REVERSE MICELLAR EXTRACTION FOR DOWNSTREAM PROCESSING OF ENZYMES/PROTEINS

A thesis submitted to the

UNIVERSITY OF MYSORE

For the award of degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

by

H UMESH HEBBAR
M. Tech (MEM)

Under the guidance of
Dr. KSMS RAGHAVARAO

Department of Food Engineering,
Central Food Technological Research Institute,
Mysore – 570 020, INDIA

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DECLARATION

I hereby declare that the thesis entitled **"Reverse micellar extraction for downstream processing of enzymes/proteins"** which is submitted herewith for the degree of **Doctor of Philosophy in Biotechnology** of the University of Mysore, 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 the guidance of Dr. KSMS Raghavarao, during the period 2003-2007.

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

Umesh Hebbar H

Date: 13th December 2007

Place: Mysore

CERTIFICATE

I hereby certify that this Ph.D thesis entitled "**Reverse micellar extraction for downstream processing of enzymes/proteins"**, submitted by **Mr. Umesh Hebbar H** for the degree of **Doctor of Philosophy in Biotechnology** of the University of Mysore, 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 2003-2007.

(Dr. KSMS Raghavarao)

Date: 13th December, 2007

Place: Mysore

ACKNOWLEDGEMENT

I express my deep sense of gratitude to my guide Dr. KSMS Raghavarao, for his excellent guidance and support during the course of the work. His inspiring suggestions, encouragement and scrupulous supervision at every stage of the work are the stepping stones for this success. His wonderful qualities such as simplicity, accessibility and willingness to help are simply unparallel and are worth imbibing. Dear Sir, I wish to thank you for all the guidance and support.

I wish to express my sincere thanks to Dr. V. Prakash, Director, CFTRI, for his untiring support and encouragement, which made this journey a very pleasant one. Dedication and commitment to work, meticulous planning and passion to excel are the qualities unique to him and have greatly inspired every individual. Dear Sir, I sincerely thank you for providing all the support and encouragement to carryout this work.

I wish to thank Mr. A Ramesh and Mr. H Krishna Murty, former Heads of Food Engineering department, CFTRI, for their encouragement and support, particularly at the early stages of my research career. An immense faith kept on me by both of them had helped me in developing a greater sense of responsibility and nurture a desire to accomplish higher goals.

Sincere thanks to my friends from Food Engineering Laboratory, Sumana B and Hemavathi AB for their cordial support without which the successful completion of this task would have been impossible. I also wish to thank Kavitha, Sudheer Kumar, Gopinath G and Sarvanan, Sandesh, Vishwanthan HK, Ravindrababu, Chetana S, Navin Nagaraj, Ganapati Patil B, Narayan AV, Raghavendra SN, Rajendra Bharate, Chethan Nayak, Manjula, and for their kind support.

With all sincerity and sense of gratitude I would like to thank my colleagues from the Department of Food Engineering for their moral support

during my pursuit to this goal. Special thanks to Dr. Venkatesh Murthy, Dr. R Subramanian, Dr. N K Rastogi, Dr. Suvendu Bhattacharya, Dr. Udaya Shankar Mr. Chakkaravarthi and Late Dr. M N Ramesh for the interactions and suggestions.

I wish to express my thanks to Dr. N G Karanth, Dr. Sridevei Annapurna Singh, Dr. Lalitha R Gowda, Mr. K K Bhat Dr. Susheelamma, Dr. S G Prapulla, and Mr. Sukumar Debnath, colleagues from other departments of CFTRI for the interactions and support in the analytical work.

My deepest gratitude goes to my family for their unflagging love and support throughout my life; this doctoral work was simply impossible without their support. My wife Sudha, daughters Medha and Shraddha are the ones who had to bare most of the brunt for all these years, sacrificing their personal life; but still stood by me steadfast and supported me in my endeavor. I thank my parents, Mr. Nagaraj Hebbar and Mrs. Rukmini Hebbar for providing the best possible environment to grow up and for the support extended to pursue higher studies. I am indebted to my in-laws, Dr. Raghupathi Rao and Mrs. Prabha R Rao who gave the much needed moral support, particularly during the crunch period, which enabled me to handle such situations in a better way and successfully achieve this task. My sincere thanks to all my relatives, friends and well wishers who have directly or indirectly contributed towards achieving this goal.

I thank almighty for blessing me with all the happiness and joy in my life and giving me the strength to successfully accomplish this task.

Umesh Hebbar H

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Abbreviation	Description
A	Interfacial area
AOT	Aerosol-OT
A_s	Surface occupied by polar head of surfactant molecule
A_{sm}	Molar area of surfactant head groups
ATPE	Aqueous two phase extraction
ATPS	Aqueous two phase system
BDBAC	N-benzyl-N-benzyl-N-dodecyl-N-bis (2-hydroxyl ethyl) ammonium chloride
BSA	Bovine serum albumin
c	Concentration of the residue
C_0	Initial concentration of solute in aqueous phase
C_{org}	Concentration of solute in organic phase at time 't'
C^0_{aq}	Initial concentration of solute in aqueous phase
C_{aq}	Concentration of solute in the aqueous phase at time 't'
C_{aqi}	Concentration of solute at the interface of aqueous phase
C_{orgi}	Concentration of solute at the interface of organic phase
C_B	Equilibrium concentration of bromelain in the bottom phase
CD	Circular dichroism
CDU	Casein digestion unit
CMC	Critical micelle concentration
C_T	Equilibrium concentration of bromelain in the top phase
CTAB	Cetyltrimethyl ammonium bromide
Da	Dalton
dC_{aqu}/dt	Change in concentration of solute in aqueous phase with time
dC_{org}/dt	Change in concentration of solute in organic phase with time
DTAB	Dodecyltrimethyl ammonium bromide
D_Z	Translational average diffusion coefficient
EDTA	Ethylene diamine tetra acetic acid disodium salt
f_{CTAB}	Surfactant area at the surface of the reverse micelle
HRP	Horse radish peroxidase

J_P	Solute flux
k	Boltzman constant
ka	Overall mass transfer coefficient
K_{aq}	Mass transfer coefficient in the aqueous film
K_{org}	Mass transfer coefficient in the organic film
k_{bo}	mass transfer coefficient during back extraction
k_{fo}	mass transfer coefficient during forward extraction
K_{f0} a	Overall mass transfer coefficient during forward extraction
K_{f0} b	Overall mass transfer coefficient during back extraction
k_s	Solubilizing rate constant
l	Length of the light path
LLE	Liquid-liquid extraction
m	Partition coefficient/Equilibrium constant of solute
M_0	Molecular weight of empty reverse micelle
m_b	Partitioning equilibrium constant during back extraction
M_{CTAB}	Molecular weight of CTAB
M_f	Molecular weight of filled reverse micelle
m_f	Partitioning equilibrium constant during forward extraction
M_w	Molecular weight of water
M_{ws}	Molecular weight of solute
N	Avagadro's number
N_{ag}	Aggregation number of surfactants
N_e^s	Aggregation number of surfactant in empty micelles
N_e^w	Aggregation number of water in empty micelles
N_f^s	Aggregation number of surfactant in filled micelles
N_f^w	Aggregation number of water in filled micelles
N_m	Number of reverse micelles
PAGE	Polyacrlamidegelelectophoresis
PEG	Polyethylene glycol
pI	Isoelectric point
PPO	Polyphenoloxidase

PVP	Polyvinyl pyrrolidone
R_h	Hydrodynamic radius
R_m	Radius of reverse micellar core
RME	Reverse micellar extraction
R_{me}	Radius of empty reverse micelle including the surfactant head
R_{mf}	Radius of filled reverse micelle including the surfactant head
RMS	Reverse micellar system
SDS	Sodium dodecyl sulfate
t	Duration
T	Absolute temperature
TOMAC	Trioctyl-methyl ammonium chloride
UF	Ultrafiltration
V	Volume of the phase
V_{aqu}	Volume of aqueous phase
V_m	Molar water volume
V_{org}	Volume of organic phase
V_p	Protein volume
V_s	Polar head volume of surfactant
V_t	Total volume of water
V_w	Water molecule volume
W_0	Water content, molar ratio of water to surfactant in organic phase
α	Fraction of surfactant molecules in the interface
θ_{obsd}	Observed elipticity
ρ_w	Density of water
$[H_2O]$	Molar concentration of water in microemulsion
[S]	Molar concentration of surfactant in microemulsion
μ	Solution viscosity

CONTENTS

SYNOPSIS

Recent developments in biotechnology have opened up new avenues towards the production of biomolecules such as enzymes and proteins of importance. Application of enzymes/proteins is rapidly increasing in food, fermentation, pharmaceutical and other related industries, thereby increasing the demand for their production. Although, there has been tremendous research in this area, the technology for separating biological products from the media has not kept pace with the advances in upstream processing. In view of the recognized fact that downstream processing accounts for a large share of the final product cost in many biotechnological processes, there has been an increased interest in the development of efficient downstream processing methods for separation, concentration and purification of biomolecules.

One of the important goals in the area of biosepartions is to achieve efficiency and selectivity in separation and purification. The separation techniques such as aqueous two phase extraction (ATPE) and reverse micellar extraction (RME) have been recognized as efficient and easily scalable methods, and hence could find many applications in biotechnology. Extraction of enzymes/proteins by organic solvents using reverse micelles is relatively a new concept. The reverse micellar extraction offers a number of unique features that makes it a promising biosepartion technique. Reverse micelles are thermodynamically stable, optically transparent, submicroscopic aggregates of surfactant molecules in non-polar solvent. These systems can provide a gentle and biocompatible environment for the separation of biomolecules. The self-assembling, labile nature of micelles enables one to control and optimize partitioning. The micelles can simultaneously offer hydrophilic and hydrophobic environment to the solutes. Recovery of biomolecules from the reverse micellar phase can be facilitated by exploiting the de-assembling nature of reverse micelles in aqueous media. After the separation, the organic phase can be recycled without any significant loss of efficiency. The

desired biomaterial can be solubilized within and recovered from the reverse micellar system by retaining all the original characteristics.

Although a few reports have been published on the application of RME technique for downstream processing of enzymes/proteins, there exists a need for a comprehensive study on various aspects of RME. A systematic study of the various process parameters such as type of surfactant, surfactant concentration, aqueous phase pH, ionic strength, micelle size etc., on the efficiency of the process is to be undertaken. A successful RME should include both forward and back extraction processes at their optimized conditions. Most of the works reported on RME have focused only on forward extraction, assuming that the conditions that normally prevent protein uptake in the forward transfer, would promote their release in the back extraction, which is not always true. Hence, there exists a need for a more detailed study on the back extraction to optimize the conditions. The extraction and purification of proteins by employing reverse micelles have been reported for model systems, where as the study on the real systems is limited. As the real systems such as fermentation broth, plant extracts etc., are complicated compared to model systems, there is a scope for further investigating the extraction behaviors of proteins present in these real systems. Also, an integrated approach such as combining RME with ATPE and membrane processes is expected to give better results in terms of improving the overall productivity of downstream processing of proteins/enzymes. On the laboratory scale, RME of proteins/enzymes is well established for model systems. However, there are only a few reports on scale up studies and process integration. Therefore there is a need to investigate scale up and process integration for the extractive recovery from real/natural systems.

In view of these knowledge gaps, attempts have been made in the present investigation to address these issues. Detailed studies have been carried out on the effect of forward and back extraction conditions on the overall process efficiency for model as well as natural systems. Application of nonionic surfactants

for the extraction and purification of proteins/enzymes have been attempted as nonionic surfactants are considered to be mild and non-interactive but still not extensively studied as compared to ionic surfactants. Attempts have been made to integrate RME with membrane processing for improving the overall efficiency. Also, the comparison studies of RME with other downstream processes such as ammonium salt precipitation and ATPE have been carried out, which are not reported so far. Application of acoustic filed for enhancing the back extraction, scale-up studies with RME, estimation of reverse micellar size and studies on mass transfer kinetics are the other areas in which detailed studies have been carried out in the present investigation.

The subject matter of the thesis has been organized and presented in the following chapters.

CHAPTER 1: GENERAL INTRODUCTION

This chapter presents the literature pertaining to fundamentals and engineering aspects of RME, discussed under the following subheadings:
Downstream processing; Reverse micellar extraction; Biomolecules and model/natural systems; Aqueous two phase extraction and Membrane processing; Mathematical modeling; Aim and scope of the present work.

The aim and scope of the present investigation have been clearly defined, keeping in view the following objectives:-

1. Study of factors affecting the forward and backward transfers in RME of enzymes/proteins from natural/real sources.
2. Comparative studies of reverse micellar systems with Aqueous Two Phase System.
3. Mathematical modeling of partitioning of biomolecules during RME

4. Integration of RME with processes such as ATPE, and membrane processes for improving the overall productivity of downstream processing of selected proteins/enzymes.

An outline of the studies conducted with these objectives forms the subject matter of the ensuing chapters. In each chapter, different sections such as Introduction, Materials and Methods, Results and Discussion and Conclusions, Tables and Figures have been included to provide the details systematically.

CHAPTER 2: CHARACTERIZATION OF REVERSE MICELLES AND SCHEME FOR EXTRACTION

The efficiency of RME mainly depends on the reverse micellar system selected to extract a given biomolecule and the processing conditions employed. Physicochemical characteristics of the reverse micellar system components, namely, organic solvent, surfactant and co-solvents could be used as useful tools for the selection of most suitable combinations. The charge on the polar head of the surfactant, Hydrophilic-lipophilic balance (HLB) value, critical micelle concentration (CMC), pH stability, solute-surfactant interaction, etc., are some of the properties, which govern the selection of surfactant for an extraction process. In case of organic solvents and co-solvents the non-polar nature, dielectric constant, molecular weight, density etc., are considered critical for the selection. The water content (W_o) of the reverse micelle, defined as the ratio of water molecules to that of the surfactant molecules per reverse micelle is used as an index of the reverse micellar size. In many a cases the size of the reverse micelle determines the extraction efficiency and also could be conveniently used for size based selective separation of solutes. The selection of suitable reverse micellar system components and optimizing the processing conditions for RME plays a major role in determining the efficiency of the process. All the above topics have been presented in the following sections.

Section A: Physicochemical characteristics

In present section the physicochemical characteristics of the reverse micellar system components, namely, organic solvent, surfactant and co-solvents employed in the present investigation are discussed in relation to the effect on RME. The surfactant properties such as charge on the polar head of the surfactant (anionic, cationic or nonionic), HLB value, CMC, pH stability, solute-surfactant interaction have been discussed. Attempts have been made to determine the CMC value of surfactants in organic solvents employing different techniques, such as absorption (optical density) at known wavelength, conductivity, and haze measurement. It was observed that even though the estimation methods were different, the CMC value obtained for the surfactant did not vary appreciably. Also, the CMC values did not change greatly with the organic solvent used for the formation of reverse micelles. The conductivity measurement method was not suitable for the measurement of CMC value of nonionic surfactants as conductivity did not vary at the CMC region. This emphasized the need for proper selection of estimation method for a given surfactant.

Type of organic solvents and co-solvents and their properties (non-polar nature, dielectric constant, molecular weight, and density) are also critical parameters and are discussed in the present section.

Section B: Structural characteristics

The size of the reverse micelle is directly related to the amount of water in the reverse micellar pool. The water content (W_o) of the reverse micelle is mostly used for the estimation of reverse micellar size. W_0 strongly depends on the relative solubility of the surfactant in the polar and non-polar solvents, expressed as HLB value of surfactant and it increases with HLB. The processing conditions such as salt and surfactant concentrations, presence of co-solvents, aqueous phase pH etc., also influence the size of the reverse micelles. Many empirical models derived for different reverse micellar systems and models based on

geometric conditions of reverse micelle have been reported. The present section discusses these models.

Section C: Scheme for reverse micellar extraction

The process for the separation and purification of enzymes/proteins from natural sources employing RME technique involves mainly two stages, namely, i) preparation of crude enzyme/protein extract from natural source and ii) reverse micellar extraction of the targeted solute from the crude extract. Selection of suitable process parameters for the preparation of crude extract with high enzyme activity/solute concentration, selection of reverse micellar system components and optimizing processing conditions for RME plays a major role in determining the efficiency of the process. The present section discusses various aspects of RME for the application of enzyme extraction from natural sources.

CHAPTER 3: REVERSE MICELLAR EXTRACTION EMPLOYING MODEL SYSTEMS

Model systems could be ideally used to understand the mechanism of RME and to study the effect of processing conditions on the forward and back extraction efficiencies. Two model solutes, namely, bovine serum albumin (BSA) and horse radish peroxidase (HRP) were selected for the study. Different reverse micellar systems were used and studies on the effect of processing conditions on the extraction efficiency were carried out. The results are discussed in the following sections.

Section A: Extraction of bovine serum albumin (BSA)

Bovine serum albumin (BSA) has been widely studied as a model protein because its structure and physicochemical properties are well characterized. BSA was selected in the present study, as a few earlier reports have indicated the difficulty in extracting larger molecular weight solutes such as BSA using RME. The suitability employing nonionic surfactant polyoxyethylene p-t octylphenol (Triton-X-100), which is ‘nondenaturing’ and ‘nontoxic’ in combination with organic

solvent toluene, was attempted. Effect of processing conditions during forward (aqueous phase pH, Triton-X-100 and NaCl concentrations, and phase volume ratio) and back extraction (aqueous phase pH, KCl concentration, alcohol addition) have been discussed. Extraction studies with surfactant mixture Sodium bis (2-ethyl-1-hexyl) sulfosuccinate (AOT)/Triton-X-100) and effect on reverse micellar size are also discussed.

It was found that extraction efficiency of BSA depends upon the reverse micellar system selected as well as the processing conditions adopted for forward and back extractions. Although, the size of the Triton-X-100/toluene reverse micelle was large enough to host BSA, micellar size appeared not to be a sufficient criterion for reverse micelles to host BSA in large amounts**.** Lower extent of extraction efficiency with Triton-X-100 was attributed to lack of strong driving force for the diffusion of large molecules of BSA into the nonionic reverse micellar core. AOT/toluene reverse micellar system resulted in complete forward and back extraction without affecting the protein structure. This emphasized the need for the selection of appropriate reverse micellar system for the solute targeted.

Section B: Extraction of horse radish peroxidase (HRP)

Horse radish peroxidase is one of the most widely used enzymes in analytical applications. HRP has a molecular weight of around 44 kDa and the iso-electric points (pIs) of the iso-enzymes range from 3 to 10. The present investigation was aimed at employing the reverse micellar system of non-ionic surfactant Triton-X–100 in toluene for the extraction of commercial grade HRP. The process parameters that affect the extraction efficiencies during forward (aqueous phase pH, NaCl concentration, buffer type and concentration) and back (aqueous phase pH, KCl concentration) extractions were altered to obtain better results.

It was found that the extraction efficiency greatly depends on the processing conditions adopted and a marginal change in processing conditions results in a drastic variation in the extraction efficiency. As reported in many of the

works, the extent of back extraction efficiency was found to be very less compared to forward extraction. The poor extraction efficiency with nonionic surfactant system was attributed to the lack of strong electrostatic interaction with the solute. The study emphasized the need for selection of suitable reverse micellar system for the given solute and it indicated that the strong electrostatic or hydrophobic interaction is needed for extracting the relatively higher solute like HRP using reverse micelles.

CHAPTER 4: REVERSE MICELLAR EXTRACTION EMPLOYING NATURAL SYSTEMS

Although, there are many reports on the application of RME for model systems, extraction of proteins/enzymes from natural/real systems such as plant extracts/fermented broth are very rare. Further, RME from such systems was reported to be much more complicated compared to model systems, due to the presence of various other impurities that hinders the extraction. Hence, in the present investigation application of RME for extraction of bromelain and peroxidase from crude extracts of pineapple wastes (core, peel, extended stem and leaves) and radish roots respectively, was attempted. The following sections discus various issues addressed during RME of these two enzymes.

Section A: Extraction of bromelain from pineapple wastes

Bromelain, a proteolytic enzyme found in the tissues of plant family *Bromeliaceae* of which pineapple (*Ananas comosus* L. Merryl*)* is the best known source. The stem bromelain (EC 3.4.22.32) and fruit bromelain (EC 3.4.22.33) obtained from the pineapple stem and pulp, respectively are finding wide applications in pharmaceutical and food industry. Bromelain is reported to be present also in pineapple wastes such as core, peel, and leaves, though relatively in smaller quantities as compared to stem. However, practically no reports on the extraction of bromelain from the above wastes are available, despite the fact that large amounts of pineapple waste is produced at the pineapple processing industries. Hence, in the present study an attempt has been made to use RME

technique for the extraction of bromelain from these wastes. Application of RME for extraction and purification of bromelain from pineapple wastes (peel, core, leaves and extended stem), and a detailed study on the effect of forward and back extraction parameters on enzyme activity recovery and purification from pineapple core extract are discussed in this section. Reverse micellar systems of cationic surfactant cetyltrimethylammonium bromide (CTAB) and anionic surfactant AOT were selectively used in the study.

It was inferred from the study that RME could be successfully applied for the isolation and primary purification of bromelain from pineapple wastes. The bromelain activity and the total protein content in the crude extracts of different wastes were varying and activity was high in core and crown extracts. Like in other RME studies, the processing conditions greatly affected the extraction efficiency and emphasized the need for optimizing the conditions for extraction based on the solute characteristics.

Section B: Scale-up, integrated of processes and comparative studies with other methods

Although, several reports on the application of RME for separation and purification of biomolecules are available, there are only a few reports on the process scale-up. Practically, no reports are available on the comparison and integration of RME with other downstream processes. Hence, in the present study, an attempt was made to scale up RME for separation and purification of bromelain from pineapple core extract and couple with ultrafiltration for further concentration/purification. Most of the studies have indicated that the back extraction of solute is relatively difficult compared to forward extraction and is the bottleneck for the scale up of the process. Attempts have been made to apply acoustic filed (MHz range) to enhance the release of solute from reverse micelles during back extraction. One of the important features of RME is the ability of reverse micelles to selectively extract solutes based on their size or charge. Differential partitioning of bromelain and polyphenol oxidase (PPO) based on size

and charge has been attempted. As the cost of organic solvent is one of the factors that directly affects the process economics at higher scale, the feasibility of recycling spent organic phase for fresh extraction has been attempted. The performance of RME for the extraction of bromelain has been compared with of ammonium salt precipitation and ATPE techniques.

Scale up studies of RME with pure reverse micellar components resulted in good extraction efficiency. It was observed that RME could be coupled conveniently with ultrafiltration to obtain better results. It was found that by manipulating the aqueous phase pH during RME, it could be possible to selectively separate and purify enzymes (bromelain and PPO) in two aqueous phases. Results of the investigation indicated that exposure to acoustic filed in MHz range, could be used as an alternative to conventional agitation method to enhance back extraction efficiency. It was observed that spent organic phase could be recycled for fresh extraction Application of ATPE technique for bromelain extraction was encouraging, although the efficiency was not as high as that obtained in RME.

Section C: Extraction of peroxidase from radish roots

Radish root, Chinese cabbage, carrot hairy root cell culture and turnip are some of the commonly used plant sources of peroxidase. A few reports on application of RME for extraction and purification of peroxidase from natural systems are available. However, most of these works have employed anionic surfactants for the extraction, which are sometimes tend to denature the enzyme. In the present investigation, the performance of nonionic polyoxyethylene sorbitan trioleate (Tween 85) and Trition-X-100 surfactants for the extraction of peroxidase from the crude extract of radish *(Armoracia rusticana)* roots has been studied apart from using anionic revere micellar system. An attempt has been made to combine the conventional ammonium salt precipitation with RME.

It was found that the nonionic system of Triton-X-100 did not give good results as compared to the model system of HRP. Tween 85, another nonionic surfactant used in the study gave good extraction results. Although, AOT/isooctane system resulted in better extraction efficiency as compared to Triton-X-100/toluene system, it was not as efficient as Tween85/toluene system. Integrated process having RME followed by ammonium salt precipitation did not show an encouraging result, which may be due to the presence of trace amount of salts in the feed used for RME.

CHAPTER 5: MATHEMATICAL MODELING

Mathematical modeling of the solubilization of biomolecules in reverse micelles is essential for an in-depth understanding and effective use of RME in downstream processing. However, the quantitative modeling of protein solubilization in reverse micelles is a complex problem and relatively fewer studies on the modeling of protein containing revere micellar systems have been reported. Most of the models are associated with the estimation of the size of reverse micelles and solute distribution during forward and back extraction of solutes. Several models (empirical and based on geometric conditions) reported in the literature are employed for the estimation of the radius of reverse micellar core under different processing conditions.

Rate of protein transfer to or from a reverse micellar phase and factors affecting the rate are important for the practical application of RME for extraction and purification of proteins/enzymes and for the scale-up of the process. Many models for the estimation of mass transfer coefficient during forward and back extractions have been proposed for different systems employed for RME. In the present investigation, the overall mass transfer co-efficient has been estimated for both forward and back extraction of bromelain during conventional phase contact method and compared with that of acoustic field treatment. The content of the Chapter is presented in the following two sections.

Section A: Estimation of reverse micellar size

Models proposed in the literature for protein solubilization range from simple geometric models (assuming reverse micelles to be of spherical shape) to more rigorous molecular thermodynamic models. Several experimental methods (using instruments) and empirical models (using W_0 value) have been reported for the determination of the radius of reverse micellar core.

The geometrical models predicted higher values for the reverse micellar size as compared to empirical models. The size of filled reverse micelles was found to be bigger as compared to unfilled ones when surfactant concentration was varied. However, no definite trend was observed for salt concentration variation and change in aqueous phase pH. The correlation between water content and reverse micellar size derived was almost close to some of the reported models and showed a good fit for different CTAB concentrations. The estimation of surfactant aggregation number revealed that with an increase in surfactant concentration, the number of revere micelles formed increases, while maintaining the aggregation number almost the same. The area occupied by the surfactant molecule at the surface of a reverse micelle, estimated based on the aggregation number was close to the value reported for ionic surfactants.

Section B: Mass transfer kinetics

The mass transfer study revealed that most of the bromelain transfer across the phases will take place in the initial period of phase contact. As reported in the earlier studies, the mass transfer coefficient for forward extraction was higher as compared to back extraction. With the application of acoustic field, the solute transfer was found to be higher than that of conventional agitation. The mass transfer rate obtained with the application of acoustic was found to be nearly 2.4 times higher than that of conventional agitation employed for back extraction.

The suggestions for future work and lists of reference and publication are presented at the end.

CHAPTER 1

GENERAL INTRODUCTION

1A. DOWNSTREAM PROCESSING

Biotechnology in recent years, has caught the imagination of industrialists, investors, and the general public as probably no other branch of technology. It has been described as the last technical innovation of the twentieth century. The growth predicted for the biotechnological industries seems to be overwhelming with the world biotechnology market expected to grow at a phenomenal rate of 50% annually [Milton *et al.,* 2007]. Factors such as cost effective and efficient process technologies, development of equipment for large scale and continuous operation, and availability of trained personnel are expected to play a major role in the growth of this industry.

Biological products can be obtained from many sources such as human and animal tissues, body fluids, plant material, microbial fermentations, raw broth from enzyme bioreactors etc. The process of recovering biological products from these sources, termed as downstream processing, involves many steps. Some of the industrial scale bioseparation operations are presented in Table 1A.1 [Chisti 1998]. Separation process in biotechnology can be categorized into three types, namely, mechanical processes, rate controlling and equilibrium processes. Filtration and centrifugation are the typical mechanical separation processes, while chromatography and electrophoresis could be classified as rate controlling. Extraction, precipitation, distillation and crystallization are called as equilibrium processes [Lee, 1989].

Recent developments in biotechnology have opened up new avenues towards the production of many biomolecules of importance of research, pharmaceutical/clinical and industrial usage. Highly selective and economical separation methods are becoming more and more important in commercial processes. Although, there has been considerable research in the area of production of biological products, the technology for separating them from the media has not kept pace with the upstream processing (production of biomolecules). The separation of many biomolecules from the product stream is

still performed by batch mode, small scale processes such as column chromatography, salt and solvent precipitation and electrophoresis for which scale up poses considerable problem, making them uneconomical unless the product is of high value [Harikrishna *et al.,* 2002]. Although, the affinity based chromatographic separations have excellent selectivity and being carried out on a large scale; for the most part, such systems operate discontinuously and the economy of scale has not often been realized [Ohlson *et al.,* 1989]. In view of the fact that product recovery costs become critical in the overall economics of modern biotechnological process, there has been an increased interest in the development of efficient and scaleable downstream processing methods for separation, concentration and purification of biomolecules.

Developing a downstream process to recover a biological product in large quantities occurs in two stages, namely, design and scale-up. The process design is concerned with selection of steps for separation of a product from contaminants and impurities, and normally requires a series of purification steps, each removing some of the impurities and bringing the product closer to the final specification. These form the basis for scale-up and define the type of equipment, its size, and the operating parameters [Milton *et al.,* 2007]. Separation parameters used in large-scale protein purification and typical purification factor ranges are provided in Table 1A.2. The cost of product purification is determined by the requirements of product quality, reliability and stability. Quality is usually measured in terms of product purity or the absence of impurities. The cost of a purified product is also influenced by the time required to produce it. Because of the wide range of complex impurities and the sensitivity of biological solutes to factors such as pH, temperature, pressure, shear and ionic strength, downstream processing of the biomolecules requires additional approaches than those used in the traditional chemical separations. Handling the shear and temperature sensitive material, separation of intracellular products, controlling the protelytic degradation are some of the issues to be addressed during downstream processing. The major challenges faced in downstream processing are selecting the precise separation

scheme, optimizing the process variables and controlling product stability [Keller *et al.,* 2001].

Liquid-liquid extraction (LLE) is a traditional chemical engineering unit operation for which the design and scale up of both batch as well as continuous processes are already accomplished. In recent years, LLE using aqueous two-phase systems (ATPS) and Reverse micellar systems (RMS) have been recognized as superior and versatile for downstream processing of biomolecules [Albertson, 1986]. High capacity, biocompatible environment, low interfacial tension, high yields, lower processing time and energy, ease of scale-up and scope for continuous operation are some of the advantages of aqueous two phase extraction (ATPE) and reverse micellar extraction (RME) [Harikrishna *et al.,* 2002].

Table 1A.1 Some of the industrial scale bioseparation operations

Solid-liquid separations	• Centrifugation • Filtration • Flocculation • Floation • Sedimentation
Membrane separations	• Diafiltration and dialysis • Microfiltration • Ultrafiltration • Reverse osmosis • Perevaporation
Extractions	• Leaching • Solvent extraction • Aqueous two-phase extraction • Liquid membrane extraction • Reverse micellar extraction • Supercritical fluid extraction
Chromatographic methods	• Affinity • Gel permeation • Hydrophobic interaction • Ion exchange
Thermal operations	• Distillation • Drying • Evaporation • Lyophilization
Miscellaneous	• Cell disruptions/rupture • Adsorption • Crystallization • Electrophoresis • Precipitation (salt, solvent)

[Chisti, 1998]

Table 1A.2 Separation parameters used in large-scale protein purification and typical purification factor ranges

Parameter	Process	Typical range of purification factor
Temperature	Heat denautration	2-20
Solubility	Salt precipitation	
	Solvent precipitation	2-20
	Polymer precipitation	
	Aqueous two-phase partitioning	
Size and shape	Gel filtration	2-20
	Ultrafiltration	2-5
Net charge	Gel electrophoresis	2-10
	Free electrophoresis	2-5
	Ion-exchange chromatography	2-40
Iso-electric point	Chromatofocusing	2-10
Hydrophobicity	Hydrophobic interaction chromatography	2-30
	Reversed-phase chromatography	2-200
Function	Biospecific affinity chromatography	50-1000
Antigenicity	Immunosorption	20-100
Carbohydrate content	Lectin affinity chromatography	2-10
Content of free–SH	Covalent chromatography	2-10
Exposed histidine	Metal chelate affinity chromatography	2-10
Exposed metal ion	Chelate affinity chromatography	2-10
Group specific	Hydroxyapatite chromatography	2-10
	Dipolar chromatography	2-40
	Dye affinity chromatography	2-40
	Charge transfer chromatography	2-20

[Milton *et al.,* 2007]

1B. REVERSE MICELLAR EXTRACTION (RME)

Reverse micelles are thermodynamically stable, optically transparent, nanometer size droplets of an aqueous solution stabilized in an apolar environment by the surfactant present at the interface. Surfactants are a special group of lipids that possess both hydrophilic (head) and hydrophobic (tail) parts and are also termed as amphiphiles or amphipathics. They adsorb at surface or interface and change the interfacial free energy associated with the building of an interface. The reverse micelles formed in ternary surfactant-water-organic solvent mixture are also called as water-in-oil emulsion (Winsor-2 system). The surfactants form aggregates in organic solvents in such a manner that their hydrophobic moieties are in contact with the solvent and the hydrophilic moieties are turned away from it. The schematic representation of a spherical reverse micelle and a surfactant molecule is shown in Fig. 1B.1. These systems can provide a gentle and biocompatible environment for the separation of biomolecules. In these systems, the biomolecules are solubilized inside the polar core of surfactant shell that protects them from denaturation by organic solvent. The complex nature of polar moieties is responsible for the abnormal behavior of amphiphiles of solution, namely, formation of aggregates. Although, reverse micelles mainly form spherical shape, various other forms of surfactant aggregates are also formed (Fig. 1B.2). Surfactants are classified either based on their solubility (soluble, insoluble) or on the surface charge of the head groups (anionic, cationic, nonionic, zwitterionic).

In general the reverse micellar phase consists of an organic solvent (~80-90%), water (~1-10%), and a surfactant (<10%) [Harikrishna *et al.,* 2002]. Organic solvent, which occupies the major part of the reverse micellar system greatly influences the water solubilization capacity of reverse micelles. The size of reverse micelles, which affects the extraction efficiency, also depends on the nature of the organic solvent used for the formation of reverse micelles. Co-solvents also termed as co-surfactants favor the formation of stable reverse micelles by improving the solubility of surfactants in organic solvent.

1B.1. Characteristic properties of reverse micellar systems

The important properties of reverse micellar systems are

- Thermodynamic stability (no phase separation with time)
- Spontaneous formation
- Lower interfacial tension ($<10^{-2}$ mN m^{-1})
- Transparent nature (nanometer size <100 nm),
- Large surface area (several hundred square meters per cubic centimeter)
- Viscosity comparable to pure organic solvents
- Capability to dissolve polar substances

Although, reverse micelles are thermodynamically stable, they are highly dynamic in nature. In addition to the normal Brownian motion, a number of relaxation processes characterize the monomer. The reverse micelles constantly collide with each other and occasionally a collision results in the fusion of two reverse micelles temporarily. When micelles collide with each other they may build a transient dimmer with a communication channel (fusion), which permits the exchange of materials. Exchange of the contents of reverse micelles is believed to occur by means of a collision-fusion-fission process [Jiquan *et al.,* 1998; Kolisis, 1999].

Application of RME technique for downstream processing of biological products has been explored, as many biochemicals including amino acids, proteins, enzymes, antibiotics, steroids, and nucleic acids can be solubilized within and recovered from such solutions without the loss of native activity. In addition to the above hydrophilic biomolecules, microbial cells can also be incorporated into the water core of reverse micelles, facilitating their extraction.

1B.2. Mechanism and methods of solute solubilization during RME

There are three commonly used methods (Fig. 1B.3) to incorporate biomolecules such as proteins and enzymes into reverse micelles, namely, injection of a concentrated aqueous solution, addition of dry lyophilized protein to a

reverse micellar solution, and phase transfer between bulk aqueous and surfactant-containing organic phases [Matzke *et al.,* 1992].

1B.2.1. Injection method: In the injection method, the protein already solubilized in a concentrated stock aqueous solution (typically, 7% by volume) is added to the surfactant containing organic solvent (dry or slightly hydrated). The resulting mixture is vigorously shaken until an optically transparent solution is obtained. This method allows controlling of the amount of water present in the system. This procedure, which is commonly used in biocatalytic applications, is simple and most effective. In this method, reverse micelles are forced to form with the protein already inside. Hence, micelle sizes do not significantly affect the protein solubilization. For small micelle sizes, the injection method solubilized more protein than dry-addition method.

1B.2.2. Dry addition method: It consists of the initial introduction of the required amount of water into the surfactant solution in an organic solvent in order to attain the required surfactant hydration degree. A dry (lyophilized) protein can be dissolved in the resulting solution under vigorous shaking. This method is commonly used in biocatalytic applications and is well suited to hydrophobic proteins. One of the drawbacks of this method is prolonged contact between the enzyme/protein molecule and the organic solvent/surfactant that may lead to a partial denautration of the former. In this method, protein solubilization is strongly dependent on micelle size. In fact, the protein is appreciably solubilized only when the diameter of the reverse micelle is either similar to or larger than that of protein. The reason is that the energy barrier for solubilization of a large protein in small micelle is too large to overcome. But, in contrast, for a larger micelle, since the micelle is not required to rearrange its contents to incorporate a protein, the energy barrier is lower and the protein is solubilized.

1B.2.3. Phase transfer method: The phase transfer method of protein solubilization is fundamentally different from the other two methods. In this

method, there are two bulk phases (aqueous and organic), which are brought to equilibrium. Under certain conditions, the protein molecules are transferred from the aqueous phase to the surfactant containing organic phase. Although, the enzyme solubilization is slow in this method, large amount of solute solubilization is possible with minimum values of water content. The phase transfer method is suitable for extraction of solutes form dilute aqueous solutions. The pH of the aqueous phase, size and iso-electric point of the protein, and the surfactant type were shown to have significant effect on the protein solubilization by phase transfer method. The main driving forces for solubilization of biomolecules in reverse micelles are the hydrophobic and electrostatic interactions. The different interaction established between the solute and the reverse micellar interface can be explored to selectively separate solute.

When biomolecules such as enzymes are solubilized in reverse micelles, they can be localized in different microcompartments, depending on their chemical structure or relative solubility in these microphases (Fig.1B.4) The degree of hydrophobicity of the biomolecule plays an important role in the preferential localization among the various microenvironments (water core, bound water, surfactant head and organic solvent). Hydrophilic enzymes are entrapped in the water core, whereas the one with an amphiphilic character can be anchored to the surfactant layer, penetrating even in the continuous organic phase [Martinek *et al.,* 1986].

1B.3 Reverse micellar extraction by phase transfer method

Phase transfer method of reverse micellar extraction consists of two fundamental steps, namely, forward and back extraction. During forward extraction the biomolecule is transferred from an aqueous solution into a reverse micellar organic phase and during back extraction the biomolecule is released from the reverse micelles and transferred into an aqueous phase (stripping phase) [Brandani *et al.,* 1996a]. The recovery of biomolecules from the reverse micellar phase can be facilitated by exploiting the deassembling nature of reverse micelles

in aqueous media. The biomolecules recovered, which retain their functional activity in aqueous phase may be subjected to further concentration/purification process. The schematic representation of forward and back extraction stages of RME is given in Fig. 1B. 5. The driving forces responsible for the extraction of biomolecule by RME are [Hong *et al.,* 2000].

- Electrostatic interaction between charged surfactant head group and the biomolecule
- Hydrophobic interaction between the hydrophobic tail of surfactant, solvent and biomolecule
- Steric interaction – The size factor (relative size of solute and reverse micelle) responsible for the rejection/selection of solute.

1B.3.1. Forward extraction: Forward extraction involves diffusion of biomolecule such as protein from bulk aqueous solution to interface, formation of a biomolecule containing micelle at the interface and diffusion of biomolecule containing micelle into the organic phase. One of the key factors that determine protein partitioning into a reverse micellar phase during forward extraction is the size of the protein. Wolbert *et al*. (1989) reported that as protein size increases, the partition of protein into reverse micelle becomes much more difficult. Because larger protein requires a reversed micelle larger than what would be thermodynamically stable. This can be solved by the addition of co-surfactants or by manipulating organic continuous phase. Also, larger proteins require a larger number of charged residues on their surface in order to be transferred into reversed micelles. This could be achieved by maintaining a higher difference between the pH of the aqueous phase and pI of the protein (a higher net charge on the protein) for solubilization to occur than small proteins. The solubility and structural integrity of the protein in the reverse micelle are closely related to the properties of the surfactant, aqueous solution and organic solvent used [Samana et al.,1987]. Forward extraction efficiency is controlled by various process parameters such as concentration and type of surfactants, pH and ionic strength of the aqueous phase, concentration and type of

co-surfactants, salts, charge of the protein, temperature, water content, size and shape of the reverse micelles etc. By manipulating these parameters selective separation of the targeted biomolecule can be achieved [Wolbert *et al.,* 1989].

1B.3.2. Back extraction: Back extraction involves coalescence of biomolecule filled reverse micelles with the interface to transfer the biomolecule to aqueous phase (also termed as stripping phase). In general, the forward extraction of proteins into reversed micelles has a high efficiency and the process step that is low yielding and thus requiring attention is the back transfer step [Spirovska and Chaudhuri, 1998]. Kinetics of the back extraction was reported to be much slower as compared to that of forward extraction and in fact it was reported to be three orders of magnitude slower than the forward extraction [Dungan *et al.,* 1991]. It was reported that the back extraction rate could be enhanced (more than 100 times) with the addition of counter-ionic surfactant [Jarudilokkul *et al.,* 1999]. The counter-ionic surfactant was reported to interact with the oppositely charged surfactant molecules and facilitate the release of solutes back into the stripping phase. During back extraction pH value of the stripping solution was adjusted to prevent the protein-protein interaction (similar to the iso-electric point of the protein to be purified) and salt at high concentration was added to reduce electrostatic interaction between the surfactant and protein [Kinugasa *et al.*, 1992].

Alternative methods suggested for back extraction include addition of dewatering agents such as isopropyl alcohol [Carlson and Nagarajan, 1992] adsorption on to silica [Leser *et al.,* 1990] and temperature shifts [Dekker *et al.,* 1991], use of ion exchange columns [Choudhuri and Spirovska, 1994], dehydration of reverse micelle with molecular sieves to recover the protein [Gupta *et al.*, 1994], addition of large amount of a second organic solvent, such as ethyl acetate to destabilize the reverse micelle and hence to release the protein [Wolbert *et al.,* 1989], formation of clathrate hydrates via pressurization [Phillips *et al.,* 1991] and back extraction with the aid of a counterionic surfactant [Jarudilokkul *et al*., 1999]. The manipulation of phase behavior by temperature or pressure for

back extraction avoids the usual high-salt buffer usage and subsequent need to desalt the aqueous phase containing the protein.

1B.4. Effect of process parameters on RME

1B.4.1. Aqueous phase pH: The aqueous phase pH determines the ionization state of the surface-charged groups on the protein molecule. Solubilization of protein in reverse micelles is found to be dominated by electrostatic interaction between the charged protein and the inner layer of the surfactant head groups. Solubilization of protein is favored at pH values above pI of protein in case of cationic surfactants, while the opposite is true for anionic surfactants. For proteins with small molecular weight such as cytochrome C, lysosomes, and ribonuclease (MW range 12000-14500 Da), the pH-pI value required for optimum solubilization is much lower (<2) when compared to that of larger proteins such as α-amylase (MW 48000 Da). This can be explained with the reasoning that as the protein size increases, size of the reverse micelle also has to increase in order to incorporate the protein molecule. To increase the size of the reverse micelle, higher energy is required, which can be provided by increasing the number of charged groups on the protein. This increase in charge density on the protein molecule can be accomplished by manipulating the pH of the aqueous solution (i.e. by increasing the pH much higher than the pI of the protein). For small proteins whose size is smaller than the size of the water pool inside a reverse micelle, solubilization occurs as soon as the net charge is opposite to that of the reverse micellar interface. An empirical relation between pI, molecular weight of the protein and the pH value at which maximum transfer efficiency could be realized was developed by Wolbert *et al.* (1989) on the basis of experimental data of Goklen and Hatton (1987) for sodium bis (2-ethyl-1-hexyl) sulfosuccinate (AOT) reverse micelles, which is given below.

$$[(\text{pH(optimum)} - \text{pI}]) = \left(+ 0.12 \text{x} 10^{-3}\right) \text{M}_{\text{WS}} - 1.07 \qquad \text{(1B.1)}$$

Similarly, the forward transfer data obtained by Wolbert *et al*. (1989) for 19 proteins using the cationic trioctyl-methyl ammonium chloride (TOMAC) reverse micelles was used to obtain the following correlation [Carlson and Nagarajan, 1992]

$$[(\text{pH(optimum)} - \text{pI}]) = \left(+ 0.11 \text{x} 10^{-3}\right) M_{WS} - 0.97 \qquad \text{(1B.2)}$$

These empirical models help in designing an appropriate separation system.

1B.4.2. Ionic strength and type: The influence of ionic strength (KCl/NaCl concentration) on the solubilization of proteins in reverse micelles is explained purely as an electrostatic effect The electrostatic potential of a protein molecule in an electrolyte is inversely proportional to the ionic strength of the solution and is characterized by Debye length [Harikrishna *et al.,* 2002]. In general, it was observed that as the ionic strength of the aqueous solution increases, the protein intake capacity of the reverse micelles decreases [Aires-Barros and Cabral, 1991]. Two reasons were given to explain this phenomenon. First, increasing the ionic strength decreases the Debye length there by reducing the electrostatic interaction between the charged protein molecules and the charged surfactant head groups of the reverse micelles. Second, increasing the ionic strength reduces the electrostatic repulsion between the charged head groups of the surfactants in a reverse micelle, there by decreasing the size of the reverse micelle. The smaller reverse micelles will have larger curvature, which increases the density of the surfactant monolayer near the surfactant head groups, resulting in a gradual expulsion of protein molecules residing inside the reverse micelles, which is termed as a squeezing-out effect. Although, lower side of the ionic strength favors the protein transfer, experiments can not be performed at very low ionic strengths as the solution becomes cloudy under these conditions. Apart from ionic strength, the type of the ions also plays an important role in determining the partition behavior of proteins in reverse micelles. Naoe *et al.* (2002) reported the efficacy of guanidium salts in the recovery of lysozyme from reverse micellar organic media.

