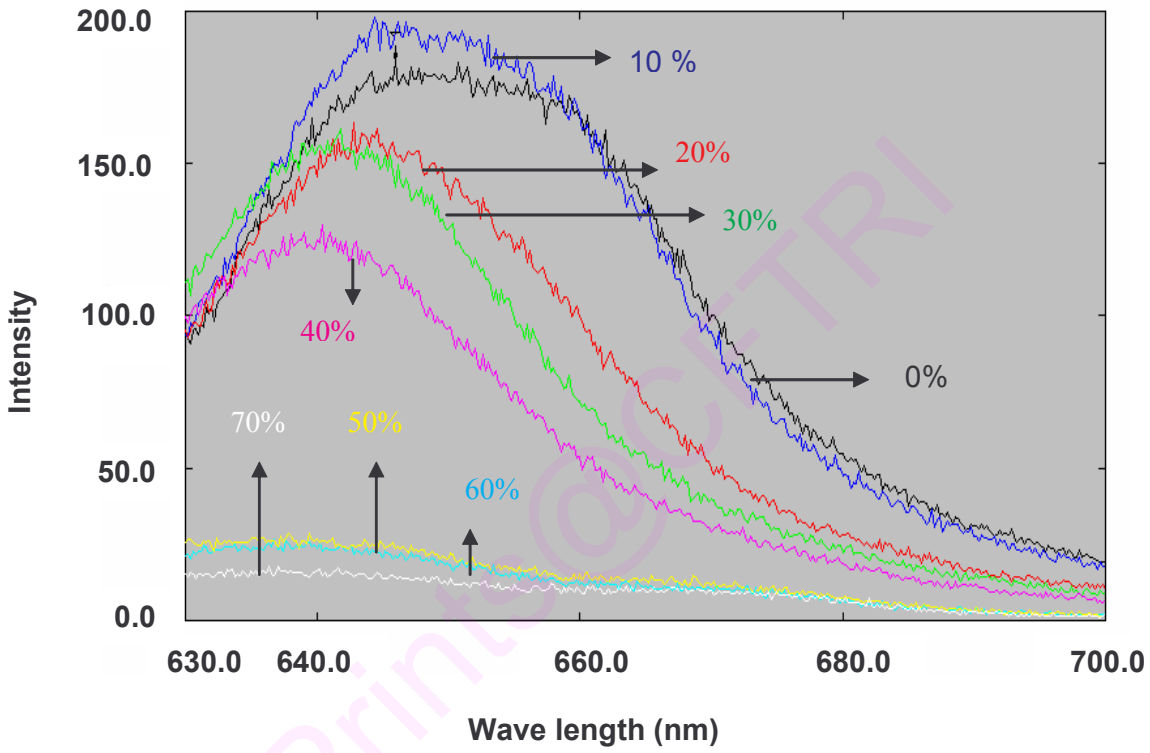

Figure 3B.2. Fluorescence intensity of C-phycocyanin after 2 hours of incubation in t-butanol of different concentrations at 25ºC

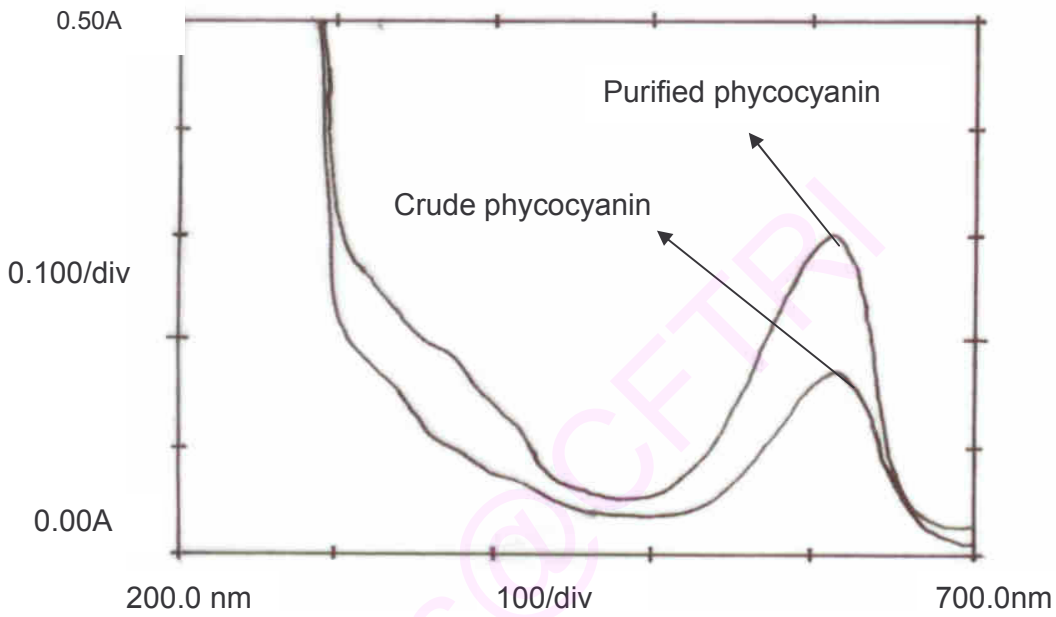

Figure 3B.3. Absorption spectra of crude and purified phycocyanin by aqueous two phase extraction (PEG 4000/pottasium phosphate)

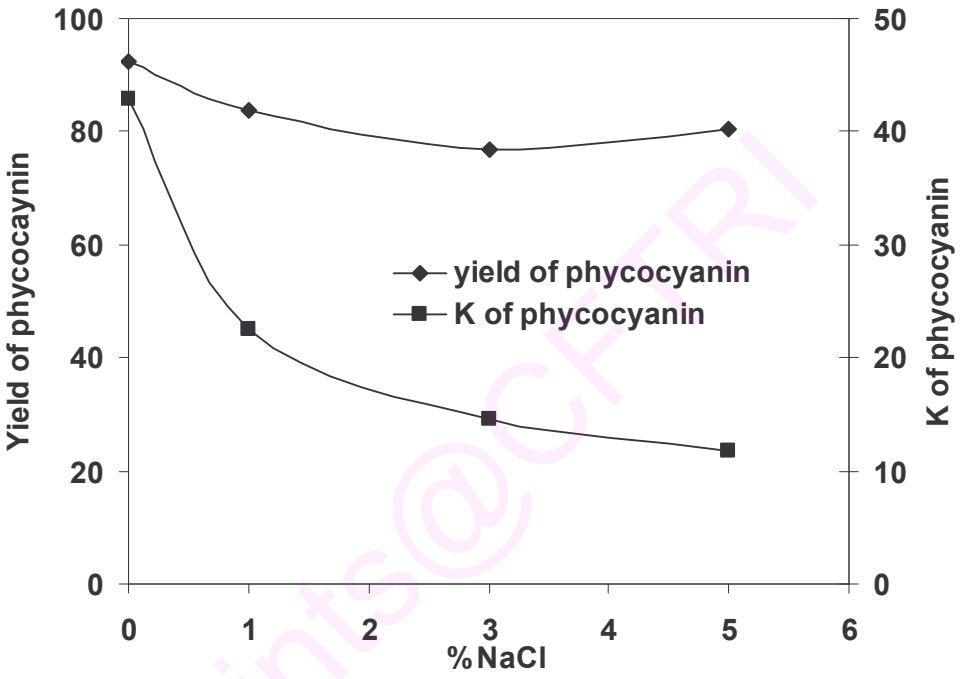

Figure 3B.4. Effect of NaCl concentration on yield and partition coefficient of C-phycocyanin during second extraction of PEG 4000 and potassium phosphate system