Effects of bile salts (sodium taurocholate and 3-1-propane sulfonate) on percolation size of AOT reversed micelles by Yang *et al.* (2003). Kinugasa *et al.* (2003) studied the effects of ion species on RME of protein and reported the classification of ions as water structure forming (WSF) and water structure breaking (WSB). The effect of cation species (KCl, $BaCl_2$ and $CaCl_2$) on extraction equilibrium of reversed micellar extraction of lysozyme was studied by Nishii *et al.* (2004).

1B.4.3. Surfactant type: The protein distribution is mainly dependent on the interaction between the protein and the surfactant head groups. When other effects are insignificant, pH of the protein solution determines the distribution behavior of protein in reverse micelles stabilized by charged surfactants. In addition to the charge, other surfactant–dependent parameters such as the size of reverse micelles, the energy required to enlarge the reverse micelles, and the density of the inner surface of the reverse micelles may also influence the protein distribution. Protein denaturation was observed in case of reverse micelles of ionic surfactants. Recently, many studies have been reported on the protein transfer using nonionic surfactants such as Tween-85, Span-60, Triton-X-100 etc [Ayala *et al.,* 1992; Subramani et al., 1995; Vasudevan *et al.,* 1996; Jiquan *et al.,* 1998; Rodriguez *et al.,* 1998; Naoe *et al.,* 1999, Hossain *et al.,* 1999]. It was demonstrated that nonionic surfactant has an apparent advantage over the ionic surfactant due to the absence of strong charges at the aqueous/organic interface, which provides a suitable environment for the protein. In many cases the effect of temperature, pressure, and ionic strength were found to be opposite to those observed in ionic surfactant based systems.

1B.4.4. Surfactant concentration: The minimum concentration of surfactant required to form reverse micelles in an organic solvent is termed as critical micelle concentration (CMC). The concentration of surfactant has been shown to have little effect on the structure and size or aggregation number of the reverse micelles. However, it changes the number of reverse micelles formed, which in

turn increases the protein solubilization capacity of the reverse micelles. At surfactant concentration above a certain value, the reverse micellar interactions may occur, which leads to interfacial deformation of reverse micelles and percolation of solute [Harikrishna *et al.,* 2002]. The reverse micellar clustering decreases the interfacial area available to host the biomolecules causing a decrease in the solubilization capacity of the reverse micelles [Cardoso *et al.,* 1999].

1B.4.5. Size of the reverse micelle*:* Micelle size is dependent on the ratio of water to surfactant molecules in a reverse micelle. The monodispersed, small sized reverse micelles can accommodate only proteins of certain dimensions. Hence micelle size may be conveniently used to include or exclude certain proteins. However, it should be noted that several micelle can regroup to form larger micelles when certain operating conditions are altered. It was also hypothesized that a protein can create around itself a new larger micelle of a requisite size to facilitate solubilization [Wolf and Luisi, 1979]. As the ionic strength increases, the reverse micellar size decreases due to decrease in the electrostatic repulsion between the head groups of the surfactants leading to squeezing-out effect. Besides the ionic strength, the type of the solvent and surfactant used also influences the reverse micelle size.

1B.4.6. Phase volume ratio: (V_{org}/V_{aqu}) is a critical parameter in the extraction and concentration of enzymes. Ideally, this ratio should be low for forward extraction and high for back extraction to achieve concentration of the solutes. However, the change in the volume ratio could also adversely affect the extraction efficiency of the system.

1B.4.7. Co-surfactant: Use of co-surfactant, also termed as co-solvent may enhance the solubilization kinetics, stability of a reverse micelle, and even selectivity. Reports on the addition of medium-chain length alcohols like isopropanol and hexanol for improving the extraction efficiency are available.

Addition of co-surfactants is needed for cationic surfactants to increase the extraction efficiency.

1B.4.8. Temperature: The effect of temperature on the water uptake capacity of anionic and cationic surfactants has been reported and the variation in water uptake was attributed to a change in the aggregation number of the surfactants. Huang and Chang (1995) reported the effect of temperature on enzyme activity and yield during forward and back extraction. Increased temperature is reported to favor the back extraction more than the forward extraction. The disruption of reverse micelles and higher rate of migration of solute containing reverse micelles towards the interface are reported to be the reasons for enhanced recovery during back extraction at higher temperatures. Hasmann *et al,* (1999) used response surface methodology (RSM) to optimize the aqueous phase pH and temperature for β-xylosidase recovery by reverse micelles.

1B.4.9. Phase contact time: In addition to the chemical and biochemical factors, protein transfer also depends on physical aspects such as duration of phase contact and the area of contact between two phases. Higher contact area favors the extraction/release of solutes during RME. However, prolonged contact time may also lead to the reduction in enzyme activity. Also, the equipment used for contacting or the nature of phase contacting influences the extraction efficiency. Use of magnetic stirrer without vortexing was reported to be better compared to vortexing or stagnant organic phase (agitation of aqueous phase alone) [Paradkar and Dordick, 1994] for higher degree of process efficiency.

1B.5. Other approaches using reverse micelles

Many other approaches such as affinity based RME and separations for high selectivity of protein extraction [Senstad and Mattiasson, 1989; Woll and Hatton 1989; Coughlin and Baclaski, 1990; Paradkar and Dordick, 1991, 1993; Choe *et al.,* 1998, 2000; Sun *et al.,* 1999; Liu *et al.,* 2006], reverse micellar

extraction in hollow fibers for higher mass transfer [Yang and Cussier, 1986; Dahuron and Cussler, 1988; Luthi and Hatton, 1991], micellar enhanced ultrafiltration to separate dissolved organic compounds from aqueous streams [Bhat *et al.,* 1987; Tzeng *et al.,* 1999], micellar electrokinetic capillary chromatography for the separation of neutral and partially charged species [Tangen *et al.,* 1997; Boyoe and Spickett, 1999] and, reverse micellar assisted supercritical fluid extraction (SCFE) [Ayala *et al.,* 1992; Rao *et al.,* 1992; Phillips *et al.,* 1991; Goetheer *et al.,* 1999; Yonker *et al*., 2003] have been attempted. Synthesis of nanoparticles employing water in oil emulsion for a variety of novel applications such as catalysis for fuel cells, food applications, nanoprobes for fluorescent bioassys etc, are also reported [Estoe *et al.,* 2006]. Reverse micelles were used for the controlled synthesis of precursor dipeptides [Chen *et al.,* 1999]. The studies on the structural and catalytic properties of enzymes [Hochkoeppler and Palmieri, 1992; Creagh *et al.,* 1993; Jene *et al.,* 1997; Melo *et al.,* 1998; Carvalho *et al.,* 1999a, 1999b; Nagayama *et al.,* 1999], esterification reaction catalyzed by enzyme [Rees and Robinson, 1994] and efficiency of protein refolding [Goto *et al.,* 2000] in reverse micelles have been reported. Singh *et al,* (1999) used reverse micelles to entrap photosynthetic bacterium and enhance H_2 production rate, as compared to cells suspended in normal aqueous medium.

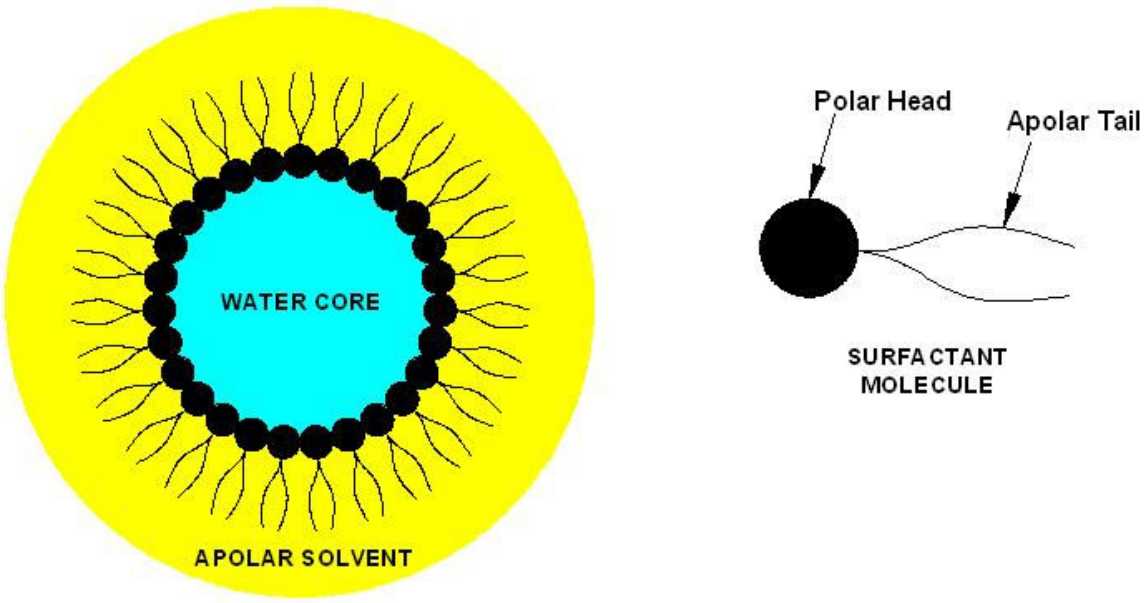


Fig.1B.1 Spherical shaped reverse micelle and a surfactant molecule [Kilkian *et al.*, 2000]

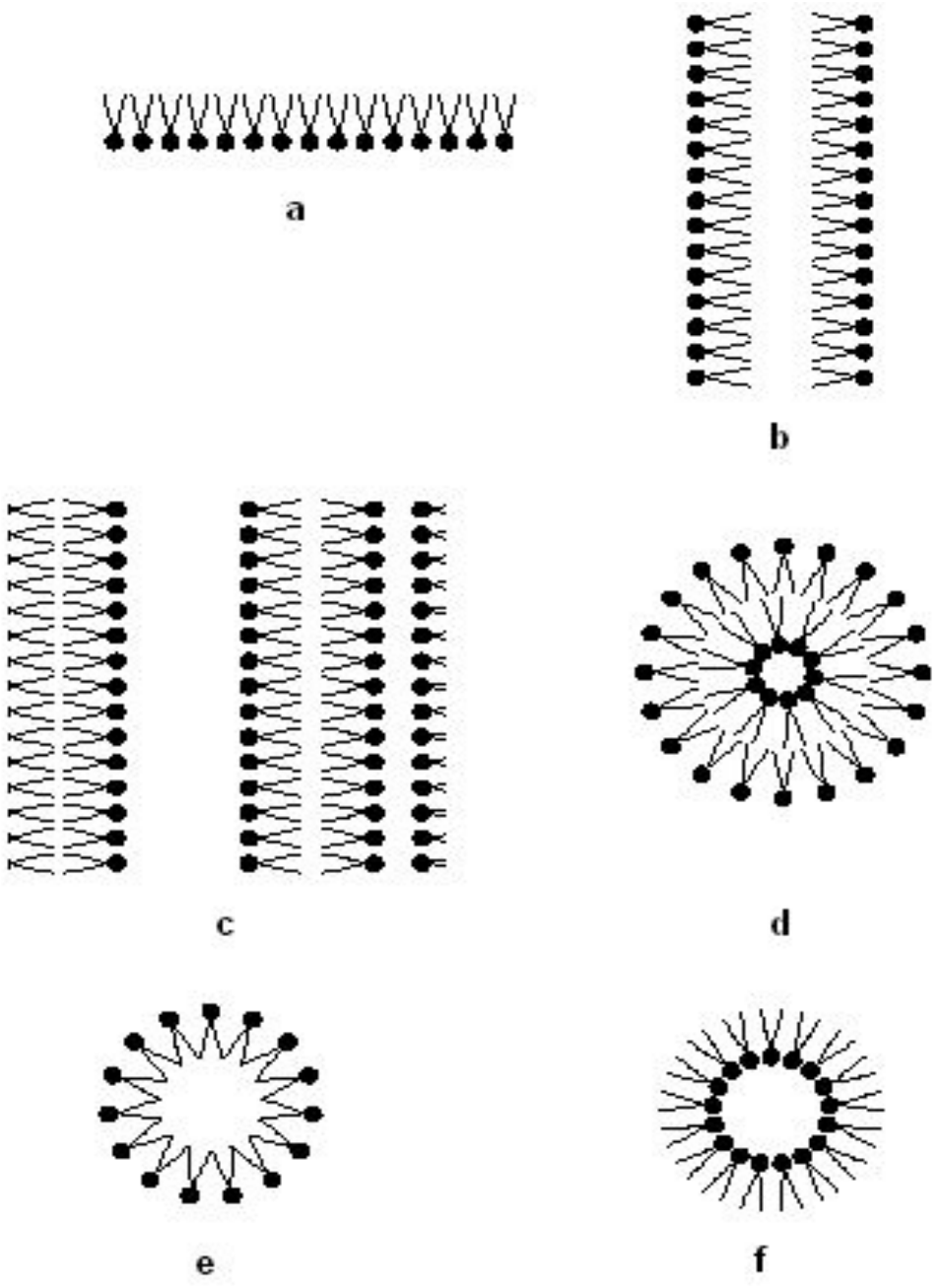


Fig.1B.2: Various forms of surfactants aggregations in solution: a) monolayer b) bilayer c) liquid crystalline phase (lamellar) d) vesicle (liposome) e) micelle and f) reverse micelle [Matzke *et al.,* 1992]

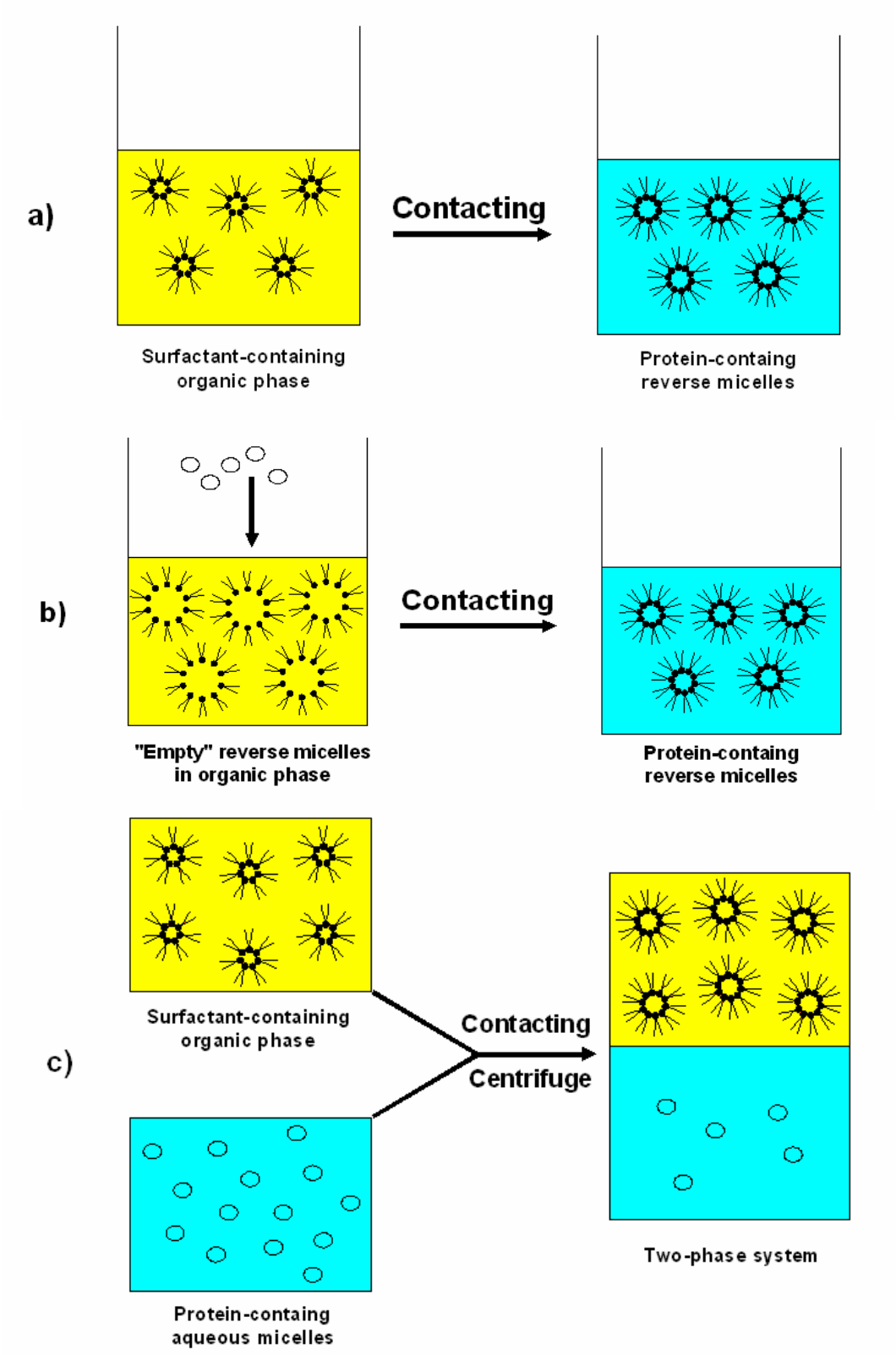


Fig.1B.3.Methods of carrying out RME: (a) injection of aqueous phase containing solute (b) addition of dry powder (c) phase transfer [Matzke *et al.,* 1992]

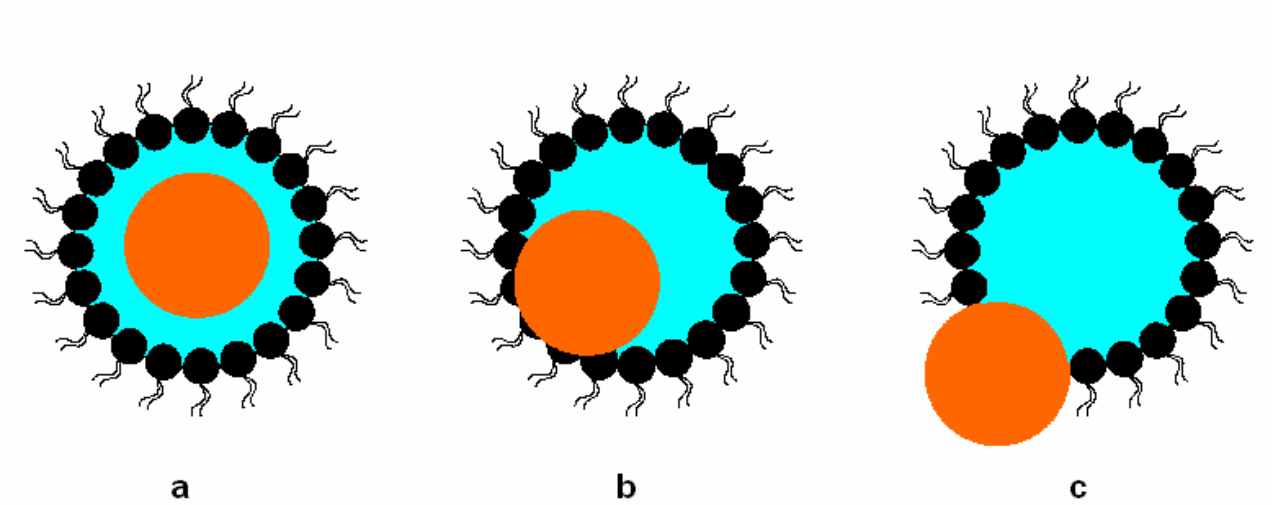


Fig.1B.4 Solubilization of different biomolecules in reverse micelles: (a) hydrophilic (b) surface-active and (c) hydrophobic [Martinek *et al.,* 1986]

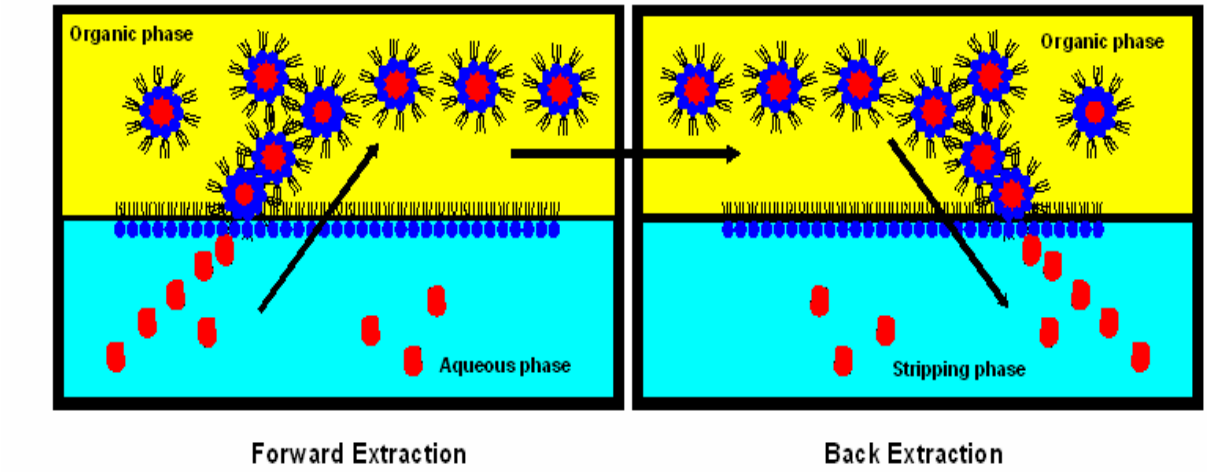


Fig.1B.5 Schematic diagram of forward and back extraction of solute during RME by phase transfer method

1C. BIOMOLECULES AND MODEL/NATURAL SYSTEMS

Several reports on the application of RME for separation and purification of biomolecules are available for model systems (commercial samples of proteins and enzymes) [Leser *et al.,* 1986; Armstrong, and Li, 1988; Brochette *et al.,* 1988; Dekker *et al.,* 1989; Marcozzi *et al.,* 1991; Ayala *et al.,* 1992; Ichikawa *et al.,* 1992; Chang *et al.,* 1994; Aftleck *et al.,* 1994; Wang *et al.,* 1995; Chang and Chen, 1995; Hilhorst *et al.,* 1995; Hu and Gulari, 1996; Hentsch *et al.,* 1996; Regalado *et al.,* 1996; Brandani *et al.,* 1996a; Imai *et al.,* 1997; Fadnavis *et al.,* 1997; Lee and Dungan, 1998; Lazarova and Tonova, 1998; Noritomi *et al.,* 1999; Cardoso *et al.,* 1999; Zhang *et al.,* 2002; Shin *et al.,* 2004; Dovyap *et al.,* 2006; Mutalik and Gaikar, 2006; Akbar *et al.,* 2007; Raikar *et al.,* 2007]. Most of these studies have focused on the effect of processing conditions on forward extraction efficiency and are also restricted to laboratory scale studies. Many of the RME reports deal only with forward extraction of biomolecules with an assumption that back extraction could be easily carried out by employing the conditions which do not favor forward extraction, which is not always true. Ionic surfactants have been extensively used for the extraction studies and there are only a few reports on the application of 'mild', non-interfering nonionic surfactants for RME. Only a few studies on RME of biomolecules from natural systems were reported earlier [Goklen and Hatton, 1985, 1987; Giovenco *et al.,* 1987; Rahaman *et al.,* 1988; Leser et al., 1989; Pfammatter et al., 1989; Aires-Barros and Cabral, 1991; Krie *et al.,* 1995; Forney and Glatz *et al.,* 1994, 1995; Regalado *et al.,* 1996; Pessoa and Vitolo, 1997; Krieger *et al.,* 1997; Shiomori *et al.,*1998; Hasmann *et al.,* 1999]. However, in the recent past many research groups working in this area have reported their results [Gaikar and Kulkarni 2001; Mutalik and Gaikar, 2003; Shin *et al.,* 2003; Liu *et al.,* 2004; Ferreira, *et al.,* 2005; Monterio *et al.,* 2005; Noh *et al.,* 2005; Chen, 2006; Hasmann *et al.,* 2007].

The present work focuses on the application of RME technique for both model [bovine serum albumin (BSA, 66 kDa) and horse radish peroxidase (HRP)] and natural systems [bromelain from pineapple wastes (peel, core, stem and

crown) and peroxidase from radish roots]. Studies on model systems have been carried out mainly to understand the mechanism of RME, extraction behavior of different reverse micellar systems and effect of process parameters on the both forward and back extraction efficiencies. A relatively large protein BSA was selected in the study, as RME of large molecular weight solutes was reported to be difficult. The enzyme HRP is reported to have many isoenzymes and maximization of the extraction efficiency is a challenge using RME technique. Natural systems such as pineapple waste and radish roots were used for the extraction of bromelain and peroxidase, respectively.

1C.1. Model systems

1C.1.1. Bovine serum albumin (BSA)

Type	:	Globular protein
Molecular weight	:	66.3 kDa
pI	:	4.9
Stability range	:	4.0-9.0
Water solubility	:	Highly soluble

BSA has been widely studied as a model protein because its structure and physicochemical properties are well characterized. BSA is a relatively large molecular weight (66.3 kDa) globular protein [Kelley and McClements, 2003] that is naturally found in the blood and milk of cows [Kinsella and Whitehead, 1989]. It consists of 607 amino acids with 17 disulfide bonds and one free cysteine group. It has relatively high water solubility because it contains a large number of ionizable amino acids [Gaincola *et al.,* 1997]. Serum albumins bind many different types of amphiphilic biological molecules, which are believed to play an important role in determining their physiological function. The reported value of isoelectric point (pI) for BSA is 4.9 [Wolbert *et al.,* 1989].

1C.1.2. Horse radish peroxidase (HRP)

Type	:	Glycoprotein
Molecular weight	:	44 kDa
pI	:	3 to 10
Stability range	:	5.0 to 10.0
Water solubility	:	Highly soluble

Horse radish peroxidase is one of the most widely used enzymes in analytical applications. HRP (E.C. 1.11.1.7) has a molecular weight of around 44 kDa [Motlekar and Bhagwat, 2001]. The isoenzymes of HRP have isoelectric points ranging from 3 to 10, with some showing higher activity than others. They are glycoproteins with a single polypeptide chain containing a heam group [Motlekar and Bhagwat, 2001]. Peroxidase in highly purified form has been widely used as a combined antigen and immunological marker in biological systems to localize cellular constituents or for quantitative analysis. HRP is also used in coupled reactions with oxidases to measure a wide variety of clinically and chemically important compounds [Regalado *et al.,* 1994]. HRP is widely used as an enzyme label in medical diagnostics and research applications. Universal covalent conjugate of proteins, antibodies and other molecules with HRP offer a wide range of amplifying possibilities. They are useful and versatile tools for ultra–sensitive detection in immunoassays and histo and cytochemical applications. The ability of HRP to catalyze the free-radical formation of a variety of aromatic pollutants followed by spontaneous polymerization can be potentially used in bioremediation and wastewater treatment. HRP with hydrogen peroxide (H_2O_2) was used to remove phenolic compounds from effluents.

1C.2. Natural systems

1C.2.1. Pineapple wastes for the extraction of bromelain

Enzyme	:	Bromelain
Type	:	Proteolytic

Molecular weight	:	28-32 kDa
pI	:	4.6
Stability range	:	4.5-9.5 for fruit bromelain and 7.0-10.0 for stem bromelain
Water solubility	:	Highly soluble

Bromelain is the collective term for proteolytic enzymes derived from the ripe and unripe fruit, as well as the stem and leaves of the pineapple plant (*Ananas comosus* L. Merryl). Pineapple belongs to the family of Bromeliaceae and is a herbaceous, perennial, monocotyledonous plant [Chadha *et al.,* 1998]. The stem bromelain (EC 3.4.22.32) and fruit bromelain (EC 3.4.22.33) obtained from the pineapple stem and pulp, respectively are finding wide applications in pharmaceutical (digestive aid, as a cleansing agent to improve the texture of the skin and to promote the healing of wounds) and food industry (meat tenderizer and dietary supplement) [Rowan *et al.,* 1990]. Bromelain is mainly comprised of cysteine proteases, with smaller amounts of acid phosphatase, peroxidase, amylase and cellulase. Bromelain contains at least four distinct cysteine proteases. Stem bromelain is a basic glycoprotein with a molecular weight of 28000 Da and isoelectric point (pI) of 9.5, whereas, the fruit bromelain is an acidic glycoprotein with a molecular weight of 31000 Da and pI of 4.6 [Yamada *et al.,* 1976]. Bromelain is reported to be present also in pineapple wastes such as core, peel, and leaves relatively lesser in quantity as compared to stem [Sriwatanapongse *et al.,* 2000]. Fruit and stem bromelain are stable at pH range 4.5-9.5 and 7.0-10.0 respectively [Haq *et al.,* 2002].

1C.2.2. Radish roots for the extraction of peroxidase

Enzyme	:	Peroxidase
Type	:	Glycoprotein
Molecular weight	:	44 kDa
pI	:	3 to 10 (for different iso-enzymes)

Stability range	:	4.5-9.0
Water solubility	:	Highly soluble

Peroxidases are mostly extracted from plant cells and some animal organs and tissues. They are widely distributed in the leaf, stem and root of radish (*Armoracia rusticana*) with highest activity being found in the roots. They are also found in Chinese cabbage, carrot hairy root cell culture, turnip etc. They belong to the group of Heme proteins, which are a diverse group of enzymes that perform a wide variety of functions in living organisms ranging from oxygen transport and storage (hemoglobin and myoglobin), electron transport and energy production (Cytochromes), to oxygen transfer (monoxygenases), the single electron oxidation of substrates (peroxidases), the dismutation of hydrogen peroxide (H_2O_2)(catalases), and synthetic transformations (various synthases). It is reported that peroxidase extracted from plant sources have different isoenzymes having their pI values in the range 3-10. Wolbert *et al.* (1989) reported the pI value as 6.9 for isoenzyme ‘C’, which is the major type of peroxidase present in horseradish.

1D. AQUEOUS TWO PHASE EXTRACTION AND MEMBRANE PROCESSING

1D.1. Aqueous two phase extraction (ATPE)

Liquid-liquid extraction using ATPS has been recognized as superior and versatile technique used for downstream processing of biomolecules [Albertson, 1986; Diamond and Hu, 1989; Raghavarao *et al.,*1995]. Aqueous two phase extraction is a method that has been known for quite some time but its importance and applications are being realized only in the recent years. No loss of native function/activity, low interfacial tension, ease of scale up, and potential for continuous operation are some of the advantages of ATPE. Gentle, non-toxic aqueous environment of ATPS are suitable for the extraction of cells, particles and macromolecules, which are sensitive to temperature, pH and solvent conditions. The physical properties of ATPS such as density, viscosity and interfacial tension, determine the phase separation time and also contribute to the biomolecule partition behavior. The major hindrances for the practical application of ATPE in industrial practice are the high cost of phase forming components and slow demixing of the equilibrated phases. The use of ATPE in downstream processing, of many enzymes such as xylanase [Gaikaiwari *et al.,* 1996], amylases [Andersson, 1985], amino acids [Li *et al.,* 1997], amyloglucosidase [Tanuja *et al*., 1997], potato polyphenol oxidase [Vaidya *et al*., 2006] and plant peroxidase [Srinivas *et al.,* 1999] have been reported. In some applications, ATPE has been also used as a primary purification technique to reduce the bulk of the processing stream, to be followed by more selective steps such as chromatography, electrophoresis etc [Sikdar *et al.,* 1991; Raghavarao *et al*., 1995,1998]. Application of ATPE for the selective extraction of solute was studied in the present work and compared with the performance of RME.

1D.2. Membrane processing

Membrane processing has been extensively used in chemical process industry and its application in food/bio industry is now becoming more popular. Some of the typical food applications include purification of water, concentration and clarification of fruit juices, milk products, alcoholic beverages and

concentration of proteins and enzymes. Membrane processes provide a means of separation at the molecular and fine-particle level and solute concentration. The family of liquid phase pressure driven processes, namely, reverse osmosis (RO), ultrafiltration (UF), cross flow (micro) filtration (CFF), and the concentration driven process such as dialysis can be used to separate a range of species found in the complex system like fermentation broth. Low temperature processing, good selectivity, retention of volatiles, lower energy cost, concentration and purification in single step, and capacity to operate at higher scale on continuous mode are some of the advantages of membrane processing. The physical and chemical characteristics of the membrane i.e. pore size and pore size distribution, nature of the solution being processed, the operating temperature and pressure, and the fluid mechanical environment provided by the membrane module affect the flux and efficiency of separation. The research in the membrane science area is focused on developing membranes that are non-fouling, more selective, and more chemical and temperature resistant. Future applications of membranes in downstream processing will involve the synergistic combination of the membrane with other separation processes that will result in more effective, highly selective separation technique [Fane and Radivich, 2007]. The present work includes process integration by coupling ultrafiltration with RME to explore the possibility of improving the overall efficiency of the process.

1E. MATHEMATICAL MODELING

1E.1. Estimation of reverse micellar size

Mathematical modeling of the solubilization of biomaterials in reverse micelles is essential for an in-depth understanding and effective use of RME in downstream processing of biomolecules. However, the quantitative modeling of protein solubilization in reverse micelles is a complex problem and relatively fewer studies on the modeling of protein containing revere micellar systems have been reported. Many of the parameters such as hydrophobic interaction of ions with biomolecule and surfactant, the free energy changes associated with the change in size of reverse micelles on protein uptake, and the distribution of charged groups of the protein molecule are unknown. Models proposed in the literature for protein solubilization range from simple geometric models [Bonner *et al.,* 1980; Levashov *et al.,* 1982; Sheu *et al.,* 1986; Zampieri *et al.,* 1986] to more rigorous molecular thermodynamic models [Sheu *et al*.,1986; Bratko *et al.,*1988; Caselli *et al*.,1988a; Woll and Hatton, 1989; Rahaman and Hatton*,* 1991; Li *et al.,* 1999]. Shell and core model proposed by Bonner *et al.* (1980) was the first model to be proposed for the estimation of filled and unfilled radii. Many Models have been proposed based on the volume of the protein included in the reverse micelles. Rahaman and Hatton (1991) developed a thermodynamic model for the prediction of the sizes of the filled and unfilled reverse micelle as a function of ionic strength, protein charge, and size, water content and protein concentration incase of phase transfer and injection methods of RME. Measurement of the actual size of the reverse micelles using instruments such as Small Angle Neutron Scattering (SANS), Small Angle X-ray Scattering (SAXS), and by ultracentrifugation methods to compare or validate the predicted values of the model has been reported. In the present study attempts have been made to estimate the size of the reverse micelles using reported models (empirical and geometrical models).

1E.2. Mass transfer kinetics

The rate of transfer of biomolecules such as protein/enzyme to or from a reverse micellar phase and factors affecting the rate are important for the practical applications of RME for the extraction and purification and for scale-up. During the forward extraction the protein diffuses in the bulk aqueous solution to the interface and forms a reverse micelle at the interface. Further, the protein containing reverse micelle diffuses into the organic phase. The coalescence of protein filled reverse micelles with the interface releases the protein into the striping phase during back extraction. The overall mass transfer rate during an extraction process depends on which of these steps is rate limiting.

Model for the estimation of mass transfer coefficient and concentrations of protein in hollow fibers was proposed by Dahuron and Cussler (1988) for both co-current and counter current flows. Dekker *et al.* (1989; 1990) proposed mathematical model to satisfactorily describe the time dependency of the concentration of active enzyme in all the phases (forward and back extractions), based on the flow, mass transfer coefficient and first order inactivation kinetics. Fraaije *et al.* (1990) reported the thermodynamic model for protein partitioning and the co-partitioning of electrolytes. Model for prediction of interfacial mass transfer coefficient for transfer of proteins between aqueous and organic phases was proposed by Dungan *et al.* (1991). This study showed that charge interactions play a dominant role in the interfacial forward transport kinetics. Mass transfer characterization in forward and back extractions of lysozyme by AOT-isooctane reverse micelles across a flat liquid-liquid interface was reported by Nishiki *et al.* (1998). Liu *et al.* (2006) studied the partitioning equilibria and the kinetics of lysozyme and bovine serum albumin. Kinetic analysis on membrane based reverse micellar extraction of lysozyme from aqueous solutions was reported by Juang *et al.* (2006).

1F. AIM AND SCOPE OF PRESENT WORK

Although, there are a few reports on the application of RME for downstream processing of enzymes/proteins, most of the studies have focused only on forward extraction. In most of these studies, it was assumed that the conditions that normally prevent protein uptake during forward extraction, would promote their release in the back extraction, which is not always true. A successful RME should include both forward and back extraction processes in their optimized conditions. Hence, there exists a need for a comprehensive study on various aspects of forward and back extractions. A systematic study of the effect of process parameters such as type and concentration of surfactant, aqueous phase pH, ionic strength and type, water content, phase volume ratio, micelle size etc., is to be carried out to optimize the process efficiency and to understand the system behavior. The extraction and purification of proteins by employing reverse micelles have been reported for model systems, where as the study on the real/natural system is limited. As RME from real systems such as crude plant extracts and fermentation broth are much more complicated compared to model systems, there is a scope for investigating the extraction behaviors of enzymes/proteins from such systems. Although, ionic surfactants have many advantages, in some cases they are reported to cause protein denaturation due to the strong electrostatic interaction that exist between their head groups and protein. This problem could be overcome by employing nonionic surfactants. Hence, there is a scope for studies on the use of nonionic surfactants that are non-interactive and non-toxic. An integrated process by combining RME with membrane processes or ATPE is expected to improve the overall efficiency of downstream processing. However, practically no such reports on the integrated process are available. Although, the laboratory scale RME for downstream processing of proteins is established to some extent, there are only a few reports on scale-up studies. Hence, there is a need to investigate the extractive recovery at higher scale which will provide a valuable data for the process development.

1F.1. Major objectives of the work

- Development of effective RME process for downstream processing of proteins/enzymes.
- Integration of RME with ATPE and membrane processes for improving the overall productivity of downstream processing of selected proteins/enzymes.

1F.2. Work elements

- Study of factors affecting the forward and backward transfers in RME of enzymes/proteins from natural/real sources.
- Comparative studies of reverse micellar systems with ATPS.
- Mathematical modeling of partitioning of biomolecules.
- Integration of RME with processes such as ATPE and membrane processes for improving the overall productivity of downstream processing of selected proteins/enzymes.

CHAPTER 2

CHARACTERIZATION OF REVERSE MICELLES AND SCHEME FOR RME

SECTION A

PHYSICO-CHEMICAL CHARACTERISTICS

2A.1. Introduction

The efficiency of RME mainly depends on the reverse micellar system selected to extract a given biomolecule and the processing conditions employed. Physicochemical characteristics of the reverse micellar system components, namely, organic solvent, surfactant and co-solvents could be used as useful tools for the selection of most suitable combinations. The charge on the polar head of the surfactant, hydrophilic-lipophilic balance (HLB), critical micelle concentration (CMC) and pH stability of the surfactant, solute-surfactant interaction, etc., are some of the properties, which govern the selection of surfactant for a given extraction process. In case of organic solvents and co-solvents, solubility in aqueous phase, dielectric constant, molecular weight, density etc., are considered critical for the selection. The performance of reverse micellar system components is interdependent and hence selection of components needs to be done carefully. The water content (W_o) and the size of the reverse micelle formed mainly depend on the surfactant-solvent-co-solvent combinations used. The selection of reverse micellar system components is also influenced by the characteristics (surface charge, iso-electric point, pH stability, titration behavior etc.) of the targeted biomolecule. The following sections discus various components of the reverse micellar system and some of their characteristics.

2A.2. Surfactants

Surfactant molecules possess both hydrophilic (head) and hydrophobic (tail) parts (Fig. 1B.1). The head and tail portions of the surfactant molecule are two distinct chemical groups:

(i) The head which is hydrophilic (water loving)

The hydrophilic head can be simple charged groups, such as carboxylic groups in soaps, or can have a complicated structure as in lecithins or can be uncharged apolar groups as polyoxyethylene part in many nonionic surfactants.

(ii) The tail which is hydrophobic (water hating)

The hydrophobic tail may consist of a single chain (alkyl chain in soaps) or up to four chains (quaternary ammonium salts). The formation of surfactant aggregates of spherical structure depends on the length of the hydrophobic tail.

Surfactant plays a key role in the separation process as the surfactant layer prevents protein/enzyme molecules from coming in contact with the organic solvent. They adsorb spontaneously at interfaces and separate the non-polar and aqueous phases thus decreasing the interfacial tension down to very low values (10^{-2} mN m^{-1}). On the other hand, excess of surfactant molecules will cause poor phase separation.

2A.2.1. Classification of surfactants: The surfactants could be classified into the following three categories based on their properties [Harikrishna *et al.,* 2002].

i) Based on the head group charge

Based on the charge of the head group it can be classified as anionic, cationic, nonionic and zwitterionic (amphoteric). The surface-active portion of the molecule (head) bears a negative and positive charge in case of anionic and cationic surfactants, respectively. Nonionic surfactant does not bear any apparent charge, while the surface-active portion of the zwitterionic molecule bears both positive and negative charge. Table 2A.1 lists the commonly used surfactants belonging to the above categories. Selection of suitable surfactant for the extraction could be made based on the charge of the surfactant to have an electrostatic interaction (driving force for extraction) with the oppositely charged solute. Anionic and cationic surfactants could be used below and above the isoelectric point (pI) of the solute, respectively, to have the electrostatic interaction with the solute.

ii) Based on the solubility in aqueous phase

Amphiphilies differ greatly in the relative balance between their hydrophilic and hydrophobic moieties, which are reflected by their behavior in water, and

provides a basis for their classification as soluble amphiphiles (surfactants/detergents) and insoluble amphiphiles. The surfactants used for the reverse micellar extraction should have low solubility in aqueous phase and fairly high solubility in the organic solvent to form stable reverse micelles. The HLB value of a surfactant gives an indication about the relative solubility of surfactant in water and organic solvent. In the HLB scale of 1-20, lower (1-10) and higher (above 10) values of HLB indicate lower and higher solubility of surfactant in aqueous phase, respectively [Sanchez-Ferrer *et al.,* 1994].

iii) *Based on the formation of lyotropic mesomorphism*

The classification is made as surfactants which display lyotropic mesomorphism (i.e. they can form liquid crystals at high concentration) and those which do not display lyotropic mesomorphism (due to the presence of bulky and complicated cyclic/aromatic hydrocarbons).

2.A.2.2. Hydrophilic-lipophilic balance (HLB): It is a relative measure of surfactants' hydrophobicity. The HLB has to be within a narrow range (typically 8 to 11) for the surfactant to form water-in-oil microemulsions. Properties of surfactant with relation to the HLB value are presented in Table 2A.2. The HLB of the surfactant is a function of surfactant head group, number of ethyl group per surfactant molecule and structure of the surfactant. The HLB value of some of the commonly used cationic surfactants was reported to be increasing in the following manner; TOMAC<DTAB<BDBAC<CTAB [Krie and Hustdet, 1992]. At constant values of temperature, solvent properties and surfactant head group, an increase in HLB of a surfactant was reported with a decrease in the number of alkyl chains per surfactant molecules.

In the present study cationic surfactant (CTAB), anionic surfactant (AOT), and nonionic surfactants (Triton-X-100 and Tween 85) have been used. The properties and structure of these surfactants are presented in Table 2A.3 and Fig. 2A.1, respectively.

2A.3. Critical micelle concentration

A phenomenological observation for most surfactant systems is the existence of critical micelle concentration (CMC). It is the minimum concentration of surfactant necessary for the formation of reverse micelles, and represents the concentration of free or unaggregated surfactant in equilibrium with the reverse micelles. The CMC is a characteristic of the system and dependent on the operating temperature and pressure, type of solvent used and structure of surfactant [Kadam 1986]. The critical micelle concentration (CMC) represents the fundamental surfactant concentration needed for the self-aggregation of amphiphilic molecules in solution [Li *et al.,* 2004] and probably is the simplest way of describing the colloid and surface behavior of a surfactant solution. The CMC is not always a single value, and it is more convenient to express a CMC range rather than a single value. The properties used in determining CMC are surface tension, optical turbidity, electrical conductivity, osmotic coefficient, density, sound velocity, diffusion, viscosity, solubilization, ultracentrifugal sedimentation and NMR chemical shifts. These physicochemical properties of a solution containing surfactant change abruptly when CMC is exceeded. This is due to the fact that the properties of a solution depend upon whether the surfactant molecules are dispersed as monomers or micellar aggregates [McClements, 1999]. Usually, the selected property or some function of a surfactant is plotted against amphiphile concentration or some function of it. Then the intersection of two lines (indicating different dependency) in a plot of surfactant concentration versus the measured property could be taken as CMC [Li *et al.,* 2004]. Fluorescence spectroscopy was utilized to determine the CMC values of anionic surfactants by external and internal probes and values were compared with that obtained by surface tension method [Goon *et al.,* 1997]. The water content of the organic phase was used to determine the CMC value by Naoe *et al.* (1999). Ghosh *et al.* (2001) measured the CMC of surfactant mixtures in aqueous medium using surface tension as the property. Subramanian *et al.* (2001) used optical density to determine the CMC value of phosphatidylcholine and surfactant mixture in high oleic sunflower oil and crude soyabean oil, respectively. In the same study, surface tension of the oil-

surfactant mixture was also used to determine the CMC and it resulted in values similar to that obtained from optical density measurement. The change in fluorescence was used as criteria to determine the CMC value of sodium cholate micelles at different temperatures [Sugioka *et al.,* 2003]. Kelley and McClements (2003) used the change in enthalpy of the buffer solution with the addition of surfactants to identify the CMC value of DTAB and SDS surfactants. Change in aqueous phase pH as a function of surfactant concentration was used to determine the CMC value of sodium dodecyl sulfate (SDS) and dodecyltrimethylammonium bromide (DTAB) by Bayrak (2003). Although, the trend of the change in aqueous phase pH values was not identical for these surfactants, appreciable change was observed in the vicinity of CMC. Recently, Li *et al.* (2004) reported the development of Resonance Rayleigh Scattering (RRS) technique to determine the CMC of surfactant. This technique was used to determine the CMC of Triton-X-100 in aqueous solution and β-cyclodextrin solution and the values obtained agreed closely with the values reported in the literature. The specific conductivity of surfactant was also used as the property for the determination of CMC of dodecyldimethylbenzylammonium (C12BBr) bromide in aqueous solution [Perez *et al.,* 2004].

Although, it is reported that the CMC value of surfactant does not change appreciably for different solvents, it is recommended to determine this value for a given surfactant-solvent system to select the surfactant concentration for extraction. Hence, in the present study, the CMC values of surfactants used (AOT, CTAB, Triton-X-100 and Tween 85) were determined. The properties such as optical density, conductivity or turbidity (% haze) of the reverse micellar phase (organic phase) were used as criteria for the determination of CMC.

2A.3.1. Materials and Methods

2A.3.1.1. Materials

Surfactants AOT, CTAB, Tween-85 and Triton-X-100 were obtained from Rohm and Haas, USA, Merck, Germany, Fluka chemicals, Switzerland and BDH

laboratory, UK, respectively and used without further purification. Organic solvents Toluene (UV spectroscopy) and Iso-octane (HPLC grade) were obtained from Loba Chemie Pvt. Ltd., Mumbai and Merck, India, respectively. Co-solvents Hexanol and n-butanol were obtained from SRL Loba chemicals, India. Various buffers used were Glycine–HCl, acetic acid–sodium acetate, phosphate, and glycine-NaOH. The buffer concentration was 50 mM in all the systems. All other reagents used were of analytical grade.

2A.3.1.2. Method

Preparation of reverse micellar system

Known quantity of surfactant was dissolved in respective organic solvent (5 ml) by mixing for 3 min using a magnetic stirrer (Cintex, Mumbai, India) at 500 rpm. The above organic phase was contacted with an equal volume of aqueous phase (buffer of known pH and salt concentration) for 15 min at 500 rpm followed by centrifugation (MP 400 R, Eltek, Mumbai, India) at 3000 *g* for 10 min. The phases were separated and organic phase was used for the analyses. For all the surfactants studied, the concentration range was selected below and above the reported CMC value. The organic solvents (isooctane and toluene) used in RME experiments were employed for the present study.

2.A.3.1.3. Analyses

i) Conductivity

Conductivity of the organic phase containing reverse micelles was measured using a conductivity meter (DI 290, Digisun, India). The conductivity was expressed as μS/cm. abrupt change in the conductivity value was taken as the CMC value.

ii) Optical density

The optical density of the organic phase was measured using a UV-Visible Spectrophotometer (Shimadzu UV-160, Japan) at a known wavelength. The point of interception of the tangent drawn to the curve was taken as the CMC value.

iii) Haze (turbidity)

The haze of the solution was measured using UV-Visible Spectrophotometer (Shimadzu UV-160, Japan) attached with MPC 3100 compartment and expressed as percentage haze. Abrupt change in the haze value was considered as the CMC value.

2A.3.2. Results and Discussion

2A.3.2.1. Anionic surfactant-AOT

i) Conductivity

AOT concentration in isooctane was varied in the range 1 to 8 mM. The conductivity increased with an increase in AOT concentration (ionic surfactant) up to a concentration and then dropped drastically (Fig. 2A.2). It is assumed that the increase in the concentration of charged surfactant molecules (monomer state) increases the conductivity below the CMC level. And above CMC, the clustering/aggregation of the surfactant molecules takes place to form reverse micelles with charged surfactant heads towards the polar core, thereby reducing the availability of charge of the ions for contributing to conductivity. This might have led to a drastic reduction in the conductivity values. A sudden change in conductivity values was observed at surfactant concentration of 5 mM, which was taken as CMC. The value obtained was close to the reported CMC value of 4.9 mM for AOT [De and Maitra, 1995]. The conductivity remained the same above the CMC value and was found to be close to the conductivity (0.18 μS/cm) of isooctane (solvent).

The CMC value of a given surfactant was reported to be also dependent on the solvent used, although, it does not vary drastically. To study the affect of solvent, studies were carried out with organic solvent toluene (Fig. 2A.3). The trend was found to be similar to the one observed with AOT/isooctane system. However, for the given AOT concentration, the conductivity values were found to be much lower than that of AOT/isooctane system. The abrupt drop in conductivity was observed at 4 mM and was taken as the CMC value. The value was found to

be slightly lower than the reported CMC value as well as the value obtained for isooctane system.

ii) Optical density

In the spectrophotometric method, the optical density was measured at 216 nm [Zhang *et al.*, 2000] and plotted against the surfactant concentrations (Fig. 2A.4). The point of intersection of tangents drawn to the curve was considered as the CMC value [Subramanian *et al.*, 2001]. The CMC value obtained was slightly higher (5.2 mM) than the reported value of 4.9 mM.

2A.3.2.2. Cationic surfactant-CTAB

i) Conductivity

The experiments were carried out with CTAB/isooctane system (Fig. 2A.5). The concentration of CTAB was varied from 0.2 to 1.6 mM. The trend observed was similar to that of AOT. The conductivity dropped abruptly close to a concentration value of 0.8 mM and remained almost the same. The above value is close to the reported CMC value (0.83) for CTAB [Myers, 1991].

ii) Optical density

A plot of optical density measured at 207 nm for different concentrations of CTAB in isooctane was used for the estimation of CMC value (Fig 2A.6). This wavelength (207 nm) was selected based on the spectrum obtained (peak value) in UV-Visible range for different CTAB concentrations during preliminary studies. The CMC value obtained from the above plot as the point of intersection of two tangents drawn to the curve was found to be1.30 mM, which was slightly higher than the reported value (0.83).

Although, no definite trend was observed with the two methods employed for the estimation of CMC for ionic surfactants, it has shown that both the methods could be used for the estimation of CMC value with fairly goof degree of accuracy.

2A.3.2.3. Non-ionic surfactant Triton-X-100 and Tween 85

i) Conductivity

Similar to ionic surfactants, the conductivity of nonionic surfactant Triton-X-100 in organic solvent toluene was measured. The conductivity did not show any change (Fig. 2A.7) over the range of Triton-X-100 concentrations (0.05 mM to 0.6 mM) studied and the value was same as that of blank solvent (toluene). Absence of surface charge on the surfactant head groups could be the reason for the constant conductivity values at different surfactant concentrations. Similar results were obtained (Fig. 2A.8) with another nonionic surfactant Tween 85 in organic solvent toluene for the concentration range (0.056 mM to 1.12 mM) studied. Kaushik *et al.* (2007) observed no change in specific conductivity in case of Triton-X-100/cyclohexane system until appreciable water was added to the system and with further increase in water addition, drastic increase in conductivity was observed. In this study it was inferred that the main barrier to the conductance was the interface between the aqueous phase and the bulk phase resulting in lower conductivity initially. The increase in conductivity on further addition of water was attributed to the increased amount water in the core of reverse micelles. However, in the present study, no such trend was observed even though the aqueous phase (excess) was in contact with the organic phase. Rodriguez *et al.* (1998) also reported that with dry Triton-X-100/toluene solutions no CMC could be measured.

ii) Optical density `

The optical density values for Triton-X-100 and Tween 85 were measured at 287 nm [Li *et al.,* 2004] and 280 nm [Bollag *et al.,* 1996], respectively (Figs. 2A.9 and 2A.10). The CMC values obtained for Triton-X-100 from the plot was 0.2 mM, which is close to the reported CMC value of 0.25-0.30 mM [Black, 1998] and 0.13 mM [Dow Chemicals catalogue]. In case of Tween 85, a wide variation in the CMC value has been reported. The reported values are 0.29 μM [Hsu and Nacu 2003], 1.0 mM [Capek, 2005], and 0.464 mM [Yeh *et al.,* 1999]. In the present study, the CMC value of 0.6 mM was obtained, which lies between the values reported by Yeh *et al*. (1999) and Capek, (2005).

iii) Haze measurement

The change in solution turbidity or haze could be used as a tool to determine the CMC value. Different concentrations of Triton-X-100 in distilled water were prepared and the optical density values measured at 610 nm were reported as percent haze (Fig. 2A.11). Initially, a gradual rise in haze values was observed with an increase in Triton-X-100 concentration. The value increased sharply at a particular concentration of Triton-X-100. The CMC value obtained from the above plot was 0.25 mM, which is very close to the reported CMC value (0.24 mM) for Triton-X-100. Above CMC, the increase in haze values was marginal. Bayrak (2003) observed that starting at the CMC, the solution's light scattering ability (turbidity) rises sharply. It was reported that, above CMC, a substantial portion of the solute ions are aggregated to form units of colloidal size, which increase the turbidity. Li *et al.* (2004) also reported an increase in light scattering ability of surfactant aggregates as compared to monomers and attributed it to the higher ability of spherical shaped reverse micelles to scatter light.

2A.4. Solvents

Organic solvent occupies about 80-90% of the reverse micellar phase. Water solubilization capacity of the reverse micelles, for a given surfactant, is strongly dependent on the type of the solvent used. Lang *et al*. (1991) observed that micellar size decreased with an increase in the molecular size of the solvent. According to the above study, as the molecular size of the solvent increases their ability to penetrate into the interfacial surfactant layer decreases thereby increasing the inter-micellar attraction between surfactant tails, and reducing the size of reverse micelles. In other words, an increase in solvent molecular size reduces the contribution of the entropy of dispersion favoring the formation of smaller micelles. In one of the studies reported [Harikrishna *et al.,* 2002], maximum water solubilization (water content, W_0=65) was obtained with n-heptane/AOT reverse micellar system. The water content value decreased significantly (W_0=5) when a solvent of higher carbon number such as hexadecane replaced n-heptane. Mutalik and Gaikar (2003) demonstrated the need for the selection of organic solvents during the study on the selective permeabilization of *Escherichia Coli* cells to extract penicillin acylase. Solvents commonly used for RME studies are given in Table 2A.1. The physicochemical properties of the solvents used in the present study are given in Table 2A.4.

2A.5. Co-solvents

Co-solvent (also termed as co-surfactant) is a type of solvent, which can help surfactants to get dissolved in organic solvent and form reverse micelles thereafter. The properties such as dielectric constant, molecular weight (long-chain/short chain) and water solubility are some of the factors, which determine the suitability of a co-solvent. The exact mechanism by which the co-solvent helps the reverse micelles to form is still not clear. However, it has been suggested that co-solvent molecules get inserted between surfactant molecules and lower the strong repulsive ion-ion interaction between the surfactant head groups, there by allowing their close packing to form the inner core of reverse micelle. Hence, generally, not-so-short chain alcohols (n-pentanol, n-hexanol, n-heptanol, n-

octanol and n-decanol), which can penetrate to a greater depth, are found to be more effective. Also, the co-solvents change the dielectric constant of the solvent favorably, i.e. enhance the dielectric constant to facilitate the transfer of solute. The type of co-solvent used also plays a major role in deciding the extraction efficiency. Chang and Chen (1995) and Chang *et al.* (1997) observed that among the various n-alcohols tested, n-butanol was the best suited co-solvent for Aliquat 336 reverse micelles. Co-solvents with low solubility in water are to be used for the formation of reverse micelles. According to Krei *et al.* (1995) the co-solvents acts by increasing the solubility of the surfactant in organic phase. Hong *et al.,* (2000) studied the effect of addition of alcohol to improve the back extraction efficiency of proteins. Carlson and Nagarajan (1992) reported a drastic improvement in the back extraction efficiency with the addition of co-solvent isopropyl alcohol (10-15%). Lee *et al.,* (2005) showed that with an addition of small amount of alcohol, Bovine carbonic anhydrase can be back extracted completely without the need for high salt concentration. A review of the work reported on the role of solvents, co-solvents in controlling the reverse micellar size has been presented by Eastoe *et al.* (2006). In another review, the role of alcohols in the back extraction of various proteins/enzymes from reverse micellar phase has been highlighted [Mathew and Juang, 2007]. Some of the properties of co-solvents used in the present study are given in Table 2A.5.

2A.6. Equilibrium phase diagrams

The solubilization properties of surfactants are often expressed by a three-or-four-component phase diagram, after determination of regions of optical transparency. From these diagrams, it is easy to calculate the volume of each component and mix the aqueous buffer solution with organic solvent having surfactant in order to obtain water-in-oil emulsion. Figure 2A.12 depicts the phase diagrams of AOT/isooctane/water [De and Maitra, 1995] and CTAB/isooctane/water [Hilhorst *et al.,* 1984] showing the reverse micellar phase region. The phase diagrams will be very useful in determining the composition of new reverse micellar systems and also in obtaining a transparent organic phase

containing reverse micelles in the case of injection method of RME. In the present study, the composition of the reverse micellar system was selected based on the literature reports and also by preliminary trials for obtaining transparent phase (reverse micellar phase).

Table 2A.1. Commonly used surfactants, solvents and co-solvents for RME

➢ SURFACTANTS
● *Anionic*
Sodium bis (2-ethyl-1-hexyl) sulfosuccinate (Aerosol OT, AOT); sodium do-decylbenzene sulfonate (SDBS); sodium di-2-ethyl hexyl phosphate (NaDEHP); dioleyl phosphoric acid (DOLPA); di (tridecyl) phosphoric acid (DTDPA); sodium dodecyl sulfate (SDS); 1,3-dilauryol glycerol-2-disodium phosphate (2-modified 1,3-diacyl glycerol)
● *Cationic*
Cetyltrimethyl ammonium bromide (CTAB); dodecyltrimethyl ammonium bromide (DTAB); tetradecyltrimethyl ammonium bromide (TTAB); trioctyl-methyl ammonium chloride (TOMAC); N-benzyl-N-benzyl-N-dodecyl-N-bis (2-hydroxyl ethyl) ammonium chloride (BDBAC); cetyl pyridinium chloride (CPC); quaternary ammonium salt with carbon atoms of R ranging from 8-10 (CH_3 R_3 N^+ CL^-) (Aliquat 336)
● *Nonionic*
Polyoxyethylene sorbitan trioleate (Tween 85); sugar ester (DK-F-110); tetraoxyethylene monodecyl ether ($C_{10}E_4$); polyoxyethylene-p-t-octyl phenol (Triton X-100) pentaethylene glycol dodecyl ether, Tergito NP-4, Rewopal HV5, Span 60
● *Zwitterionic*
Soybean lecithin, Betaine
➢ SOLVENTS
C_7-C_{15} n-alkanes; isooctane; n-decane; carbon tetrachloride; cyclohexane; octane; n-heptane; n-hexane; hexadecane
➢ CO-SOLVENTS
Isopropyl alcohol; butanol; hexanol; octanol; decanol; pentaethylene oxide

[Kilikian *et al.,* 2000 ; Harikrishna *et al.,* 2002)

Table 2A.2. HLB values and properties of surfactants

HLB Value	Properties
<10	Oil soluble
>10	Water soluble
4-8	Antifoaming agent
7-11	Water-in-oil emulsifier
12-16	Oil-in-water emulsifier
11-14	Wetting agent

(FAQs, www.dowchemicals.com)

Table 2A.3. Physicochemical properties of surfactants used in the study

AOT { Sodium bis (2-ethyl-1-hexyl) sulfosuccinate }		
Surfactant Charge	:	Anionic
Molecular weight (g/mol)	:	444
CMC (mM)	:	4.90
Cetyltrimethylammonium bromide (CTAB)		
Surfactant Charge	:	Cationic
Molecular weight (g/mol)	:	355
CMC (mM)	:	0.83
Triton-X-100 (polyoxyethylene p-t-octylphenol)		
Surfactant Charge	:	Nonionic
Molecular weight (g/mol)	:	613
CMC (mM)	:	0.25-0.30
HLB	:	13.4
Tween-85 (Polyoxyethylene sorbitan trioleate)		
Surfactant Charge	:	Nonionic
Molecular weight (g/mol)	:	1870
CMC (mM)	:	2.4×10^{-4} to 0.464
HLB	:	11.0

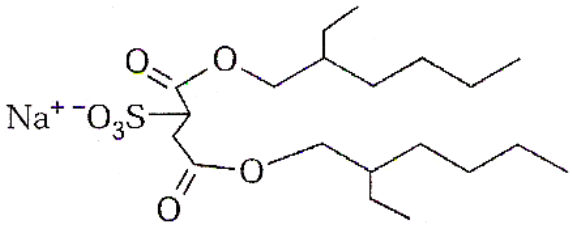
Table 2A.4. Physicochemical properties of solvents used for RME

Isooctane		
Molecular weight (g/mol)	:	113
Density (g/cm^3)	:	0.69
Dielectric constant	:	1.96
Miscibility with water	:	Immiscible
Toluene		
Molecular weight (g/mol)	:	92
Density (g/cm^3)	:	0.86
Dielectric constant	:	2.37
Miscibility with water	:	Low
Hexane		
Molecular weight (g/mol)	:	86
Density (g/cm^3)	:	0.68
Dielectric constant	:	1.90
Miscibility with water	:	Immiscible

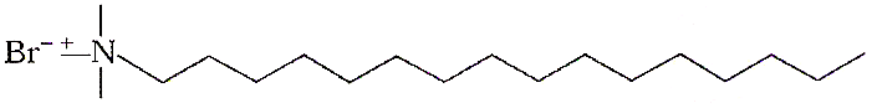
Table 2A.5. Physicochemical properties of co-solvents used for RME

Hexanol		
Molecular weight (g/mol)	:	102
Density (g/cm^3)	:	0.82
Dielectric constant	:	12.5
Miscibility with water	:	Low
Butanol		
Molecular weight (g/mol)	:	74
Density (g/cm^3)	:	0.80
Dielectric constant	:	17.7
Miscibility with water	:	Low
Isopropyl alcohol		
Molecular weight (g/mol)	:	50
Density (g/cm^3)	:	0.78
Dielectric constant	:	13.8
Miscibility with water	:	Low

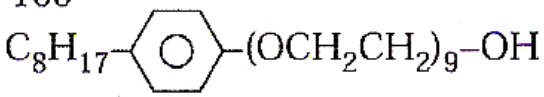
AOT (bis(2-ethylhexyl) sodium sulfosuccinate)



CTAB (cetyltrimethyl ammonium bromide)



Triton X-100



Tween 85

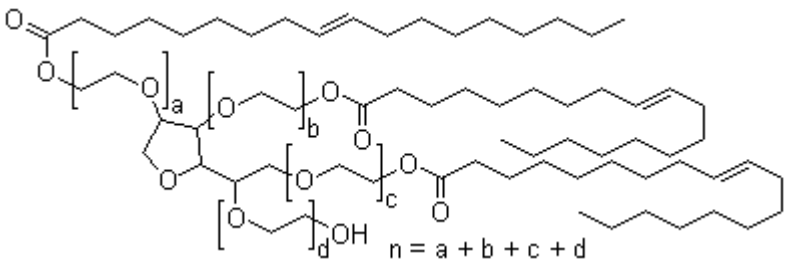


Fig.2A.1 Structure of surfactants used in the study [Kolisis, 1999]

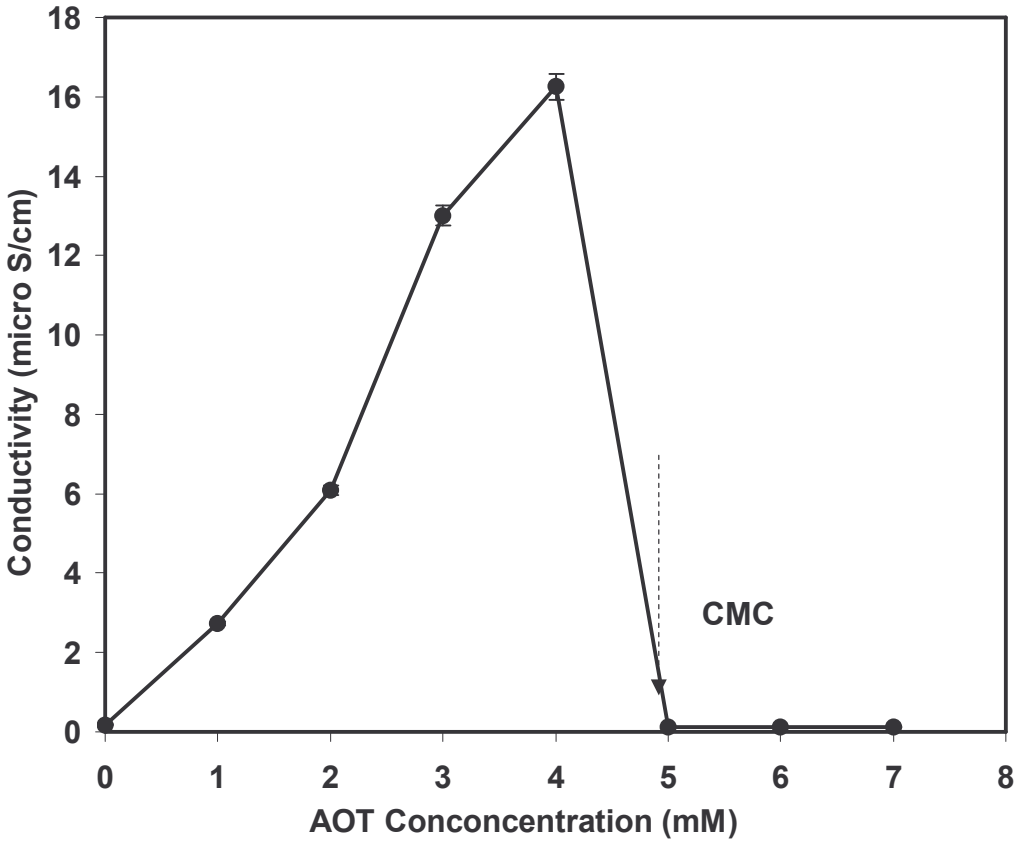


Fig.2A.2. Conductivity of organic phase (AOT/isooctane) at different AOT concentrations

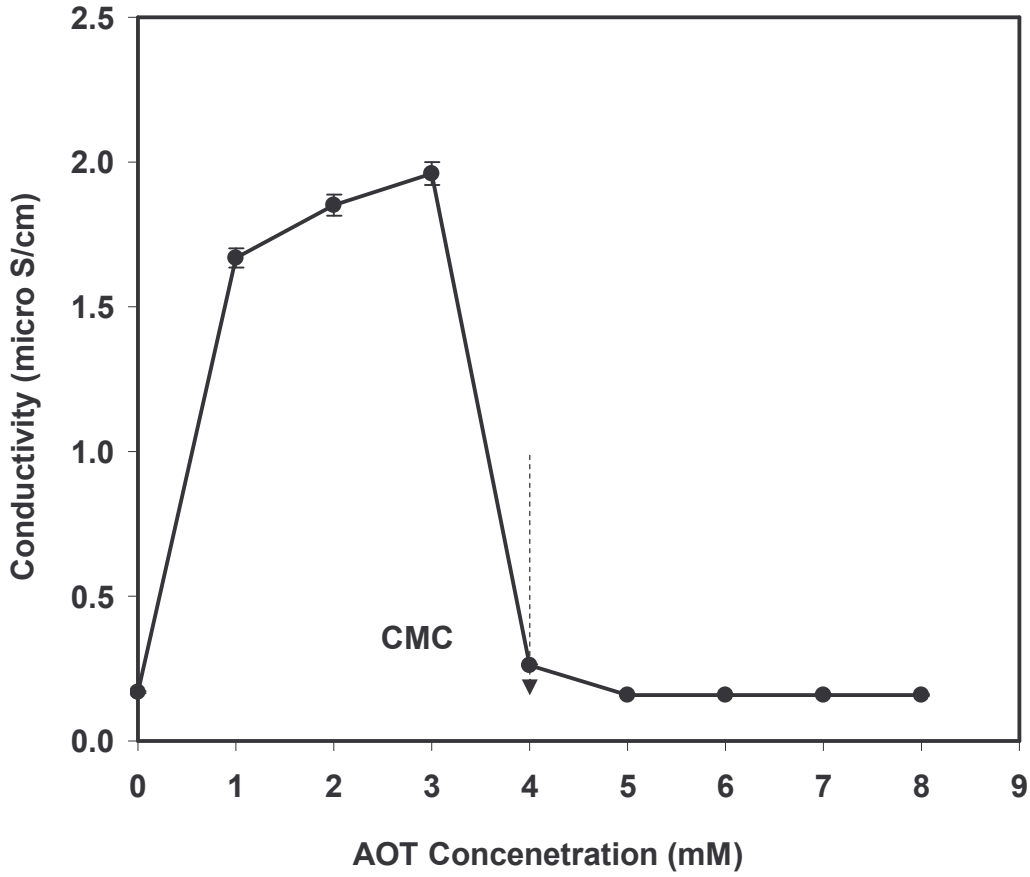


Fig.2A.3. Conductivity of organic phase (AOT/toluene) at different AOT concentrations

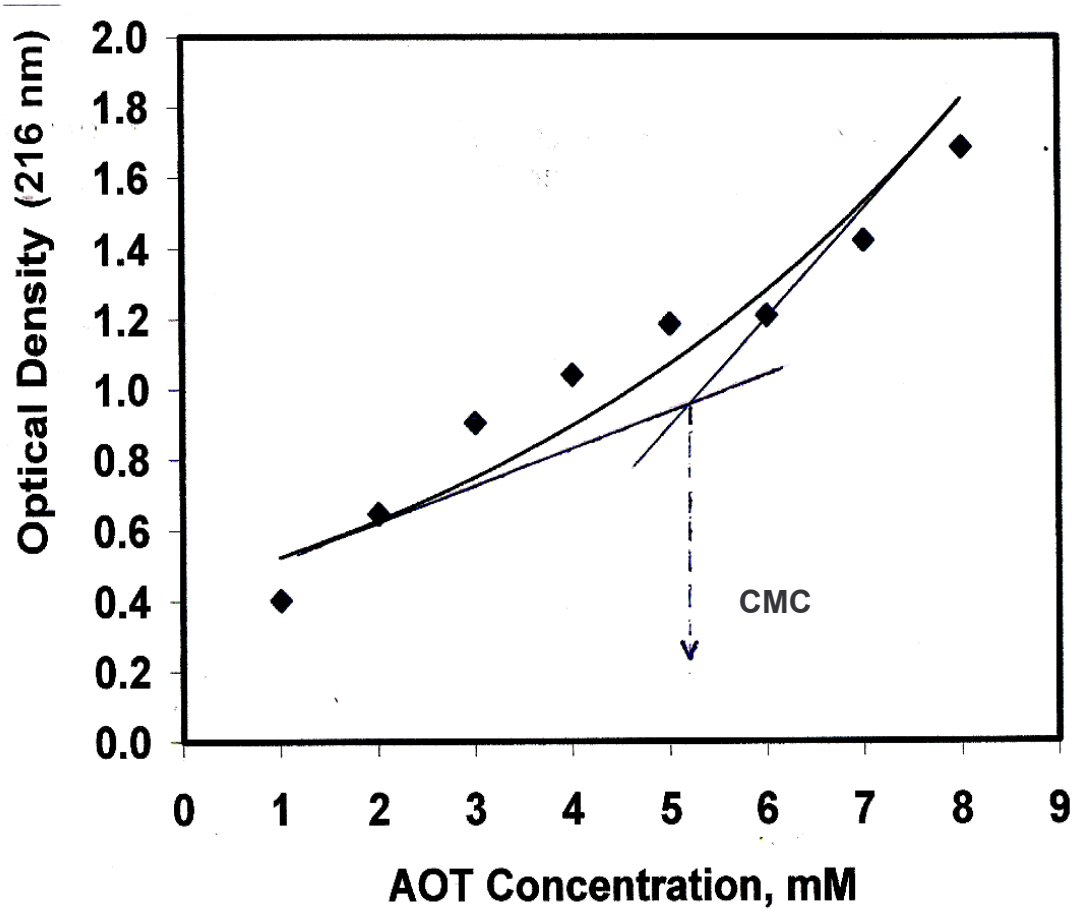


Fig.2A.4. Optical density (216 nm) at different AOT concentrations in isooctane

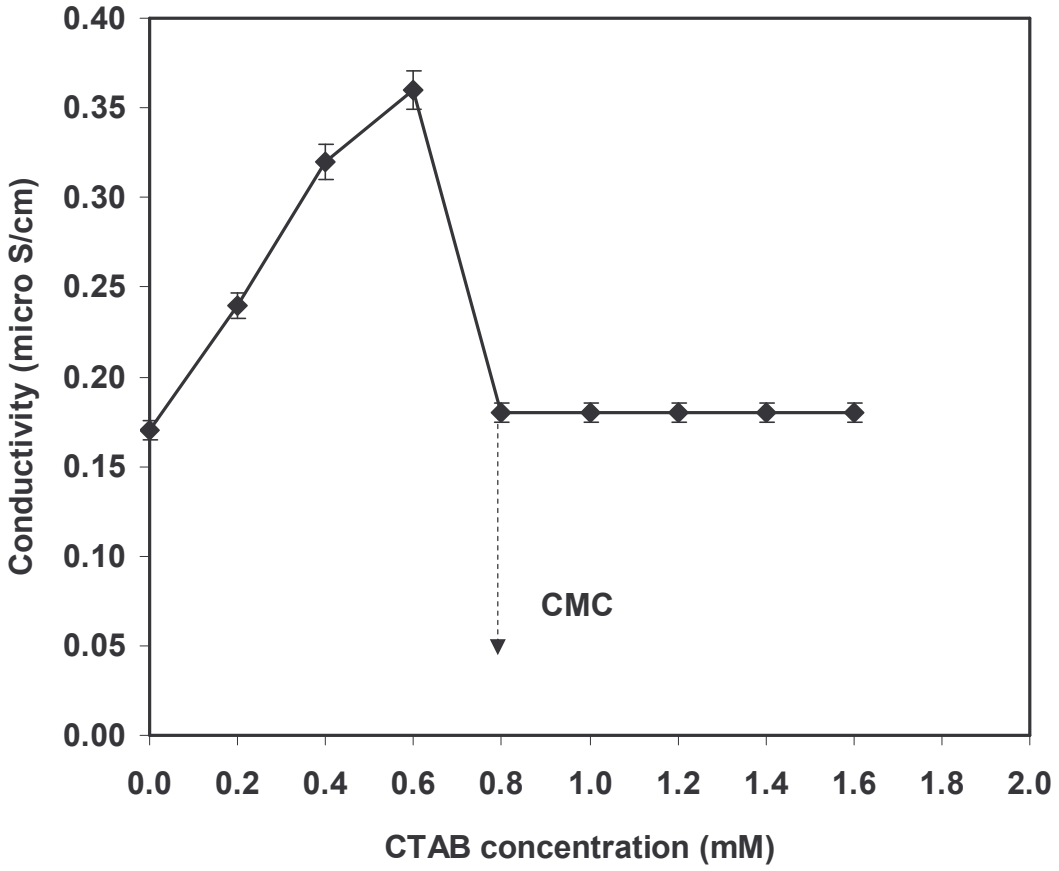


Fig.2A.5. Conductivity of organic phase (CTAB/isooctane) at different CTAB concentrations

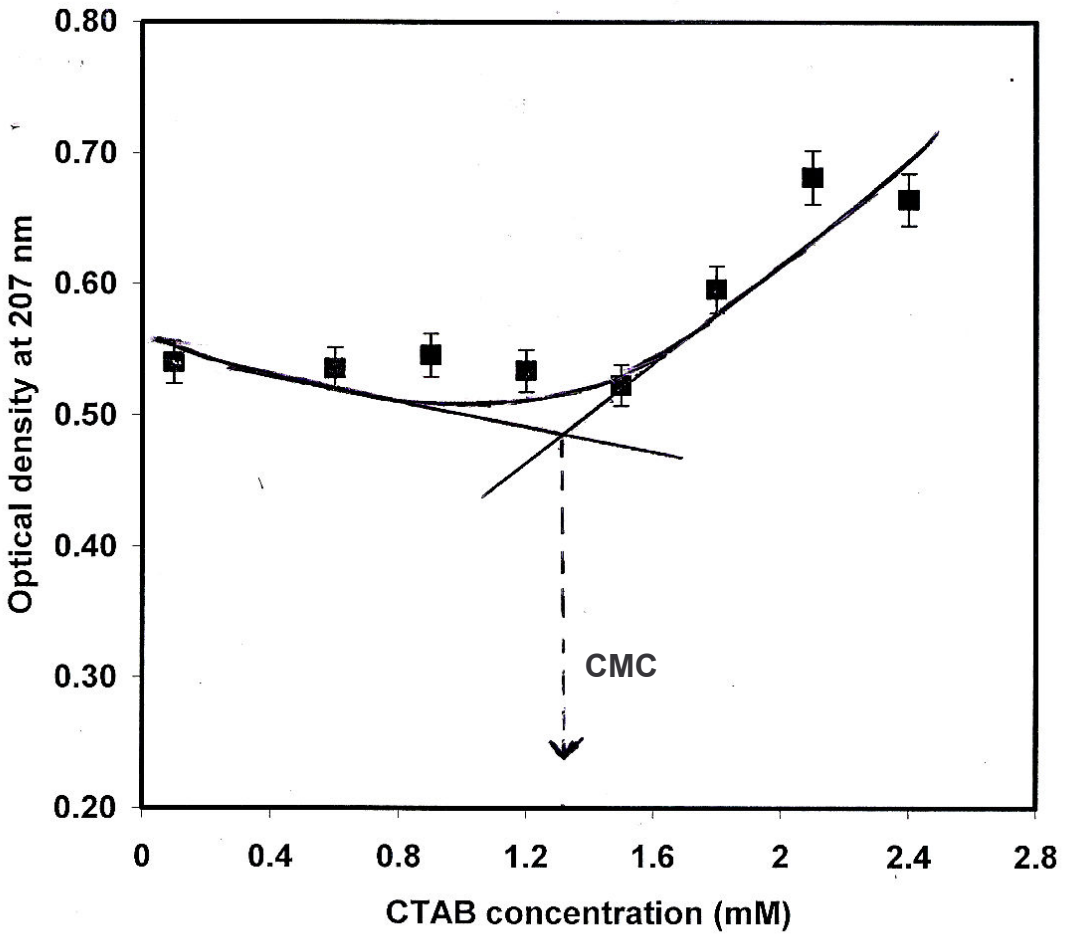


Fig.2A.6. Optical density (207 nm) at different CTAB concentrations in isooctane

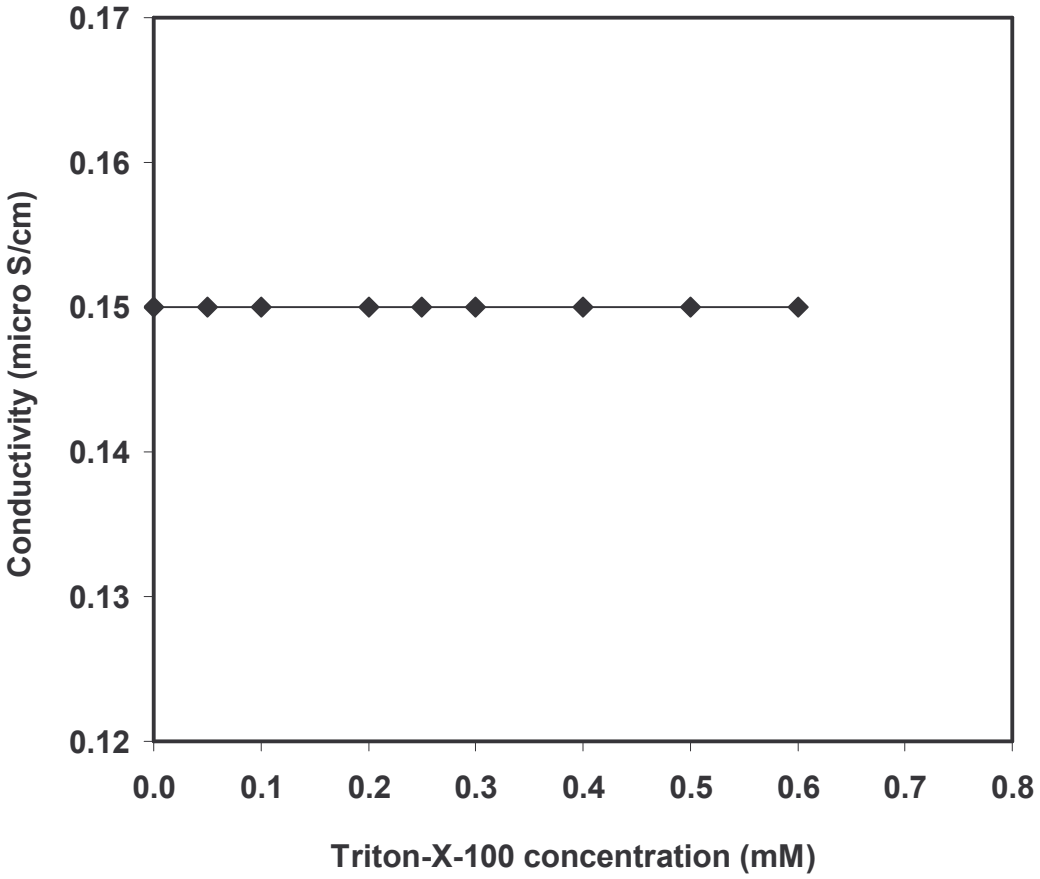


Fig.2A.7. Conductivity of organic phase (Triton-X-100/toluene) at different Triton-X-100 concentrations

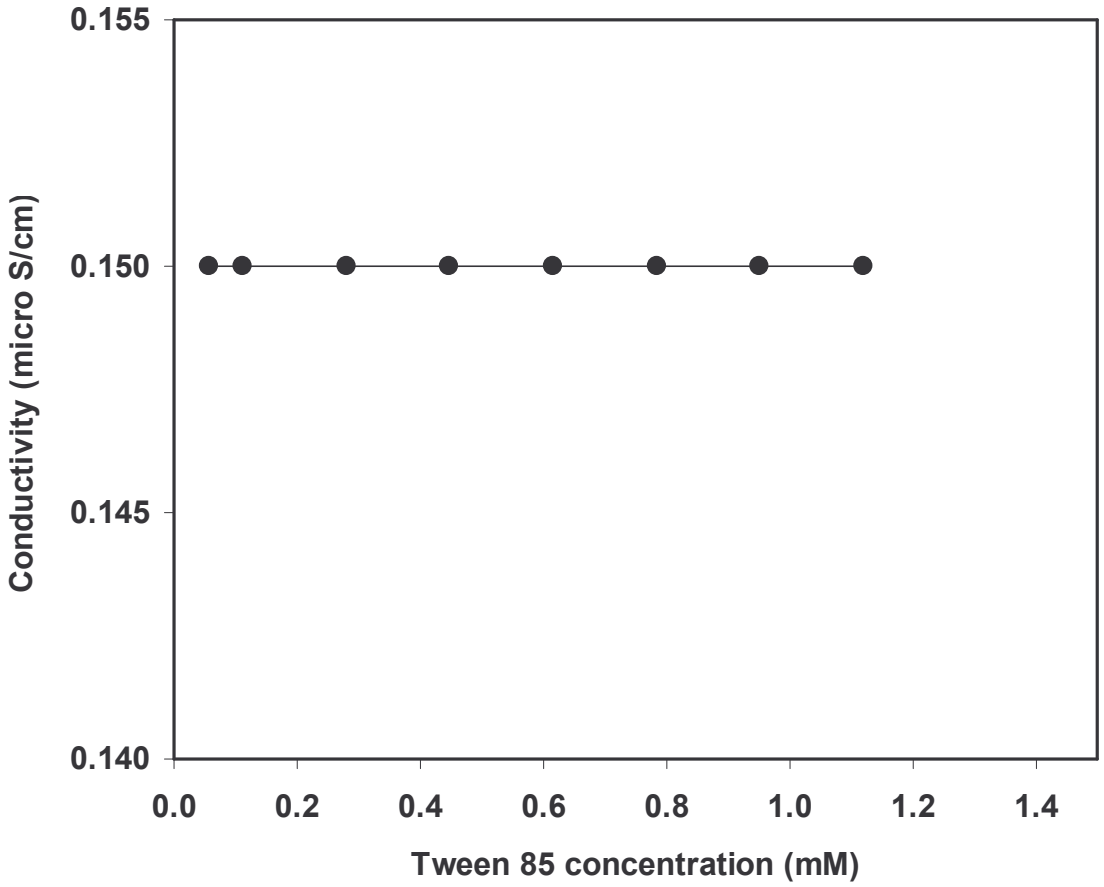


Fig.2A.8. Conductivity of organic phase (Tween 85/toluene) at different Tween 85 concentrations

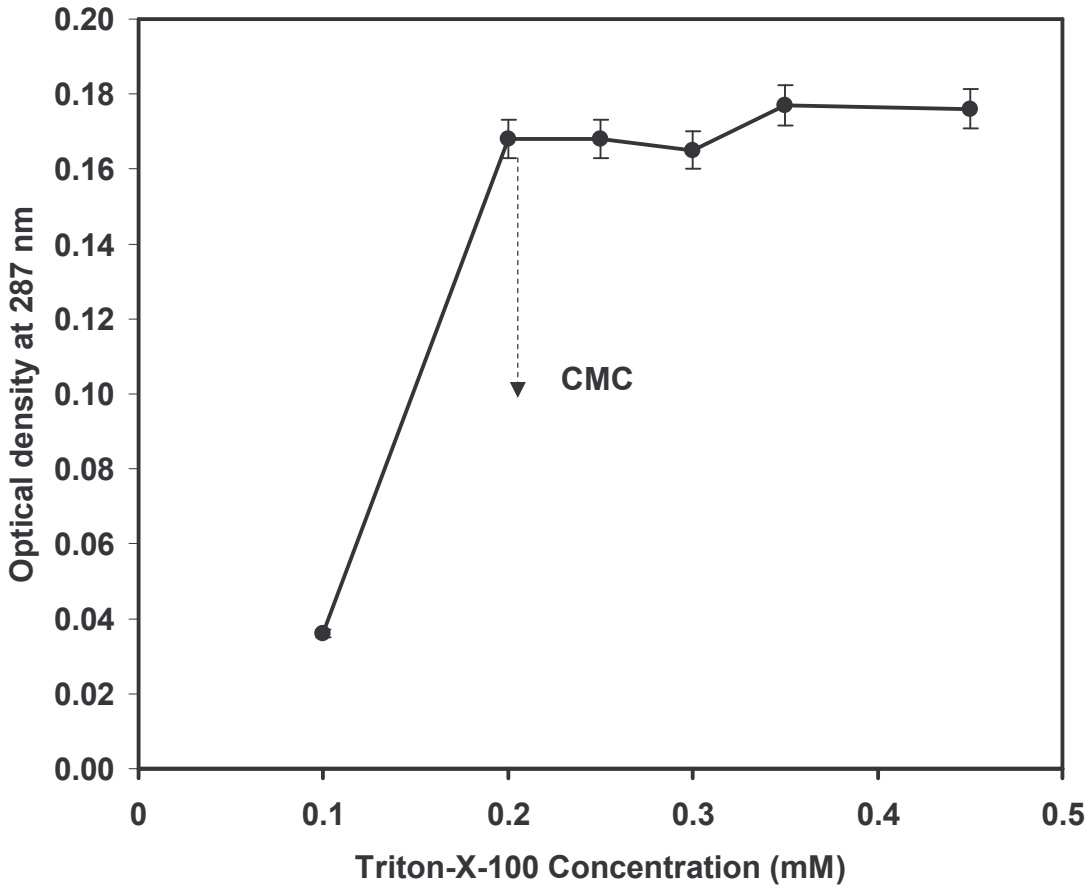


Fig.2A.9. Optical density (287 nm) at different Tritin-X-100 concentrations in toluene

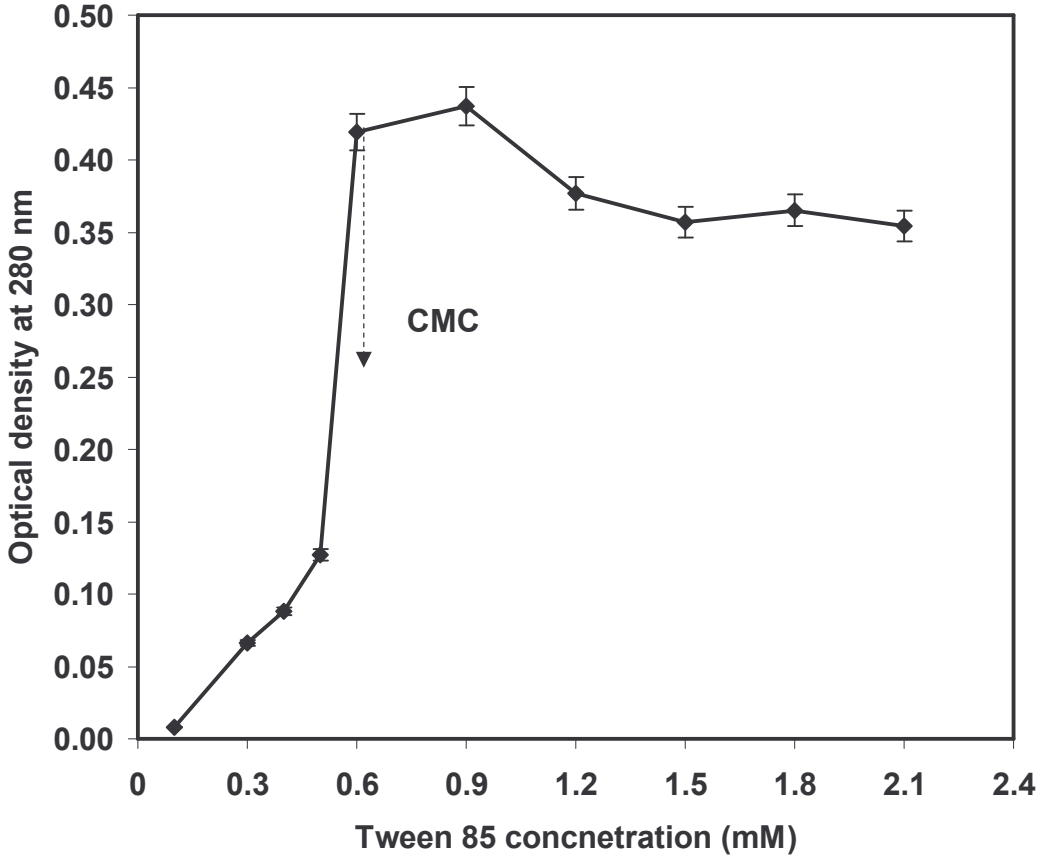


Fig.2A.10. Optical density (280 nm) at different Tween 85 concentrations in toluene