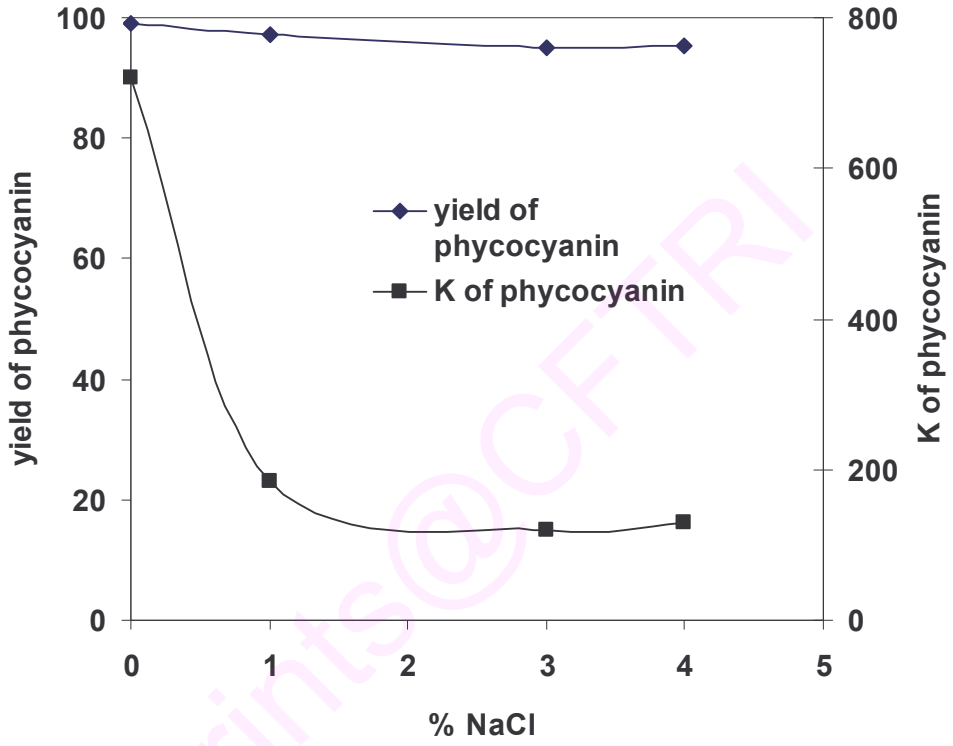

Figure 3B.5. Effect of NaCl concentration on yield and partition coefficient of phycocyanin during second extraction of PEG 1000 and sodium sulfate system

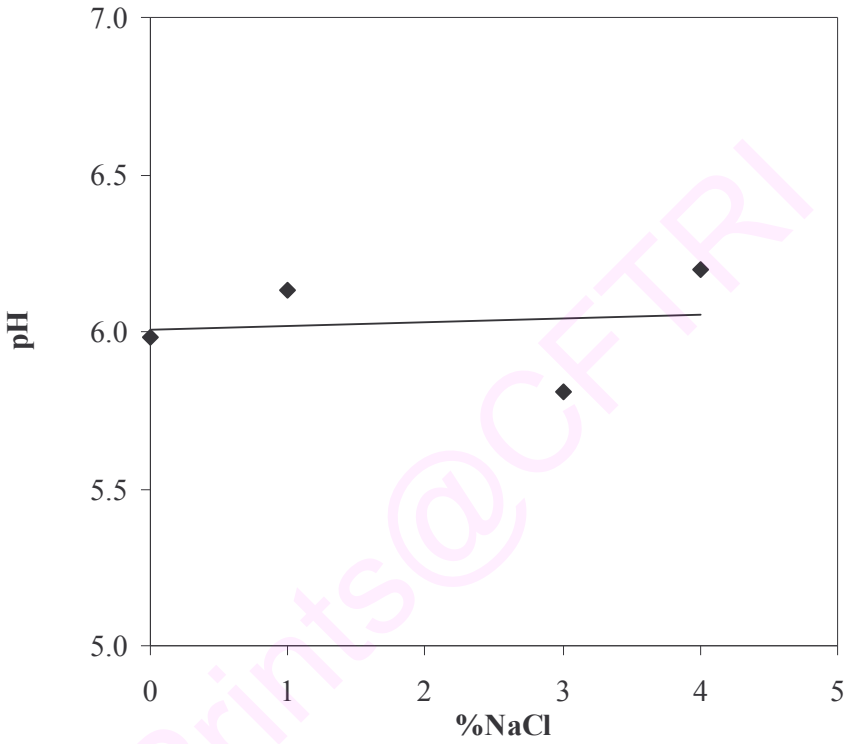

Figure 3B.6. Effect of NaCl concentration on the pH of top phase in PEG 1000 and sodium sulfate system

Chapter- 4

EXTRACTION AND PURIFICATION OF ORYZANOL FROM RICE BRAN OIL SOPAPSTOCK

Section- A

EXTRACTION BASED PROCESSES

4A.1. Introduction

Rice is the second largest cultivated crop produced worldwide with current annual world production (December 2005) being approximately 632 million metric tons (www.fao.org). Rice bran is one of the valuable byproducts of the rice processing industry (Shin et al., 1997). In many instances, it is economically feasible to extract oil from rice bran and purify it by physical or chemical refining for either food or industrial use. Due to several technical and non-technical problems in oil refining, the actual annual production of rice bran oil (RBO) is far below the demand (Gingras, 2000). The major difficulties faced in processing crude RBO for edible purposes are the high levels of free fatty acids (FFA), waxes, gums and pigments. Accordingly, most RBO is used in non-food applications. Alkali refining of RBO produces soapstock as byproduct. The typical soapstock from RBO contains about 65-70 wt% water, 20-22 wt% soap, 2-2.5 wt% glycerides (mainly triglycerides) and 7-7.5 wt% unsaponified matter. The unsaponified fraction contains about 42% sterols, 24% higher fatty alcohols, 20% oryzanol (as ferulic acid esters), 10% hydrocarbons and 2% unidentified compounds (Akiya, 1962). Oryzanol represents about 15% (20% of 7.5% wt %) of the unsaponified matter. At present, the major use of the soapstock is the generation of soap for the toiletry and detergent industries. It could also be used for production of therapeutically active components like oryzanol and tocopherols (Orthoefer, 1996).

Chemically, oryzanol is reported as a mixture of ferulic acid esters of triterpene alcohols (phytosterols), i.e., ferulate (4-hydroxy -3-methoxy

cinnamic acid) (Seetharamaiah and Prabhakar, 1986). The components of oryzanol were identified as □^{7}-stigmastenyl ferulate, stigmasteryl ferulate, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, □^{7}-campestenyl ferulate, campesteryl ferulate, □^{7}-sitotenyl ferulate, sitosteryl ferulate, campestanyl ferulate and sitostanyl ferulate (Xu and Godber, 1999). Important rice bran ferulates are cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate.

Kaimal (1999) summarized the beneficial health effects of oryzanol. A purported application of oryzanol in pharmaceuticals includes hypocholesterolemic activity (Seetharamaiah and Prabhakar, 1986). Oryzanol also has a protective role in lipid peroxidation and finds applications in sunscreen agents, as an antioxidant and preservative in cosmetics and food preparations, the treatment of atopic dermatitis, senile xeroderma and prevention of skin dryness (Kaimal, 1999). A safety assessment of oryzanol indicated no genotoxic or carcinogenic activity (Tsushimota et al., 1991). Because of these beneficial effects of oryzanol on human health, a global interest in developing facile methods for separating oryzanol from RBO soapstock has developed.

The extraction of oryzanol is complicated due to the presence of the surface active impurities. The problems encountered during extraction of oryzanol are mainly due to variations in the compositions of RBO soapstock, which include surface-active impurities such as soaps, phospholipids, waxes, glycolipids, etc. The process conditions used during oil refining and seasonal

variations in oilseed composition dictate the type and quantity of the impurities extracted with the soapstocks during chemical refining of RBO. Thus, the amount of individual impurities, which are to be removed, vary from one soapstock to the other. Hence, an isolation procedure developed for one soapstock does not necessarily work for another soapstock. The impurities that stabilize the soapstock dispersion (Solid-Liquid and Liquid-Liquid ratios) vary depending on the type and amount present. Impurities also affect processing conditions and hamper the equilibria governing the separation process steps such as extraction, isolation and purification. Narayan et al. (2006) have discussed some of the problems encountered in large scale extraction of oryzanol from RBO and RBO soapstock and their solutions.

The major impurities removed during isolation and recovery of oryzanol from soapstock or acid oil derived therefrom include FFA, soap, glycerides, phospholipids, waxes, sterols, glycolipids and other impurities such as resinous matter, tocopherol derivatives, and pigments.

Leaching is probably the simplest operation for the separation and purification of oryzanol. Leaching is extraction of soluble components from an insoluble permeable solid phase using a suitable solvent. Leaching operations typically reported in literature are for two purposes: (a) leaching of impurities from the oryzanol enriched fraction and (b) leaching of oryzanol from the dried soapstock. Examples of the category (a) from literature include the patented processes of Masao and Yoshizane (1968), Takeshi (1969) while that of

category (b) include that of Indira et al. (2005). The principles behind these patented processes are discussed.

Masao and Yoshizane (1968) reported a patented process for obtaining 85% pure oryzanol and 2.5% yield from RBO soapstock using two-step leaching process. In the first step, the major impurities, mainly soap, were separated from the soapstock by solvents such as methanol or ethanol while simultaneously sparging carbon dioxide through the mixture. Sparging with carbon dioxide facilitated preferential leaching of the soap, leaving oryzanol in the purified medium. Thus carbon dioxide sparging improved selectivity of extraction and reduced oryzanol losses in the leaching medium. In the second leaching step, weak alkali salts such as sodium bicarbonate or sodium carbonate (which were associated with the residue during the first leaching step) were removed by methanol or ethanol washing. This process has the advantage of using fewer unit operations and sufficient quantity of solvent for efficient removal of impurities.

Takeshi (1969) reported a patented process involving eight steps for obtaining oryzanol from soapstock. RBO soapstock was first converted to acid oil with sulfuric acid, then the acid oil was subjected to esterification with a mixture of sulfuric acid and methyl alcohol, and the fatty acid methyl esters removed by distillation. The residue remaining after distillation of the esters was subjected to successive leaching with n-hexane, methanol and methanolic alkali. In each of the leaching steps, the residue obtained from the previous step was taken as the raw material for the next step. Here the

distillation process was improved by esterifying FFA, since the esters distill off at a lower temperature than FFA, which results in an energy saving process. The major limitation of this process is the number of unit operations involved.

Recently, Indira et al. (2005) reported a process for obtaining oryzanol of 40-45% (w/w) purity and 80% (w/w) recovery. The raw material (soapstock saponified for second time and dehydrated) was subjected to a leaching step using solvents such as ethyl acetate, acetone or their mixtures to extract the oryzanol. This process offers several advantages such as reduced scale of operation (as considerable reduction of moisture in soapstock is achieved by dehydration), increased interfacial area for efficient leaching by micromixing and hence, ease of scale up. It was noted that milder operating conditions, with regard to temperature and pH, could be used to minimize degradation of oryzanol during processing. The limitation of this process is that it results in oryzanol of varying purity (33-43% w/w) and recovery (57-80% w/w).

The performance of Liquid-Liquid extraction (LLE) based processes mainly depend on the differential partitioning of oryzanol and the other soapstock components into the two immiscible liquid phases. The criteria for selection of the phase system and conditions for LLE are differential partitioning of the product and impurities into separate phases, followed by rapid phase separation after mixing. Important process parameters that need to be considered during LLE are polarity of the solvents used, solid content of the two phase system, ratio, temperature and tie-line-length, which is a line joining the two points of a binodial in a phase diagram describing the two

solvent phases. The tie-line-length gives the composition of solvents required for LLE. Processes based on LLE discussed are that of Tsuchiya et al. (1957) and Seetharamiah and Prabhakar (1986).

Tsuchiya et al. (1957) obtained oryzanol of 60% (w/w) purity and 2.5% (w/w) yield from RBO (100kg). In this process two saponification steps are used, namely, hydrolysis of the residue obtained after a second saponification with HCl and a two-step LLE with ether and alkali. In the first saponification step (171.30% of NaOH) RBO was converted to soapstock while in the second saponification step, oryzanol containing neutral oil was converted into its salt form. The residue remaining after the second saponification step was subjected to acidification, thus converting the salts of oryzanol into their free acid forms. The product obtained after hydrolysis was subjected to a two-step LLE using ether and aqueous alkali. In the first extraction, the impurities were extracted into the ether phase while the aqueous phase was hydrolyzed. In the second extraction the target product (oryzanol) was extracted into the ether phase. The drawback of this patented process is that there are a number of steps and the yield of oryzanol is low.