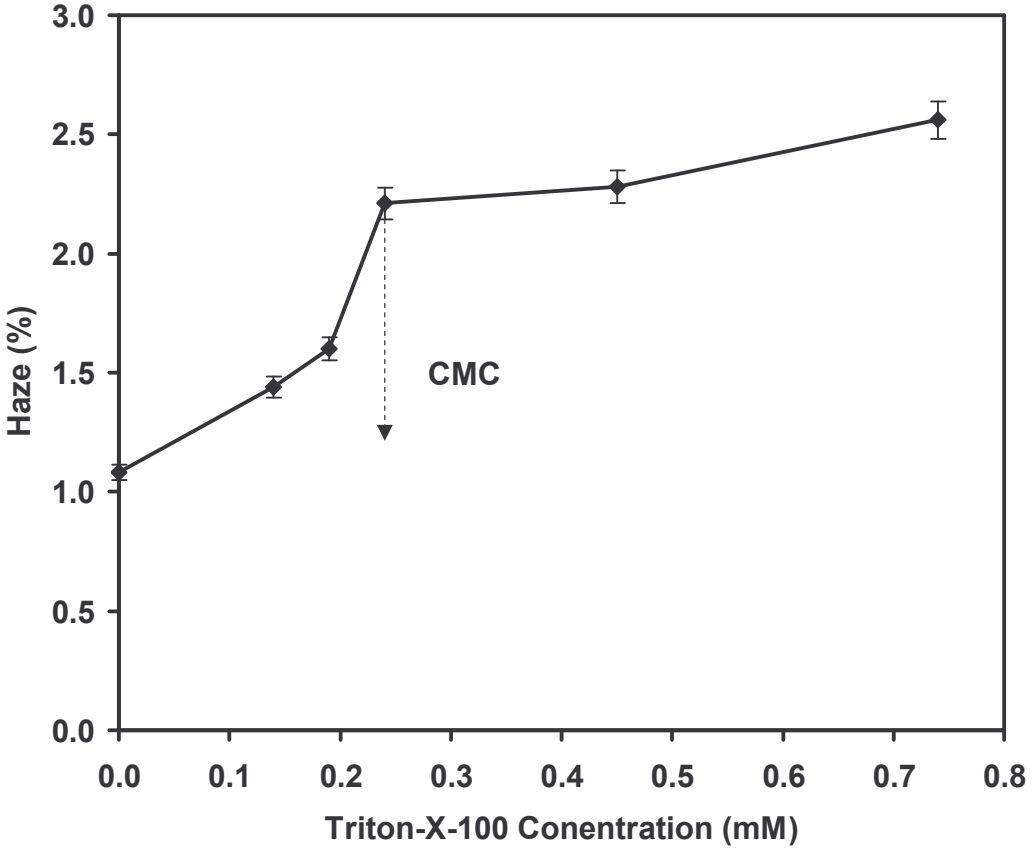


Fig.2A.11. Haze values at different Triton-X-100 concentrations in isooctane

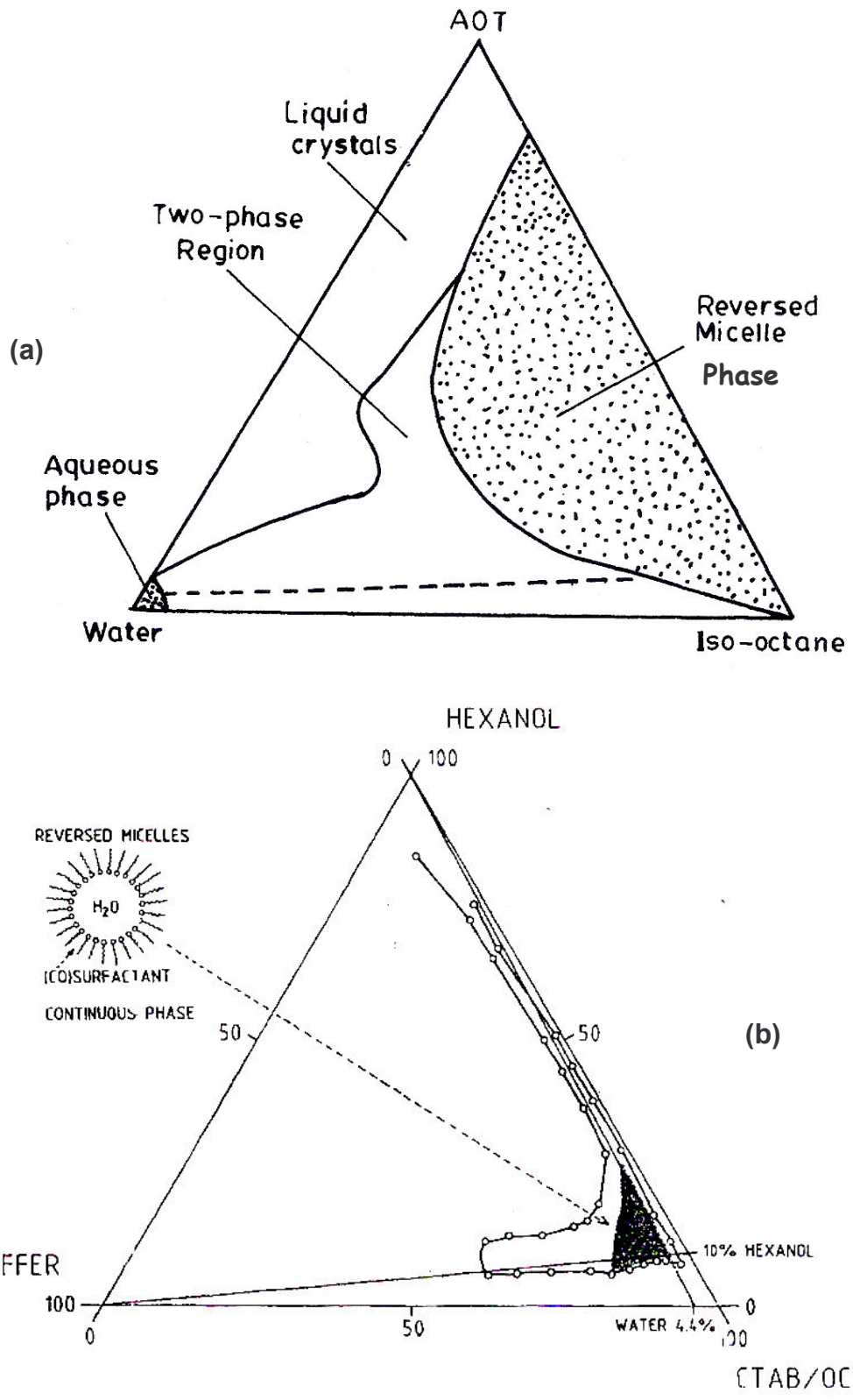


Fig. 2A.12 Equilibrium phase diagram of (a) AOT/isooctane/water [De and Maitra, 1995] and (b) CTAB/isooctane/water [Hilhorst *et al.,* 1984]

SECTION B

STRUCTURAL CHARACTERISTICS

2B.1. Water content of the reverse micelle

The size of the reverse micelle is directly related to the amount of water in the reverse micellar pool. The water content (W_o) of the reverse micelle, defined as the ratio of water molecules to that of the surfactant molecules per reverse micelle [Harikrishna *et al.,* 2002] is used as an index of the reverse micellar size. Increase in water content increases the size of the reverse micelle and W_0 value is being widely used in the estimation of the radius of the reverse micellar core. W_0 strongly depends on the relative solubility of the surfactant in the polar and non-polar solvents, expressed as HLB of the surfactant and it increases with HLB value. A few studies have revealed that W_0 has significant influence on the extraction of solutes during RME. Krei and Hustdet (1995) studied the partitioning behavior of α-amylase in different reverse micellar systems containing cationic surfactants. Below a certain critical water content value (W_0 = 40), extraction was observed to be insignificant. Further it was reported that a high W_0 (~120) was required to achieve almost complete solubilization of insulinase into BDBAC/isooctane/hexanol reverse micelles [Pessoa and Vitolo, 1998]. In general, it was observed that the cationic surfactants form very small micelles (W_0<3) and thus requires the addition of co-surfactants to enhance the water content value [Pessoa and Vitolo, 1998]. Anionic surfactants normally form large micelles (W_0 = 20-115) and does not require the addition of co-solvents.

The nature of the water in the core of the reverse micelle is of great importance since proteins/enzymes and other biomaterials reside in this water pool. The water pool is generally regarded to be a composite of two different types, bound water (lining the interior wall) and free water (remaining). The water entrapped in reverse micelles, particularly the bound water behaves differently from bulk water and is more similar to water present in the vicinity of biological membranes with regard to its physicochemical properties [Luisi *et al.,* 1988]. According to Politi and Chaimovich (1986), an accepted physical model for reversed micelles describes the water in the pool using a two-state model. Water that is close to the interface is very viscous would be in equilibrium with free water,

which exhibits properties similar to that of bulk water. The unusual behavior (depressed freezing point, changed spectroscopic parameters, and restricted mobility) of bound water has been attributed to its strong interaction with the head groups of the surfactant as well as to an overall disruption of the three-dimensional hydrogen-bonded network usually present in bulk water.

2B.2. Size of reverse micelles

The structural characteristics of the reverse micelle, namely, the size and shape vary significantly with the type of surfactant-solvent combinations. There is a general agreement in the literature regarding the existence of pre-micellar aggregates which may adopt the role of nuclei in the framework of the micellar pseudo-phase model. Here, it is hypothesized that initially linear arrangement of pre-micellar aggregates transforms into closed aggregates [Kadam, 1986]. The reverse micelles are smaller than micelles and the surfactant aggregation number is reported to be lower than 50. Although, reverse micelles might form different shapes, in most of the cases it would be spherical in shape and the hydrophobic interactions between surfactant and solvent determine its curvature which, in turn influences its size [Kadam, 1986]. The size of the reverse micelles is dependent on its water content (W_0). The monodisperse small sized reverse micelles can accommodate only proteins of certain dimensions. Hence micelle size may be used to include or exclude the certain proteins. However, it was also reported that several micelles can regroup to form larger micelles when certain operating conditions are altered. It was also hypothesized that a protein can create around itself a larger micelle of a requisite size to facilitate solubilization [Kadam, 1986]. The reverse micelle size is normally expressed in terms of radius of reverse micelle or aqueous (water/hydraulic) core, which is the distance between the centre of the core and the inner surface of surfactant head. The hydrodynamic radius of the reverse micelle would also include the thickness of the surfactant head or it is the distance from centre of the core to the outside edge of the surfactant layer.

Apart from the surfactant-solvent combination, the reverse micelle size is a function of ionic strength of the system, temperature, and surfactant concentration also. The selection of a suitable solvent becomes an important task and several solvents could be tested for the performance. Goklen and Hatton (1987) studied the effect of solvent structure on reverse micelle size and W_0. Hexane, isooctane and octane were reported to have much higher W_0 values (75-115) compared to dodecane, cyclohexane, xylenes, carbon tetrachloride, or chloroform (5-20). As the ionic strength of the system increased the micellar size decreased due to reduction in the electrostatic repulsion between the head groups of the surfactants. This results in the gradual expulsion of the protein molecule from the reverse micelles, which is termed as squeezing-out effect. Increase in temperature is reported to increase the size of reveres micelles [Regalado *et al.,* 1994; Dekker, 1990]. Although, a few studies [Levashov *et al.,* 1982; Matzke *et al.,* 1992] indicated that the size of the reverse micelle is unaffected by the surfactant concentration, many others reported [Bonner *et al.,* 1980; Caselli *et al.,* 1988b; Sheu *et al.,* 1986; Zampieri *et al.,* 1986; Chang *et al.,* 1994; Krei *et al.,* 1995] an increase in the size of reverse micelle.

Several experimental methods and empirical models have been employed to determine the size and shape of micellar aggregates. The radius of aqueous core, which will be of nanometer scale could be measured using instruments such as Dynamic light scattering (DLS), Small angle neutron scattering (SANS), Small angle X-ray scattering (SAXS) or ultracentrifugation [Harikrishna *et al.,* 2002]. The hydrodynamic or stokes radii of non-protein containing reverse micelles can be determined using the following equation [Hayes and Gulari, 1990].

$$R_h = \frac{k\,T}{6\pi\mu D_Z} \qquad (2B.1)$$

Several empirical equations, which use W_0 value for the estimation of the radius of aqueous core were also reported [Sheu *et al.,* 1986; Dekker *et al.,*1991;

Krei and Hustedt 1992; Gaikar and Kulkarni 2001; Kinugasa *et al*., 2003]. Kinugasa *et al.* (2002) measured the size of reverse micelles based on viscosity measurement of the microemulsion. The comparative study indicated a good agreement with the size reported using other methods for the same surfactant/solvent combination.

The equations reported based on geometrical conditions and empirical equation for the estimation of reverse micellar size are given in Table 2B.1.Some of the equations are derived based on the assumption that reverse micelles are spherical in size and the total volume of water in the reverse micelle is equal to that of the reverse micellar core. Several empirical equations have been reported based on the experimental results for the given reverse micellar system.

Table 2B.1 Models reported in the literature for the estimation of radius of reverse micellar core

Geometrical models

S. No.	References	Equation
1.	**Levshavo *et al.,*** (1982)	$R_m = \{\frac{3}{4\pi}(N^0_{ag}\, W_0 V_w)\}^{1/3}$ R_m in Å

Levshaov *et al.* (1982) proposed a model for the estimation of radius of reverse micellar core for unfilled and filled reverse micelles assuming the reverse micelle to be spherical in shape. The aggregation number of surfactants and W_0 values were used for the estimation of size. With the change in water content values for filled and unfilled reverse micelles, the size of the reverse micelle is expected to be different for both.

2.	**Sheu *et al.,*** (1986)	$R_{me} = \left\{\frac{3}{4\pi}\left(N_e^w V_w + N_e^s V_s\right)\right\}^{1/3}$ $R_{mf} = \left\{\frac{3}{4\pi}\left(N_f^w V_w + N_f^s V_s + V_p\right)\right\}^{1/3}$ R_m in nm

The authors have proposed models for the estimation of empty and filled reverse micelles based on the geometrical conditions. The micelles have been assumed to be spherical and the protein was assumed to be located concentrically within the filled micelles. It was also assumed that only a single protein molecule occupies each reverse micellar core. The volume of the protein (V_p), water molecule (V_w) and surfactant (V_s) were assumed to be constant. The radii measured with this equation refer to the outer shell surface, which also include the surfactant head groups and, not just the polar core of the reverse micelle. The authors have carried out SANS studies to determine the characteristic dimensions of revere micelles formed under different processing conditions.

3.	**Dekker *et al.*** (1991)	$R_m = \frac{3W_0 M_{ws}}{A_s \alpha}$ R_m in nm

The model was derived based on geometric conditions (assuming reverse micelles to be spherical). The shape factor α was employed in the equation to estimate the reverse micellar radius. However, the report did not analyze the suitability of the above equation for the extraction of alpha-amylase enzyme, which was studied in this work.

4. **Krei and Hustedt** (1992)

$$R_m = \frac{3W_oM_{ws}}{A_sN\rho_w}$$

R_m in nm

The model was proposed by equating the water content of the reverse micelle to the radius of the aqueous core of the reverse micelle. The study was mainly focused on the effect of process parameters on partitioning co-efficient for alpha-amylase with cationic surfactants. The work discusses only the importance of W_0 values for selective separation of solutes.

5. **Regalado *et al.,*** (1994)

$$R_m = \frac{3V_mW_0}{A_{sm}}$$

R_m in nm

The geometrical conditions were taken into consideration while deriving the model. It was assumed that all the surfactant molecules are used in the formation of reverse micelles. It was also proposed that since the molar water volume and surfactant head group area per mole are not changing over the temperature range studied, the change in water content was mainly due to change in aggregation number. However, the suitability of this model for both empty and filled reverse micelles was not discussed. No comparative data on theoretical and experimental values were available in the report.

6. **Jolivalt *et al.,*** (1993)

$$R_m = \frac{3V_wW_0}{A_s}$$

R_m in nm

In this study, the incorporation of solute into the reverse micelles did not change the water content values and hence the size of the reverse micelle, contrary to the reports of increase in water content and size with the inclusion of solute.

Empirical models

1. **Bru *et al*.,** (1989)

$$Rm = 0.175W_0$$

$$R_h = 0.175W_0 + \text{ Surfactant tail length}$$

R_m in nm

The empirical models were proposed mainly for determining the reverse micellar core and hydrodynamic radii for reverse micelles formed by surfactant AOT in isooctane. The hydrodynamic radius of the reverse micellar core was determined by adding the tail length of the surfactant to the aqueous core radius. However, no comparative study of the estimated and actual (experimental) radii was reported in the study. The

reverse micellar core radius estimated with the help of empirical equation was used for estimating the surfactant aggregation number.

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2. **Motlekar and Bhagwat,** (2001) $R_m = 0.15W_0$

R_m in nm

An empirical model was used for the estimation of the size of reverse micelle required to solubilize the solute intended. However, it was not clear whether the model could be used for both empty and filled micelles. No comparative data on theoretical and experimental values were available in the report.

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3. **Gaikar and Kulkarni,** (2001) $R_m = 1.64W_0$

and

R_m in Å

Hasmann *et al.,* (2003)

An empirical model was presented based on the water content value for the estimation of the size of the reverse micelle. However, the suitability of the model for both empty and filled micelles was not discussed in the report. No comparative data on theoretical and experimental values were available in the report.

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4. **Kinugasa *et al.,*** (2003) $R_m = 0.145W_0 + 0.57$

R_m in nm

The model proposed for the estimation of the size of reverse micelle was based on the water content of the reverse micellar core. However, it was not clear whether the equation could be used for both empty and filled micelles. No data on the goodness of fit of the proposed model was reported.

SECTION C

SCHEME FOR REVERSE MICELLAR EXTRACTION

2C.1. Introduction

The process for the separation and purification of enzymes/proteins from natural sources employing RME technique involves mainly two stages, namely, i) preparation of crude enzyme/protein extract from natural source and ii) reverse micellar extraction of the targeted solute from the crude extract. Selection of suitable process parameters for the preparation of crude extract having good enzyme activity/solute concentration, selection of reverse micellar system components and optimizing processing conditions for RME plays a major role in achieving the process efficiency. The following sections discuss various aspects involved in the overall process and its application to reverse micellar extraction of bromelain from pineapple wastes.

2C.1.1. *Preparation of crude extract of enzyme/protein* from natural source

Step.1: Selction of suitable source for the targeted enzyme/protein is done based on many factors such as enzme/protein content, stability/activity of the enzyme, raw material avialbility and cost etc. The selection of extraction steps depends on whether the idenitfied enzme/protein is intracellular or extracellular.

Application in the present study: In the present study extraction of Bromelain from pineapple wastes was attempted. Bromelain is a collective term for proteolytic enzymes derived from the ripe and unripe fruit, as well as the stem and leaves of the pineapple plant. Although, bromelain is commercially extracted from stem of the plant, bromelain is found in appreciable quantity in parts such as pulp, stem, leaves, and peel. Bromelain is an intracellular enzyme and is released by cell rupture. Although, pineapple is a seasonal fruit, the wastes (peel, core, crown) generated in the pineapple processing plant could be used as the source for the large scale production. Bromelain is also reported to be present in grapefruit, fruit, cherries, peppers, apricots, lemons, oranges, prunes, and black currants, however, in minute quantities.

Step. 2: In many of the processes used for the preparation of crude extract from natural sources, primary processing such as sorting, ize reduction etc., is needed. Some of these operations would help in obtaining higher degree of extraction of enzyem/protein.

Application in the present study: Sorting/selection of the pineapple fruit is needed as the bromelain content vary with the degree of maturity. Partly ripened fruits were used in the study to obtain higher protein yield and bromelain activity. Pineapple fruit was washed thoroughly and wastes (peel, core, crown etc.) were separated from the pulp for extraction. The pineapple wastes were chopped into small pieces to facilitate higher degree of extraction from crude.

Step. 3: Release of the intracellular enzyme/protein could be done by rupturing the cells with different methods such as homogenization, ultrasonification, thermal lysis, simple crushing in a mixer/blender, crushing in a bead mill etc., Homogenization with water or buffer of known pH could facilitate the extracted enzyme to retain higher activity.

Application in the present study: Cut pineapple wastes were crushed in a mixer/blender with the addition of known quantity of water to get a homogenate. Addition of phosphate buffer of pH 6.0 in the predetermined ratio facilitated proper blending and better extraction of bromelain from the cells.

Step. 4: Cell debris need to be separated from the extract and this could be achieved by centrifugation, sedimentation, filtration etc.

Application in the present study: The homogenate containing cell debris was subjected to cloth filtration followed by centrifugation to obtain clarified dilute extract.

Step. 5: Some of the natural extracts contain minerals, and they are to be removed as they adversely affect the efficiency of RME. This could be achieved by dialysis, membrane processing or by passing through ion-exchange columns.

Application in the present study: The extracts from the pineapple wastes did not contain the minerals in appreciable quantity and hence the above step was not employed.

A general scheme for separation of enzyme from plant sources and preparation of crude extract is presented in Fig. 2C.1.

2C.1.2. Reverse micellar extraction of the targeted solute from the crude extract

The crude extract, normally a dilute solution containing the desired solute along with other impurities is to be further processed for purifying the targeted enzyme/protein. Many parameters needs to be considered while selecting the reverse micellar system components and also while optimizing the extraction conditions. These parameters have been discussed in detail in Table 2C.1 with reference to the application in the present work.

Table 2C.1. Scheme for the selection of RME parameters for extraction from natural sources

	Details		Application in the present study (RME of Bromelain)
Step 1.	**Identification of the enzyme/protein properties**		
● *Iso-electric point (pI)* To manipulate the surface charge of the solute, which decides the selection of surfactant for electrostatic interaction			Fruit bromelain: 4.6 Stem bromelain: 9.1
● *Temperature and pH stability of solute* To determine suitable forward and back extraction conditions			pH : 4-9 Temperature: up to 65°C
● *Molecular weight and size* To select the method of solute addition (phase transfer, injection or dry addition). Lower molecular weight solute (<30 kDa) could be easily extracted with phase transfer method. Injection method is suitable for large molecular weight solutes. Strong interactions (electrostatic/ hydrophobic) are needed for extracting bigger size solutes			Molecular weight: 28–32 kDa Size: ~1.6 nm (Radius estimated assuming the molecule to be spherical)
● Hydrophobic/Hydrophilic nature *Selection of surfactants and RME conditions is done based on the nature of the solute. Strong electrostatic interactions are needed for the extraction of hydrophilic solute, whereas hydrophobic interactions could play a major role for hydrophobic solute.*			Bromelain is hydrophilic in nature and requires a strong electrostatic interaction for extraction into reverse micellar core
● Sensitivity of the solute for other RME conditions *Sensitivity to salt and co-solvent (if needed) added during forward and back extraction conditions*			Bromelain was found to be stable with CTAB, salts (NaCl & KBr) and co-solvents (Hexanol & Butanol) used. No complex formation was observed during the study.

Step 2.	**Selection of reverse micellar components**		
➢ ***Surfactant***			
● *Charge (anionic, cationic, nonionic or zwitterionic)* To select suitable surfactant to have electrostatic interaction with charged solute. Ionic surfactants are suitable for the extraction of hydrophilic solutes due to electrostatic interaction. Nonionic surfactants are better suited for hydrophobic solutes in order to use hydrophobic interaction as the driving force for extraction.			Anionic-AOT: To be used at pH lower than pI (4.6). Since, AOT is relatively unstable below 4.5, it was not used for fruit bromelain extraction. Cationic-CTAB: To be used at pH higher than pI and was used in the present study.
● *pH stability range* Solute should not get precipitated or react under RME conditions			CTAB is stable in the acidic/alkaline pH range of operation (5-10).
● *Critical micelle concentration (CMC)* Amount added to be more than CMC to form reverse micelles. Low CMC indicates lower addition of surfactant, which is a significant factor incase of high cost surfactants.			The degree of addition was maintained above CMC of CTAB (0.83 mM) and optimized during the study.
● *Hydrophilic-lipophilicb balance (HLB)* HLB value in the range of 8-11 is preferred for the formation of water-in-oil system. Mixture of surfactants can be used to get a favorable HLB value. Nonionic surfactants have a fixed HLB value, whereas it varies for ionic surfactants with the solvent.			HLB of CTAB varies with the solvent and not determined in the present study. However, it was reported to be more soluble in organic solvent and considered to be a good surfactant for RME.
● *Ability to form stable and reasonably bigger sized reverse micelles in combination with organic solvent* Since, the size of the reverse micelle formed and the water content of micelle depends on the surfactant-solvent combination, apart from the conditions employed for extraction, a suitable combination of reverse micellar components to be selected. Cationic surfactants require addition of co-solvent to form bigger sized reverse micelles.			Although, CTAB/isooctane combination does not provide a reverse micelle of favorable size, with co-solvents it forms a very good reverse micellar system with respect to size.

• Non-interaction with solute *The surfactant selected should not interact (denaturing proteins and reducing the activity of enzymes) with the solute to be extracted. It should not form a complex with the solute to form an interface during RME*		CTAB is non-interactive with bromelain (No-complex formation /precipitation was observed during the preliminary study).
• Cost *Low cost surfactants reduce the processing cost. Alternatively mechanism for recovery/reuse of surfactants to be evolved especially in case of expensive surfactants.*		Pure grade CTAB is relatively expensive. Laboratory scale studies were carried out with pure grade CTAB, whereas the scale up studies were carried out using analytical grade surfactant, which is not expensive.
➢ **Organic solvent**		
• *Miscibility with aqueous phase* Organic solvent should be non-polar in nature and should have the capacity to dissolve the surfactant selected.		Isooctane is non polar in nature and reported to form stable reverse micelles with many of the surfactants including CTAB.
• *Dielectric constant* Higher dielectric constant indicates lower resistance to mass transfer and hence higher ability of solute transfer. It can be increased by adding solvent/co-solvent mixtures with higher dielectric constant.		1.96
• *Molecular weight* Water solubilization capacity of the reverse micelles, for a given surfactant, is strongly dependent on the type of solvent used. Lower molecular weight solvents increase the water solubilization capacity.		113 g/mol
• Density *Lower ratio of solvent to water density indicates the easy separation of aqueous and organic phase after phase equilibration.*		0.70

➢ **Co-solvent**			
• *Miscibility with aqueous phase* Co-solvent should have low solubility in water and generally long chain alcohols are used for RME. Use of co-solvent may enhance the solubilization kinetics, stability of a reverse micelle, and even selectivity.			Hexanol and butanol have low solubility in water and the combination forms a stable reverse micelle with CTAB/isooctane.
• *Dielectric constant* Higher dielectric constant is favorable for the formation of stable reverse micelles.			Hexanol - 12.5 Butanol - 17.7
Step 3.	**Selection of salt for forward and back extraction**		
• *Water structure-forming and water structure-breaking* Water structure-forming (like Na^+) and water structure-breaking salts (K^+) to be used for forward and back extraction, respectively.			NaCl - Water structure forming salt was used for forward extraction KBr - Water structure breaking salt was used for back extraction
• *Size of the ions* Larger ions such as K^+ cause more screening, and hence results in less solubilization, than smaller ions such as Na^+. Hence, Na^+ salts are to be used for forward extraction while K^+ ions are to be used for back extraction.			NaCl and KBr were used for forward and back extraction, respectively.
Step 4.	**Selection of forward and back extraction conditions**		
• *Aqueous phase buffer and pH* Type of buffer used depends on the pH requirement and compatibility with the solute. Lower buffer concentrations are to be used for forward extraction as compared to back extraction. Aqueous phase pH value depends on the solute stability, iso-electric point, surfactant type (charge) and the favorable pH–pI values.			Preliminary approximation based on the equation $[(pH(optimum) - pI]) = (+0.11 x 10^{-3}) M_{ws} - 0.97$ As per the above equation, the optimum pH was found to be 6.93. However, the aqueous phase pH was optimized during the study.

• *Ionic strength* To be optimized for the given solute/reverse micellar system. Minimum concentration needed to lower phase turbidity/cloudiness, while concentration beyond the level leads "squeezing out" effect.		Degree of addition varied from minimum concentration to a level where best results are obtained.
• *Surfactant concentration* Concentration to be higher than CMC. To be optimized for the given system. Very high concentration leads to lower extraction due to micelle-micelle interaction.		Concentration used was well above the CMC of CTAB (0.83) and level of addition optimized during the study.
• *Phase volume ratio (V_{org}/V_{aq})* Lower for forward extraction and higher for back extraction are favorable for the concentrating the solute. However to be optimized for the given system.		Volume ratio of 1:1 was selected as literature reports favor the above ratio.
• *Temperature* To be selected depending on the stability of the solute. Higher temperature is favorable for back extraction. Low temperature increases the viscosity of phases.		Bromelain is stable at 25±2°C, and hence used for forward and back extraction.
• *Phase equilibration and phase separation (method & duration)* To be optimized based mainly on the solute characteristics. Larger molecular weight solutes require higher phase contact time. Prolonged contact time could reduce the enzyme activity.		For laboratory scale trials, phase equilibration and separation were carried out using magnetic stirrer and centrifuge, respectively. For scale-up studies pitched blade turbine was used. Duration of equilibration and phase separation was optimized based on literature reports and results of the study.
• Co-solvent addition *Degree of addition may be optimized for the given system*		Hexanol/buatanol in the ratio 8.5:1.5 (%v/v). The degree of addition was optimized based on the previous study.

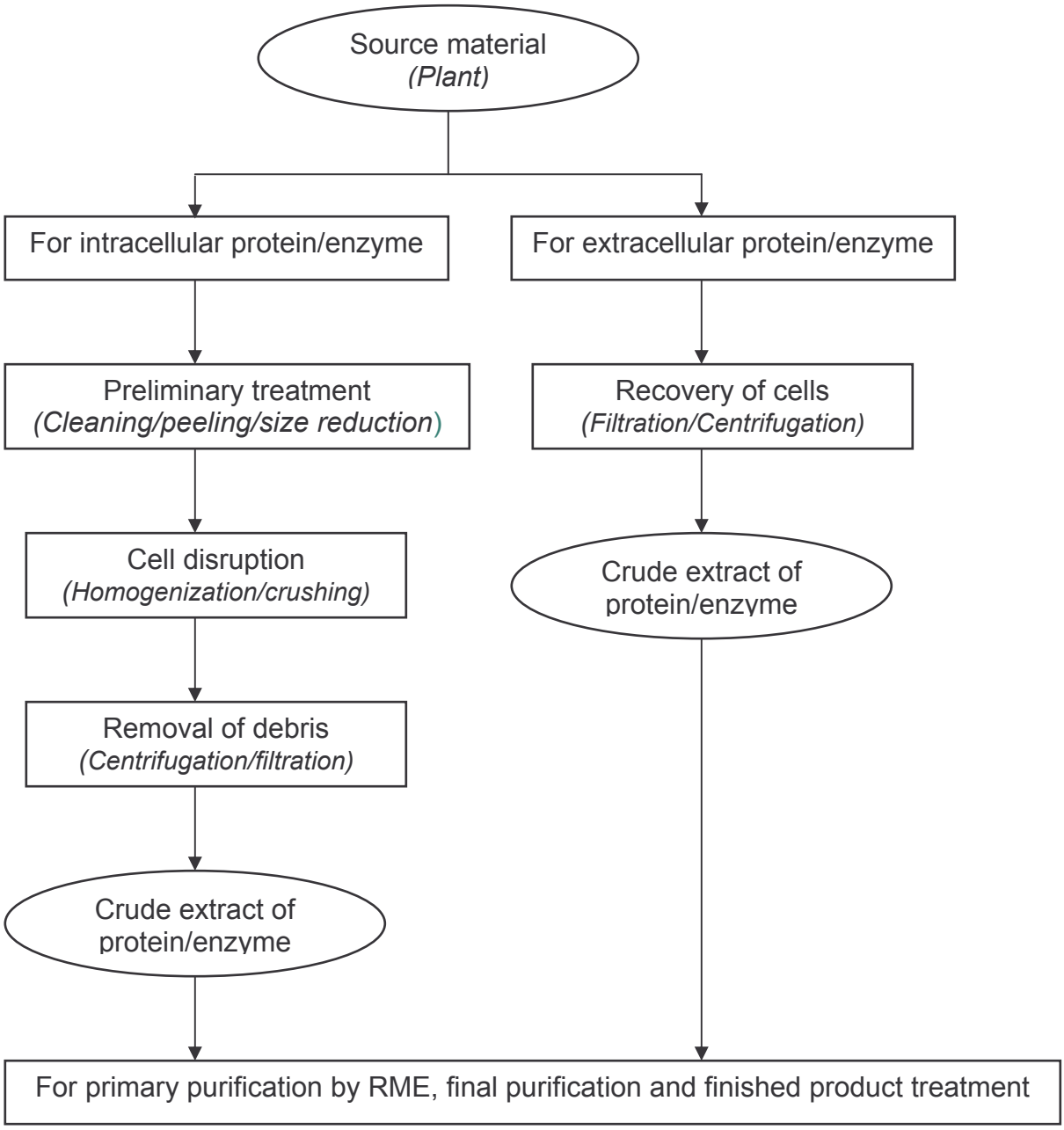

Fig. 2C.1 Scheme for separation of enzyme from plant sources and preparation of crude extract

CHAPTER 3

REVERSE MICELLAR EXTRACTION EMPLOYING MODEL SYSTEMS

SECTION A

EXTRACTION OF BOVINE SERUM ALBUMIN (BSA)

3A.1. Introduction

Reverse micellar extraction of BSA has been observed to be difficult owing to its large molecular size (3.5-3.6 nm, 66 kDa) compared with the average size of reverse micelles. Although, a few reports on RME of BSA are available, many have reported an unsuccessful extraction. Wolbert *et al.* [1989] attributed the unsuccessful extraction of BSA in AOT/isooctane reverse micellar system to the extraction conditions employed that were not sufficient enough to provide the required quantity of energy (pH-pI) for extraction. The affinity extraction of proteins with reverse micellar system composed of cibacron blue (CB)-modified lecithin was studied and concluded that the failure to extract BSA was due to its large molecular weight [Sun *et al.,* 1998]. Addition of alcohol to the aqueous phase during back extraction of BSA enhanced the extraction efficiency of CTAB/CB/hexane reverse micelles [Zhang *et al.,* 1999]. An overall extraction of nearly 90% without affecting the structure of the protein was achieved with AOT/isooctane system [Shimori *et al.,* 1998]. This is the sole report wherein a higher degree of extraction of BSA using reverse micelles has been achieved. However, the optimized extraction conditions of the above study were different from that employed by Wolbert *et al.* [1989]. The extraction of BSA was greatly enhanced by the addition of alkyl halides to the reverse micellar system of cationic surfactant CTAB in hexanol [Zhang *et al.,* 2002]. This system achieved almost complete back extraction at low salt concentration. The effect of addition of different alcohols on the extraction of BSA using AOT/isooctane reverse micelles was reported [Hong *et al.,* 2000].

Most of the reports on RME deal with ionic surfactants and only a few reports [Ayala *et al.,*1992; Barrabin *et al.,* 1993; Vasudevan and Wiencek, 1995; 1996; Naoe *et al.,*1998; Hossain *et al*., 1999; Adachi *et al.,* 2000] are available on the application of nonionic surfactants (Triton-X-100, Span 60 and Tween 85, Tween 20). Extraction of large solutes with nonionic surfactants was reported to be difficult due to lack of strong driving force [Harikrishna *et al.,* 2002]. It was reported

that the nonionic surfactants do not denature/alter the structure of the solute during extraction as compared to some of the ionic surfactants [Adachi *et al.,* 1998; 2000]. Attempts have been made in the present study to use nonionic surfactant for the extraction of BSA. In addition, the performance of surfactant mixture (AOT and Triton-X-100) was also studied.

Objectives of the present work are i) to study the performance of nonionic surfactant Triton-X-100, which is 'nondenaturing' and 'nontoxic' in the extraction of large molecular weight solute such as BSA, which has not been reported so far, ii) use of organic solvent toluene in place of widely studied isooctane to form reverse micelles, iii) extraction studies with AOT/Triton-X-100 mixture, and iv) to study the effect of process parameters on BSA extraction in some of the above reverse micellar systems.

3A.2. Materials and Methods

3A.2.1. Materials

AOT and Triton-X-100 were obtained from Rohm and Haas, USA and BDH laboratory, UK, respectively and used without further purification. Toluene (UV spectroscopic grade) and BSA were obtained from Loba Chemie. Pvt. Ltd., Mumbai, India. All other reagents used were of analytical grade. Various buffers used were Glycine–HCl, acetic acid–sodium acetate, phosphate, and glycine-NaOH. The buffer concentration was 50 mM for Triton-X-100 system and 10 mM for AOT, AOT/Triton-X-100 systems.

3A.2.2. Methods

i) Forward extraction

Forward extraction experiments were carried out by contacting aqueous and organic phases using a magnetic stirrer (Cintex, Mumbai, India) for 15 min at 500 rpm under controlled temperature (25±2ºC). BSA concentration was

maintained at 1 mg/ml unless mentioned otherwise and the aqueous phase was prepared by dissolving the solute in buffer of known pH and salt (NaCl) concentration. For all the experiments, the phase volume and phase volume ratio were 15 ml and 1 (except phase volume ratio study), respectively. The organic phase had known amount of surfactant dissolved in it. Phase separation was carried out in a laboratory centrifuge (MP 400 R, Eltek, Mumbai, India) at 3000 *g* for 30 min. The organic phase separated from the mixture after the forward extraction was subjected to back extraction.

ii) Back extraction

Back extraction was carried out by contacting the organic phase (10 ml) of the forward extraction with an equal volume of stripping phase (buffer of known pH and KCl concentration) using a magnetic stirrer for 30 min at 500 rpm at controlled temperature (25±2ºC). The two phases were separated by centrifugation at 3000 *g* for 45 min and subjected analysis.

In all the AOT/toluene experiments, a blank run (without BSA) was carried out and used as the control. All the experiments were carried out in duplicate.

3A.2.3. Analyses

i) BSA concentration

For AOT/toluene system, BSA concentration in feed and in organic as well aqueous phases of forward and back extractions was determined by spectrophotometry at 280 nm (UV-160A, Shimadzu, USA). In case of Triton -X-100/toluene and AOT/Triton-X-100/toluene systems, Bradford [1976] method was used since Triton-X-100 has good absorption at 280 nm. Protein concentration readings were taken in triplicate and average values were reported. Efficiencies of forward and back extraction were estimated using the equations given below.

$$\text{Forward extraction efficiency}(\%) = \frac{\text{BSA concentration in organic phase after forward extraction (mg/ml)}}{\text{BSA concentration in feed (mg/ml)}} \text{x}100 \quad \text{(3A.1)}$$

$$\text{Back extrction efficeincy}(\%) = \frac{\text{BSA concentration in back extracted aqueous phase (mg/ml)}}{\text{BSA concentration in forward extracted organic phase (mg/ml)}} \text{x}100 \quad \text{(3A.2)}$$

In case of experiments with phase volume ratio variation, the volume factor was taken into consideration while estimating the extraction efficiencies.

ii) Circular Dichroism (CD) analysis

The CD analysis was carried out to study the effect of extraction conditions on the solute structure using a spectropolarimeter (Model 2310, JASCO, USA). The results are expressed as mean residue ellipticity (θ), which is defined as [Shiomori, *et al.,* 1998]

$$\theta = \frac{100\theta_{obsd}}{lc} \quad \text{(3A.3)}$$

The CD spectra were measured at the protein concentration of 0.25 mg/ml with cell of 1 mm path length.

iii) Water content measurement

The water content in the organic phase of forward extraction was measured using Karl Fischer auto titrator (DL 32, Mettler Toledo, Switzerland). The W_0 values were estimated as molar ratio of water to surfactant in organic phase. The reverse micellar core radius (R_m) was estimated using the following empirical equation [Bru, *et al.,* 1989].

$$R_m = 0.175W_0 \quad \text{(3A.4)}$$

where, W_0 is the water content of the reverse micelle, defined as molar ratio of water to surfactant. Higher W_0 value indicates the higher amount of water in the core, which in turn points out the bigger size of the reverse micellar core.

3A.3. Results and Discussion

In addition to the type of reverse micellar system selected, the efficiency of extraction depends on the processing conditions employed [Pires *et al.,* 1996]. The processing conditions were varied during forward and back extractions employing Triton-X-100/toluene and AOT/toluene systems however, not in the case of mixed surfactant systems to study the effect on extraction efficiency.

3A.3.1. Triton-X-100/toluene system

3A.3.1.1. Forward extraction

i) Effect of aqueous phase pH

The aqueous phase pH was varied from 3.0 to 9.0 at two different concentrations (8 and 14 mM) of Triton-X-100 (Fig. 3A.1). The concentrations selected were higher than the critical micellar concentration (0.25-0.30 mM) of Triton-X-100. The NaCl concentration was 0.5 M in both the cases. BSA extraction efficiency increased with an increase in aqueous phase pH, reaching a maximum at pH 7.0 in both the cases. The surfactant concentration of 14 mM gave slightly higher extraction efficiency (52.5%) as compared to that obtained with 8 mM (41.5%). Extraction efficiency decreased with further increase in pH. Although, nonionic surfactants' head groups do not have any net charge, it was reported that they do exhibit surface charge (positive or negative) at neutral pH, which induces a weak electrostatic interaction between surfactant and solute, leading to solute extraction [Vasudevan and Wiencek, 1996]. Vasudevan et al. [1995] reported that the extraction of solute into reverse micelles depends on the ability of the surfactant to form stable reverse micelles that have sufficiently large size to accommodate the solute. In the present study, the radius of the empty Triton-X-100/Toluene (14 mM) reverse micelles was measured using the water content (W_0) values (Table 3A.1). The estimated radius of the reverse micellar core (5.67

nm) for this system was found to be large enough to host BSA molecule that has an approximate radius of 3.5-3.6 nm (assuming it to be spherical). The estimated radius of the reverse micellar core in Triton-X-100/toluene was close to the value (5.52 nm) as measured by Rodriguez et al. [1998] using Dynamic Light Scattering instrument. Although, the size of the reverse micelle was large enough to host BSA, reverse micellar size was observed to be not a sufficient parameter to allow BSA to get entrapped in the reverse micelles in large amounts. Lack of strong electrostatic driving force between the solute and surfactant might have resulted in the low forward extraction efficiency. The size (0.67 nm) of reverse micelle formed by Triton-X-100 in isooctane, most widely used organic solvent for RME, was found to be much smaller than that formed with toluene (Table 3A.1). This may be due to the higher ability of toluene (relatively lower molecular weight and more polar) to penetrate between surfactant molecules to increase the size of the reverse micelles. Similar observation was made by Rodriguez et al. [1998] while studying Triton-X-100 system with toluene and propylbenzene.

ii) Effect of surfactant concentration

The surfactant Triton-X-100 concentration in the organic phase was varied (Fig. 3A.2) in the range of 2 to 17 mM, while maintaining the aqueous phase pH and NaCl concentration at 7.0 and 0.5 M, respectively. The extraction efficiency increased with the surfactant concentration and the maximum extraction efficiency (52.5%) was obtained at 14 mM. It was observed that when the surfactant concentration is above 14 mM, the turbidity of the phases increased, making the separation of the phases difficult. An increase in the extraction efficiency with surfactant concentration followed by a decrease is in line with the observations made by other researchers during the forward extraction of different biomolecules [Shiomori *et al*., 1998; Cardoso *et al*, 1999; Huang and Lee 1994]. It was reported that the increase in surfactant concentration increases the number of reverse micelles in the organic phase, favoring higher solute extraction. De-assembling of reverse micelles due to inter-micellar collision and hindrance to the diffusion of

solute by surfactant aggregates were reported to be the reasons for lower extraction at very high surfactant concentrations [Harikrishna *et al.,* 2002].

iii) Effect of ionic strength

The influence of ionic strength on the diffusion of proteins into reverse micelles was explained mainly as an electrostatic effect [Harikrishna *et al.,* 2002]. In general, it was observed that as the ionic strength increases, the protein uptake capacity of reverse micelles decreases [Cardoso *et al.,* 1999]. However, it was also reported that a minimum salt concentration is needed for the extraction to take place [Harikrishna *et al.,* 2002]. Water structure-forming salt such NaCl, which causes lesser 'screening' of solutes is favorable for forward extraction and hence selected in the study. The effect of ionic strength (0.15 to 1.0 M NaCl) on degree of extraction is shown in Fig. 3A.3. The surfactant concentration and aqueous phase pH were maintained at 14 mM and 7.0, respectively. It can be observed that increased salt concentration enhanced the degree of extraction initially, and a maximum extraction (~52.5%) was obtained at 0.5 M NaCl concentration. The variation in extraction efficiency with salt concentration indicated the existence of electrostatic attraction between the solute and surfactant. The extraction efficiency decreased slightly with further increase in salt concentration (0.6, 0.75 and 1.0 M), although the extent of decrease was not appreciable. The expulsion of the solute from the core due to reduction in reverse micellar size with increased salt concentration (squeezing out effect) and reduction in Debye length [Harikrishna *et al.,* 2002] might be reasons for this lower extraction efficiency at higher salt concentrations.

iv) Effect of phase volume ratio

Volume ratio (V_{org}/V_{aqu}) of phases is a critical parameter in extraction and concentration of enzymes. Ideally, this ratio should be low for forward extraction and high for back extraction to achieve concentration. Preliminary trials were carried out with volume ratios of 0.10 and 0.20 (Table 3A.2). Since, the forward extraction efficiency was extremely low (<5%), the experiments were carried out at

0.75 and 0.50 volume ratio, without altering the surfactant concentration. Forward extraction efficiency increased to 59% and 42%, respectively at the above volume ratios. Change in surfactant to solute ratio under these conditions could be the reason for the above observation. Shin *et al.* (2003b) observed that with the decrease in volume ratio, the amount of ions coming in contact with the organic phase containing surfactant increases, which in turn reduces the extraction efficiency.