Seetharamiah and Prabhakar (1986) reported a four step process including LLE, column chromatography, crystallization and recrystallization to extract oryzanol from RBO Soapstock. The solvents used for LLE were a mixture of diethyl ether and methanol. In this step, most of the soap was extracted with methanol. The ether rich phase was extracted repeatedly with aqueous alkali to isolate most of the oryzanol in the aqueous phase and the

remaining impurities were removed by ether extraction. Finally the alkaline oryzanol extracts were pooled, neutralized with acetic acid, and oryzanol extracted back into ether and the ether phase desolventized to obtain an oryzanol concentrate. This fraction was then subjected to column chromatography on alumina and eluted with solvent such as hexane, petroleum ether-methanol (9:1 v/v) and diethyl ether-methanol (20:1 v/v) and the resulting oryzanol product crystallized. Two crystallizations were carried out, one with methanol and other with a mixture of methanol and acetone (2:1v/v), respectively. At the end of the chromatography step, the yield of oryzanol was 75.7% (w/w) with a purity of 51.4% (w/w). However, in this article, the authors did not mention the purity and yield of oryzanol at the end of the recrystallization steps. The limitation of this process is that in the LLE repeated extraction needs to be carried out, which increases the number of extraction steps and decreases the yield of oryzanol. Hence this method is limited for scale-up production.

In the present study, leaching of oryzanol from dried RBOS was carried out using ethyl acetate and acetone, each separately as well as their mixtures. The product after leaching (unsaponified matter) was subjected to LLE in which the solvents used were hexane and ethyl alcohol.

4A.2. Materials and Methods

Soapstock was procured from M.K.Agro Ltd., Srirangapatna, Mysore Dist., Karnataka, India. Ethyl acetate and acetone were procured from

Ranbaxy Chemicals, New Delhi. Hexane, NaOH, rectified spirit and HCl were procured from Rankem, New Delhi. All the chemicals used were LR grade.

4. A.2.1. Pretreatment of soapstock

The soapstock was pretreated as described by Indira et al. (2005). A known quantity of NaOH (based on stoichiometry) was added and mixed at 70-90°C for about 30 min to saponify the neutral oil present. Micromixing was carried out in colloid mill for about 10 min in order to increase the interfacial area of the soapstock. The soapstock was then dried in a conventional drier to remove the water content.

4A.2.2. Experimental Procedure

Predetermined quantity of dried soapstock was leached with ethyl acetate, acetone or their mixture by mixing in a magnetic stirrer for 1 hour. The extract was then filtered and insolubles separated. The supernatant was desolventized, dried in vacuum and the oryzanol content estimated.

The product obtained after leaching (unsaponified fraction) was treated with a mixture of predetermined amount of alkali and rectified spirit (ethyl alcohol) and mixed for 1 hr (in order to convert oryzanol to its salt form which easily solubilizes in ethanol). Hexane and methanol were the solvents used for LLE. Hexane was added and mixed for about 30 min which lead to the formation of homogeneous phase. The mixture was kept at 4°C for about 2 hrs in order to facilitate phase separation. Since the phase separation did not occur, a predetermined quantity of water added to increase the polarity and

thus induce phase separation. After complete separation of phases the top and bottom phases were separated and bottom phase was neutralized with HCl, filtered and dried in vacuum to recover oryzanol.

4A.2.3. Estimation of oryzanol

Oryzanol content in soapstock was estimated by the procedure described by Seetharamiah and Prabhakar (1986). 100 mg of dried soapstock was dissolved in 1ml HCl (1N) and 1ml petroleum ether. 10 ml of hexane was added and stirred and the mixture left for about 30 min for separation into two phases. The top layer was separated and extracted with hexane twice to recover oryzanol. The absorbance was measured at 314-315 nm by using spectrophotomer (Shimadzu UV 1601).

The oryzanol content in the unsaponified matter was estimated as follows. About 2-3 mg of unsaponified matter was taken and dissolved in 10 ml chloroform. The mixture was then diluted suitably with chloroform and the absorbance taken at 320 nm. The extinction coefficient of oryzanol was taken as 358.9 for the estimation of oryzanol.

The following equation was used for the calculation of % oryzanol:

$$\%\text{oryzanol} = \frac{\text{measured absorbance x dilution factor}}{358.9 \text{ x weight of the sample}} \times 100$$

4A.3. Results and Discussion

4A.3.1. Solid-liquid extraction (leaching)

Ethyl acetate, acetone and their mixtures were used as solvent for leaching of oyzanol from soapstock. With an increase in ratio of ethyl acetate to acetone content, the purity and yield remained initially constant and at 50:50 (w/w %) decreased [Figure 4A.1]. At ratios of ethyl acetate to acetone 50:50-75:25 (w/w %) purity decreased by about 15-25% and the yield by 21% Ethyl acetate and acetone have relative polarities of 0.228 and 0.355 (Reichardt, 1988). The decrease in purity and yield at 50:50 and 75:25 is because of the decrease in selectivity of extraction of oryzanol which may possibly because of the high polarity of acetone. 100 % w/w ethyl acetate resulted in 34% purity and about 100% recovery of oryzanol, while 100 %w/w acetone showed comparable purity with low yield (58%). Hence, ethyl acetate was found suitable leaching solvent than acetone for the extraction of oryzanol.

4A.3.1. Liquid-liquid extraction

Hexane is the solvent commercially used for the extraction of oil while alcohols (short-chained ones) extract the non-glyceride and unsaponifiable compounds (Hu et al., 1996). Hexane and methanol are the commonly used solvents in LLE for preferential extraction of oil, vitamin-E and oryzanol from rice bran (Hu et al., 1996). In the present study, in order to find the suitability of hexane and methanol to extract oryzanol from unsaponified fraction (obtained after leaching of soapstock), these solvents were used in LLE. With an increase in purity of unsaponified fraction (25-36%), it was found that the

recovery of the oryzanol varied by about 10% while the purity remained constant [Figure 4A.2]. Overall purity and recovery of the product (oryzanol) achieved was about 40-42% and 55-64% respectively. The low yield of oryzanol is because of the surface active agents such as waxes and gums (phospholipids). The surface active agents caused microemulsion formation (thermodynamically stable microsize emulsion stabilized by phospholipids, waxes and mono or di-esters of fatty acids or micellar salvation of solute) during LLE that decreased the yield of oryzanol.

4A.4. Conclusions

Leaching of oryzanol from soapstock resulted in comparable purity with LLE. However, the recovery achieved in leaching was remarkably better than LLE because of the selectivity of extraction by ethyl acetate. In LLE, after the first step of addition of hexane and alcoholic NaOH, the bottom phase (alcoholic NaOH) is further subjected to subsequent steps of addition of water, neutralization, filtration of residues and finally desolventization that decreases the yield. The other reason for decrease in yield in LLE is the microemulsion formation by the surface active agents.

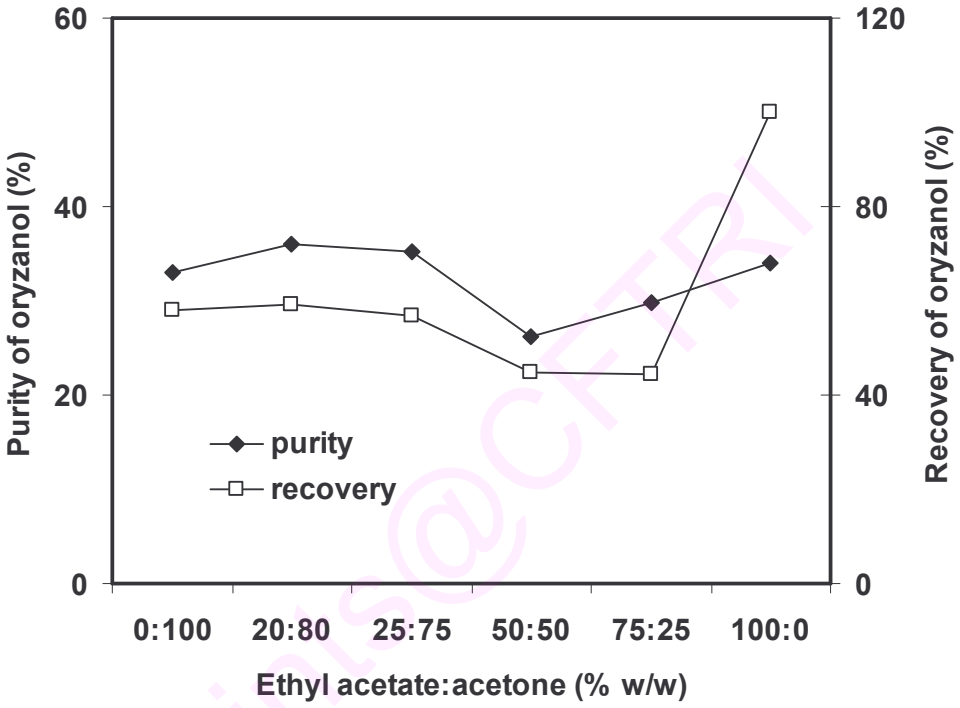

Figure 4A.1. The effect of different proportions of ethyl acetate to acetone on purity and yield of oryzanol

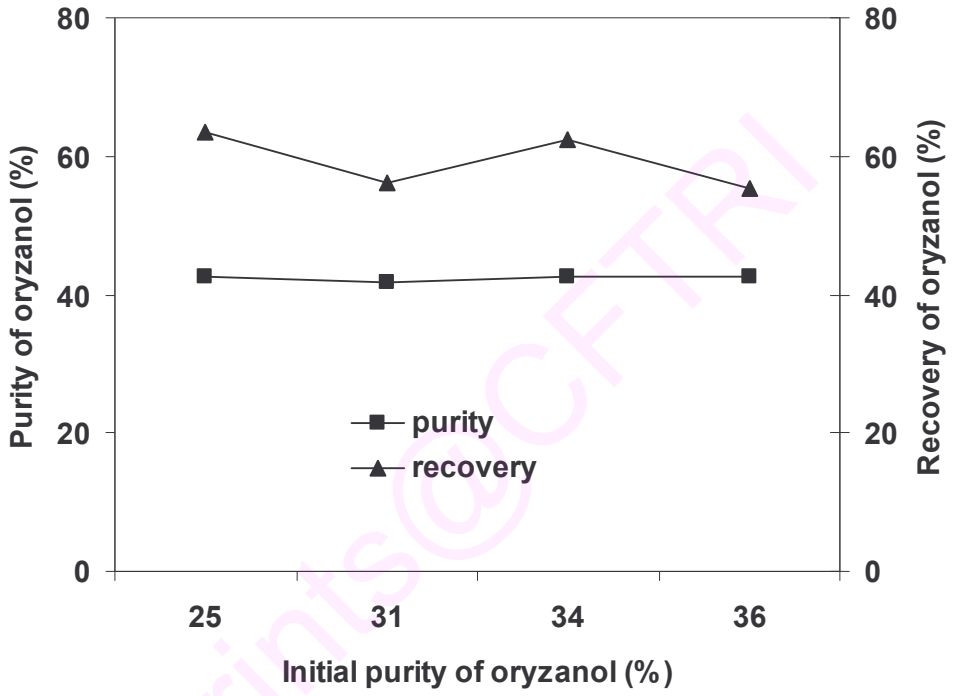

Figure 4A. 2. Effect of initial purity of unsaponified fraction on the purity and recovery of product after LLE

Section- B

ENZYMATIC DEGUMMING

4B.1. Introduction

Phospholipids (gums) can be classified as hydratable and nonhydratable. Gums are present in soapstock and their relative proportions vary depending upon the efficiency of the degumming step. The main component of hydratable gums is phosphatidylcholine (PC) whereas non-hydratable gums are calcium and magnesium salts of phosphatidic acid (PA) and phosphatidylethanolamine (PE) (Young et al., 1994). The gums, especially the nonhydratable phospholipids are the most interfering impurities affecting oryzanol separation, probably due to their high surface activity. During solvent extraction of RBO soapstock, the gums stabilize the soapstock microemulsion and thereby decrease the rate of phase separation. To reduce their interference during the extraction step (Solid-Liquid and Liquid-liquid) used for purification of oryzanol, efficient degumming of RBO is desirable prior to the deacidification step.

The hydratable gums in RBO can be removed by either water, acid degumming or by the use of surface-active agents such as lauryl suphate, sodium oleate, alkylated phenol ethylene oxides, or alkyl aryl sulphonates (Bhattacharyya and Bhattacharyya, 1985). These methods however are not capable of reducing phosphorous content in the RBO below 10 ppm. Methods for the removal of the nonhydratable phospholipids from RBO include superdegumming and enzymatic degumming. A superdegumming method patented by Unilever has been developed and is based on the principle that the nonhydratable phospholipids present in the RBO can be converted to a hydratable form by heating the oil to 70-80°C and treating with citric acid for

about 20 minutes at this temperature (Ringers and Segers, 1977). The hydratable phospholipids formed are allowed to crystallize as calcium and magnesium salts, waxes and glycerol. Final neutralization gives oil with a phosphorous content of about 5 ppm.

An enzymatic degumming method has been proposed for treating the nonhydratable phospholipids of vegetable oils such as rapeseed, soybean, sunflower seed (Buchold, 1993). The enzyme, phospholipase (fungal origin), hydrolyzes the ester bond of the phospholipids at the oil-water interface thereby converting the non-hydratable phospholipids into fully hydratable phospholipids and FFA (Sambasiva Rao, 2002). The hydratable phospholipids are then removed by conventional water degumming. This enzyme degumming method was extended to RBO and after bleaching the oil, had a phosphorous content of about 5 ppm (Roy et al., 2002). Phospholipase from microbial origin has been used for degumming rapeseed oil and its performance has been found better than that of pancreatic phospholipase (Clausen, 2001). The microbial phospholipase requires low water content thus eliminating the need of sludge recycling.