3A.3.1.2 Back extraction

i) Effect of aqueous phase pH and salt concentration

The organic phase obtained from the forward extraction (aqueous phase pH 7.0, 14 mM Triton-X-100 and 0.5 M NaCl) was used for back extraction of BSA. KCl, a water structure-breaking salt, is used for the back extraction. KCl concentration and aqueous phase pH were altered during back extraction and results are presented in Table 3A.3. Since, it was reported that the back extraction of solute from reverse micelles to the aqueous solution is relatively slow due to high interfacial resistance to the mass transfer [Dekker *et al.,* 1986; Dungan *et al.,* 1991], the phase contact time was maintained higher (45 min) as compared to forward extraction (15 min). However, the back extraction efficiency in all the cases was very low with a maximum of 15.5% at aqueous phase pH 8.0 and KCl concentration of 2 M. It appears that the back extraction conditions adopted did not favor the de-assembling of reverse micelles leading to poor extraction efficiency.

ii) Effect of alcohol addition

Carlson and Nagarajan [1992] reported an increase in back extraction of porcine pepsin and bovine chymosin with the addition of isopropyl alcohol. The co-solvent addition was assumed to facilitate the release of solute back into the aqueous phase. However, in the present study the extraction efficiency did not enhance appreciably with the addition of isopropyl alcohol. It may be noted that

addition of isopropyl alcohol (5% and 10% (v/v)) to the aqueous phase during back extraction resulted in an extraction efficiency of 19.3% and 23.2%, respectively at pH 8.0 and KCl concentration of 2 M.

3A.4. AOT/toluene reverse micellar system

3A.4.1. Forward extraction

i) Effect of surfactant concentration

AOT concentration in the organic phase was varied (40-200 mM) to study its effect on extraction efficiency (Table 3A.4). The above concentration range is nearly 8 to 40 times the CMC value (4.9 mM) of AOT. The aqueous phase pH and NaCl concentration were 5.5 and 0.5 M, respectively. With an increase in AOT concentration, extraction efficiency increased and resulted in good extraction at concentrations above 80 mM. Complete extraction of BSA was achieved at 160 mM concentration. When the surfactant concentration was increased to 200 mM, the extraction efficiency slightly decreased (89%). The decrease in extraction efficiency at higher surfactant concentration may be attributed to the de-assembling of reverse micelles due to inter-micellar collision. The forward extraction efficiencies of 86% and 91% for BSA with AOT/isooctane system, using NaCl and $CaCl_2$ systems, respectively was reported by Shimori *et al.* [1998]. AOT concentration in the above repot was ~200 mM, which is slightly higher than that (160 mM) in the present study.

ii) Effect of aqueous phase pH

Aqueous phase pH was varied in the range (4.5 to 6.5) close to the isoelectric point (*pI*) of BSA (4.9) to observe its effect on the extraction efficiency. It was expected that with the use of anionic surfactant, extraction would be taking place at pH lower than the *pI* (where the protein would be positively charged). However, as can be seen from the Table 3A.4, the extraction was maximum at pH (5.5), which is higher than the isoelectric point. Significant variation in extraction efficiency with marginal changes in aqueous phase pH indicated the sensitivity of the system to pH change. Extraction of BSA at pH higher than the *pI* indicated that

the driving force responsible for extraction in the present case could be the hydrophobic interaction. The presence of side chains on amino acids like glutamate, aspartate etc., having terminal groups that exhibit high hydrophobicity at pH higher than isoelectric point of BSA might have contributed for the above result. Shimori *et al.* [1998] also reported maximum extraction of BSA at pH 5.0-5.5 with AOT/isooctane system. Size (4.33 nm) of the reverse micelle formed by AOT in isooctane was found to be little larger than that formed (3.36 nm) by AOT/toluene system (Table 3A.1), contrary to the observation made with Triton-X-100 where there was a large difference in micelle size with a change in solvents. This indicates the strong dependency of the reverse micellar size on the surfactant/solvent combination. This can be observed in the mixed micelle systems as compared to the parent surfactant in both solvents. The reverse micellar size did not vary much with toluene systems, while, it was large with the isooctane system where the two surfactants behave very differently in the same solvent. Although, the size of the reverse micelle is close to the size of the solute, the strong hydrophobic interaction between surfactant and solute might be the reason for higher forward extraction efficiency.

iii) Effect of ionic strength

NaCl concentration was varied (Table 3A.4) from 0.25 to 1.0 M without altering the other parameters. Extremely high extraction efficiency was obtained at all salt concentrations. The results indicated that the ionic effect in the present case was insignificant, and the major driving force for extraction may be hydrophobic interaction. Shiomori *et al.* [1998] reported that the salt concentration affects the hydrophobicity of both surfactant and protein and maintained that the hydrophobicity of AOT reverse micelles remains almost constant at NaCl concentration below 1.0 M. Hence, it may be inferred that the increased extraction efficiency observed in the present study is due to the increase in hydrophobicity of BSA. Complete extraction of BSA was obtained at 0.75 as well as 1.0 M salt concentrations.

3A.4.2 Back extraction

Back extraction of BSA from the reverse micellar organic phase of forward extraction was carried out by contacting it with a fresh aqueous phase (buffer with salt). The organic phase obtained from the forward extraction, which had given maximum extraction (AOT: 160 mM, NaCl: 0.75 M and aqueous phase pH: 5.5) was subjected to back extraction. During back extraction, the salt concentration and aqueous phase pH were varied to study their effect on back extraction efficiency.

i) Effect of ionic strength and aqueous phase pH

It was reported that addition of high amount of salt during back extraction destabilizes the reverse micelles and transfers the solute into the fresh aqueous phase. Complete extraction of BSA was obtained at 2 M KCl (aqueous phase pH 5.5) although no extraction took place at 1 M concentration (Table 3A.5). The drastic increase in back extraction efficiency with increase in salt concentration indicated the sensitivity of the system for ionic strength.

pH of the fresh aqueous phase used for the back extraction was altered (5.0 and 5.5), maintaining KCl concentration the same (2 M). Complete extraction of BSA was obtained at pH 5.5, while there was no extraction at pH 5.0 (Table 3A.5). Higher aqueous phase pH combined with increased salt concentration might have destabilized the reverse micelles resulting in higher back extraction efficiency.

3A.5. Effect of BSA concentration on the performance of AOT/toluene system

Concentration of solute in the aqueous phase also influences the extraction efficiency of the system. To study the performance of AOT/toluene system at higher concentration of BSA, experiments were carried out at a concentration of 12 mg/ml without changing the other conditions of forward and back extractions. While the forward extraction efficiency dropped to 52% the back extraction was

near complete (98%). An interface (could be BSA-AOT complex) formation was observed during the forward extraction. Reduction in surfactant-to-solute ratio at higher concentration might be responsible for the reduction in the forward extraction efficiency, which indicates the need for optimizing the solute concentration. Shimori *et al.* [1998] reported that with an increase in BSA concentration, the forward extraction efficiency increased up to a certain concentration level. At higher concentrations precipitation (BSA-surfactant complex) was noticed.

3A.6. Effect of employing mixture of surfactants

To study the feasibility of partial replacement of AOT by Triton-X-100, a surfactant mixture was used. The optimum concentration of Triton-X-100 (14 mM) and AOT concentration (80 mM) that has resulted in fairly high extraction was selected for the surfactant mixture. The other extraction conditions were same as that of AOT/toluene system. The forward extraction efficiency was found to be 66% compared to the 90% observed at AOT concentration of 80 mM, while the back extraction efficiency was nearly 23%. Yamada *et al.* [1993] reported that with the addition of nonionic surfactants such as Triton-X-100, Tween-85 etc., to AOT, a reduction in the hydrophobicity of the system occurs. Since, the hydrophobic interaction is considered to be the major driving force for the extraction with AOT system, the reduced hydrophobicity with the addition of Triton-X-100 might have resulted in lower extraction efficiency. The size of the reverse micelle in the mixed system (Table 3A.1) did not vary much as compared to AOT/toluene system.

3A.7. Circular dichroism (CD) analysis

Successful reverse micellar extraction aims at obtaining high extraction (forward and back) efficiency without affecting the structure/activity of the biomolecule. The CD spectrum in the far-UV region, which reflects the secondary structure of a protein [Hong *et al.,* 2000], was compared with that of feed BSA (Fig. 3A.4). As can be seen from the spectrum, the ellipticity of the extracted BSA was

almost similar to that of the feed BSA over the observed range. This indicates the maintenance of the structural integrity of BSA during reverse micellar extraction.

3A.8 SDS-PAGE Analysis

The aqueous phase containing BSA obtained from RME was subjected to SDS-PAGE analysis for confirmation. The concentrated sample was loaded to 10% gel along with standard BSA (Sigma) sample. The SDS lane pattern of BSA matched well with the standard (Fig. 3A.5) and the band obtained was found to be close to 66 kDa, which is the reported molecular weight of BSA.

3A.9. Conclusions

Reverse micellar system as well as the processing conditions for both forward and back extraction were found to have effect on the extraction efficiency of BSA was found to depend upon the Size appeared not to be a sufficient criterion for reverse micelles to host BSA at higher concentrations. Lower extraction efficiency with Triton-X-100 could be attributed to lack of strong driving force for the diffusion of large molecules of BSA into the nonionic reverse micellar core. Nano sized AOT/toluene reverse micellar system resulted in complete forward and back extraction without affecting the protein structure. Forward extraction at pH higher than isoelectirc point indicated the hydrophobic interaction between BSA and AOT. The significant variation in extraction efficiency with slight change in processing conditions showed the sensitivity of the system and emphasized the need for optimizing the conditions for an efficient extraction process for the given candidate. Addition of Triton-X-100 to AOT decreased the extraction efficiency as compared to AOT alone, which may be attributed to the reduced hydrophobic interaction.

Table 3A.1. Estimated size of the reverse micellar core for different surfactants in toluene and isooctane solvents

Surfactant	Concentration mM	Reverse micellar core radius, R_m (nm)
Toluene		
Triton-X-100	14	5.67
AOT	160	3.36
AOT+Triton-X-100	80 +14	3.39
Isooctane		
Triton-X-100	14	0.67
AOT	160	4.33
AOT+Triton-X-100	80 +14	1.08

Table 3A.2. Effect of phase volume ratio on forward extraction of BSA

Phase volume ratio (V_{org}/V_{aqu})	Forward extraction efficiency (%)
0.10	2.5
0.20	4.3
0.50	59.0
0.75	42.0
1.00	52.5

System: Triton-X-100/Toluene
Forward extraction conditions - BSA: 1mg/ml, aqueuses pH: 7.0, Triton-X-100: 14 mM, NaCl: 0.5 M)

Table 3A.3. Effect of processing conditions on back extraction of BSA

Aqueous phase pH	KCl concentration (M)	Back extraction efficiency (%)
7.0	1.0	8.70
	2.0	10.20
8.0	1.0	12.40
	2.0	15.50
9.0	1.0	5.40
	2.0	13.70

System: Triton-X-100/toluene
Phase volume ratio: 1

Table 3A.4. Effect of processing conditions on forward extraction of BSA

Process parameter	Value	Forward extraction (%)
AOT Concentration (mM) (Aqueous phase pH:5.5, NaCl:0.5 M)	40	25.2
	80	93.5
	120	96.5
	160	100.0
	200	89.0
Aqueous phase pH (AOT:160 mM, NaCl:0.5 M)	4.5	24.0
	5.0	72.2
	5.5	100.0
	6.0	85.5
	6.5	80.4
NaCl Concentration (M) (AOT :160 mM, Aqueous phase pH:5.5)	0.25	98.0
	0.50	98.3
	0.75	100.0
	1.00	100.0

System: AOT/toluene

Table 3A.5. Effect of processing conditions on back extraction of BSA

Process parameter	Value	Back extraction efficiency (%)
Aqueous phase pH*	5.0	Nil
	5.5	100
Salt concentration(M)**	1.0	Nil
	2.0	100

System : AOT/toluene
*KCl concentration : 2 M, **Aqueous phase pH : 5.5, Phase volume ratio : 1

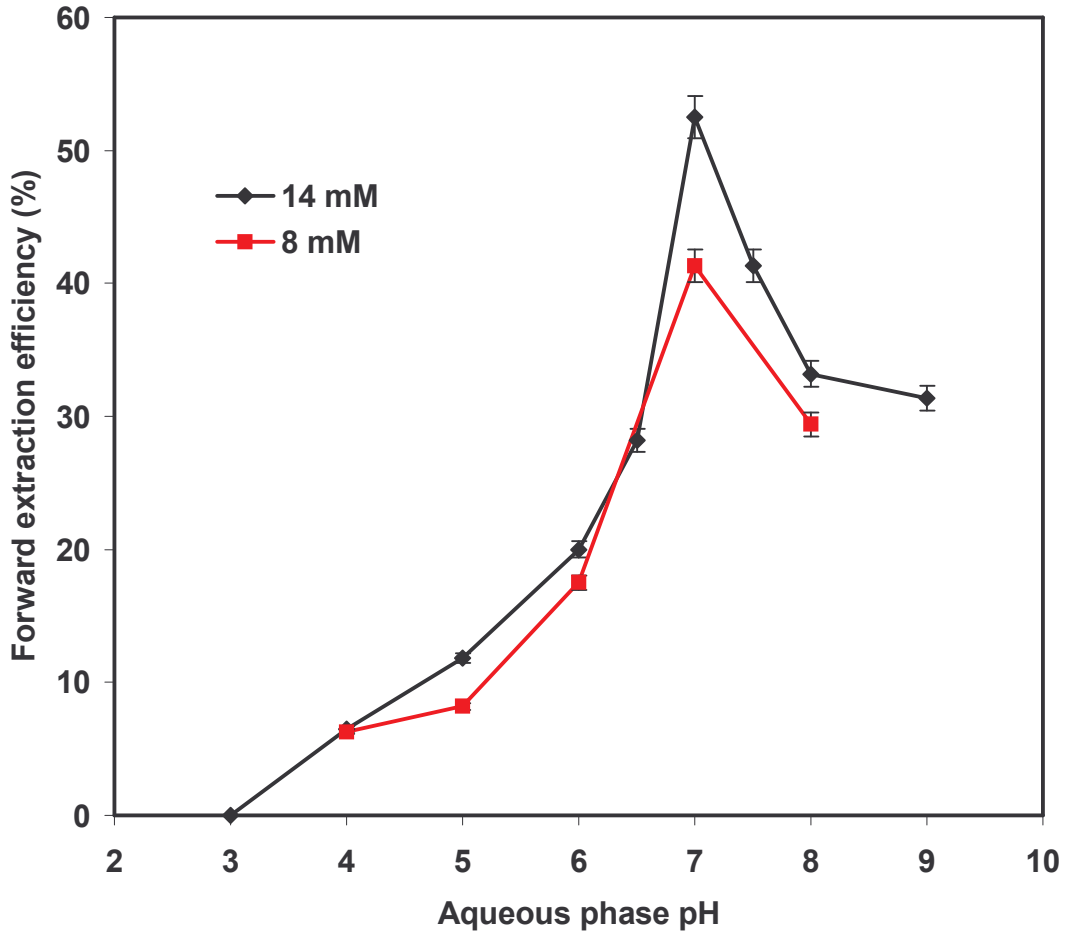

Fig. 3A.1. Effect of aqueous phase pH on the forward extraction of BSA in Triton-X-100/toluene reverse micellar system

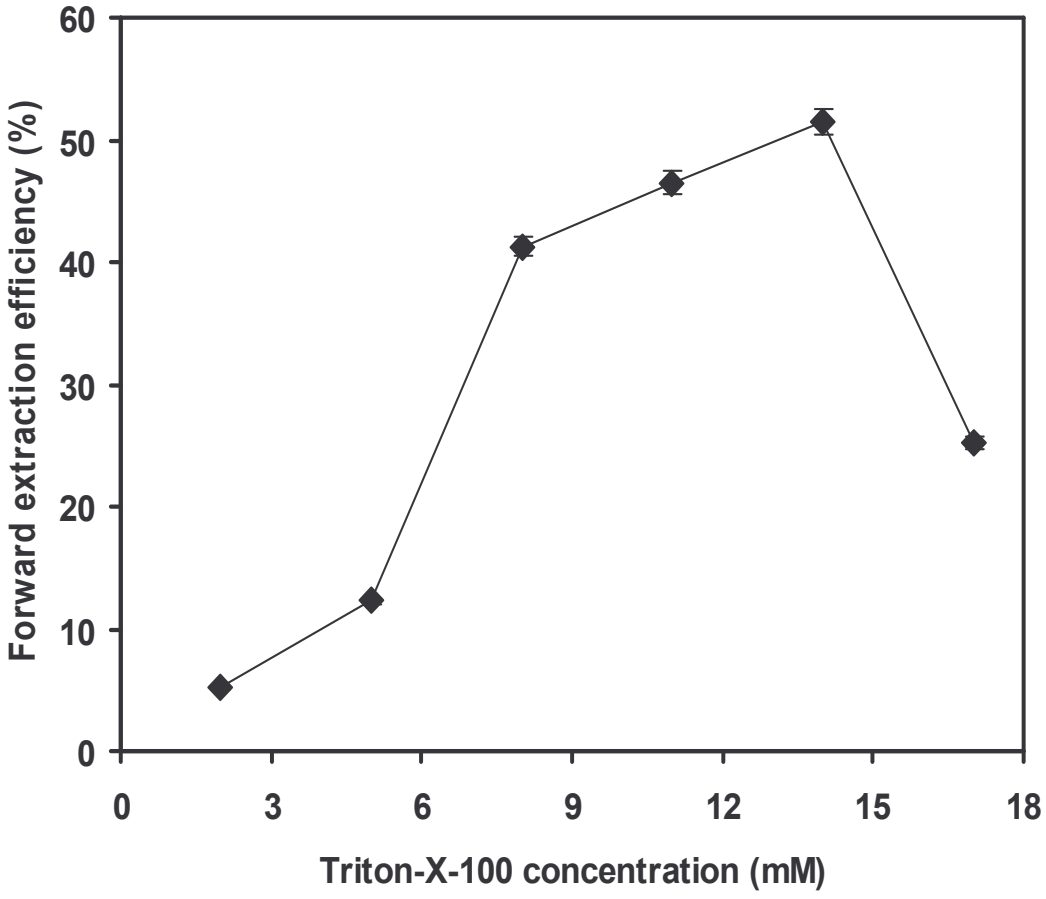

Fig. 3A.2. Effect of surfactant concentration on forward extraction of BSA in Triton-X-100/toluene reverse micellar system

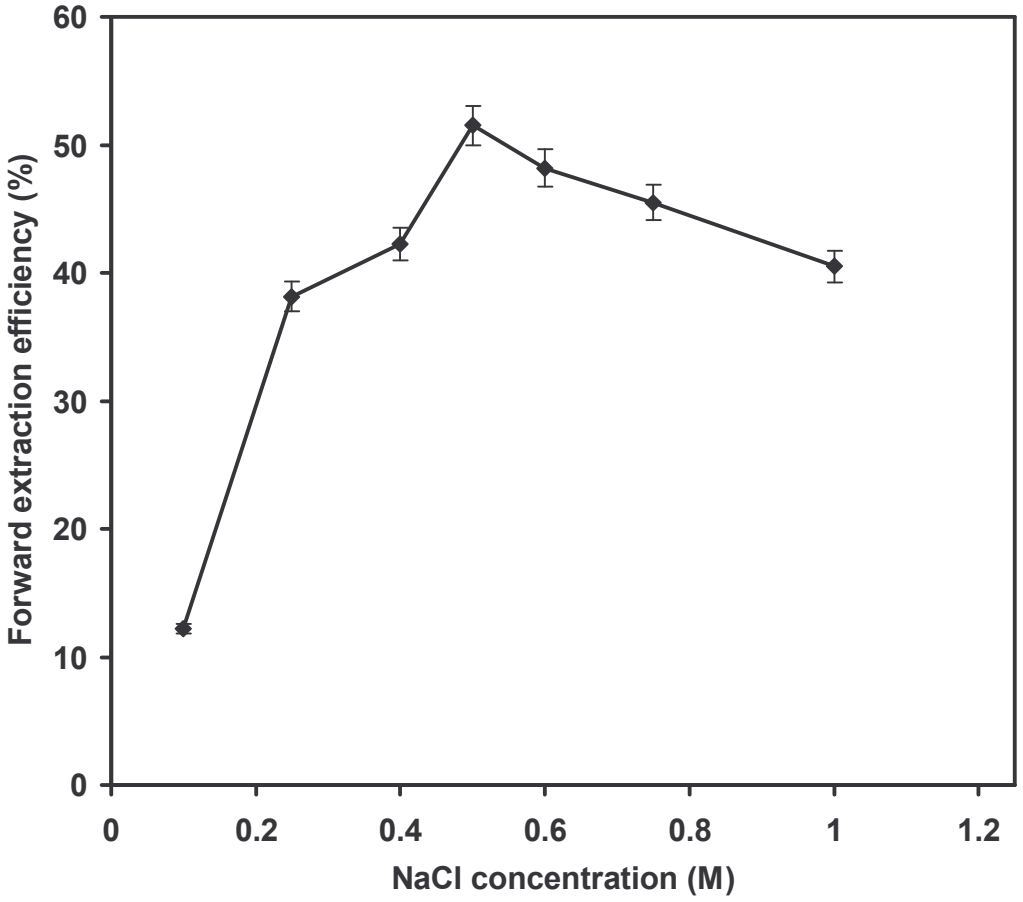

Fig. 3A.3. Effect of NaCl concentration on forward extraction of BSA in Triton-X-100/toluene reverse micellar system

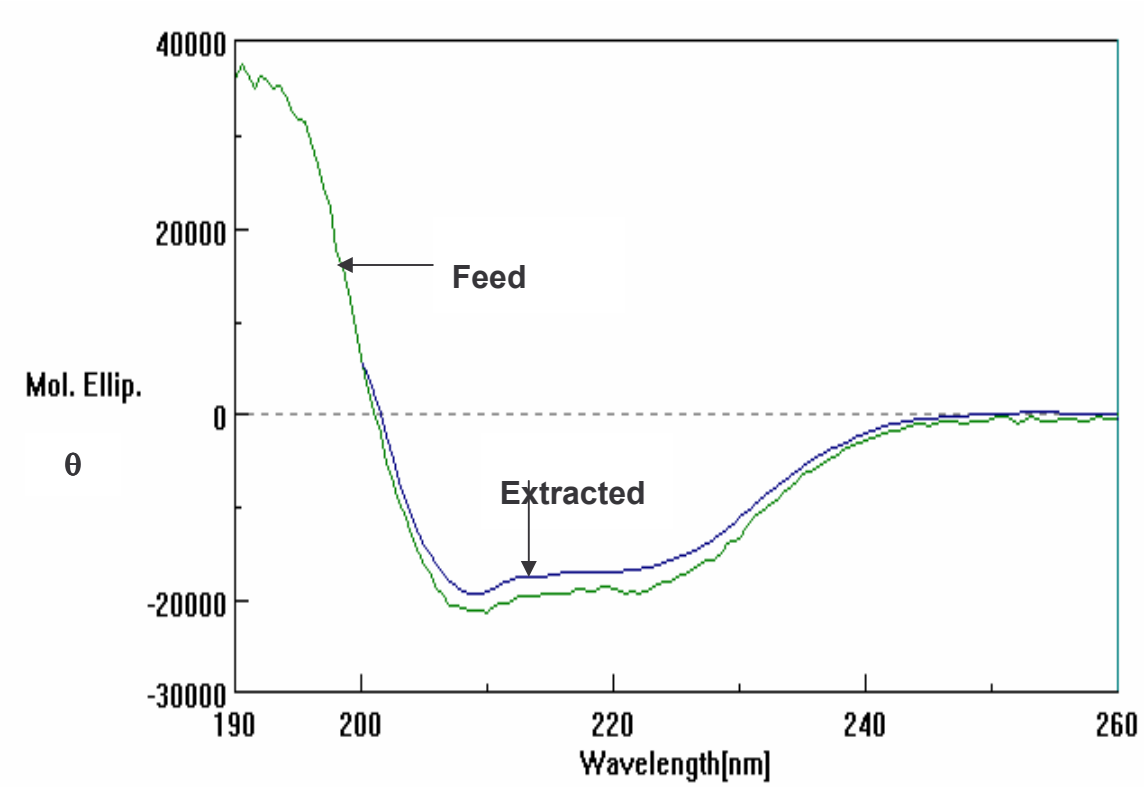

Fig. 3A.4. Circular Dichroism (CD) spectra of feed and AOT/toluene reverse micellar extracted BSA

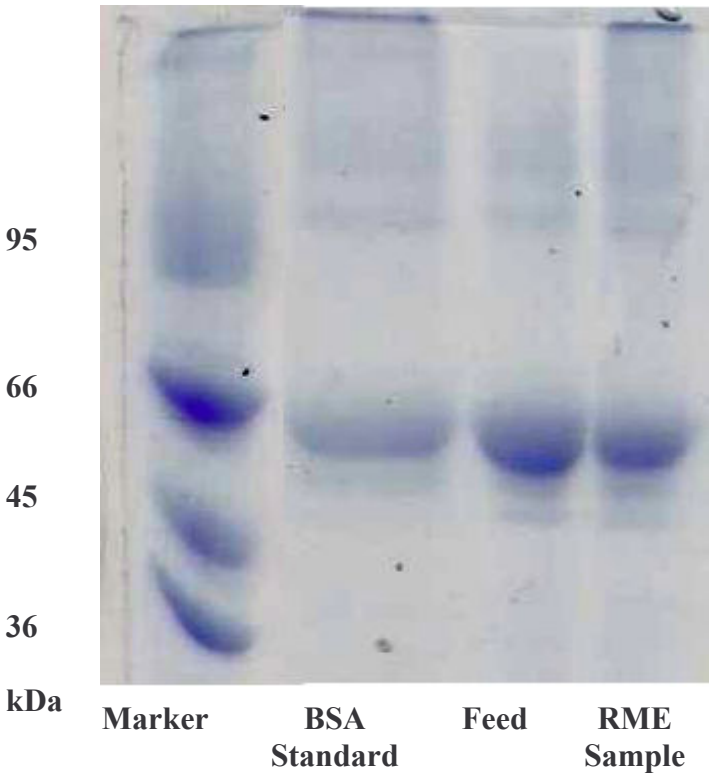

Fig. 3A.5. SDS-PAGE analysis of BSA samples

SECTION B

EXTRACTION OF HORSE RADISH PEROXIDASE (HRP)

3B.1 Introduction

Peroxidases are widely distributed in nature and can be easily extracted from the plant cells and, some animal organs and tissues. Because of the high catalytic activity, it has been used in several applications such as synthesis of various aromatic chemicals, wastewater treatment, and the removal of peroxide from foodstuffs and industrial wastes [Kim and Yoo, 1996]. A few reports on application of RME of peroxidase are available. Phase transfer studies on the solubilization properties of Horseradish peroxidase (HRP) using the anionic surfactant Aerosol-OT (AOT), dissolved in isooctane has been reported [Wolbert *et al.,* 1989] where an overall extraction efficiency was found to be 35%. Paradkar and Dordick (1991) reported an extraction efficiency of 83% when HRP was purified from a synthetic mixture with alkaline phosphatase, using lectin as an affinity ligand. Regalado *et al.* (1994) studied the recovery of commercial horse radish peroxidase (HRP) using AOT/isooctane reverse micellar system. Activity recovery in the range 87% to 136% was obtained with different phase volume ratios. Regalado *et al.* (1996) also reported the purification of peroxidase from horse radish roots by adopting two-stage reverse micellar extraction technique as a single step reverse micellar extraction was not efficient. AOT/isooctane system used for the extraction was able to result in a purification factor of 80. The separation and purification of horse radish peroxidase from *Armoracia rusticana* root was reported by Huang and Lee (1994). In the single stage extraction, all the proteins except the target solute i.e. peroxidase partitioned to the organic phase to get 20 fold purification. A few studies have been reported on the use of nonionic surfactants as an alternative to ionic surfactants. Vasudevan and Wiencek (1996) reported the work on use of micro emulsions of nonionic surfactants such as Neodol 91-2.5 and Tween 85. Effect of process conditions on degree of Cytochrome C extraction with nonionic surfactant span 60 was studied by Naoe *et al.* (1998).

RME has been successfully used for the purification of various other proteins and enzymes like BSA [Shiomori *et al.,* 1998], α-amylase [Chang and Chen, 1995; Chang *et al.,* 1997], chymotrypsin [Chang *et al*., 1994; Goto *et al.,* 1995]

cytochrome C [Ichikawa *et al.,* 1992]. In most of the reported extractions of proteins/enzymes, ionic surfactant AOT has been used, because of its higher ability to form reverse micelles and higher water pool. Only a few RME studies have been carried out using nonionic surfactants since they are 'mild' and 'less interactive'.

Present work is aimed at exploring the possibilities of extracting the commercial grade HRP enzyme from aqueous phase using a revere micellar system of Triton-X–100, a nonionic surfactant in organic solvent, toluene. The process parameters that affect the extraction efficiencies during forward and back extractions were optimized.

3B.2. Materials and Methods

3B.2.1 Materials

HRP (80 U/mg) was purchased from Himedia laboratories, India and Triton-X-100 was obtained from BDH laboratory, UK and used without further purification. Toluene (UV spectroscopy) was obtained from Loba Chemie., Mumbai, India. All other reagents used were of analytical grade. The various buffers used were malonic, citric, phosphate, glycine and sodium carbonate. Aqueous phases and all other solutions were prepared using distilled water.

3B.2.2. Methods

3B.2.2.1. Forward extraction

The forward extraction experiments were carried out by contacting aqueous and organic phases using a magnetic stirrer (Cintex, Mumbai, India) for 15 min at 500 rpm at controlled temperature (25±2ºC). The aqueous phase (30 mM) was prepared by dissolving the known quantity of HRP in buffer of known pH and salt (NaCl) concentration. The phase transfer was conducted under controlled temperature (25±2ºC). The phase ratio (V_{aqu}/V_{org}) and phase volume were maintained at 1 and 10 ml, respectively, unless otherwise mentioned. The organic phase had known amount of surfactant dissolved in the solvent toluene. Phase

separation was carried out in a laboratory centrifuge (MP 400 R, Eltek, India) at 5000 rpm for 15 min. The organic phase separated from the mixture after forward extraction was subjected to back extraction. In all the experiments the concentration of HRP was maintained at 0.33 mg/ml.

3B.2.2.2. Back extraction

Back extraction was carried out by contacting the organic phase (8 ml) of forward extraction with an equal volume of fresh aqueous phase (80 mM buffer of known pH and KCl concentration) using a magnetic stirrer for 30 min at 500 rpm at controlled temperature (25±2ºC). Centrifugation at 5000 rpm for 30 min separated the two phases. The two phases were then collected for analyses.

3B.2.3. Analyses

Protein content and enzyme activity measurement

The total protein content and the enzyme activity were measured in the aqueous phase by the standard commassie brilliant blue G-250 method [Bradford, 1976] and guaicol method [Kim and Yoo, 1996], respectively. Protein concentration readings were taken in triplicate and average values are reported. Efficiencies of forward and back extractions and specific activity of the enzyme were estimated using the equation given below.

$$\text{Forward extraction efficiency}\,(\%) = \frac{\text{Protein concentration in organic phase after forward extraction}\,(\text{mg/ml})}{\text{Protein concentration in feed}\,(\text{mg/ml})} \text{x}100 \qquad (3B.1)$$

$$\text{Back extraction efficiency}\,(\%) = \frac{\text{Protein concentration in back extracted aqueous phase}\,(\text{mg/ml})}{\text{Protein concentration in forward extracted organic phase}\,(\text{mg/ml})} \text{x}100 \qquad (3B.2)$$

$$\text{Activity recovery (\%)} = \frac{\text{Peroxidase activtity in aqueous phase after back extraction}}{\text{Peroxidase activty in the feed}} \text{x}100 \quad \text{(3B.3)}$$

Specific activity (U/mg) =

$$\frac{\text{Total activity of HRP in back extracted aqueous phase}\,(\text{U/ml})}{\text{Protein concentration in back extracted aqueous phase}\,(\text{mg/ml})} \quad \text{(3B.4)}$$

$$\text{Purification fold} = \frac{\text{Specific activity of peroxidase after RME}}{\text{Specific activity of peroxidase in the feed}} \quad \text{(3B.5)}$$

3B.3 Results and Discussion

3B.3.1 Forward extraction

i) Effect of aqueous phase pH

Vasudevan and Wiencek (1996) reported that the nonionic surfactants will have surface charge at neutral pH, which induces a weak electrostatic interaction between charged solute and surfactant. Hence, extraction was carried out at aqueous phase pH 7.0. However, the forward extraction efficiency was poor (23.2%) at this aqueous phase pH and it indicated the non-existence of sufficient electrostatic interaction to extract HRP. Hence, studies were carried out over a range of aqueous phase pH from 2.5 to 7.0 (Fig. 3B.1). The details of other processing conditions employed for extraction are shown in Table 3B.1. At pH 3.0, the extraction was higher (35.3%) compared to other pH values. However, further increase in pH marginally decreased the extraction and it remained almost the same up to pH 7.0. Hydrophobic interaction may not be playing a major role in the present system as the surfactant used (Triton-X–100) is relatively more hydrophilic in nature (HLB-13.4) and, also the amino acids in the enzyme are expected to be hydrophilic in nature below the iso-electric point. Hence, it is inferred that the steric effect might be the reason for extraction in the aqueous pH range studied. Earlier studies by Hebbar and Raghavarao, (2007) have indicated that Triton-X-

100/toluene reverse micelles forms a relatively larger sized reverse micelles (5.67 nm at Triton-X-100 concentration of 14 mM), which may be sufficient to incorporate HRP molecules (44 kDa, ~2.75 nm) into the hydraulic core. Since, aqueous phase pH 3.0 gave relatively higher forward extraction, the same was maintained in further experiments.

ii) Effect of salt (NaCl) strength

The influence of ionic strength on the solubilization of proteins in reverse micelles reported to be purely an electrostatic effect. Figure 3B.2 shows the enzyme uptake at aqueous pH 3.0 at different salt concentrations (0 to 0.45 M). Details of the other processing conditions employed are presented in the Table 3B.1. Although, an increase in salt concentration favored the extraction, higher concentration of salt resulted in a decreased yield. It was observed that as the ionic strength of the aqueous phase increases, the protein uptake capacity decreases (Aires-Barros and Carbal, 1991; Marcozzi *et al.,* 1991). Decrease in Debye length due to an increase in ionic strength, in turn reducing the electrostatic interactions was considered to be one of the reasons for the above observation. The other reason being the decreased electrostatic repulsion between charged head groups of surfactants at higher ionic strength, leading to the squeezing out effect. It was observed that at very low ionic strength the extraction was very low and phases were a little turbid.

iii) Effect of buffer concentration

It may be noted that, the strength of the buffer used for the aqueous phase preparation also plays a role in deciding the extraction efficiency. The effect of the buffer (citrate-phosphate) concentration on the extraction of HRP was studied. The results are shown in Table 3B.2. As can be seen from the table, the maximum yield was obtained at 30 mM concentration although no significant change in forward extraction efficiency was observed at 50 mM. It has been reported that the increase in buffer concentration beyond a certain value, decreases the extraction efficiency due to reduction in micelle size [Ayala *et al.,* 1992; Naoe *et al.,* 1996].

However, such a trend was not observed in the present study for the concentration range studied.

iii) Effect of buffer type

Apart from the buffer strength, the type of buffer used also influences the extraction efficiency. Different buffers (Malonic-NaOH, Citrate-Phosphate and Glycine-HCl) having 30 mM concentration were used to get the desired aqueous phase pH (3.0). The forward extraction efficiencies with these buffers are presented in Table 3B.3. It can be observed that the citrate-phosphate and glycine-HCl buffers resulted in almost the same results (~35%), whereas with malonic buffer the extraction was comparatively less (22.1%). The variation in enzyme extraction with different buffers may be attributed to the physicochemical interactions among the enzyme, the buffer and the surfactant molecules near the reverse micellar wall, resulting in incomplete enzyme uptake. Similar observations were made by Regalado *et al.* (1994), while studying the effect of type of buffers on back extraction of HRP.

3B.3.2 Back extraction

i) Effect of aqueous phase pH

HRP extracted into the reverse micelles during forward extraction was recovered in the back extraction by bringing the organic phase of forward extraction in contact with the fresh aqueous phase. Forward extraction conditions employed were: aqueous phase pH 3.0, citrate-phosphate buffer 30 mM, NaCl 0.15 M, and phase volume ratio 1. The salt concentration (KCl) was maintained at 1.0 M. The back extraction efficiency was very low (Fig. 3B.3) at all aqueous phase pH values studied (8.0 to 10.0) and maximum back extraction (18%) was obtained at aqueous phase pH 10.0. The overall extraction thus obtained was found to be very low (~6.5%) under these conditions.

ii) Effect of salt (KCl) concentration

To study the effect of salt concentration on back extraction, the KCl concentration in aqueous phase was varied (0.1, 1.0 and 2.0 M). The aqueous phase pH was maintained at 10.0 in all the cases. The extraction efficiency increased with the KCl concentration, and a maximum extraction efficiency of 37.2% was obtained at 2 M KCl (Table. 3B.4). The specific activity of HRP in the back aqueous phase of back extraction was measured at the above conditions and a maximum activity of 162.7 U/mg was obtained at 2 M KCl, which corresponds to a purification of nearly 2 fold. However, the overall extraction was only 13%, corresponding to the above back extraction, which is low.

3B.4. Conclusions

The efficiency of forward and back extractions was observed to depend mainly on the processing conditions adopted. A forward extraction efficiency of 35.3% was obtained at pH 3.0 and salt (NaCl) concentration of 0.15 M. Steric effect was assumed to be the most probable reason for forward extraction of HRP. Aqueous phase pH during back extraction did not significantly affect the extraction efficiency and a maximum of only 18% extraction could be obtained at pH 10.0. Increase in salt concentration enhanced the extraction efficiency and at 2 M KCl nearly 37.3% extraction with two-fold increase in enzyme activity was obtained. The study emphasized the need for selection of suitable reverse micellar system for the given solute and it indicated that the strong electrostatic or hydrophobic interaction is needed for extracting the relatively high molecular weight solute like HRP using reverse micelles.