Gums are partially soluble in hot acetone but insoluble in cold acetone. The extent of solubilization of phospholipids in hydrocarbon solvents decreases in the order of pentane, hexane and n-heptane.

In the previous studies, degumming using phospholipase has been carried out using the vegetable oils (rapeseed, soybean, sunflower, RBO) as

the starting material. In the present study, the degumming was attempted by lecitase using the unsaponified fraction (obtained after leaching) as the starting material under different experimental conditions.

4B.2. Materials and Methods

Lipase Novo was procured from Novozymes, Denmark. NaOH and ethyl acetate were purchased from Ranbaxy, New Delhi, India.

4B.2.1. Experimental procedure

The unsaponified fraction (obtained after the leaching of soapstock) was dissolved in ethyl acetate and lecitase was added (20000 units of enzyme was added calculated based on stoichiometry). The pH was maintained constant at 5.5 by a mixture of buffer (citric acid and NaOH). This miscella (containing unsaponified matter with lecitase) was further stirred at 40°C for about 6 hrs in a mechanical stirrer for the hydrolysis of phospholipids. After 6 hr, the mixture was transferred to a separating funnel and after complete separation of phases, the top and bottom phases separated. The top phase (product rich phase) was divided into two equal portions. The first portion was dried, saponified and leached to obtain oryzanol (Protocol 1). The second portion containing solvent miscella was saponified directly to obtain oryzanol (Protocol 2). The flow chart for the experimental procedure is shown in Figure 4B.1. Oryzanol was analyzed as described in section 4A.2.3.

4B.3. Results and Discussion

The degumming of the vegetable oils is effective at 45-50°C and pH of 5.5 (Buchold, 1993, Dahlke and Buchold, 1995). Hence, in the present study, similar experiments as to that of conventional enzymatic degumming of the vegetable oils were carried out with unsaponified fraction as the starting material. In all the experimental conditions, it was found that recovery was in the range 69-100%. The purity of oryzanol in experimental conditions 1-3 (Protocol 1 and 2) is low as compared to the initial purity of crude (Table 4B.1). The purity and the recovery decreased in experimental condition (1) (Protocol 1 and 2) as compared to the control (experimental condition 2). In experimental condition 1, since the temperature was not controlled it is possible that the degree of enzyme degumming was less effective in the unsaponified fraction (starting material), thus reducing the purity and yield of oryzanol (Table 4B.1).

In Protocol 2, it is observed that control shows highest purity and recovery (experimental condition 2). In experimental condition 3, Protocol 2, the purity and yield of oryzanol decreased as compared to control. The reason may possibly due to the less degree of degumming. The reduced degree of saponification (quantity of NaOH is decreased by 25% as compared to control) did not have any significant effect on the yield in experimental conditions 3 and 4 (Table 4A.1).

4B.4. Conclusions

On comparing protocols 1 and 2, for all the experimental conditions, control showed highest purity and recovery compared to other conditions. The

main objective of enzyme degumming is to break the non-hydratable phospholipids. This results show that degree of degumming is not effective in the unsaponified matter since higher purity values are not observed though the recovery is acceptable.

Table 4B.1. Experimental protocols for enzymatic degumming

	Initial purity of oryzanol in soapstock (%)	Protocol –1 (drying and saponification, leaching)		Protocol –2 (direct saponification of miscella)	
Experimental conditions		purity	recovery	purity	recovery
1. pH controlled , temperature not controlled, saponification by stiochiometry	43	26.81	71.66	31.17	69.3
2. pH and temperature controlled, saponification by stiochiometry		35.83	~ 100	44.87	86.97
3. pH and temperature controlled, degree of saponification reduced		35	90	34.55	74.57
4. pH and temperature controlled, solid to solvent ratio and degree of saponification reduced	24	24	88	24	~ 100

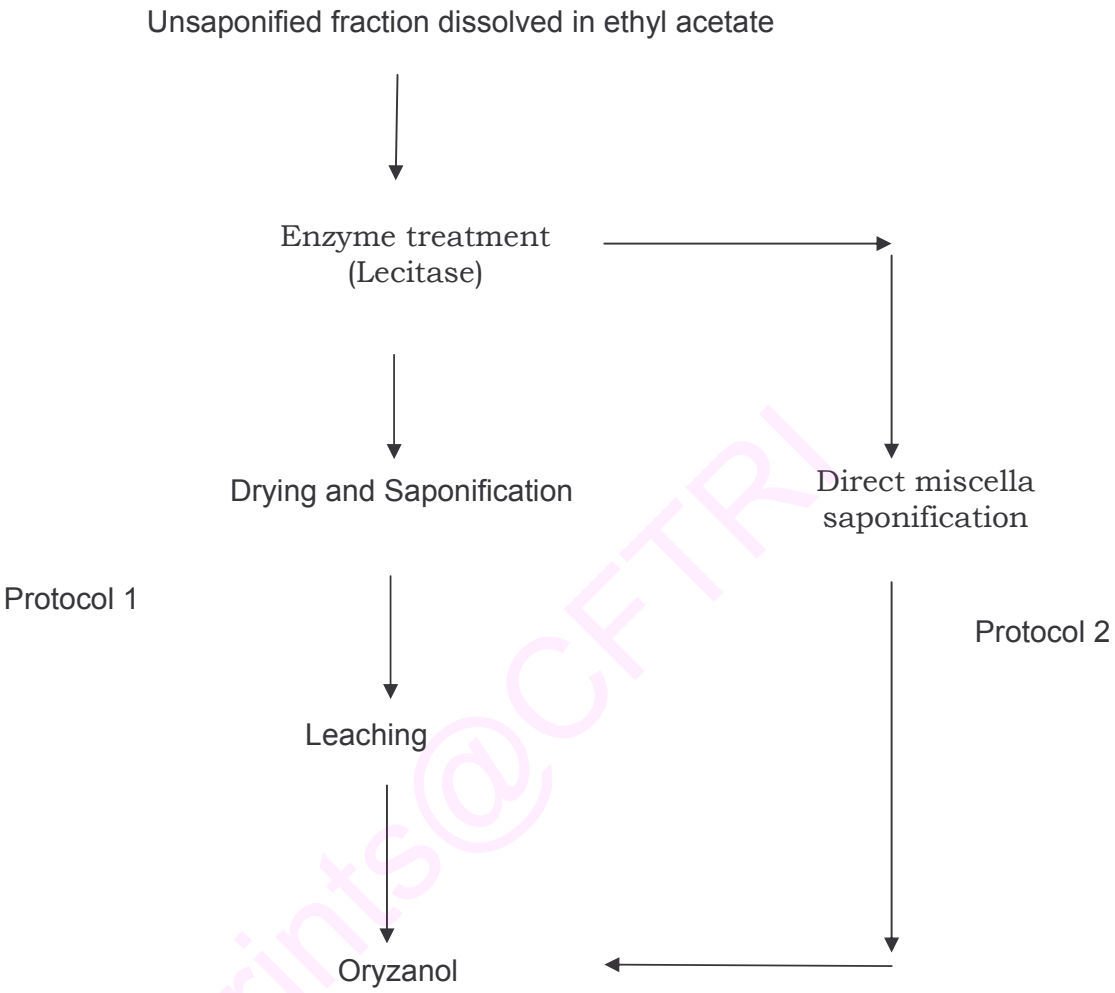

Figure 4B.1. Protocol for enzyme degumming of unsaponified fraction

Section- C

CRYSTALLIZATION

4C.1. Introduction

Crystallization of oryzanol is usually carried out using Rice bran oil (RBO), rice bran acid oil or RBO soapstock as the starting material. This unit operation is generally used as final step in oryzanol recovery after preliminary purification steps such as leaching or liquid-liquid extraction (LLE). During crystallization, the parameters considered are temperature, type of the solvent or mixture of solvents along with their proportions, rate of nucleation and rate of crystal growth. Some of the previous reports (patents and research articles) using RBO, rice bran acid oil and RBO soapstock as the starting material for the crystallization of oryzanol are discussed.

Saska and Rossister (1998) have purified degummed and dewaxed RBO by a silica based continuous chromatography followed by crystallization with heptane to obtain oryzanol with a yield of 85-90% and 90-95% purity. This countercurrent operation resulted in a continuous separation with reduced solvent and adsorbent by a factor of 2 to 5 less than a comparable batch chromatography process.

Yu et al. (2006) have purified oryzanol from RBO by fractional crystallization with acetone at different temperatures (-8 to -35°C) to result in about 95~ 100% recovery of oryzanol in a batch process. The temperature of 14°C was found the best temperature for oryzanol recovery.

Yasuo et al. (1968) employed a four step procedure for extraction of oryzanol from rice bran acid oil. The steps were esterification of RBO to

methyl ester, molecular distillation, LLE and crystallization. Final crystallization of oryzanol was carried out with hexane. The yield of oryzanol obtained was 32.6% w/w with a purity of 98.3% w/w. This process, however, has the drawback of giving low recovery of oryzanol.

Tsuchiya and Okubo (1961) reported a two step procedure for extraction of oryzanol from rice bran acid oil. The first step involves esterification of the FFA to methyl esters and subsequent distillation of the esters to obtain on unsaponified fraction. The second step involves subjecting the unsaponified fraction to column chromatography using a mixture of alcohol and ether as an eluant. The eluant was water washed and desolventized to obtain oryzanol. This process has the drawback of using column chromatography, which has problems of pressure drop in the column and channeling leading to uneven flow especially in large scale applications. The cost of the adsorbent and solvent are high when used on a large scale. The yield of the process also was low [1.75 % (w/w)] and the purity of the oryzanol was not reported.

Koji and Tokuo (1986) have described a two step procedure for obtaining a purified and concentrated form of oryzanol, namely cycloartenol ferulic acid ester. The product obtained after an initial crystallization was the starting material used that was subjected to a multi-stage recrystallization using a mixture of alcohol and hydrocarbon solvents. The alcohols were methanol, propanol or butanol, etc., and the hydrocarbons were hexane, cyclohexane or toluene, etc. The solvents for recrystallization were recovered

by distillation. The authors did not mention the initial raw material used nor the final purity and yield of oryzanol.

Mingzhi and Yanyan (1997) reported a four step procedure for extraction of oryzanol from RBO soapstock. The starting material was a second soapstock (the extra alkali added during the second alkali refining of RBO results in the formation of another soapstock fraction) of RBO, which was subjected to multiphase fractional crystallization. The purity of the oryzanol obtained at the end of crystallization processing of this soapstock was 98 %w/w with a yield of 1.9 %w/w. The authors did not mention the weight of the second soapstock fraction, hence, the total yield from the process was not known.

Narayan et al. (2005) used the unsaponifiable material of soapstock (obtained by leaching of pretreated and dehydrated soapstock) as the starting material for the crystallization of oryzanol. Crystallizing solvents used were mixtures of acetone and methanol in different proportions at the reflux temperature. Upon cooling to room temperature, mucilaginous impurities (such as wax) precipitated out. The mucilaginous impurity was separated and the eluate was further cooled to 5-10°C overnight for oryzanol crystallization. Oryzanol was obtained at 65 %w/w purity and the yield was 70 %w/w. The novelty of this patented process is the selection of the starting material (pretreated and dehydrated RBO soapstock) that had less mass transfer resistance because of the increased surface area and partial removal of interfering impurities such as gums.

Masao and Yoshizane (1968) have reported a four step procedure for the extraction of oryzanol from RBO soapstock. The steps employed were a two-step leaching process and LLE. The objective of the leaching steps was to extract impurities into solvents such as trichloroethylene, benzene, n-hexane or a mixture of benzene and n-hexane. The impurities (mainly soap) from the second leaching step were extracted with water while subjecting the mixture with CO_2 gas for acidification to facilitate leaching. The residual mixture obtained after second leaching step was subjected to LLE (with the same solvents used in the first leaching step) to extract oryzanol into the organic phase. Finally oryzanol was obtained in a crystalline form by removing the solvent. The purity of oryzanol obtained was $\geq$ 90 %w/w, with a yield of 1.35 %w/w (% in RBO soapstock). The drawback of this method is that it uses chlorinated or aromatic solvents for LLE, which are difficult to remove and require stringent safety methods for use, adding to the cost of processing.

Rao et al. (2002) used a six-step procedure for isolating oryzanol from RBO soapstock. This process includes saponification of the neutral oil present in the soapstock, conversion of the soapstock to an anhydrous material, leaching followed by crystallization, column chromatography, and recrystallization to obtain 90% (w/w) pure oryzanol in an overall yield of 70% (w/w). The method has the drawback that a number of unit operations are involved and the anhydrous porous RBO soapstock material has considerable mass transfer resistance (due to increased diffusion distance or thickness) during oryzanol extraction. Furthermore, the use of a step involving a soxhlet

extraction process reduces selectivity of leaching at higher temperatures and a column chromatography step is difficult to scale up.

Seetharamiah and Prabhakar (1986) reported a four step process including LLE, column chromatography, crystallization and recrystallization to extract oryzanol from RBO soapstock. Two stages of crystallization were carried out, the first with methanol and second with a mixture of methanol and acetone (2:1 v/v), respectively. At the end of the chromatography step the yield of oryzanol was 75.7 %w/w with a purity of 51.4 %w/w. However, in this article, the authors did not mention the purity and yield of oryzanol at the end of the recrystallization steps. The limitation of this process is that in the LLE repeated extraction needs to be carried out, which increases the number of extraction steps and decreases the yield of oryzanol. Hence this method may be limited for scale-up production.

The present study deals with the crystallization of oryzanol employing unsaponified fraction of RBO soapstock as the starting material by using a mixture of acetone and methanol in different proportions as the solvent.

4C.2. Materials and methods

Acetone, methanol and ethyl acetate were procured from Ranbaxy New Delhi, India.