Table 3B.1. Processing conditions employed for forward extraction of HRP

Aqueous phase pH	NaCl concentration (mM)	Triton X -100 concentration (mM)
2.5, 3.0, 4.0, 5.0, 6.0, 7.0	0.15	15
3.0	0.05 0.15, 0.25, 0.35, 0.45	15
3.0	0.15	5, 10, 15, 20, 25

Table 3B.2. Effect of buffer concentration on forward extraction of HRP

Buffer (Citrate-phosphate) concentration (mM)	Forward extraction efficiency (%)
10	23.1
30	35.3
50	34.2

System: Triton-X-100/Toluene
(Forward extraction conditions - Aqueous phase pH: 3.0; NaCl: 0.2 M, Phase volume ratio: 1)

Table 3B.3. Effect of type of buffer on forward extraction of HRP

Buffer	Forward extraction efficiency (%)
Malonic	22.1
Citrate-phosphate	35.3
Glycine-HCl	34.7

System: Triton-X-100/Toluene
(Forward extraction conditions - Aqueous phase pH: 3.0; NaCl: 0.2 M, Phase volume ratio: 1)

Table 3B.4. Effect of salt concentration on back extraction of HRP

KCl concentration (M)	Back extraction efficiency (%)	Specific activity (U/mg)
0.1	16.0	38.9
1	18.0	11.8
2	37.3	162.7

System: Triton-X-100/Toluene
(Back extraction conditions - Aqueous phase pH: 10.0; Phase volume ratio: 1)

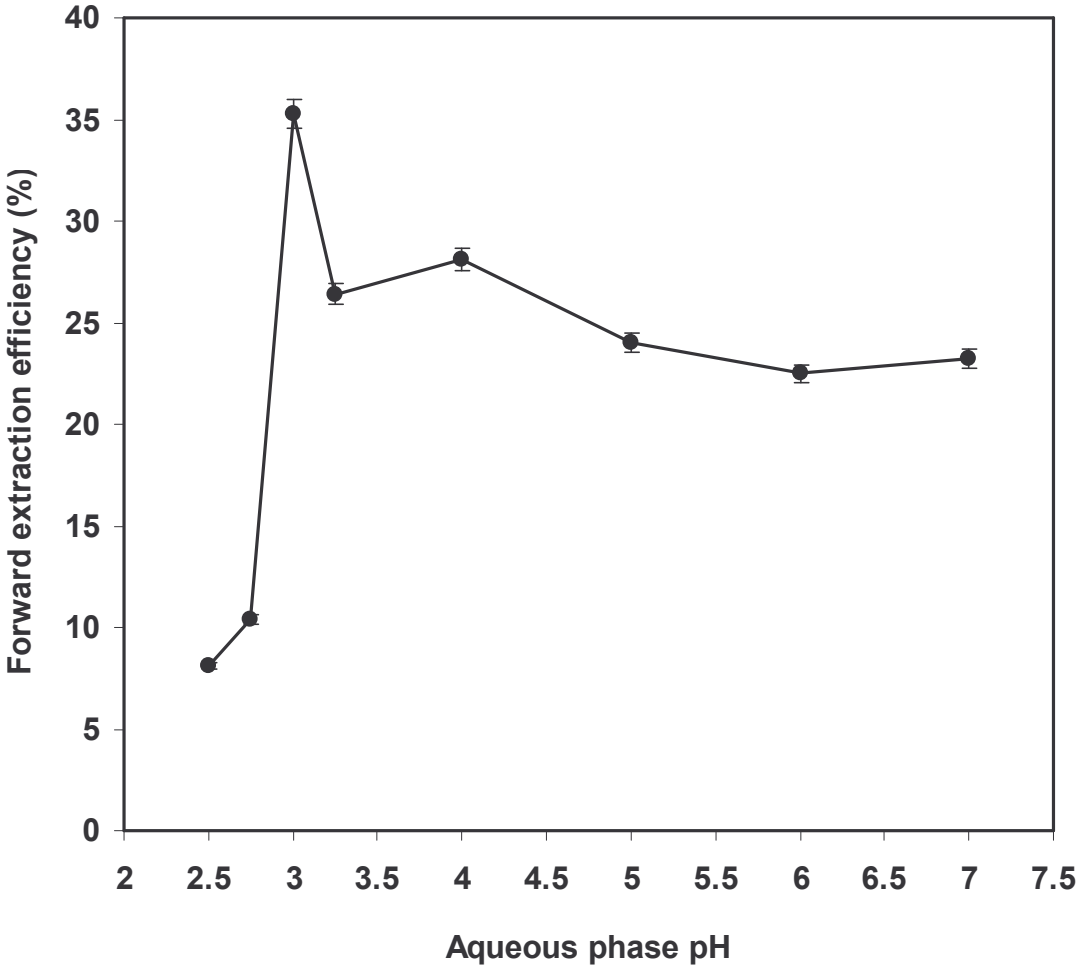

Fig. 3B.1 Effect of aqueous phase pH on forward extraction of HRP

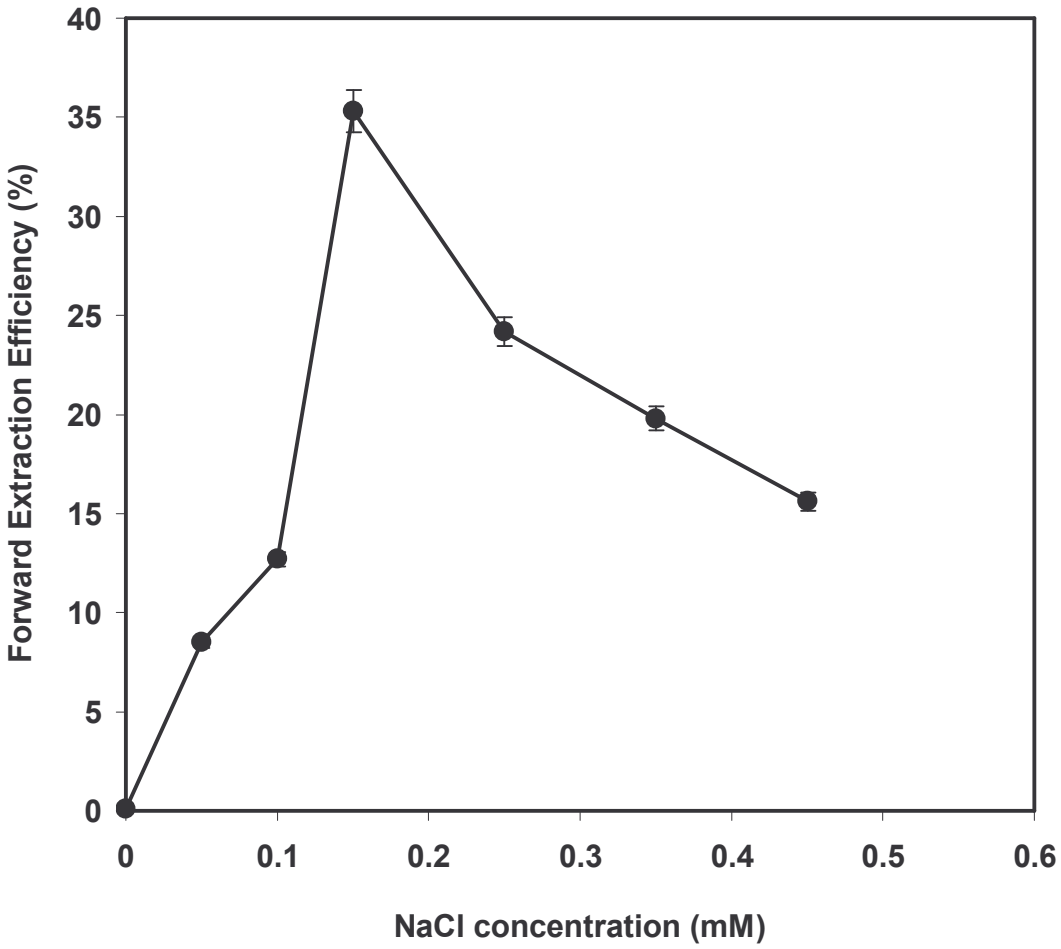

Fig. 3B.2. Effect of NaCl concentration on forward extraction of HRP

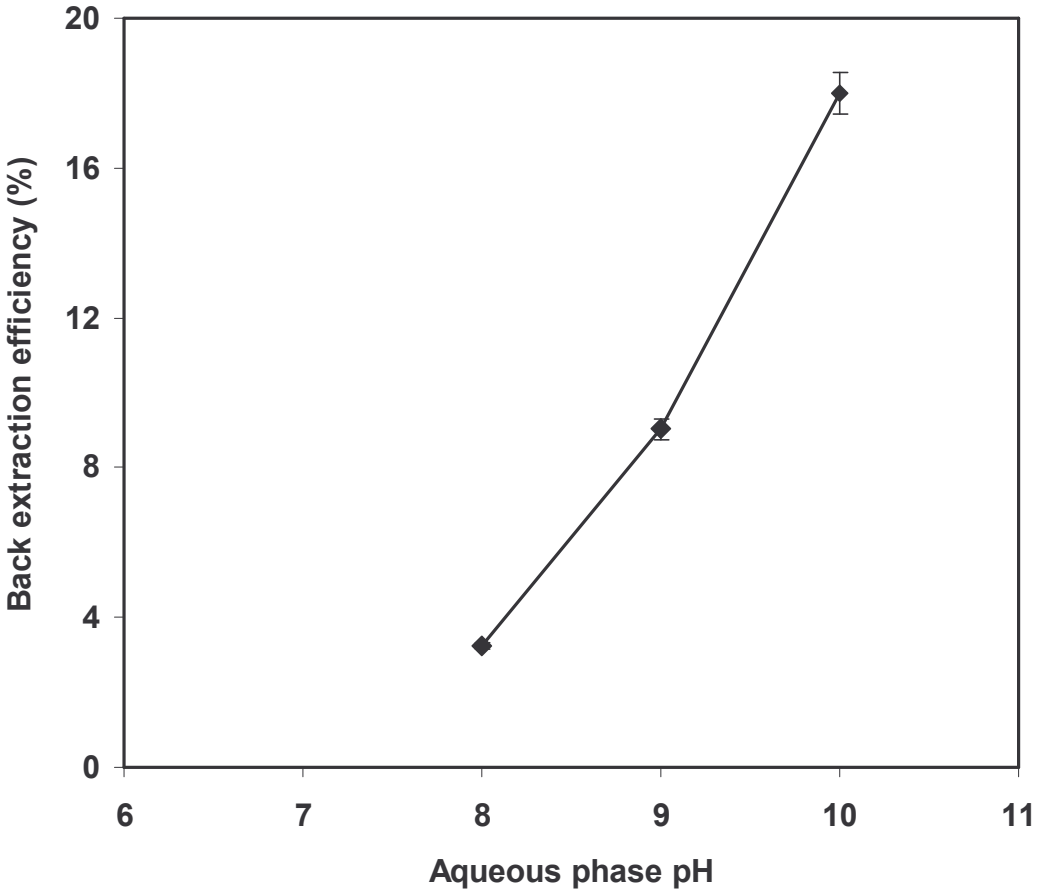

Fig. 3B.3. Effect of aqueous phase pH on back extraction of HRP

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CHAPTER 4

REVERSE MICELLAR EXTRACTION EMPLOYING NATURAL SYSTEMS

SECTION A

EXTRACTION OF BROMELAIN FROM PINEAPPLE WASTES

4A.1. Introduction

Bromelain, a proteolytic enzyme is found in the tissues of plant family *Bromeliaceae* of which pineapple (*Ananas comosus* L. Merryl*)* is the best known source. Pineapple belongs to the family of Bromeliaceae and is a herbaceous, perennial, monocotyledonous plant [Chadha *et al.,*1998]. Normal fruiting pineapple plant consists of suckers, slips, penducle and crown in addition to leaves, stem and roots (Fig. 4A.1). Crown is the miniature plant, consisting of condensed stem and leaves, growing from the apex of the fruit. The stem is short and thick, 15-25 cm long, narrow at the base and wider at the top with short internodes. The leaves are long and narrow, and are arranged in a right-or-left handed spiral on a short stem, forming a rosette. During pineapple harvesting the stem portion, which is the main source of bromelain is discarded as waste. The portions of the pineapple fruit that are regarded as wastes during processing are crown, core of the fruit and peel, which also contain bromelain. Bromelain is mainly comprised of cysteine proteases, with smaller amounts of acid phosphatase, peroxidase, amylase and cellulase. Stem protease is a basic glycoprotein with a molecular weight of 28,000 Da and an iso-electric point 9.5, whereas the fruit bromelain is an acidic glycoprotein with a molecular weight of 31,000 Da and an isoelectric point of 4.6 [Yamada *et al.,* 1976].

The stem bromelain (EC 3.4.22.32) and fruit bromelain (EC 3.4.22.33) obtained from the pineapple stem and pulp, respectively are finding wide applications in pharmaceutical and food industry [Rowan *et al.,* 1990; Ko *et al.,* 1996]. Purification and characterization of bromelain from pineapple fruit using combinations of ammonium sulphate precipitation and ion exchange column [Suh *et al.,* 1992; Ko *et al.,* 1996], and ion exchange chromatography and gel filtration [Choi *et al.,* 1992] has been reported. Bromelain is reported to be present also in pineapple wastes such as core, peel, and leaves although, relatively in smaller quantities as compared to stem [Sriwatanapongse *et al.,* 2000]. Frieman and Sabaa-Srur (1999) estimated the bromelain activity in pineapple wastes and suggested that it may be possible to produce bromelain from pineapple wastes.

However, practically no detailed reports on the extraction of bromelain from the above wastes are available, despite the fact that large amounts of pineapple waste is produced at the pineapple processing industries [Rowan *et al.,* 1990]. Hence, in the present study an attempt has been made to use RME technique for the extraction and primary purification of bromelain from these wastes. Considering the fact that pineapple generates nearly 35% of waste [Bardiya *et al.,* 1996], a successful RME could lead to the value addition to wastes that are otherwise disposed as such or used for composting. Although, report on possible utilization of these wastes for energy generation by method such as biomethanation [Bardiya *et al.,* 1996] is available, industrial scale application of such technique is not reported.

The objectives of the present study are i) RME for the extraction of bromelain from pineapple wastes such as core, peel, and leaves and extended stem (portion of the stem to which leaves of the crown are attached) and ii) detailed study on the effect of forward and back extraction parameters and process optimization to achieve higher enzyme activity recovery and purification from pineapple core extract.

4A.2. Materials and Methods

4A.2.1. Materials

i) Pineapple fruit

Mature pineapple fruits (Ananas comosus L. Merr. cv. Kew, 7-9°Brix) available in the local market were used for the extraction.

ii) Chemicals

Cetyltrimethylammonium bromide (CTAB) and sodium bis (2-ethyl-1-hexyl) sulfosuccinate (AOT) were obtained from Merck, Germany and BDH Laboratory Supplies, UK, respectively. Iso-octane (HPLC grade) was purchased from Merck,

Mumbai, India. Hexane and hexanol from SRL, Mumbai, casein (Hammerstein grade) and n-butanol from Loba chemie, India were used. All other chemicals of AR grade were used for the experiments and analyses.

4A.2.2. Methods

i) Preparation of crude extract

The peel and core portions were separated from the fruit. The extended stem and leaves were obtained from the pineapple crown. For the preparation of crude extract, known quantity of waste was crushed along with extraction buffer [0.01 M sodium phosphate buffer of pH 6.5, containing 1% PVP] at 1:1 ratio for 10 min and then filtered through a cheese cloth. The filtrate was centrifuged (MP 400 R, Eltek, India) at 10000 g for 15 min and the supernatant (crude enzyme extract) obtained was used for RME experiments.

ii) Reverse micellar extraction

Forward extraction with anionic surfactant, AOT was carried out by contacting 10 ml of the organic phase (solvent/surfactant) with equal volume of aqueous phase (enzyme extract with salt). For cationic CTAB/isooctane system, the organic phase also contained a mixture of co-slovents (5% (v/v) hexanol and15% (v/v) butanol). Back extraction was carried out by contacting the organic phase obtained from forward extraction with a fresh aqueous phase also termed as stripping phase (buffer of known pH containing KBr). The phases were mixed thoroughly for 1 h and centrifuged at 4000 g for 15 min (MP 400 R, Eltek, India) during both forward and back extraction. The aqueous phases obtained after forward and back extractions were analyzed for bromelain activity and total protein content. All the phase equilibration experiments were carried out at 25 ± 2^{0}C.

iii) Bromelain activity

Bromelain activity in aqueous phase was determined according to the casein digestion unit (CDU) method using Hammerstein grade casein (0.6%) as substrate in the presence of cysteine and EDTA [Murachi, 1976]. The assays were based on proteolytic hydrolysis of the casein substrate. The absorbance of the clear filtrate (solubilized casein) was measured at 275 nm using spectrophotometer (Shimadzu UV-160). One unit of bromelain activity is defined as 1 μg of tyrosine released in 1 min per ml of sample when casein is hydrolyzed under the standard conditions of 37°C and pH 7.0 for 10 min.

iv) Protein content

Protein content in aqueous phase was determined by measuring absorbance at 280 nm using bromelain as standard in case of experiments with CTAB system. The dye binding method of Bradford [1976] was used (using BSA as standard) when AOT system was employed. The sample analyses were performed against respective blank solutions. Protein concentration readings were taken in triplicate and average value was used for the calculation of extraction efficiencies. The forward and back extraction efficiencies, activity recovery (%) and purification fold are estimated as shown below.

$$\text{Forward extraction efficiency}\,(\%) = \frac{\text{Protein concentration in organic phase}}{\text{Protein concentration in feed}} \times 100 \qquad (4A.1)$$

$$\text{Back extraction efficiency}\,(\%) = \frac{\text{Protein concentration in organic phase}}{\text{Protein concentration in aqueous phase of back extraction}} \times 100 \qquad (4A.2)$$

$$\text{Activity recovery (\%)} = \frac{\text{Bromelain activtity in the aqueous phase after RME}}{\text{Bromelain activty in the feed}} \text{x} 100 \quad \text{(4A.3)}$$

$$\text{Purification fold} = \frac{\text{Specific activity of bromealin after RME}}{\text{Specific activity of bromelain in the feed}} \quad \text{(4A.4)}$$

4A.3. Results and Discussion

The processing conditions for obtaining crude enzyme extracts from pineapple wastes were optimized and stability of bromelain at different pH and storage conditions were studied. The forward and back extraction parameters were altered to optimize the bromelain activity recovery and purification. Purity of bromelain extracted from core was confirmed using SDS–PAGE analysis.

4A.3.1. Native PAGE analysis of peel and core extracts

To identify the type (stem or fruit) of bromelain present in core and peel extracts, native PAGE analysis of these extracts was carried out and compared (Fig. 4A.2) with the standard stem bromelain (Sigma). The native band obtained for the peel is similar to the standard used, indicating the presence of stem bromelain. However, in the case of core extract, the analysis indicated the presence of another solute besides stem bromelain, which was suspected to be that of fruit bromelain.

4A.3.2. Preparation of crude bromelain extract

Extraction medium: To study the suitability of medium, extractions were carried out using distilled water and phosphate buffer (pH 6.5). The bromelain activity and the protein concentration in crude extracts obtained from pineapple wastes (core, peel, stem and crown) are presented in Table 4A.1. In all the cases, the phosphate buffer extracts had higher specific activity as compared to the extraction with distilled water. The higher specific activity could be attributed to the salts present in the buffer. The specific activity of bromelain present in the crown

extract was higher as compared to the other three extracts, while it was found to be lowest in crude obtained from peel. For all the RME experiments the phosphate buffer extract was used.

4A.3.3. Stability studies

Storage stability: The enzyme present in the crude should be stable at least for a duration that is sufficient to carryout RME. Crude extracts obtained from the wastes were stored at 25±2°C and activity was measured at frequent intervals for a period up to 24 h (Fig. 4A.3). Decrease in specific activity with time was observed in all the extracts, although they were fairly stable up to 4 h in all the cases. The bromelain in core extract was relatively more stable as compared to other extracts. The specific activity decreased by nearly 30-40% in peel and crown extracts at the end of 24 h.

pH stability: The pH of the aqueous phase used for the reverse micellar extraction is one of the critical parameters that determines the extraction efficiency. To study the stability of bromelain, the pH of the crude extracts was altered in the range 4 to 8 (reported stable range of bromelain) and the enzyme activity was measured. The results are presented in Fig. 4A.4. The specific activity of the enzyme was fairly stable in all the extracts except in that of crown where a decrease of about 20% was observed in the pH range studied.

4A.3.4. RME of bromelain from pineapple core

The native PAGE analysis carried out earlier had indicated the similarity between bromelain present in core and that of standard fruit bromelain. Hemavathi *et al.* (2007) had reported that AOT, the commonly used anionic surfactant forms a complex with bromelain, which precipitates at pH below 4.5. Hence, the AOT system was not employed for the extraction. Fairly good extraction of bromelain from pineapple pulp was reported using CTAB/iso-octane/hexanol/butanol system by Hemavathi *et al.* (2007), and hence the same system was used in the present study. The aqueous phase pH, NaCl and CTAB concentrations were altered

during forward extraction, while aqueous phase pH and KBr concentrations were varied during back extraction.

4A.3.4.1. Forward extraction parameters

i) Effect of aqueous phase pH

Forward extraction was carried out at aqueous phase pH 5.0-10.0 in order to have an electrostatic interaction between positively charged CTAB and negatively charged bromelain (above pI - 4.6). CTAB and NaCl concentrations were maintained at 150 mM and 0.1M, respectively. The back extraction was carried out at aqueous phase pH10.0 and 0.5 M KBr. The effect of aqueous phase pH on forward extraction efficiency, activity recovery and purification fold is presented in Fig. 4A.5. The forward extraction efficiency increased with an increase in the aqueous phase pH and maximum value (69%) was obtained at aqueous phase pH 9.0. Increased electrostatic interaction between surfactant and solute molecules at higher pH-pI values [Fletcher and Parrott, 1989] could be the reason for increased extraction. The activity recovery was 132% at aqueous phase pH 9.0, with a purification of 1.7 fold. The maximum purification (5.2 fold) was obtained at pH 8.0, where the activity recovery and forward extraction efficiency were 106% and 45%, respectively. Although, at aqueous phase pH 10.0, the extraction was fairly good (~57%), activity recovery (81%) and purification (1.2 fold) were relatively low. The aqueous phase pH, which is above the reported stable pH range (4.0-9.0) for bromelain could be the reason for lowered bromelain activity. Since, the activity recovery and purification fold are the primary criteria in enzyme extraction and purification, the aqueous phase pH of 8.0 was used in further studies.

ii) Effect of surfactant (CTAB) concentration

CTAB concentration was varied (Fig. 4A.6) from 50 to 200 mM under similar processing conditions of forward extraction (aqueous phase pH 8.0 and 0.10 M NaCl). Back extraction conditions were same as that indicated in section 4A3.4.1. It was reported that an increase in surfactant concentration increases the

number of reverse micelles, which in turn enhances the forward extraction efficiency [Pessoa and Vitolo, 1998]. In the present study, as expected, the forward extraction increased with an increase in surfactant concentration. Higher forward extraction efficiency (45%), activity recovery (106%) and purification of 5.2 fold were obtained at 150 mM concentration. However, the extraction efficiency dropped from 45% to 34% at 200 mM concentration, which may be due to the lower extent of extraction caused by the increased micellar interaction and collapse of reverse micellar structure, normally observed at higher surfactant concentrations [Regalado *et al.,* 1996]. 150 mM CTAB concentration was maintained in all the remaining experiments.

iii) Effect of salt (NaCl) concentration

Kinugasa *et al*. 2003 reported that presence of water structure forming salt such as NaCl enhances the stability of the reverse micelles, resulting in higher extent of forward extraction. Hence, in the present study, NaCl concentration in the aqueous phase was increased up to 0.3 M, while maintaining the aqueous phase pH (8.0) and CTAB concentration (150 mM) the same (Fig. 4A.7). When NaCl was not added, the phases turned cloudy and the extraction was very low (~17%). The best results (45% forward extraction efficiency, 106% activity recovery and purification of 5.2 fold) were obtained at NaCl concentration of 0.1 M. When the NaCl concentration was increased above 0.1 M, the extraction efficiency, activity recovery as well as the purification fold decreased. The 'size exclusion' effect caused by the higher salt concentration [Harikrishna *et al.,* 2002] could be the reason for above observation. For all further studies, NaCl concentration was maintained at 0.1 M.

4A.3.4.2 *Back extraction parameters*

i) Effect of aqueous phase pH

Back extraction of solutes from the organic phase is affected by pH of the stripping phase used [Shiomori *et al.,* 1995]. It was reported that the aqueous phase pH, which does not favor the uptake of enzyme during forward transfer

should help in the release of solute back into the aqueous phase [Harikrishna *et al.,* 2002]. In the present study, the aqueous phase pH was varied from 3.9 to 4.5 (which is lower than the pI of bromelain), in order to facilitate the release of bromelain from reverse micelles (Fig. 4A.8). Although, the back extraction efficiency was slightly lower (~ 62%) at pH 4.2 as compared to that at aqueous phase pH 4.5, the activity recovery (106%) and purification (5.2 fold) were higher at pH 4.2.

ii) Effect of salt (KBr) concentration

The organic phase of forward extraction (aqueous phase pH 8, CTAB 150 mM, NaCl 0.1M) containing reverse micelles was subjected to back extraction to recover bromelain into fresh aqueous phase. High concentration of water structure breaking salt such as KBr during back extraction is reported to be used for destabilizing the reverse micelles, leading to the release of solute back to a fresh aqueous phase [Kinugasa *et al.* 2003]. An increase in salt concentration reduces the repulsive interactions between the surfactant head groups and there by decreases the size of the micelles. This helps in the exclusion of the enzyme from the reverse micellar core during back extraction [Harikrishna *et al.,* 2002]. In the present study, the KBr concentration was varied from 0.25 to 0.75 M (Fig. 4A.9), while maintaining the aqueous phase pH at 4.2. The back extraction efficiency increased with the increase in KBr concentration up to 0.5 M. An activity recovery of 106% and purification of 5.2 fold was obtained at this concentration. Further increase in the salt concentration to 0.75 M reduced the extraction efficiency (18%) as well as activity recovery (30%) and purification (2.1 fold).

4A3.5. RME of bromelain from extended stem

The crude extract obtained from the ‘extended stem’ was found to have good bromelain activity. Attempts were made to extract bromelain from the extended stem using anionc and cationic surfactants. Cationic CTAB and anionic AOT surfactants were used above and below the isoelectric point (9.5) of stem bromelain, respectively to have an electrostatic interaction between surfactant and bromelain molecules. The forward extraction using CTAB system was carried out

at pH values 10.0, 11.0 and 12.0 and in all the experiments CTAB and NaCl concentrations were maintained at 150 mM and 0.1 M, respectively. Back extraction was carried out at aqueous phase pH 4.2 and KBr concentration of 0.5 M. The results of the study are presented in Fig. 4A.10. The activity recovery decreased with an increase in aqueous phase pH, while the purification did not show any definite trend. Although, the activity recovery was high (102%) at pH 10.0, a relatively good activity recovery (80%) and purification (3.5 fold) were obtained at pH 11.0.

For the extraction with anionic surfactant AOT, pH range of 6.0 to 8.0 was selected. AOT and NaCl concentrations were maintained at 100 mM and 0.1 M, respectively and the back extraction was carried out at aqueous phase pH 8.0. As can be seen from Table 4A.2, although, the purification fold was more than 1.0 in all the cases; the activity recovery values were very low (<4%). The reason for the above observation could be the interaction between AOT and bromelain, which had resulted in the precipitation at the interface. This emphasizes the need for selection of suitable reverse micellar system for the extraction of desired solute.

4A3.6. RME of bromelain from pineapple peel

The native PAGE analysis indicated that the bromelain present in the peel was similar to standard stem bromelain. Hence, RME experiments were carried out at pH values (9.5-12.0) above the iso-electric point (9.5) of stem bromelain in order to have an electrostatic interaction (Fig. 4A.11). CTAB system was used for the extraction and all the other forward and back extraction conditions were maintained same as that employed for the extraction from core. The activity recovery was found to be less than 100% in all the trials and best results were obtained at aqueous phase pH 10.5 with 78% activity recovery and 2.1 fold purification.

4A.3.7. RME of bromelain from crown

The crude extract obtained from the crown was subjected to RME using CTAB/isooctane/hexanol/butanol reverse micellar system. The extraction was carried out at aqueous phase pH 10 and 11, while maintaining other conditions the same (CTAB: 150 mM; NaCl: 0.1 M; KBr: 0.5 M; back extraction aqueous phase pH: 4.2). An activity recovery of 60% and 54% with purification fold of 1.4 and 1.7 were obtained at pH 10.0 and 11.0, respectively (Table 4A.2), which are lower than that obtained from the core and extended stem.

4A.3.8. SDS–PAGE analysis

The aqueous phase of back extraction containing bromelain extracted from core using CTAB reverse micellar system was dialysed overnight at 4°C and lyophilized. The concentrated sample was loaded to 10% gel along with standard bromelain sample. The SDS lane pattern of reverse micellar extracted bromelain matched well with the standard (Fig. 4A.11). The band obtained was found to be around 26 kDa, which lies close to the reported range of the molecular weight (28 to 32 kDa) of bromelain.

4A.4. Conclusions

Reverse micellar extraction (RME) could be successfully applied for the isolation and primary purification of bromelain from pineapple wastes (core, peel, and leaves and extended stem). The bromelain activity in the crude extracts wastes varied and higher activity was found in core and crown extracts. CTAB/isooctane/hexanol-butanol system resulted in a fairly good extraction of bromelain from core extract. AOT/isooctane system used for the extraction from the extended stem did not show good activity recovery. The processing conditions greatly affected the extraction efficiency and emphasized the need for optimizing the conditions for extraction.

Table 4A.1. Bromelain activity and protein concentration in crude extracts obtained from pineapple wastes

Sample	Enzyme Activity (CDU/ml)	Protein concentration (mg/ml)	Specific Activity (CDU/mg)
Extraction solvent: Distilled water			
Core	415.12	10.29	40.32
Peel	215.65	21.10	10.22
Crown	495.10	10.02	49.41
Stem	330.46	22.45	14.72
Extraction solvent: Phosphate buffer (pH 6.5)			
Core	677.18	12.06	56.15
Peel	407.97	22.06	18.49
Crown	900.12	12.71	71.52
Stem	681.16	23.98	28.40

Table 4A.2. RME of bromelain from extended stem and crown of pineapple fruit

Aqueous phase pH	Purification fold	Activity recovery (%)
Extended stem*		
6.0	1.3	1.7
7.0	1.0	1.6
8.0	2.1	3.1
Crown**		
10.0	1.4	60
11.0	1.7	54

* AOT/isooctane system ** CTAB/Isooctane/heaxanol-butanol system

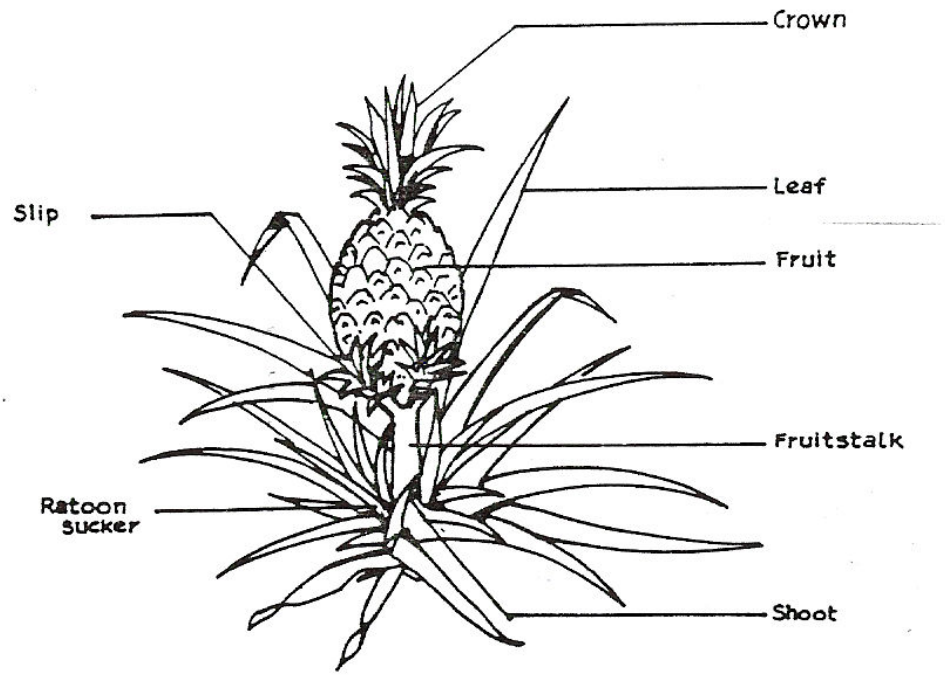
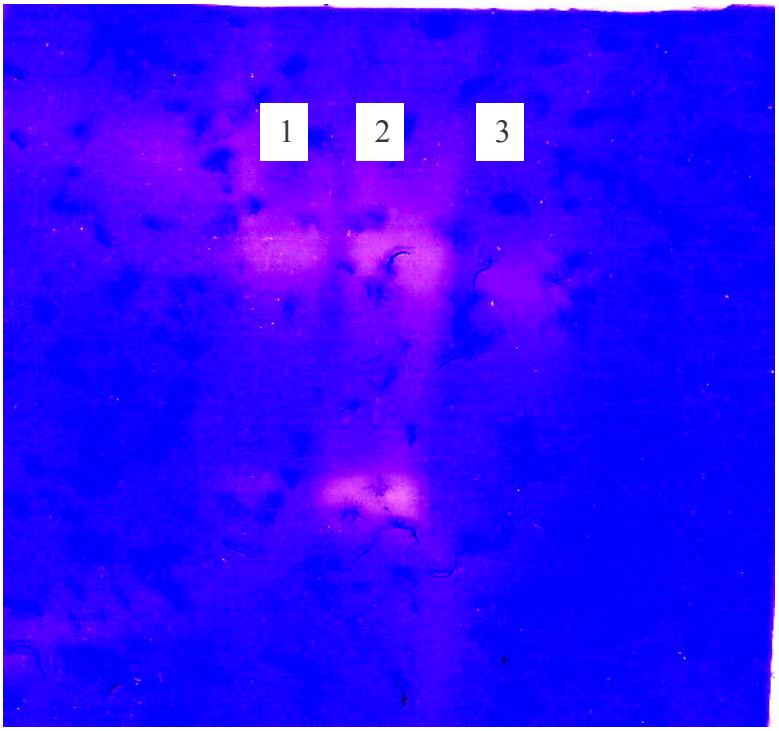


Fig. 4A.1. Parts of the pineapple fruit plant



1. Peel 2. Core 3. Stem bromelain (Standard)

Fig. 4A.2. Native PAGE for core and peel extracts

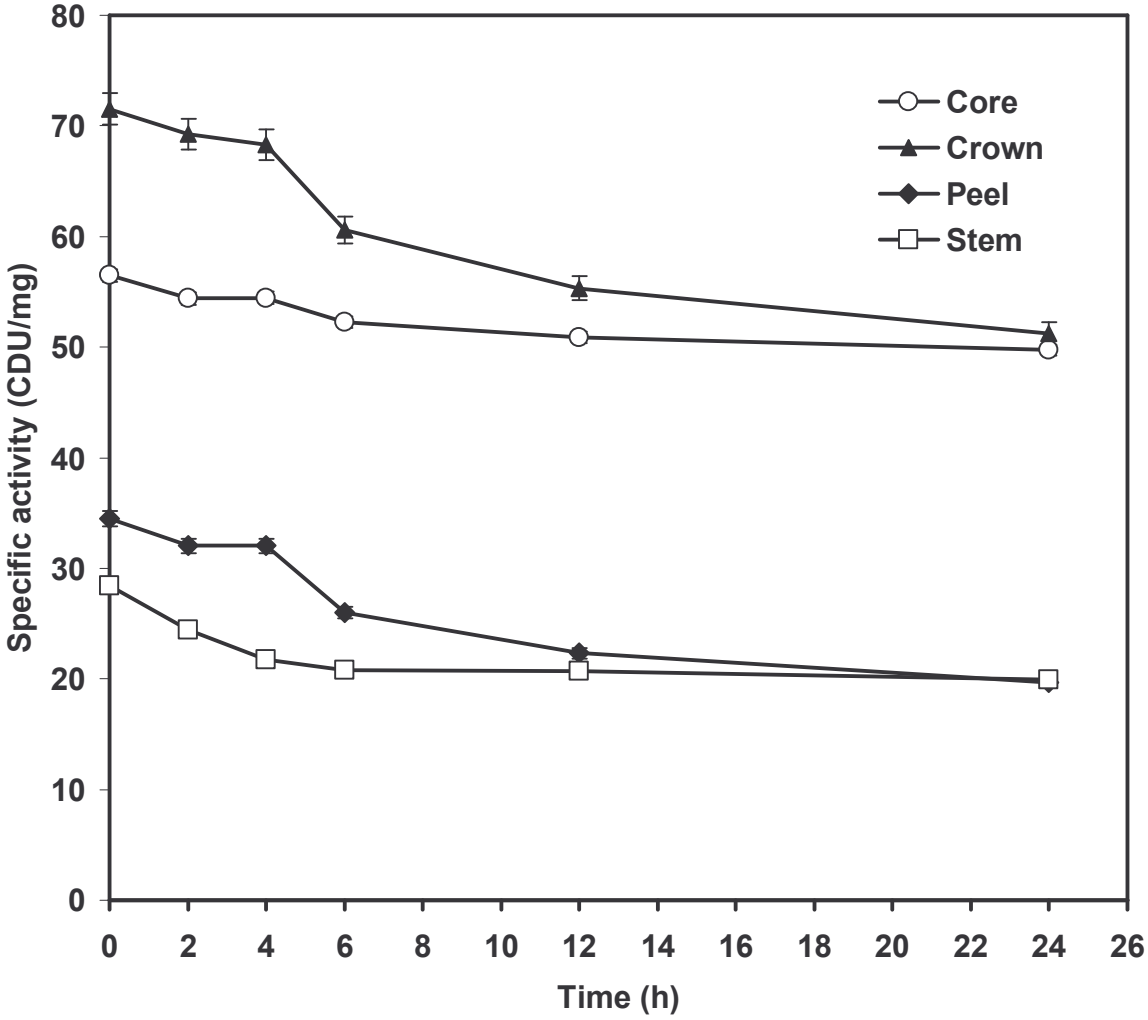


Fig. 4A.3. Specific activity of bromelain in the crude extracts stored at 25±2ºC

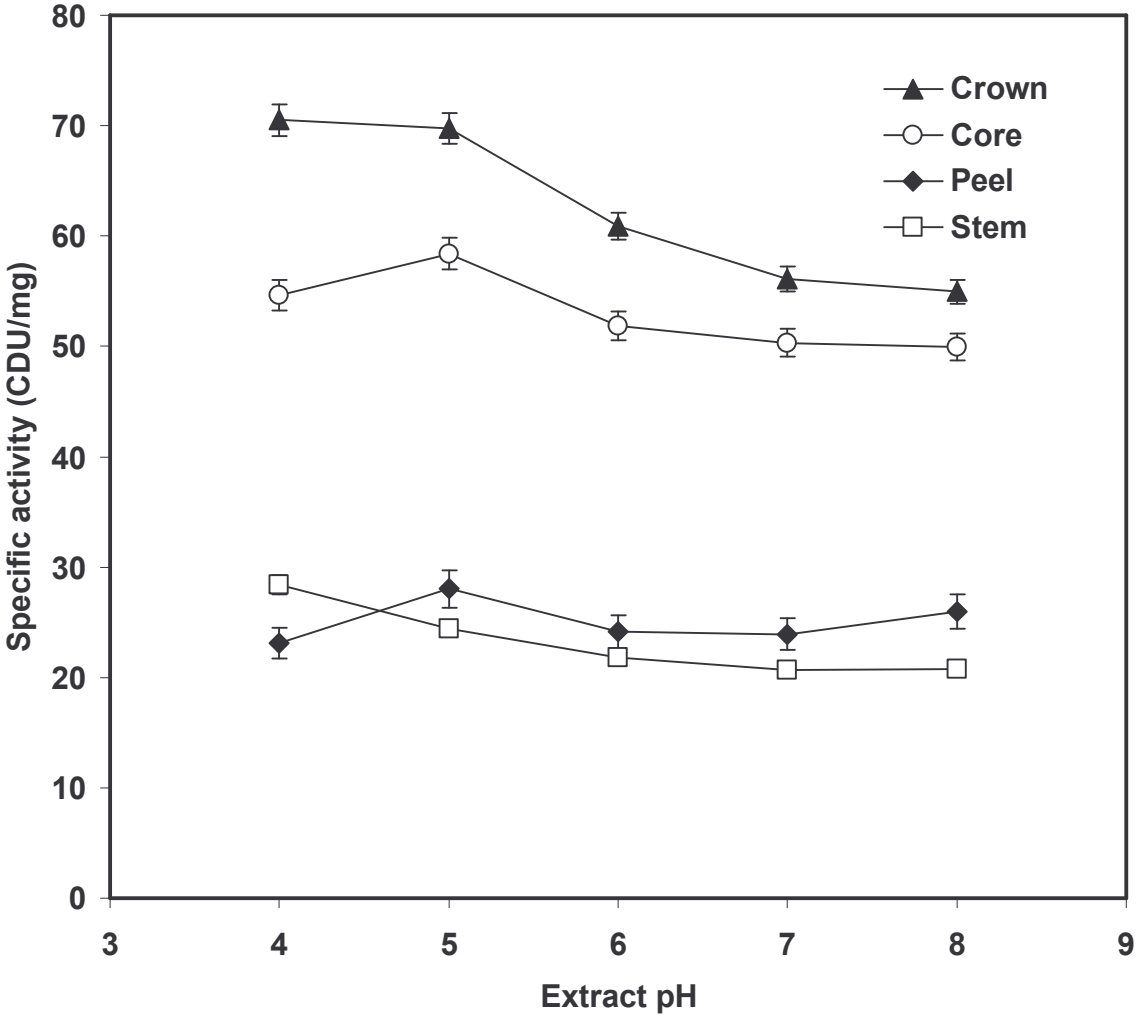


Fig. 4A.4. Specific activity of bromelain at different pH of crude extract

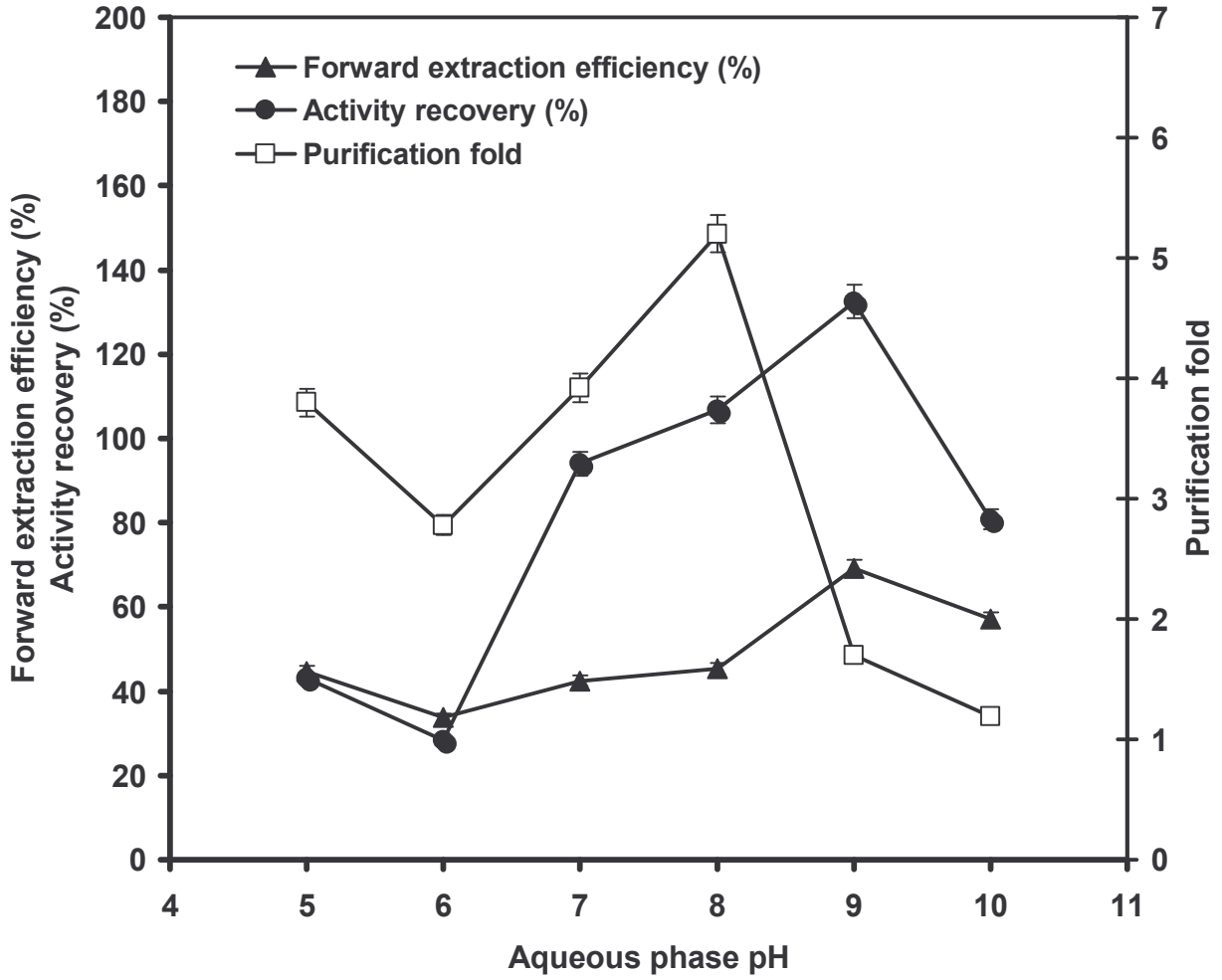


Fig. 4A.5. Effect of aqueous phase pH on forward extraction efficiency, activity recovery and purification fold during RME from core

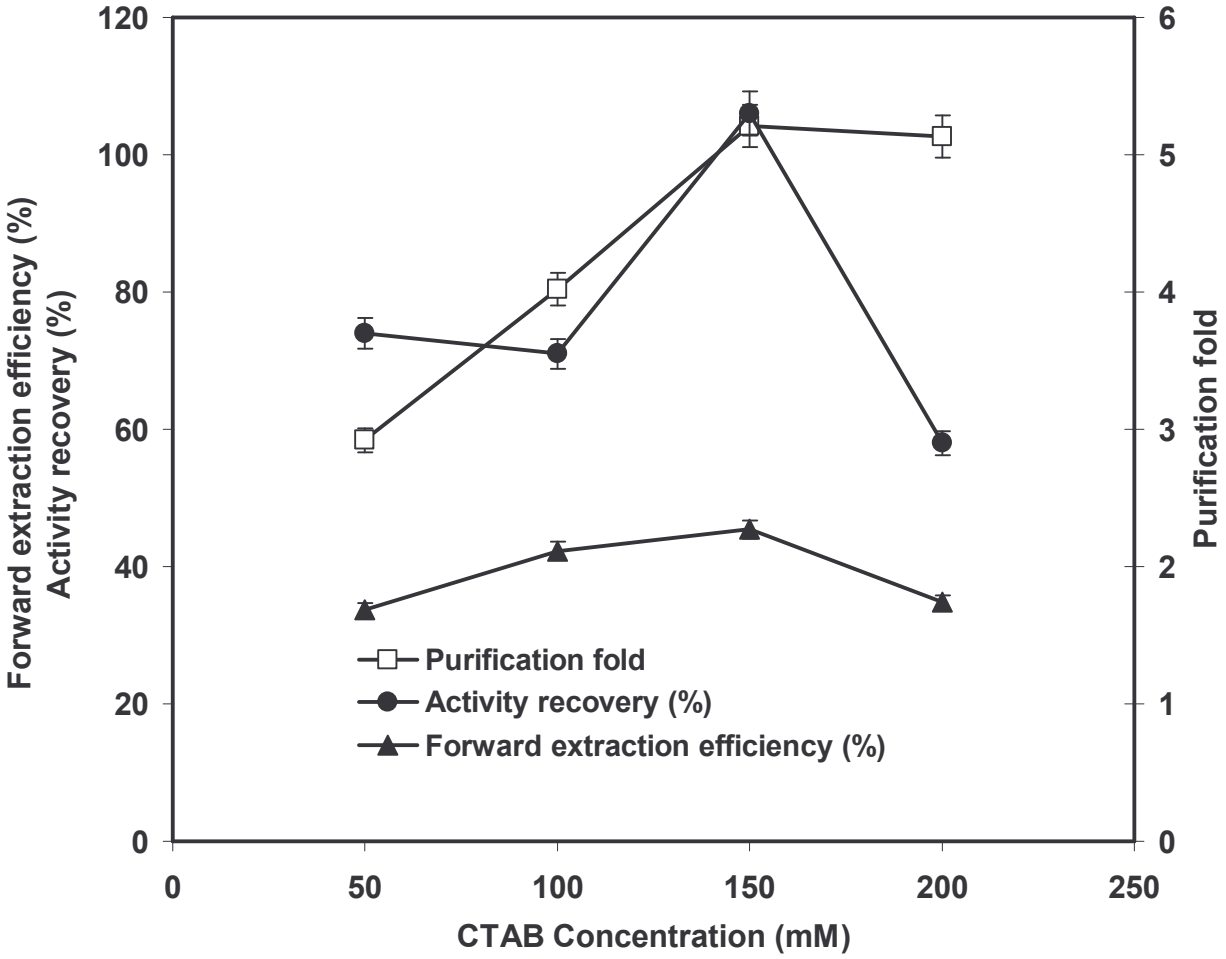


Fig. 4A.6. Effect of CTAB concentration on forward extraction efficiency, activity recovery and purification fold during RME from core

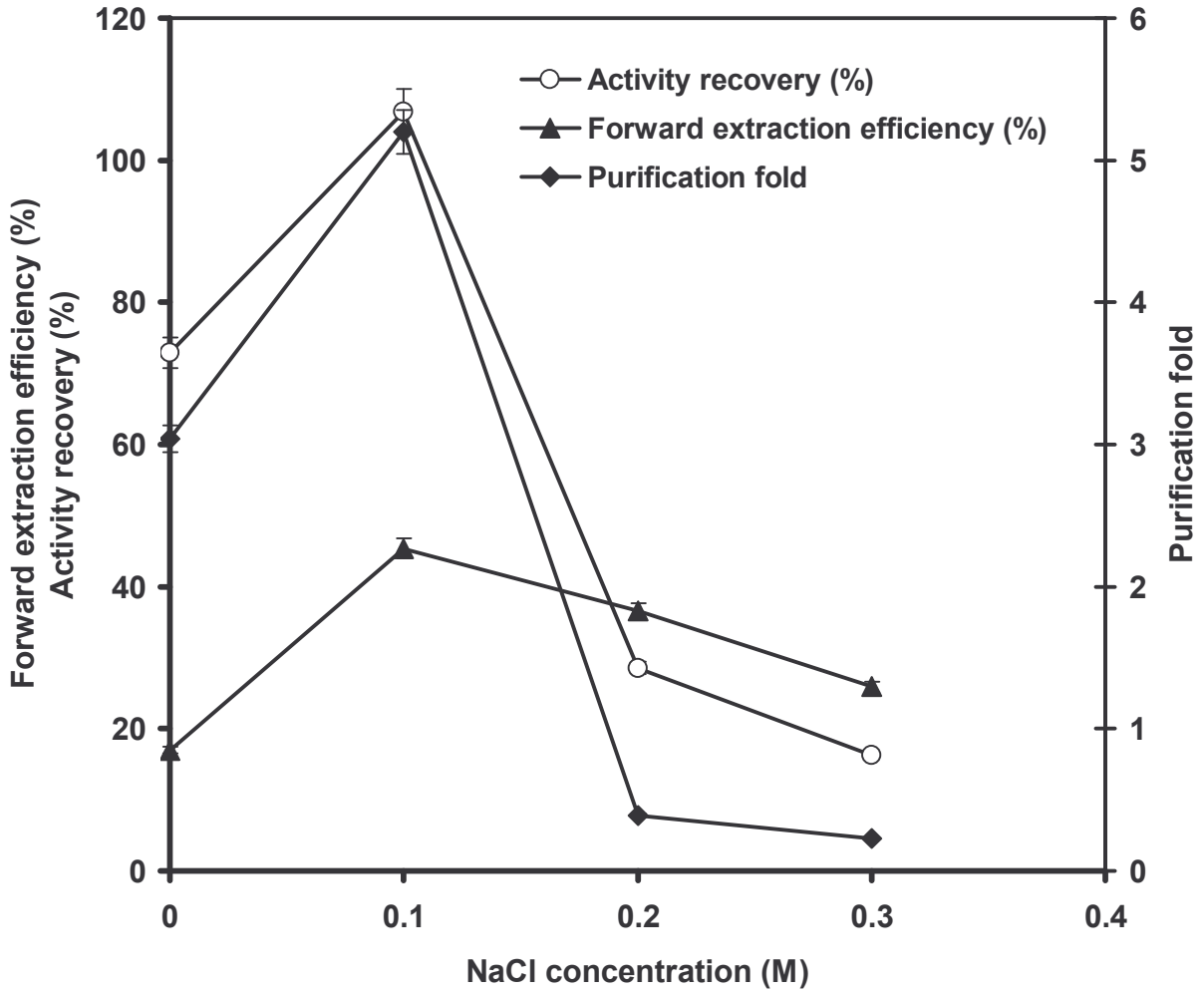


Fig. 4A.7. Effect of NaCl concentration on forward extraction efficiency, activity recovery and purification fold during RME from core

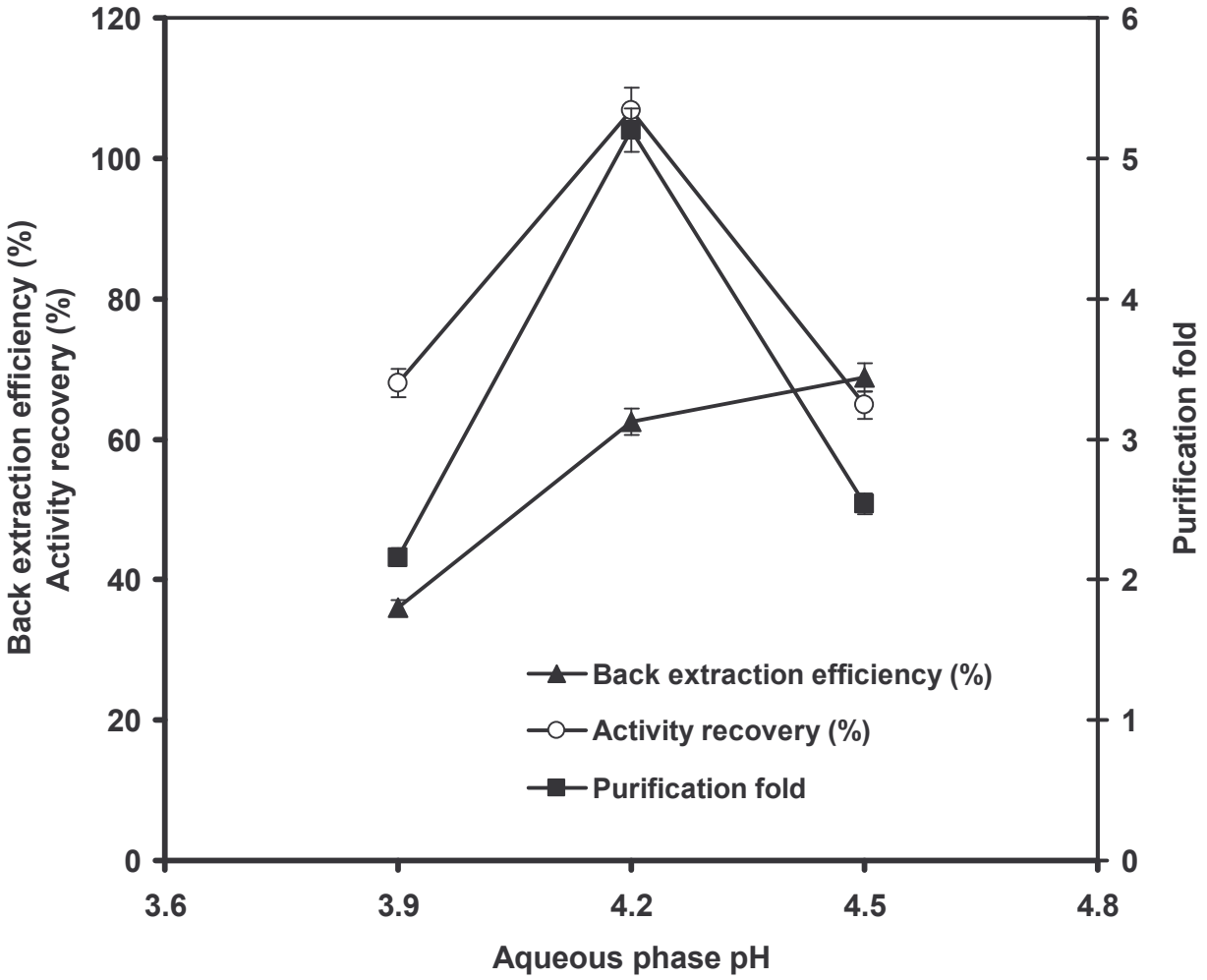


Fig. 4A.8. Effect of aqueous phase pH on back extraction efficiency, bromelain activity and purification fold during RME from core

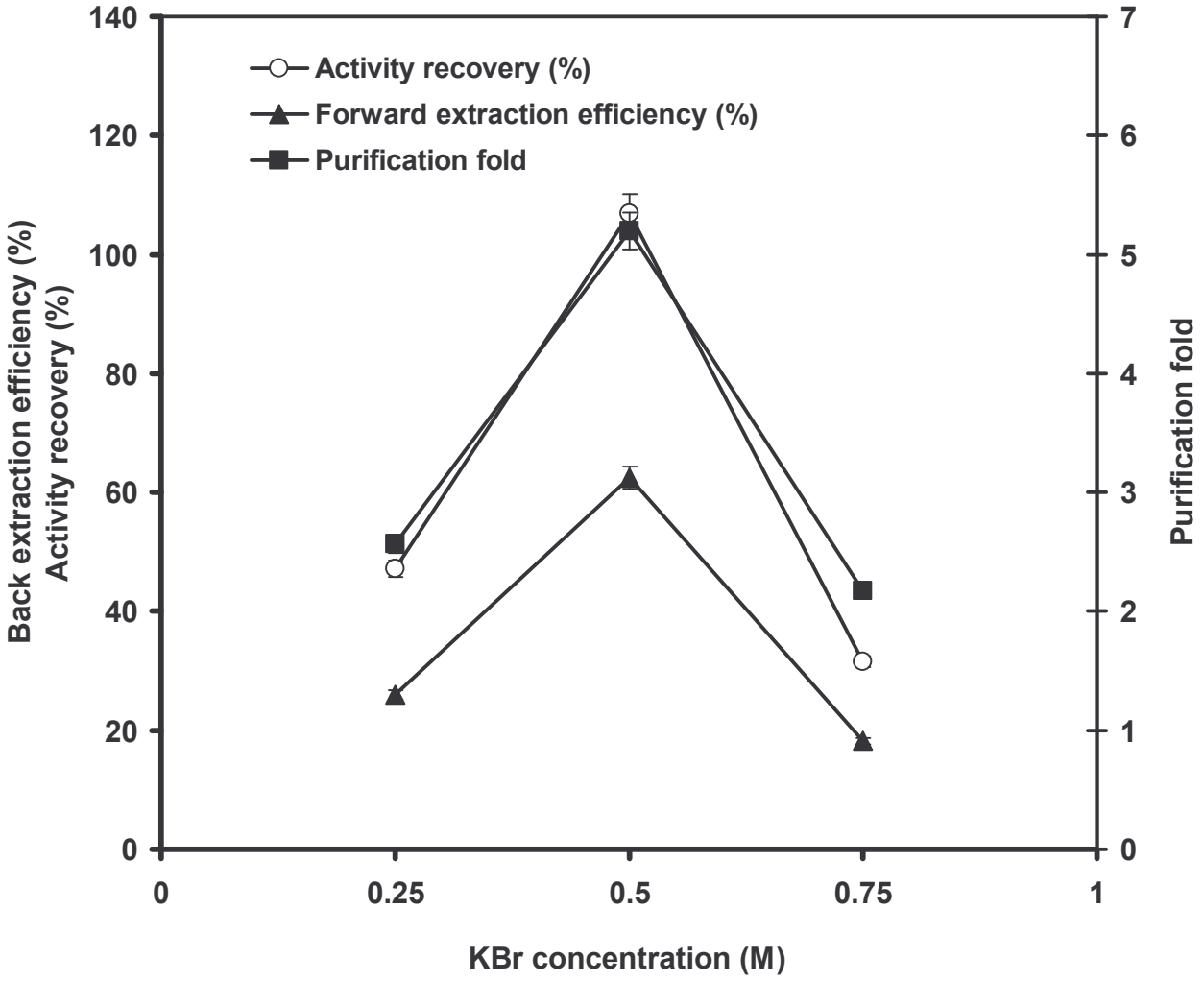


Fig. 4A.9. Effect of KBr concentration on back extraction efficiency, bromelain activity and purification fold during RME from core

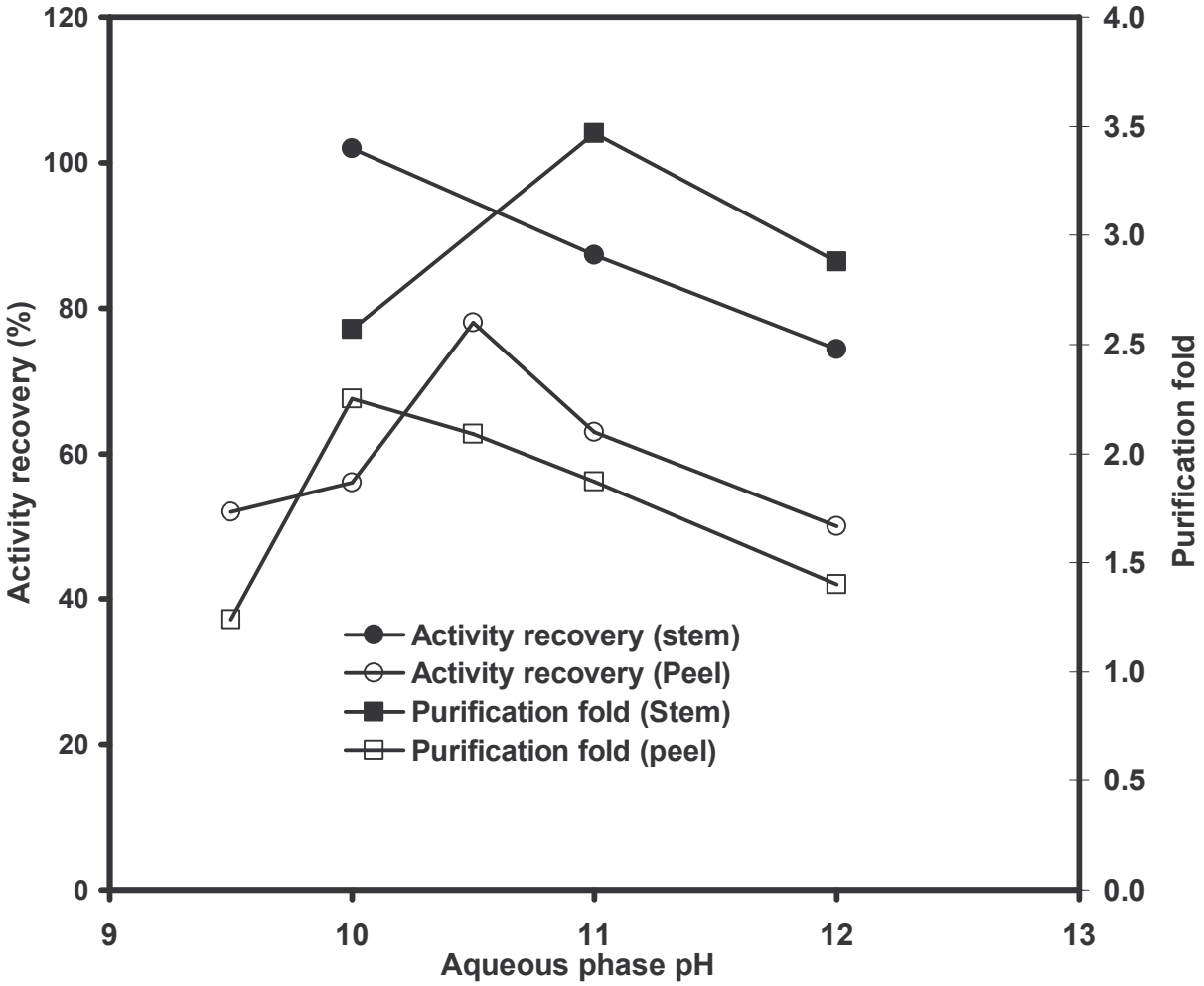


Fig. 4A.10. Effect of forward extraction aqueous phase pH on activity recovery and purification fold during extraction from stem and peel

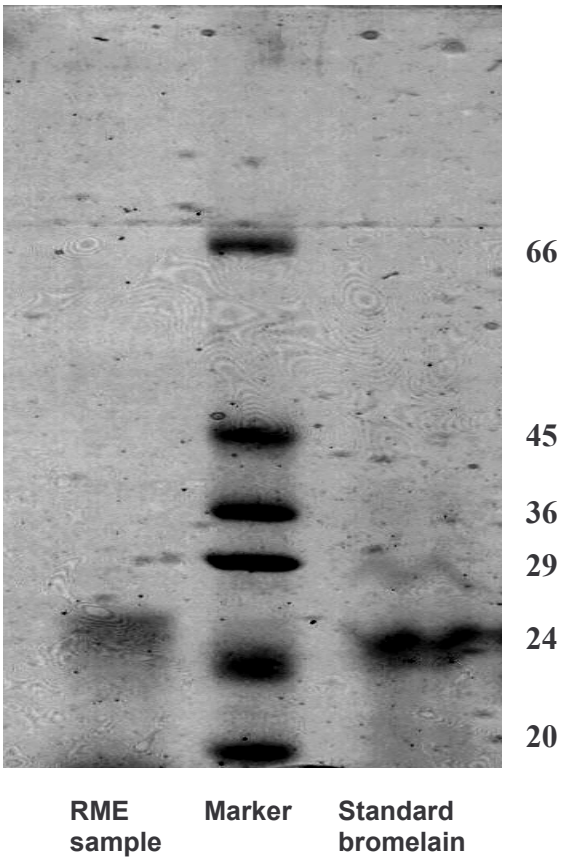


Fig. 4A.11. SDS-PAGE analysis of reverse micellar extracted bromelain from core

SECTION B

SCALE-UP, PROCESS INTEGRATION AND COMPARATIVE STUDIES WITH OTHER METHODS

4B.1 Introduction

Although, several reports on the application of RME for separation and purification of biomolecules are available, there are only a few reports on the process scale up. Although, many laboratory scale studies area available on RME, only a few reports are available on process scale up. Krie *et al.* (1995) reported the extraction of α-amylase from 2 L of clarified fermentation broth with N-benzyl-N-benzyl-N-dodecyl-N-bis (2-hydroxyl ethyl) ammonium chloride (BDBAC) microemulsion. Pessoa and Vitolo (1997) carried out the insulinase extraction study at 5 L scale. Reduction in activity yield by 15-20% was reported in both the above studies and it was partly attributed to the lower degree of phase contact at higher volume. Also, practically, no reports are available on the integration of RME with other downstream processes. Hence, in the present study, an attempt was made to scale up the RME for separation and purification of bromelain from pineapple core extract and integrate with ultrafiltration for further concentration /purification. Ultrafiltration is a gentle and non denaturing membrane separation technique for concentration/separation of macromolecules based on their size and is preferred in the biopharmaceutical industry because it is more efficient and flexible than competing technologies [Sharma *et al.,* 2005].

Transfer of solubilized solutes from the reverse micellar phase back into aqueous phase is known as back extraction and a successful RME should have optimized conditions for both forward and back extractions. Most of the studies on RME have tactically assumed that conditions, which normally are not favorable for forward extraction, would promote their release in the back extraction. However, many of the studies have indicated that the above assumption is not always true and back extraction of solute is relatively more difficult. Hence, some alternative approaches for enhanced recovery of solutes have been attempted by many researchers. They include methods include addition of dewatering agents such as isopropyl alcohol [Carlson and Nagarajan, 1992] use of silica particles for the sorption of proteins as well as surfactants and water directly from the protein-filled reverse micelles [Leser *et al.,* 1990] and temperature shifts [Dekker *et al.,* 1991],

use of ion exchange columns [Choudhrui and Spirovska, 1994], dehydration of reverse micelle with molecular sieves to recover the protein [Gupta *et al.*, 1994], addition of large amount of a second organic solvent, such as ethyl acetate [Wolbert *et al.,* 1989], formation of clathrate hydrates via pressurization [Phillips *et al.,* 1991] and using a counterionic surfactant [Jarudilokkul *et al*., 1999]. Application of high intensity acoustic radiation in food processing can be broadly classified into two main categories, namely to monitor process or product, and to directly affect a process or product [Floros and Liang, 1994]. Monitoring applications can be mainly found in quality assurance, process control and non destructive inspection, whereas, the physical and mechniacal effects of sound are mainly utilized for cleaning surfaces, enhancement of drying, inactivate microorganisms and enzymes, disrupt cells, degas liquids, accelerate extraction, mixing etc, [Floros and Liang, 1994]. Srinivas *et al,* (2000) have reported the reduction in de-mixing time in aqueous two phase systems by the application of acoustic field in the MHz range. Conventional wisdom predicts that application of acoustic filed causes mixing rather than de-mixing in the system, since it imparts energy to the system in order to achieve dynamic agitation, shear, cavitation, heating [Nagaraj *et al.,* 2002]. However, it was shown that acoustic fields of higher frequency (MHz range) results in mild circulation currents in the phase dispersion, which increases the droplet coalescence, eventually resulting in lower de-mixing time [Raghavarao and Todd, 2000]. In the present work an attempt was made to apply acoustic filed in the MHz range as an alternative to phase contact step (conventional back extraction) wherein agitation was provided using a magnetic stirrer. It was expected that the mild circulation current generated by ultrasonic waves would facilitate the deassembling of reverse micelles and facilitates the release of solute into stripping phase.

One of the major advantages of RME is the selective separation of solute based either on the charge or size. By suitably manipulating the aqueous phase pH, which determines the charge of the solute, the electrostatic interaction between the solute and surfactant could be varied. Aires-Barros and Cabral (1991)

reported the complete separation of large molecular weight (120000 Da, pI 3.7) Lipase A and relatively smaller Lipase B (30000 Da, pI 7.3) from *Chromobacterium viscosum* using AOT reverse micelles. The large difference in molecular size and selective electrostatic interaction effected due to suitable manipulation in the aqueous phase pH, were reported to be the reasons for the extraction. In the present study, attempts were made to use the above technique to selectively extract bromelain (28000 Da, pI 4.6), leaving behind the large molecular weight PPO (120000 Da, pI 6.6) from the dilute extract (feed). This could possibly lead to separation and purification of enzymes in two aqueous phases at the end of RME.

Aqueous two-phase extraction (ATPE) has been recognized as another versatile technique for downstream processing of biomolecules with advantages similar to RME. Use of ATPE for downstream processing of many enzymes such as xylanase [Gaikaiwari, *et al.,* 1996], amylases [Andersson *et al.,* 1985], amino acids [Li *et al.,* 1997], amyloglucosidase [Tanuja *et al.,* 1997], potato polyphenol oxidase [Vaidya *et al.,* 2006] and plant peroxidase [Srinivas *et al.,* 1999] have been reported. Use of immobilized metal ions for affinity partitioning of proteins in aqueous-two phase system was reported by Birkenmeier *et al,* (1991). In some applications, ATPE has been used as a primary purification technique to reduce the bulk of the processing stream, to be followed by more selective steps such as chromatography, electrophoresis etc [Sikdar, *et al.*, 1991; Raghavarao, *et al*., 1995 and 1998]. In the present work, a comparative study of the performance of ATPE and RME has been carried out.

The objectives of the present study are i) to integrate RME with ultrafiltration for enhancing the purification of bromelain from pineapple core ii) to study the suitability of the application of acoustic (ultrasonic) field for back extraction, iii) to study the differential partitioning of bromelain and polyphenol oxidase (PPO) using RME iii) to examine the feasibility of recycling spent organic phase for extraction and iv) to compare the performance RME with that of ammonium salt precipitation and ATPE method.

4B.2 Materials and Methods

4B.2.1 Materials

i) Pineapple fruit

Mature pineapple fruits (Ananas comosus L. Merr. cv. Kew, 7-9°Brix) available in the local market were used for the extraction.

ii) Chemicals

Cetyltrimethylammonium bromide (CTAB, Extra pure and AR grade) and sodium bis(2-ethylhexyl) sulfosuccinate (AOT) were obtained from Merck, Germany and BDH Laboratory Supplies, UK, respectively. Iso-octane (HPLC and AR grade) was purchased from Merck, Mumbai, India. Hexane and hexanol from SRL, Mumbai, casein (Hammerstein grade) and n-butanol from Loba chemicals, India were used. Polyethylene Glycol (PEG) 4000 and PEG 1500 were purchased from Sisco Research Laboratories (Mumbai, India) and Sigma Chemicals (St. Loius, MO), respectively. All other chemicals of AR grade were used for the experiments and analyses.

4B.2.2 Methods

i) Preparation of crude extract

The peel and core of fruit were separated from the pulp. The leaves from the crown were separated from the short stem (extended stem) to which they are attached. For the preparation of crude extract, known quantity of waste was crushed along with extraction buffer [0.01 M sodium phosphate buffer of pH 6.5, containing 1% PVP] for 10 min and then filtered through a cheese cloth. The filtrate was centrifuged at 10000 g for 15 min for separation of solids and the supernatant (crude enzyme extract) obtained was used for RME experiments.

ii) Scale up of studies with reverse micellar extraction

Scale up studies were carried out at two levels, i.e. at phase volume of 250 ml and 1000 ml.

Phase volume 250 ml

Forward extraction was carried out by contacting 250 ml of isooctane (organic phase) consisting of 150 mM CTAB/iso-octane/5% (v/v) hexanol/15% (v/v) butanol with an equal volume of aqueous phase (crude enzyme extract of pH 8.0 with 0.1 M NaCl). CTAB and iso-octane used for the study were of extra pure and HPLC grade, respectively. Back extraction was carried out by contacting the organic phase obtained from forward extraction with fresh aqueous phase (phosphate buffer of pH 4.2 and 2 M KBr) using a magnetic stirrer for 1.5 h and centrifuged at 4000 g for 15 min (MP 400 R, Eltek, India) during both forward and back extractions. The aqueous phases after forward and back extractions were analyzed for bromelain activity and total protein content. The phase contacting and separation were carried out at controlled temperature (25 ± 2^{0}C).

Phase volume 1000 ml

Equal volumes (1000 ml) of aqueous and organic phases were contacted in a glass container (3 L capacity) using magnetic stirrer and a pitched blade turbine. Analytical grade CTAB and isooctane were used in this study. The container was provided with baffles for proper mixing. The composition of organic and aqueous phases, contact time for forward and back extraction were maintained same as that employed for experiment with 250 ml. The mixture was allowed for 2 h for phase separation by gravity during both forward and back extractions, in place of centrifugal separation employed in the case of experiment with 250 ml.

iii) Ultrafiltration

250 ml of the aqueous phase containing bromelain obtained from RME was subjected to ultrafiltration using Tangential Flow Filtration system (TFF 50, Millipore, USA) shown in Fig. 4B.1. Cellulose acetate membrane (MWCO - 5 kDa) module having surface area of 50 cm^2 was used for the filtration. The trans-membrane filtration pressure was maintained at 2 bar during the experiment. The average permeate flow rate during the process was 3ml/min and the experiment was continued till a reduction in volume (retentate) of 5 fold was achieved.

iv) Aqueous two phase extraction (ATPE)

In all the experiments the phase systems were prepared on a percent w/w basis by contacting the required quantities of phase mixing solutes in dilute enzyme extract (~6.6 ml). The phases were contacted thoroughly for 30 min using a magnetic stirrer (Cintex, Mumbai) and allowed (2 h) to separate. The top and bottom phases were analyzed for bromelain activity and protein content. The pH of the phase was adjusted using either 1 N NaOH or 1 N HCl wherever required. The phase diagrams of PEG 4000 and PEG 1500 with K_2HPO_4-KH_2PO_4 system reported by Zaslavsky (1995) and Albertsson (1986) were used for the selection of phase composition.

v) Ammonium sulphate precipitation

Known quantity (50% saturation) of ammonium sulphate was added to the crude extract and the mixture was stirred for 1 h [Murachi, 1976]. The mixture was centrifuged to separate out precipitated pellets. The supernatant was again mixed with excess amount of salt to precipitate protein. The precipitate was dissolved in known quantity of water and dialyzed (16 h) against deionized water before analyzing for protein content and bromelain activity.

vi) Ultrasonication

Ultrasonic bath (HM-460, Holmer Products Corp., USA; 1.2 MHz) was used to destabilize the reverse micelles and facilitate back extraction. The water bath of the unit was filled with distilled water up to the required level and the mixture of organic (obtained from forward extraction) and fresh aqueous (buffer of known strength with KBr) phases (10 ml each) was exposed to acoustic field at different levels of intensity (low, medium and high) for a known duration. The schematic representation of the set-up used for the experiment is presented in Fig 4B.2. All other conditions of forward and back extractions were maintained same as that of laboratory scale studies.

4B.2.3 Analyses

i) Bromelain activity

Bromelain activity in aqueous phase was determined according to the casein digestion unit (CDU) method using Hammerstein grade casein (0.6%) as substrate in the presence of cysteine and EDTA [Murachi, 1976]. The assays were based on proteolytic hydrolysis of the casein substrate. The absorbance of the clear filtrate (solubilized casein) was measured at 275 nm using spectrophotometer (Shimadzu UV-160). One unit of bromelain activity is defined as 1 µg of tyrosine released in 1 min per ml of sample when casein is hydrolyzed under the standard conditions of 37°C and pH 7.0 for 10 min.

ii) Protein content

Protein content in aqueous phase was determined by measuring absorbance at 280 nm using bromelain as standard as standard in case of CTAB system. The dye binding method of Bradford [1976] was used (using BSA as standard) in case of AOT system. The sample analyses were performed against respective blank solutions. Protein concentration readings were taken in triplicate and average value was used for the calculation of extraction efficiencies. The

forward and back extraction efficiencies, activity recovery (%) and degree of purification are estimated as shown below.

$$\text{Forward extraction efficiency}(\%) = \frac{\text{Protein concentration in organic phase}}{\text{Protein concentration in feed}} \times 100 \quad \text{(4B.1)}$$

$$\text{Back extraction efficiency}(\%) = \frac{\text{Protein concentration in organic phase}}{\text{Protein concentration in back extracted aqueous phase}} \times 100 \quad \text{(4B.2)}$$

$$\text{Activity recovery}(\%) = \frac{\text{Bromelain activtity in the aqueous phase after RME or in the retentate}}{\text{Bromelain activty in the feed}} \times 100 \quad \text{(4B.3)}$$

$$\text{Purification fold} = \frac{\text{Specific activity of bromealin after RME}}{\text{Specific activity of bromelain in the feed}} \quad \text{(4B.4)}$$

iii) Polyphenol oxidase (PPO) activity

PPO was assayed according to the spectrophotometric method described by Das, *et al.,* (1997). The assay mixture consisted of 2.6 ml of 0.01 M NaPi buffer, pH 6.5, and 0.3 ml of dialyzed enzyme extract. The increase in absorbance at 420 nm was measured. One unit of enzyme activity is defined as the amount of the enzyme that causes an increase in absorbance of 0.001/min at 25°C.

iv) Determination of the partition coefficient (m)

The partition coefficient (m) of the enzyme during ATPE was calculated by the following equation.

$$m = C_T/C_B \quad \text{(4B.5)}$$

where C_T is equilibrium concentration of the enzyme in the top phase and C_B is equilibrium concentration of enzyme in the bottom phase. The bromelain activity recovery in the top phase obtained from ATPE was estimated using the following equation and reported as percentage activity recovery.

$$\text{Activity recovery}(\%) = \frac{\text{Volume of top phase x bromelain activity}}{\text{Volume of crude extract x bromelain activity}} \text{x} 100 \quad \text{(4B.6)}$$

4B.3 Results and Discussion

4B.3.1 Scale up studies of reverse micellar extraction

Laboratory scale (10 ml) studies on the optimization of processing conditions for the extraction and purification of bromelain from crude extract of pineapple core using reverse micelles has been detailed in section A of Chapter 4, and also reported by Hebbar *et al.,* 2007. Under optimized conditions of extraction (forward extraction: 150 mM CTAB; 0.10 M NaCl; aqueous phase pH 8.0, Back extraction: Aqueous phase pH 4.2, KBr 2.0 M) an activity recovery of 106% and a purification of 5.2 fold were obtained. Hence, in the present study also same conditions were maintained. The forward and back extraction pH values employed were within the stability range (3.5–8.5) reported for bromelain. In the present study, RME was carried out at a scale higher (250 ml) than the laboratory scale (10 ml). All the processing conditions except phase contact time were maintained same as that of laboratory scale study. The contact time was increased from 60 to 90 min for both forward and back extraction in order to have a good degree of phase contact. The activity recovery and purification measured at the end of back extraction are presented in Table 4B.1. Slight reduction in activity recovery was (~97%) was observed as compared to laboratory studies, while the purification increased from 5.2 to 6.5. The results indicated that RME process could be easily scaled up without affecting the performance.

To study the performance at scaled up level, RME experiments were carried out at 1000 ml level as descried in section 4B.2.2. Analytical grade surfactant and solvent were used to study the performance these components, which are relatively less costly as compared to pure components used in the laboratory scale level. Cost of the reverses micellar components is an essential parameter that determines the economic feasibility of the process at higher scale. Aqueous phase obtained after forward and back extraction were analyzed for protein content and bromelain activity and results are presented in Table 4B.2. Activity recovery (85%) and purification fold (2.1) were less as compared to that obtained with the laboratory scale studies and experiment at 250 ml phase volume. Lower degree of activity recovery and purification fold may be attributed to the use of analytical grade surfactants and solvents, which might contain some amount of impurities that hinders the extraction of solute. Replacement of centrifugal separation by gravity settling of mixed phases might have also affected the performance. Increase in CTAB concentration and duration of phase separation needs to be optimized to enhance the extraction efficiency.

4B.3.2 Ultrafiltration (UF)

RME could be used as a primary technique to reduce the bulk of the processing stream, with more selective purification steps following it. In the present study attempts were made to have an integrated process wherein reverse micellar extraction was followed with ultrafiltration to improve the overall efficiency. The aqueous phase (250 ml) obtained from RME was subjected to UF in a tangential flow membrane system. The molecular weight cut-off of the membrane (5 kDa membrane) was selected so as to retain bromelain (28-32 kDa) and separate out the other solutes having lower molecular weight. The extraction was continued (~ 100 min) till the volume of the retentate reduced to 50 ml (i.e. reduction by 5 folds). The activity of bromelain in the retentate increased to 490.81 CDU/ml against 275.5.CDU/ml in the feed (Table 4B.3). Purification of 1.3 fold was achieved during the UF step, leading to a reasonably high purification of 8.5 fold for the integrated process (Table 4B.3). Bromelain activity and protein

concentration were low in the permeate indicating the efficient separation of the enzyme from other impurities. It may be expected that any traces of CTAB, a low molecular weight (364.46) surfactant, present in the aqueous phase would be removed during UF. The above would facilitate the further purification of bromelain by conventional purification methods. With the developments in the membrane processing technology, the scale up of UF step is not a major constraint. Hence, an integrated process of RME and UF might well be used as an effective process for the extraction and purification of bromelain at higher scale. The schematic representation of the integrated process for clarification of crude, RME of bromelain and UF is shown in Fig. 4B.3.

4B.3.3 Application of ultrasonication for facilitating back extraction

Acoustic field (1.2 MHz frequency) was applied at different intensities (low, medium and high) for a period of 15 min. The mixtures were subjected to phase separation by centrifugation for 15 min. The back extraction efficiency was higher (~20%) in all the cases (Table 4B.4) as compared to conventional back extraction method. Although, low intensity mixing gave relatively lesser back extraction efficiency (53%), the activity recovery (70%) and purification fold (2.8) were higher. Vigorous agitation was noticed at high intensity and the temperature of the mixture increased by nearly 2-2.5°C. All further experiments were carried out at low acoustic intensity. Increase in temperature and exposure to high intensity acoustic might have been responsible for lower activity recovery at higher intensity.

To study the effect of exposure time to acoustic field on extraction efficiency, the duration of ultrasonication was increased from 15 min to 60 min by keeping all other parameters the same. Although, no drastic change in extraction efficiency was observed, it increased initially and dropped later with increase in exposure time (Table 4B.5). Relatively higher activity recovery (>85%) was obtained at exposure time more than 30 min. Purification fold of 4.5 was obtained at 45 min, which is almost close to the purification fold (5.2) and activity recovery

(106%) obtained with conventional extraction. This indicated that acoustic field could be effectively used for back extraction of bromelain from reverse micelles.

The effect of acoustic field and agitation was compared with the acoustic field treatment alone (Table 4B.6). Acoustic treatment for 15 min followed by agitation for 15 min in a magnetic stirrer increased the protein extraction (61%), activity recovery (85%) as well as purification fold (3.5) as compared to acoustic treatment for 15 min alone. Additional agitation provided might have resulted in the release of solute from reverse micelles. The extraction efficiency did not change considerably when separation of phases by centrifugation was not done after ultrasonication. This indicated that the application of acoustic not only helps in deassembling the reverse micelles, but also facilitates the quicker demixing of phases. The effect of counter ionic surfactant on the extraction efficiency has been reported [Jarudilokkul *et al.,* 1999]. The counter ions were reported to assist in releasing the solutes from reverse micelles due to electrostatic interaction. Anionic surfactant AOT was used as a counter ion to affect the release of bromelain from reverse micelles of cationic surfactant CTAB. Addition of AOT increased the back extraction efficiency appreciably (97%), which may be due to the release of solute from reverse micelles caused by the interaction between oppositely charged surfactants. However, the activity recovery (82%) and purification fold (1.6) decreased as compared to the experiment without the addition of counter ionic surfactant.

4B.3.4 Differential partitioning of bromelain from PPO

In the present study, attempts were made to use the above technique to selectively extract bromelain (28000 Da, pI 4.6), leaving behind the large molecular weight PPO (120000 Da, pI 6.6) from the dilute extract (feed). During the forward extraction the aqueous phase pH was maintained between the isoelectric points of bromelain (4.6) and PPO (6.5) in order to have the selective extraction of negatively charged bromelain by the cationic surfactant CTAB. Two aqueous phase pH values (5.0 and 5.5) were in used in the study and the other conditions of forward and back extractions were maintained similar to that

employed in the large scale RME study (described in section 3.1). The bromelain and PPO activity were estimated in both the aqueous phases (forward and back extraction) and results are presented in Table 4B.3. Higher activity of bromelain and PPO in the back extracted and leftover aqueous phases, respectively indicated the selective extraction of bromelain during RME. Both the conditions resulted in higher PPO activity recovery (133-136%) and purification (2.12-2.25 fold) in the aqueous phase leftover after forward extraction. The degree of bromelain purification (2.69 to 3.8 fold) was fairly good in the above conditions. At pH 8.0, the aqueous phase pH at which bromelain activity recovery and purification were maximum, the selectively extraction efficiency marginally decreased as PPO (pI 6.5) was also extracted due to electrostatic interaction. However, higher difference between aqueous phase pH and isoelectric point (pH-pI) might have resulted in better extraction of bromelain at pH 8.0.

4B.3.5. Recycling of the organic phase

Recycling of organic phase obtained after back extraction is one of the major factors that contribute to the process economy, particularly when the phase components (solvent, surfactant and co-solvents) are expensive. However, the extraction efficiency of RME might be affected with recycled organic phase and needs to be studied. In the present study, attempts were made to reuse the organic phase obtained after back extraction for further extraction with crude extract. When recycled organic phase was used as such for extraction, the phases turned turbid. Addition of co-solvents (15% butanol and 5% hexanol) to the organic phase resulted in clear phase formation. All other conditions of RME were maintained the same. The activity recovery and purification were found to be very low i.e., 18% and 1.1, respectively (Table 4B.7). Protein extraction during the forward extraction with recycled organic phase was nearly 20% less as compared to that obtained from fresh organic phase. The observed lower degree of extraction was suspected to be due to loss of some quantity of CTAB during the first extraction cycle. Hence, the experiment was carried out with an additional (25% of the original quantity) amount of CTAB to the recycled organic phase.

Although, the activity recovery was less (55%), a fairly good purification (2.7 fold) was obtained under these conditions. The results showed that recycling of organic phase could be explored in RME, may be replenishing the components such as co-solvents and surfactant. A detailed study on number of recycles *vis a vis* process efficiency and economy are to be carried out to optimize recycling.

4B.3.6. Aqueous two phase extraction (ATPE) of bromelain

Although, in ATPE, the mechanism governing the partition of biological materials is still not well understood, the observed solute partition is reported to be the result of van der Waals, hydrophobic, hydrogen bond and ionic interactions of the solutes with the surrounding phase [Gunduz and Korkmaz, 2000]. The partition coefficient is influenced by many factors, including phase composition, molecular weight of phase forming polymer, tie line lengths etc. In the present work, the above parameters were altered to study the affect on activity recovery, purification and partition coefficient and the performance was compared with that of RME.

i) Effect of PEG molecular weight

The polymer molecular weight influences protein partitioning as a direct result of interactions between two polymers. It has been found that increase in PEG molecular weight decreases the partition coefficient as the free volume in the top phase decreases with PEG molecular weight [Raghavarao, e*t al.,* 1995]. However, the effect of PEG molecular weight on partitioning of lower molecular weight proteins was reported to be very less [Silva and Franco, 2000]. Vaidya *et al.* (2006) reported that the molecular mass of PEG contributes significantly to the partitioning behavior of proteins having a molecular mass greater than 50 kDa. It was also observed that with a decrease in the molecular weight of phase forming polymers, the interfacial tension decreases [Wu et al., 1996], which would enhance the mass transfer rate of solutes. In the present study, PEG 4000 and 1500 were used under similar extraction conditions and the results are shown in Table 4B.8. Although, the activity recovery of enzyme remained almost the same, the purification increased (2.6 fold) with the higher PEG molecular weight.

However, the partition coefficient was higher (1.65) with PEG 1500 as compared to that of PEG 4000. It is expected that with the decrease in polymer molecular weight, the solutes (bromelain as well as others such as PPO) are partitioned more towards the top phase resulting in higher partition co-efficient. The lower interfacial tension caused by PEG of lower molecular mass might have also contributed to the higher partitioning of proteins. The partitioning of higher amount of solutes (other than bromelain) may be responsible for the lower purification observed with PEG 1500.

ii) Effect of phase composition

Variation in phase composition changes the physical properties such as viscosity, density and interfacial tension of the system [Nagaraj *et al.,* 2002] which in turn affect the solute partitioning. It was reported that the interfacial tension increases with higher phase composition (Tie line length) and attributed it to the higher difference between composition of top and bottom phases [Asenjo, *et al.,* 2002]. The change in free volume was reported to be the reason for variation in solute partitioning [Grossman and Gainer, 1988]. The concentrations of PEG and phosphate were selected randomly (Table 4B.8) such that the system would lie above the binodal curve to ensure phase separation. Decrease in PEG concentration increased the partition coefficient. The purification (2.6 fold) and activity recovery (147%) were maximum at 12% (w/w) PEG concentration and it got lowered above and below this concentration.

iii) Effect of aqueous phase pH

The aqueous phase pH range of 7.0-8.0 was selected in this study, as RME studies of bromelain gave good results. A change in aqueous phase pH is often used to alter the partitioning of a biomolecule and the partition co-efficient varied exponentially with the electrochemical potential difference between the phases and the net charge of the partitioned biomolecule. However, in the present study, partition co-efficient increased with the decrease in aqueous phase pH (Fig. 4B.4) with a maximum value of 1.16 at aqueous phase pH 7.0 contrary to the above

observation. This suggests that factors other than electrical charge (such as hydrophobicity of compounds) also play a role in the partitioning of the solute in the system studied. The activity recovery and purification did not show any definite trend and, higher values of activity recovery (147%) and purification (2.6) was obtained at aqueous phase pH 7.5. Although, the purification was good (2.1) at aqueous phase pH 8.0, the activity recovery decreased drastically to 92%.

iii) Effect of phase volume ratio

It is reported that as the phase volume ratio (ratio of volume of top phase to bottom phase) increases, the free volume in the bottom phase decreases, leading to partitioning of proteins to top phase, which will decrease the purity [Raghavarao *et al.,* 1995]. The effect of phase volume ratio in PEG4000/K_2HPO_4/KH_2PO_4 system was studied are results are shown in Fig. 4B.5. When the phase volume ratio increased from 0.31 to 0.77, the partition coefficient increased almost by 2 folds, indicating a higher protein transfer to the top phase. Increase in protein transfer with phase volume ratio decreased the purification and activity recovery of bromelain by nearly 3 folds and 45% receptively.

The optimized activity recovery and purification values obtained in the present study were found to be higher as compared to the bromelain partitioning in two phase aqueous system reported by Rabelo *et al.* (2004). Thermoseaparation of bromelain from pineapple fruit using poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymers aqueous solution resulted in an activity recovery around 79.5%, purification factor around 1.25 and activity partition coefficient around 1.4 were obtained.

4B.3.7. Ammonium sulphate precipitation

Ammonium sulphate precipitation technique is conventionally used for the primary purification of bromelain followed by other purification methods such as chromatography, electrophoresis etc. Although, the above method is simple, it is tedious and not expected yield high purity. The present study was undertaken to compare the performance of the above method with RME. The dialyzed aqueous

phase obtained by re-dissolving the precipitate was used for the analysis. The activity recovery and purification were 105% and 2.0, respectively (Table 4B.9). Although, the purification increased, the value was much lower than that obtained from RME.

4B.3.7. SDS – PAGE analysis

The aqueous phase containing bromelain obtained from back extraction of scale up study (detailed in section 3.1) was dialysed overnight at 4^0C and lyophilized. The concentrated sample was loaded to 10% gel along with standard bromelain sample. The SDS lane pattern of reverse micellar extracted bromelain matched well with the standard (Fig. 4B.6). The band obtained was found to be around 26 kDa, which lies close to the reported range of the molecular weight (28 to 32 kDa) of bromelain.

4B.3.8. Conclusions

Scale up studies of RME using pure reverse micellar components, resulted in good extraction efficiency of bromelain. Extraction with analytical grade components for extraction resulted in reduced activity recovery and purification fold. An integrated process of reverse micellar extraction followed by ultrafiltration was successfully used for obtaining higher bromelain activity recovery and purification. By manipulating the aqueous phase pH during RME it was possible to selectively separate and purify bromelain and PPO in two aqueous phases. Exposure acoustic field in MHz range, as an alternative to conventional agitation method enhanced the back extraction efficiency. Addition of counter ionic surfactant increased the back extraction efficiency considerably. Recycling of organic phase could be explored in RME, may be replenishing the components such as co-solvents and surfactant. Application of ATPE technique for bromelain extraction was encouraging, although the efficiency was not as high as that obtained from RME.