4C.2.1. Experimental procedure

The unsaponified fraction (obtained after the leaching of soapstock) was subjected to crystallization. Acetone and methanol were employed as binary solvents in crystallization. The ratio of unsaponified fraction to solvent was maintained constant at 1:5 for all the experiments. The unsaponifiables were mixed with different ratios of acetone and methanol [1:1 to 1:4 % w/w] and refluxed at 80°C for about an hour to facilitate non selective dissolution. After refluxing, the mixture of hot solvent mixture was left at room temperature for about 1.5-2 hrs in order to cool down thus facilitating the precipitation of interfering impurities such as wax (fraction 1). The supernatant containing miscella was decanted and kept at 4°C for about 10-15 hrs in order to facilitate crystallization of oryzanol (fraction 2). After the crystallization, the remaining supernatant was separated and desolventized (fraction 3). In all the 3 fractions, the content of oryzanol was determined.

4C.3. Results and Discussion

A mixture of two or more solvents will occasionally be favorable for crystallization (Mullin, 2002). Acetone and methanol were selected as the crystallization solvents based on the earlier works (Seetharamiah and Prabhakar, 1986, Rao et al, 2002). With an increase in ratio of acetone to methanol from 1:1-1.2:3.8, the purity of oryzanol increased upto 1.2:3.8 and then decreased, while % recovery increased and remained constant from 1:3-1.2:3.8 (Figure 4C.1). Acetone and methanol have relative polarities of 0.355 and 0.762. The higher yield of oryzanol at acetone to methanol ratios of 1.2:3.8 and 1:3 is due to the higher polarity of methanol. However, at a ratio of

acetone to methanol of 1:4, both the purity and recovery decreased but decrease in purity is significant by about 37% while the decrease in recovery is by about 11%. The purity at 1:4 decreased due to the non-selective extraction of oryzanol, that is, the impurities such as gums and waxes are also extracted.

4C.4. Conclusions

Crystallization of oryzanol from the unsaponified fraction resulted in 63% purity with ~ 100% recovery. Acetone to methanol ratio of 1:3 – 1.2:3.8 was suitable for oryzanol crystallization at about 4°C.

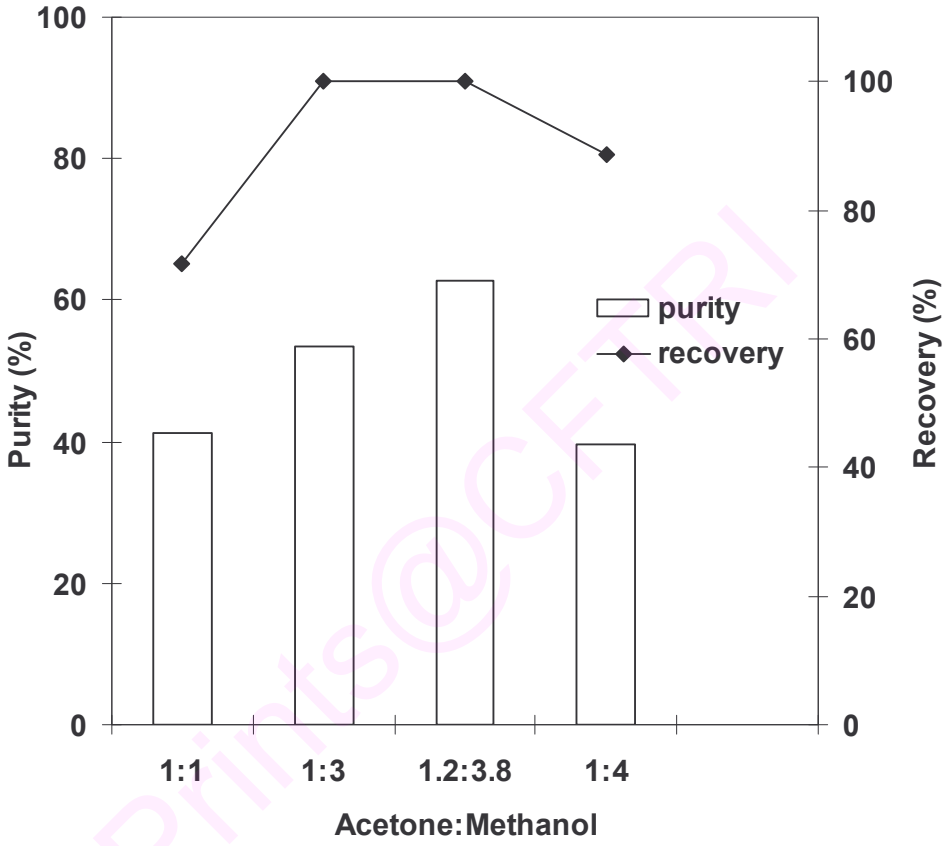

Figure 4C.1. Effect of the ratio of acetone to methanol on the purity and recovery of oryzanol during crystallization

SUGGESTIONS FOR

FUTURE WORK

SUGGESTIONS FOR FUTURE WORK

1. In ATPE, the suitability of thermoseparating polymers such as ethylene oxide- propylene oxide (EO-PO) on the partitioning of peroxidase from *I.palmata* (peroxidase is stable from 0-60°C) can be explored. Use of thermoseparating polymer can facilitate reuse of the polymer for subsequent extraction.
2. In ATPE, the use of detergent based system (non-ionic) can also be explored for the partitioning of peroxidase from *I.palmata* can be explored.
3. Affinity based extraction such as smart polymers (chitosan, alginate, eudragit) in ATPE and TPP for the extraction and purification of peroxidase from *I.palmata* can be explored.
4. The applicaton of detergent based ATPE employing non-ionic detergents such as Triton X-100, aerosol-OT (AOT, sodium bis (2-ethylhexyl) sulphosuccinate), Tween 80,. cetyltrimethylammonium bromide (CTAB) based on the molar ratio (ratio of water to surfactant) can be explored for the extraction and purification of C-phycocyanin. Use of detergent based ATPE facilitates reuse of the polymer for subsequent extraction similar to that based on thermoseparating polymers.
5. The recycling of the phase forming components (t-butanol and ammonium sulfate) in TPP can be explored. Spent alcohol (such as t-butanol) from top phase after TPP can be reused by distillation while the spent salt (such as ammonium sulfate) bottom phase can be reused after ultrafiltration.

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OUTCOME OF WORK

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POSTERS PRESENTED IN NATIONAL CONFERENCES

1) Narayan, A.V., Barhate, R.S. and Raghavarao, K.S.M.S. “Role of solute concentration and phase volume ratio on partitioning of proteins in aqueous two phase extraction” poster presented at *ICFOST 2002, CFTRI,* Mysore, Karnataka.
2) Narayan, A.V., Nagaraj, N. and Raghavarao, K.S.M.S. “A new method for the recovery of polyethylene glycol from spent aqueous two phase systems” poster presented at *5th linternational Food Convention, IFCON 2003,* CFTRI, Mysore, Karnataka.
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