Table 4B.1. Scale up studies (phase volume 250 ml) and coupling of RME with ultrafiltration (UF)

Sample	Volume (ml)	Activity (CDU/ml)	Protein Concentration (mg/ml)	Activity recovery (%)	Specific Activity (CDU/mg)	Purification (fold)
Crude extract	250	283.14	6.98	100	40.58	1.00
Aqueous phase after RME	250	275.50	1.05	97	262.38	6.5
UF Retentate	50	490.81	1.43	35	343.23	8.5
UF Permeate	200	27.138	0.86	8	31.56	1.2

Table 4B.2. Scale up studies with RME (phase volume1000 ml)

Sample	Activity (CDU/ml)	Protein Concentration (mg/ml)	Activity recovery (%)	Specific Activity (CDU/mg)	Purification (fold)
Crude extract	537.33	7.86	-	68.36	1.00
Aqueous phase after FE	335.60	4.4	62.45	75.92	1.11
Aqueous phase after BE	461.34	3.1	85	148.81	2.10

FE: Forward extraction BE: Back extraction

Table 4B.3. PPO and bromelain activity at different aqueous phase pH during RME

S. No.	Sample	Protein concentration (mg/ml)	Bromelain			PPO		
			Activity (%)	Specific Activity (U/mg)	Purification (fold)	Activity (%)	Specific Activity (U/mg)	Purification (fold)
1.	Feed (crude extract)	8.68	100	32.02	1.00	100	25.35	1.00
	FE at pH 5.0							
2.	Aqueous phase after FE	5.24	65	34.60	1.08	136	57.10	2.25
3.	Aqueous phase after BE	2.86	125	121.71	3.80	14	10.76	0.42
	FE at pH 5.5							
4.	Aqueous phase after FE	5.45	39	20.10	0.63	133	53.69	2.12
5.	Aqueous phase after BE	2.65	82	86.24	2.69	27	22.41	0.88
	FE at pH 8.0							
6.	Aqueous phase after FE	4.92	31	17.30	0.54	75	33.54	1.32
7.	Aqueous phase after BE	1.89	113	166.50	5.2	30	34.93	1.37

FE: Forward extraction; BE: Back extraction

Table 4B.4. Effect of acoustic field intensity on extraction efficiency during back extraction

Intensity of acoustic field	Back extraction efficiency (%)	Activity recovery (%)	Purification fold
Low	53	70	2.8
Medium	62	57	1.7
High	57	65	2.1

Table 4B.5. Effect of processing time on extraction efficiency during back extraction

Duration of exposure (min)	Back extraction efficiency (%)	Activity recovery (%)	Purification fold
15	53	70	2.8
30	57	92	2.4
45	47	90	4.5
60	44	85	3.9

Table 4B.6. Effect of processing time on extraction efficiency during back extraction

Method	Back extraction efficiency (%)	Activity recovery (%)	Purification fold
Acoustic field (15 min)	53	70	2.8
Conventional mixing (60 min)	50	106	5.2
Acoustic field (15 min) + conventional mixing (15 min)	61	84	3.9
Acoustic field (15 min) and without centrifugation	64	87	3.5
Acoustic field 45 min + Counter ionic surfactant + Phase separation	97	82	1.6

Table 4B.7. Recycling of organic phase for bromelain extraction from core

S. No.	Sample	Activity (CDU/ml)	Protein concentration (mg/ml)	Specific activity (CDU/mg)	Purification fold
1.	Crude extract	373.73	6.86	54.48	-
2.	Aqueous phase after RME with fresh organic phase	449.59	1.49	302.34	5.5
3.	Aqueous phase after RME with recycled organic phase and co-solvents	65.92	1.10	59.93	1.1
4.	Aqueous phase after RME with recycled organic phase (co-solvents + 25% CTAB)	205.94	1.40	147.10	2.7

Table 4B.8. Effect of phase composition and PEG molecular weight on bromelain extraction with ATPE

S. No.	Sample	Partition coefficient	Purification fold	Activity recovery (%)
Phase composition variation [PEG 4000/(K_2HPO_4/KH_2PO_4)] (%w/w)				
1.	13.5/15.0	0.63	1.2	24
2.	12.0/14.0	0.59	2.6	147
3.	11.5/11.0	0.91	1.3	53
PEG molecular weight variation				
1.	PEG 4000	0.59	2.6	147
2.	PEG 1500	1.65	1.5	152

Table 4B.9. Performance of ammonium sulphate precipitation for bromelain extraction

Sample	Activity (CDU/ml)	Protein concentration (mg/ml)	Specific activity (CDU/mg)	Purification fold	Activity recovery (%)
Feed	592.51	7.00	84.6	-	100
Ammonium sulphate precipitation	617.84	3.77	163.62	1.98	104

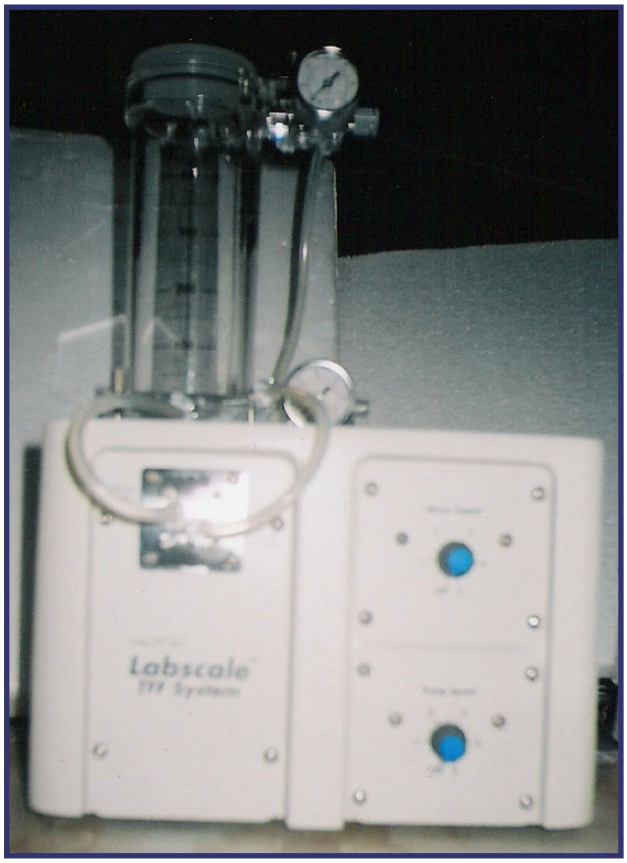


Fig. 4B.1 Tangential Flow Filtration (TFF) system used for ultrafiltration

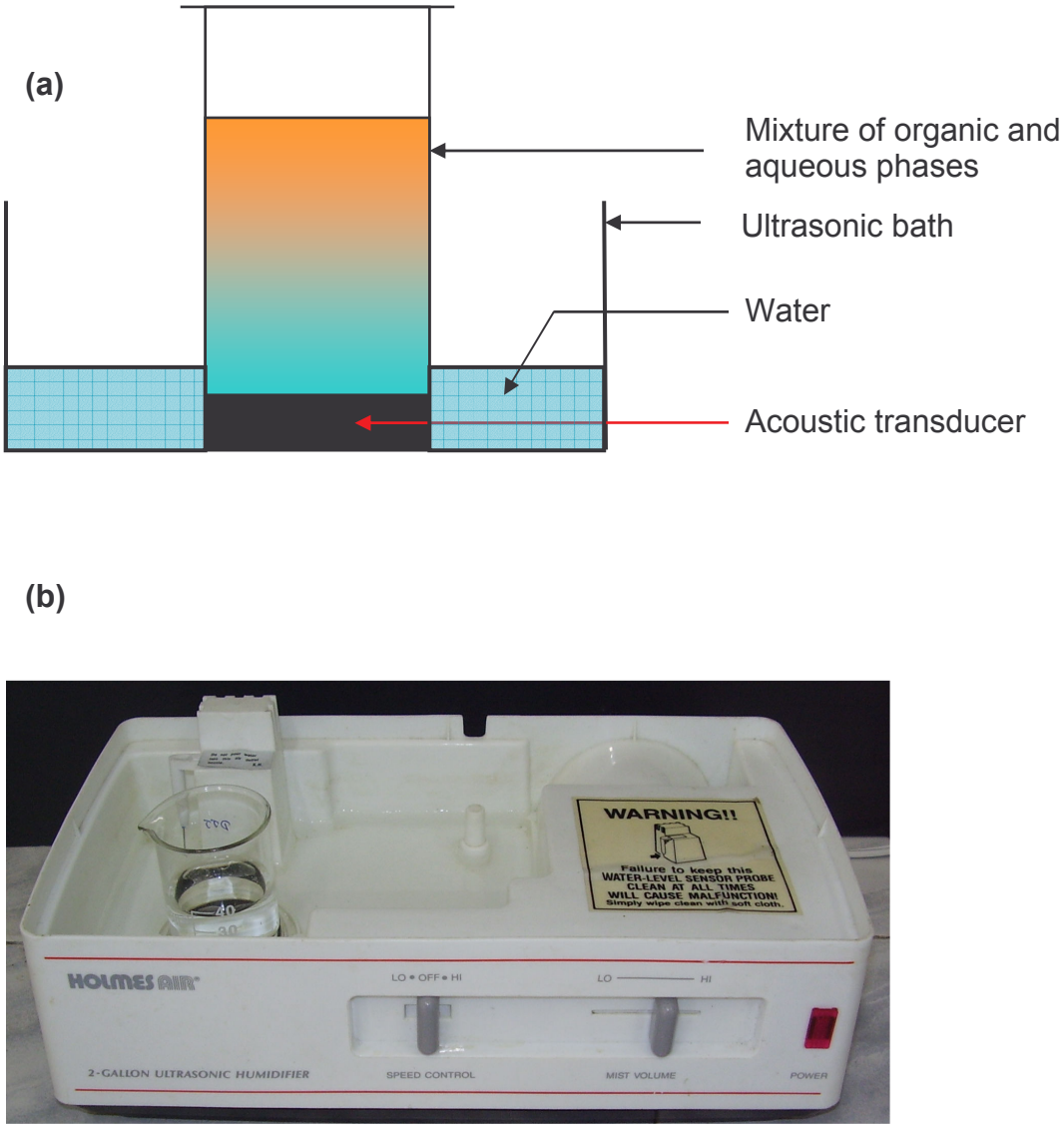


Fig. 4B.2. (a) Schematic representation of experimental set up (b) ultrasonic bath used for the treatment

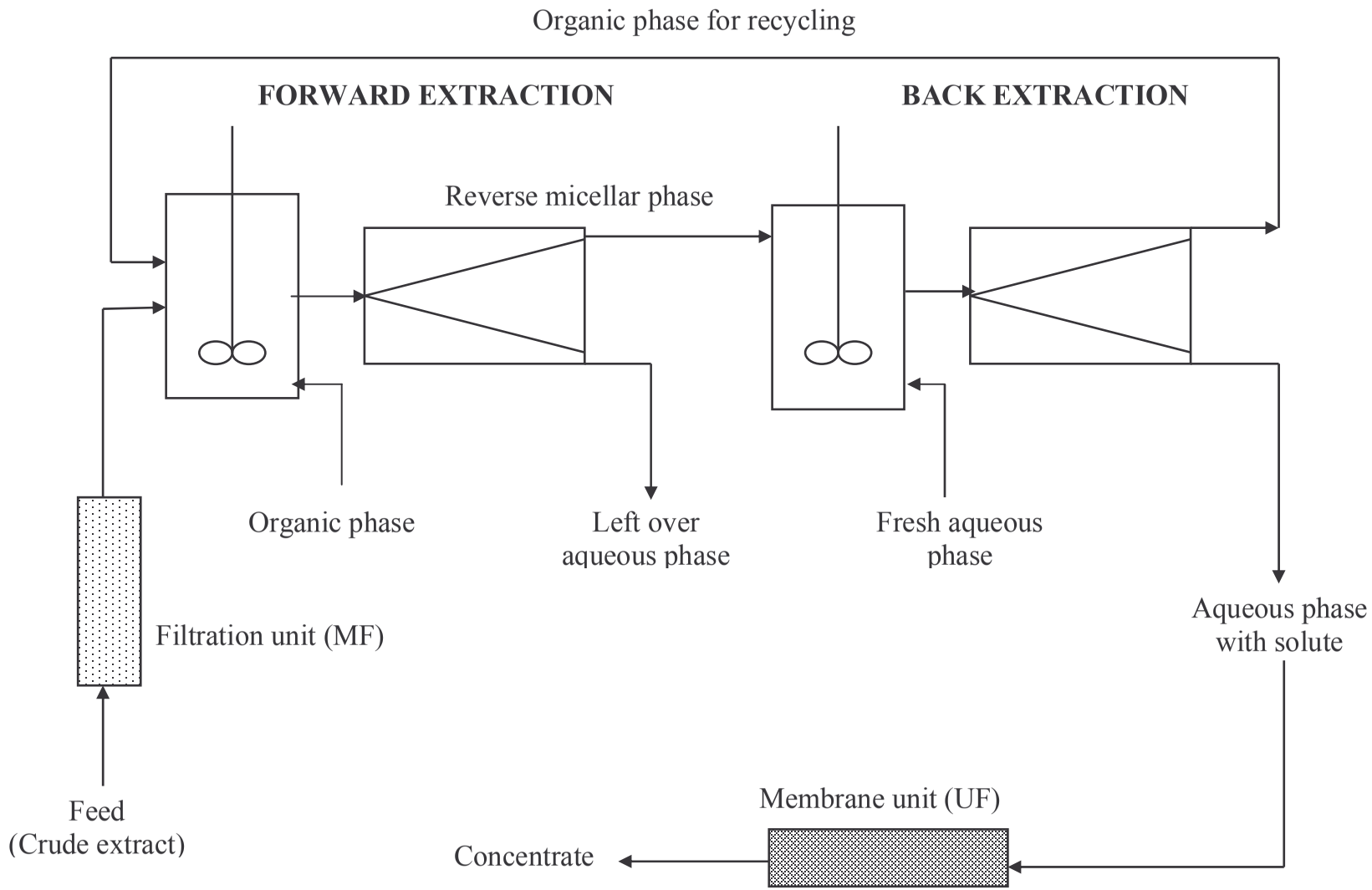


Fig.4B.3. Schematic diagram of integrated RME and membrane processes for the extraction and purification of bromelain

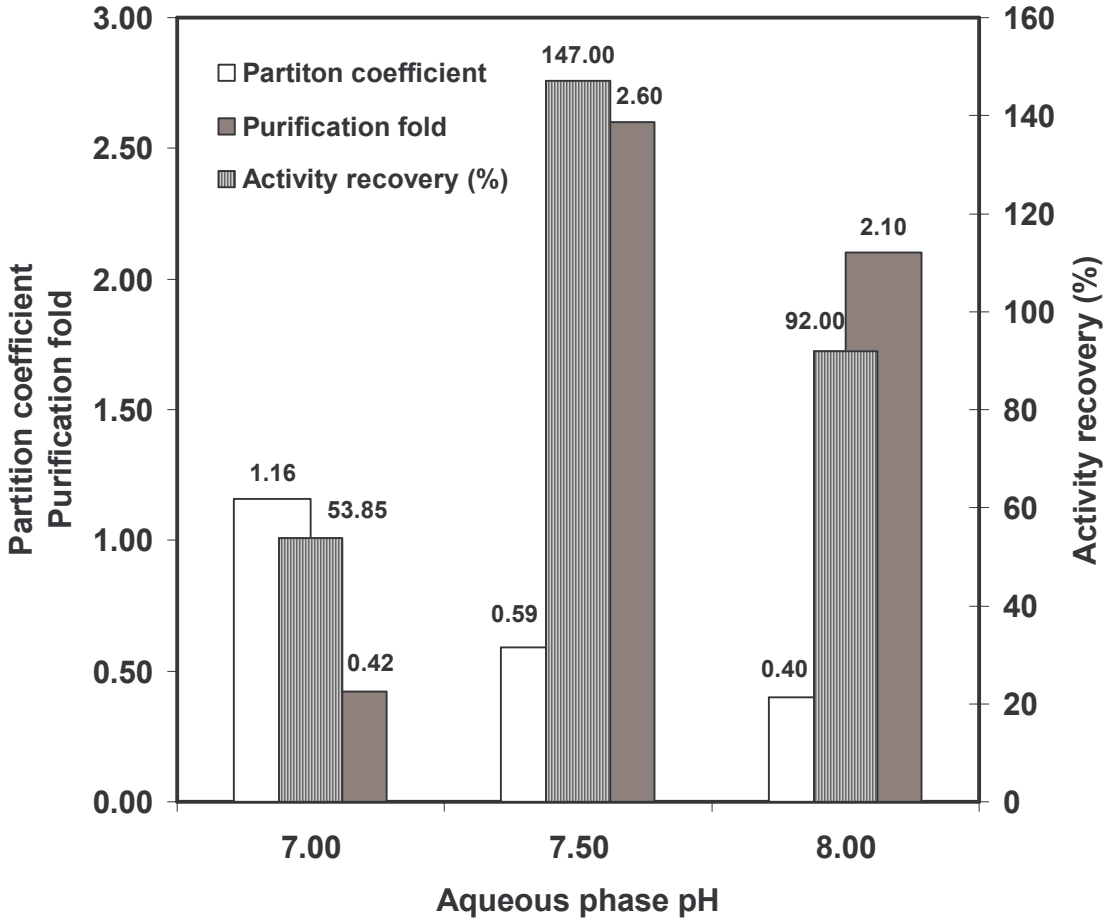


Fig 4B.4. Effect of aqueous phase pH on ATPE of bromelain

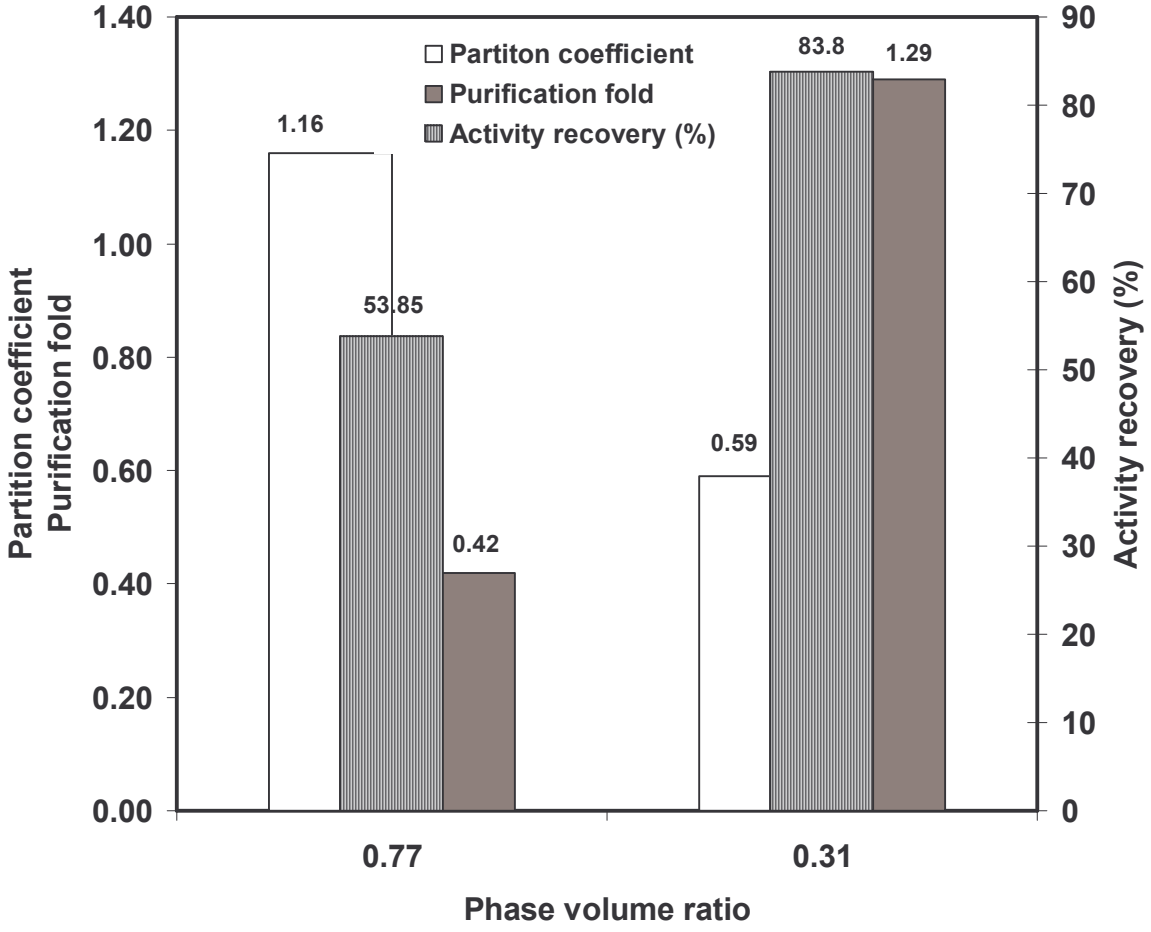


Fig. 4B.5. Effect of phase volume ratio on ATPE of bromelain

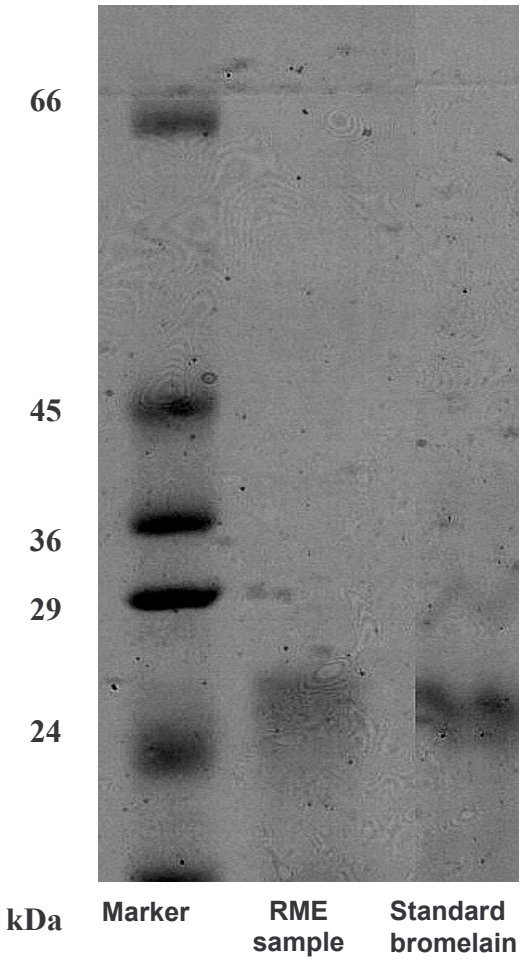


Fig. 4B.6. SDS PAGE analysis of reverse micellar extracted bromelain

SECTION C

EXTRACTION OF PEROXIDASE FROM RADISH ROOTS

4C.1. Introduction

Peroxidases are widely distributed in nature and are mostly extracted from plant cells and some animal organs and tissues. Radish root, Chinese cabbage, carrot hairy root cell culture and turnip are some of the commonly used plant sources of peroxidase. A few reports on application of RME for extraction and purification of peroxidase in model as well as natural systems are available. Wolbert *et al*. (1989) reported an overall extraction efficiency of 35% during phase transfer studies of commercial (horse radish peroxidase) HRP using AOT/isooctane system. Regalado *et al.* (1994) studied the recovery of commercial HRP at different phase volume ratios and obtained an activity recovery in the range 87% to 136%. Affinity based reverse micellar extraction and separation (ARMES) of commercial HRP and Soyabean peroxidase (from Soyabean hulls) was reported by Paradkar and Dordick (1991). Activity recovery of 30% with very high degree of purification (29.2 fold) was reported. An extraction efficiency of 83% was obtained when HRP was purified from a synthetic mixture. Motlekar and Bhagwat (2001) obtained almost 90% activity recovery with the injection method RME of commercial HRP. Huang and Lee (1994) attempted the single step extraction of HRP from *armoracia rusticana roots.* In the single stage extraction, all the proteins apart from the objective enzyme were taken to the organic phase to get 20 fold purification in the aqueous phase. Regalado *et al.* (1996) reported the purification of peroxidase from Horse radish root by adopting two-step reverse micellar extraction technique. AOT/isooctane system used for the extraction resulted in a degree of purification of 80 fold.

Most of the RME studies reported are on ionic surfactants and there are only a very few reports (Ayala *et al.,* 1992; Yamada *et al.,* 1993; Vasudevan *et al.,* 1995; Vasudevan and Wiencek, 1996; Adachi *et al*., 1998; Hossain *et al.*, 1999) on the use of nonionic surfactants for extraction. The wide usage of anionic surfactant AOT may be attributed to the ability of the surfactant to readily form reverse micelles in many of the organic solvents and also to have an electrostatic interaction with the solute, which will be driving force for RME. However, in a few

studies, the electrostatic interactions of ionic surfactants are reported to cause denaturation of enzymes, and nonionic surfactants are considered to be a good alternative to overcome the above problem.

The present work was aimed at i) to study the performance of nonionic (Tween-85 and Trition-X-100) surfactants for the extraction of peroxidase from the crude extract, which is not reported so far, ii) performance evaluation of AOT/isooctane reverse micellar system for peroxidase extraction and iii) to evaluate the performance of an integrated process of RME followed by conventional ammonium suplhate precipitation.

4C.2. Materials and Methods

4C.2.1 Materials

Fresh radish (*Armoracia rusticana*) available in the local market was used for the experiments. Triton-X-100 (isooctyl phenoxy polyehoxy ethanol), Tween-85 (Polyoxyethylene sorbitan trioleate) and AOT (Sodium bis-2-ethyl-1-hexyl sulphosuccinate) are purchased from Rohm and Haas Company, USA, Fluka chemicals, Switzerland and BDH labs, UK respectively and used without further purification. Toluene (UV spectroscopy) and isooctane (GR) were obtained from Loba Chemie., Mumbai and Qualigens India Ltd, Mumbai, respectively. All other reagents used were of analytical grade. Aqueous phase and all other solutions were prepared using distilled water.

4C.2.2 Methods

i) Preparation of crude extract

The crude extract form the radish root was obtained by crushing the washed and peeled roots with known quantity of distilled water followed by filtration and centrifugation. The clarified extract was dialyzed for 48 h using 10 kDa membrane against frequently changed de-ionized water at 4±2°C (Fig. 4C.1).

ii) Reverse micellar extraction

The forward extraction experiments were carried out by contacting known quantity of aqueous and organic phases using a magnetic stirrer (Cintex, Mumbai, India) for 30 min at 500 rpm at controlled temperature (25±2ºC). The aqueous phase (crude extract) had known salt (NaCl) concentration. For all the experimental runs the phase volume and phase ratio were 10 ml and 1.0, respectively, unless mentioned otherwise. The organic phase had known amount of surfactant dissolved in it. Phase separation was carried out in a laboratory centrifuge (MP 400 R, Eltek, India) at 3000 *g* for 15 min. The organic phase separated from the mixture after forward extraction was subjected to back extraction. Back extraction was carried out by contacting the organic phase of forward extraction with an equal volume of fresh aqueous phase. The phase contact and separation conditions were maintained similar to that of forward extraction.

iii) Ammonium sulphate precipitation

Known quantity (50% saturation) of ammonium sulphate was added to the crude extract and the mixture was stirred for 1 h [Murachi, 1976]. The mixture was centrifuged to separate out precipitated pellets. The supernatant was again mixed with excess amount of salt to precipitate protein. The precipitate was dissolved in known quantity of water and dialyzed (16 h) against deionized water using 10 kDa membrane before analyzing for protein content and peroxidase activity.

4C.2.3 Analyses

Protein content and enzyme activity measurement

Total protein content and the enzyme activity were measured in the aqueous phase by the standard comassie brilliant blue G-250 method [Bradford, 1976] and guaicol method [Kim and Yoo, 1996] respectively. All the readings were taken in triplicate and average value was reported. The efficiencies (%) of forward, back and overall extraction, activity recovery (%) and purification (fold) were estimated using the following equations.

$$\text{Forward extraction efficiency (\%)} = \frac{\text{Protein concentration in organic phase after forward extraction (mg/ml)}}{\text{Protein concentration in feed (mg/ml)}} \times 100$$

(4C.1)

$$\text{Back extraction efficiency (\%)} = \frac{\text{Protein concentration in aqueous phase after back extraction (mg/ml)}}{\text{Protein concentration in organic phase after forward extraction (mg/ml)}} \times 100$$

(4C.2)

$$\text{Overall efficiency (\%)} = \frac{\text{Protein concentration in aqueous phase after back extraction (mg/ml)}}{\text{Protein concentration in feed (mg/ml)}} \times 100$$

(4C.3)

$$\text{Activity recovery (\%)} = \frac{\text{Peroxidase activtity in aqueous phase after back extraction}}{\text{Peroxidase activty in the feed}} \times 100$$

(4C.4)

$$\text{Purification fold} = \frac{\text{Specific activity of peroxidase after RME}}{\text{Specific activity of peroxidase in the feed}}$$

(4C.5)

4C.3 Results and Discussion

The conditions for preparation of crude enzyme extract were optimized based on the specific activity of peroxidase. The storage stability of peroxidase present in the crude was studied under controlled conditions. The dialyzed extract was subjected to RME with different reverse micellar systems. Purity of peroxidase extracted was confirmed using SDS–PAGE analysis.

4C.3.1. Preparation of crude enzyme extract

The crude extract containing peroxidase was obtained from radish roots following the process detailed in Materials and Methods (Section 4C.2.2).

Extraction with distilled water gave higher (~1.7 times) specific activity as compared to extraction with buffer of same pH (6.5). Hence, for all the experiments distilled water was used for extraction. The crude extract was subjected to dialysis (for 48 h at 4±2°C) using 10 kDa membrane.

4C.3.2. Stability studies

Storage stability: The stability of the enzyme in the dialyzed crude extract at controlled temperature (4±2°C) was studied for a period of 96 h and the results are presented in Fig. 4C.2. The enzyme was found to be fairly stable during the storage and a only marginal decrease (~ 2.5%) in activity was observed at the end of 96 h. The total protein content of the crude remained the same (0.33 mg/ml) throughout the storage period. The above indicated the suitability of bulk storage of crude at low temperatures for a reasonably good period, which is essential for large scale operations.

pH stability: The specific activity of peroxidase in different crude pH (adjusted using 0.1 N NaOH and 0.1 N HCl) was measured (Fig. 4C.3) in order to study the feasibility of reverse micellar extraction at different aqueous phase pH (3.0-10.0). The specific activity was high at crude extract pH values less than 5.0 and it dropped by nearly 40-60% in higher crude extract pH values (6.0-10.0). The enzyme was found to be more active in acidic pH, which has resulted in higher specific activity.

4C.3.3. RME with nonionic surfactants

Reverse micellar system nonionic surfactants Triton-X-100 and Tween 85, which are 'non-denaturing' and 'non-toxic' were used for the extraction in organic solvent toluene. Although, the HLB value of Triton-X-100 is relatively higher (13.4), it was reported to form stable reverse micelles in organic solvent toluene [Rodriguez *et al.,* 1998]. A fairly high water core radius (~5.5 nm) for the Triton-X-100/toluene reverse micelles was reported in this study. Similar observation was made by Hebbar and Raghavarao (2007) during the study of extraction of BSA

(detailed in Section A of Chapter 3). Tween 85, another nonionic surfactant used in RME has a favorable HLB value (11.0) for RME. Study by Hebbar and Raghavarao (2004) on the extraction of commercial HRP using this reverse micellar system had reported a degree of purification of 2.0 and activity recovery of 83% (detailed in Section B of Chapter 3). Forward (aqueous phase pH 3.0, 15 mM Triton-X-100 and 0.15 M NaCl) and back extraction (aqueous phase pH 8.0, 2 M KCl, volume ratio 1:1) conditions employed in this study were taken as starting point in the present study. During the preliminary studies, the forward extraction with undialyzed crude resulted in a very low extraction efficiency (<10%). Regalado *et al.,* (1996) reported similar observation and attributed this to the "screening effect" of ions ($Mg^{2+,}$ Ca^{2+} and K^{+}) present in the crude extract of horse radish. Hence, for all further RME studies, dialyzed (as indicated in section 3.1) crude extract was used, which would be almost free from all ions that causes screening effect.

4C.3.3.1 Triton-X-100/toluene system

i) Effect of aqueous phase pH

The aqueous phase pH plays a major role in the reverse micellar extraction, particularly in case of ionic reverse micelles, as it determines the degree of electrostatic interaction with charged solutes. Although, nonionic surfactants do not posses any charge, some of the nonionic surfactants (Tween 85, Span 20 and alkyl polyglucoside), reported to exhibit zeta potential at neutral aqueous phase pH [Vasudevan and Wiencek, 1996]. The above was reported to be the reason for the weak interaction with solute resulting in higher extraction at neutral pH. Considering the above observation, and higher specific activity of peroxidase in crude extract at pH 3.0-5.0, the aqueous phase pH of was varied from 3.0 to 9.0 during forward extraction. With an increase in pH, forward extraction (%) remained almost the same up to pH 5.0 (Fig. 4C.4). A maximum extraction of 48.0% was obtained at pH 7.0. Further increase in pH, however, decreased the forward extraction efficiency. The phenomenon explained by Vasudevan and Wiencek (1996) that is weak electrostatic interaction between charged surfactant molecules

and solute, was expected to be the reason for the observed higher extraction at pH 7.0. Regalado *et al.* (1994) reported the presence of seven iso-enzymes with wide range (3.0–10.0) of iso-electric point (pI) in peroxidase extracted from horse radish. It was assumed that in the present study also iso-enzymes with charge opposite to that of surfactant at neutral pH might have lead to the above observation. Other interactions such as hydrophobic and steric (driving forces responsible for extraction) might have contributed to the extraction at other aqueous phase pH values. Although, the back extraction efficiency did not vary much with the change in forward extraction aqueous phase pH, the degree of extraction was very low (10-12%) in all the cases. It may be inferred that the back extraction conditions employed in the present study did not favor the release of solute back into the aqueous phase. Activity recovery at the end of back extraction was higher (69%) when the forward extraction aqueous pH was 7.0. Purification fold of 0.92 obtained at pH 7.0 was higher compared to that obtained at other pH values.

ii) Effect of Triton-X-100 concentration

Surfactant concentration is one of the parameters that determines the efficiency in RME. The concentration of surfactant that is required to have an efficient extraction would be normally much higher than the CMC of surfactant. The concentration of Trtiton-X-100 was varied in the range of 3 to 18 mM and its effect on forward and back extraction efficiencies as well as activity recovery were studied (Fig 4C.5). The minimum concentration selected for the study was much higher than the CMC of Triton-X-100 (0.30 mM). As expected, the forward extraction efficiency increased with an increase in surfactant concentration and a maximum forward extraction (48%) was obtained at 15 mM concentration. The back extraction efficiency remained very low (less than 13%) in all the cases and did not vary appreciably with the surfactant concentration. Increased surfactant concentration increases the number of reverse micelles formed, leading to higher forward extraction. Inter-micellar collision at higher Triton-X-100 concentration (above 15 mM) might have resulted in lower forward extraction efficiency. Activity

recovery also showed the trend similar to that of forward extraction efficiency with a maximum recovery (69%) at Trtiton-X-100 concentration of 15 mM. Purification fold remained almost the same (0.7-0.9) at concentrations in the range 7-15 mM.

4C.3.3.2 Tween 85/toluene system

Since, the extraction efficiency with Trtion-X-100 system was found to be very low, RME of peroxidase was attempted with another nonionic surfactant, namely, Tween 85. The surfactant concentration was varied by keeping all other forward and back extraction conditions same as that reported for Triton-X-100/toluene system. As, no phase separation was observed during RME when organic phase had only toluene and Tween 85, co-solvent isopropyl alcohol (10% v/v) was added to the organic phase. Both the phases were clear and complete phase separation was obtained with the addition of co-solvent. Tween 85 concentration was varied from 0.05 to 0.5 (%v/v). The overall extraction efficiency measured at the end of back extraction was maximum (47%) at 0.1% concentration (Fig. 4C.6). However, the purification was higher (2.6) at of 0.5% concentration. Higher overall extraction as well as purification fold with Tween 85 system as compared to Triton-X-100/toluene system confirmed the need for selection of appropriate reverse micellar system.

4C.3.3.3. AOT/isooctane system

Reverse micellar system of anionic surfactant is widely used for RME of biomolecules as it can readily form reverse micelles of reasonably bigger core and high stability. As AOT/isooctane combination was reported to form a good reverse micellar system, the same was used in the present study. The aqueous phase pH was varied from pH 3.0 to 6.6 to study its affect on extraction efficiencies (Table 4C.1). This aqueous pH range was selected as the earlier studies by Regalado *et al.* (1994) and Huang and Lee (1994) reported that aqueous pH of 3.0–5.0 was optimum for the extraction of peroxidase from different sources. It was expected to have electrostatic interaction at the above pH range as some of the iso-enzymes of peroxidase were found to have iso-electric point less than 7.0. The phase

volume ratio (1.0), NaCl concentration (0.15 M) and surfactant concentration (110 mM) were maintained constant during forward extraction. The back extraction was carried out at 2 M KCl using phosphate buffer of pH 8.0. Forward extraction efficiency was relatively higher at lower pH values (3.0-5.0) with a maximum extraction of 50% at pH 4.0. Electrostatic interaction between the positively charged iso-enzymes and negatively charged AOT molecules might have been responsible for relatively higher extraction at lower aqueous phase pH. The extent of back extraction was substantially high (>84%) in all the cases as compared to forward extraction, which has been rarely observed. The maximum activity recovery (44%) and purification fold (1.2) were obtained at aqueous phase pH 5.7 and 5.4, respectively.

4C.3.4. Ammonium salt precipitation

Salting out with ammonium sulfate is a commonly employed method of separating proteins. In most of the purification processes, ammonium salt precipitation is used as the primary purification step, followed by other techniques such as column chromatography, electrophoresis etc., In the present study, an attempt was made to have an integrated process wherein RME was followed by ammonium salt precipitation to explore the possibilities of an improved overall performance. The crude peroxidase extract obtained from radish was subjected to precipitation with 50% ammonium sulfate. The precipitated protein pellets were solubilized in phosphate buffer of pH 6.0 (1:3 ratio on weight basis) and dialyzed for 16 h at 4±2°C to remove the traces of salt present. Further, the dialyzed extract was subjected to RME. The optimized conditions of forward and back extractions (Forward extraction: aqueous phase pH 5.7, NaCl 0.15 M, and 110 mM AOT in isooctane; Back extraction: aqueous phase pH 8.0, KCl 2M) obtained with AOT/isooctane system were employed in the present study. The results are shown in Table 4C.2. Ammonium salt precipitation resulted in a purification fold of 1.51. However, RME of ammonium salt precipitated aqueous phase decreased the purification fold to 0.48 (with respect to feed used for RME extraction), instead of enhancing the purification. Although, the feed used for RME was dialyzed, the

presence of trace amount of ammonium sulphate in the aqueous phase might have resulted in the screening effect and lowered the extent of extraction efficiency. The results indicated that the combination of the above two processes may not be a feasible as complete removal of salt after ammonium salt precipitation is difficult or tedious.

4C.3.5. SDS–PAGE analysis

The aqueous phase containing peroxidase obtained RME using AOT/toluene was dialyzed for 16 h at 4±2^{0}C and lyophilized. The concentrated sample was loaded to 10% gel along with standard horse radish peroxidase sample and crude extract. The SDS lane pattern of peroxidase obtained from RME matched well with the standard (Fig. 4C. 7). The feed had two distinct bands one near 36 kDa and the other one close to 60 kDa. The molecular weight band obtained for the RME sample was found to be around 45 kDa, which is the reported molecular weight (44 kDa) of peroxidase. A couple of other bands with higher molecular weight were also observed in the RME sample.

4C.4. Conclusions

Reverse micellar extraction with Triton-X-100/toluene system did not result in good extraction efficiency. Back extraction efficiency was considerably low with the above system, which resulted in a poor efficiency of overall extraction. Tween 85/toluene reverse micellar system gave fairly good overall extraction (44%) and purification fold (2.6) for the conditions employed in the study. Although, AOT/isooctane system resulted in better extraction efficiency as compared to Triton-X-100/toluene system, it was not as efficient as Tween85/toluene system. Integrated process having RME followed by ammonium salt precipitation did not show an encouraging result.

Table 4C.1. Effect of aqueous phase pH on forward extraction of peroxidase

Aqueous phase pH	Extraction efficiency (%)			Activity recovery (%)	Purification fold
	FE	BE	Overall		
3.0	49	60	30	23	0.77
4.0	50	94	47	20	0.32
5.0	40	92	37	29	0.92
5.4	40	93	37	39	1.20
5.7	43	90	39	44	1.00
6.0	38	90	36	34	0.96
6.6	40	84	34	15	0.40

FE: Forward extraction, BE: Back extraction

System: AOT/isooctane

Table 4C.2. Performance of combined ammonium salt precipitation and RME (two–step purification method)

Sample	Volume (ml)	Total Activity (CDU)	Total Protein (mg)	Specific activity (CDU/mg)	Purification fold
Crude extract	600	1879200	1800	1080	-
After ammonium salt precipitation	15	98535	60.3	1634.1	1.51
Aqueous phase after FE	10	11349	44.7	253.9	0.16
Aqueous phase after BE	10	4357	0.56	778.2	0.48

FE: Forward extraction, BE: Back extraction

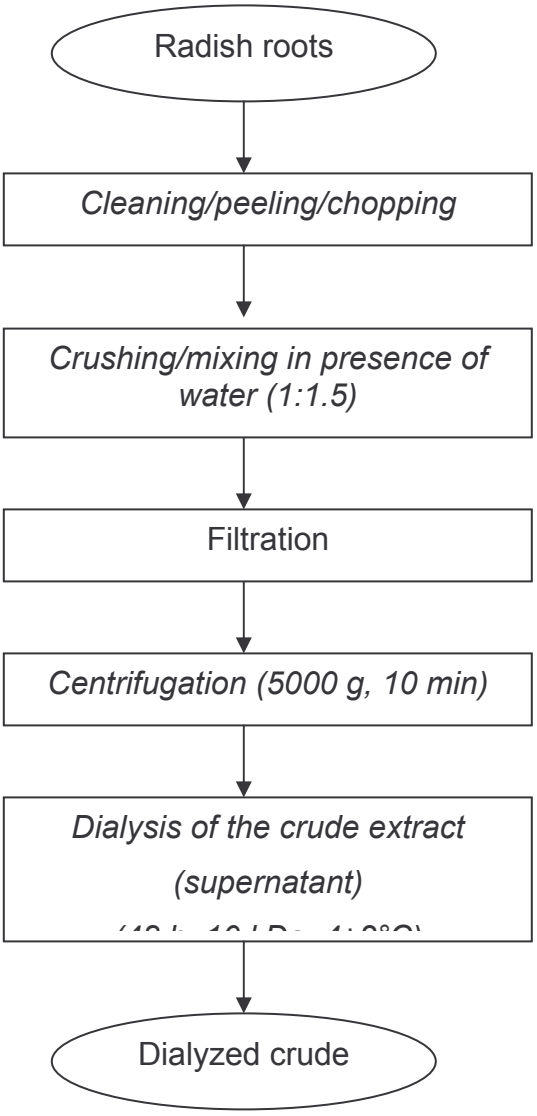


Fig. 4C.1. Flow diagram for the preparation of crude enzyme extract from radish roots

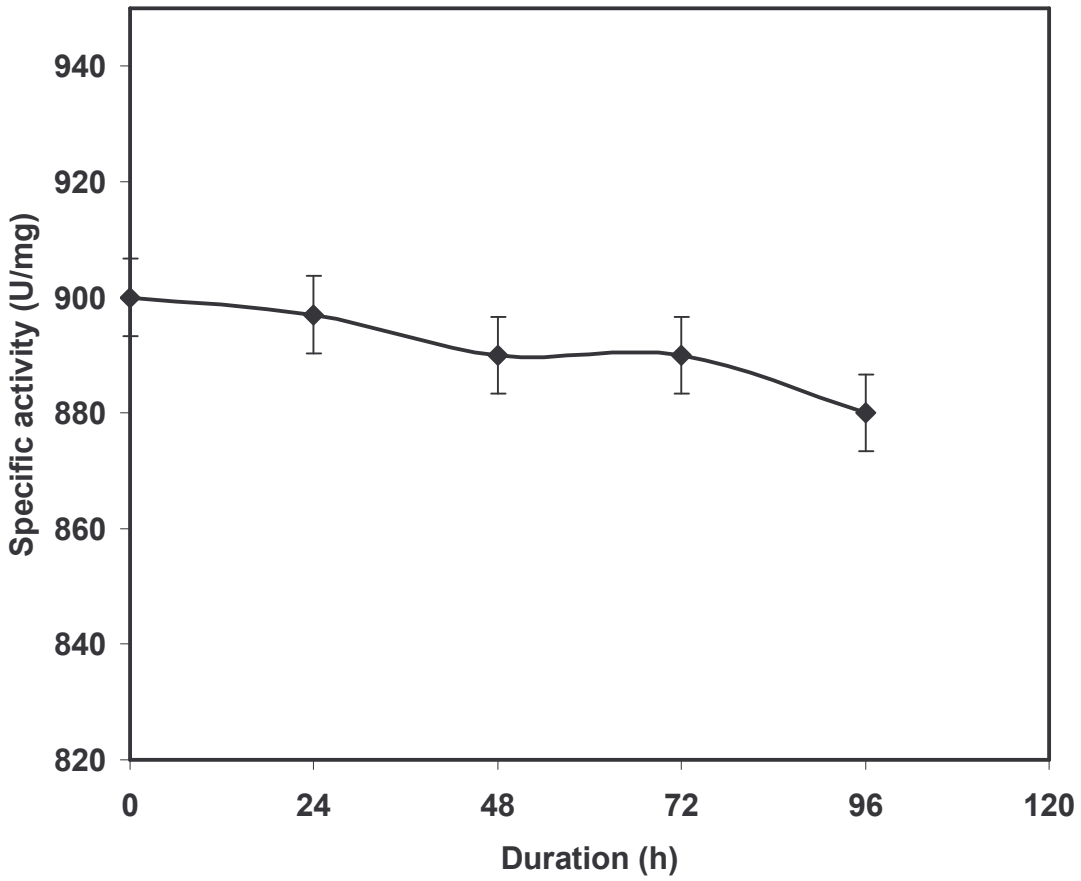


Fig. 4C.2. Stability of peroxidase in dialyzed crude extract stored at 4±2°C

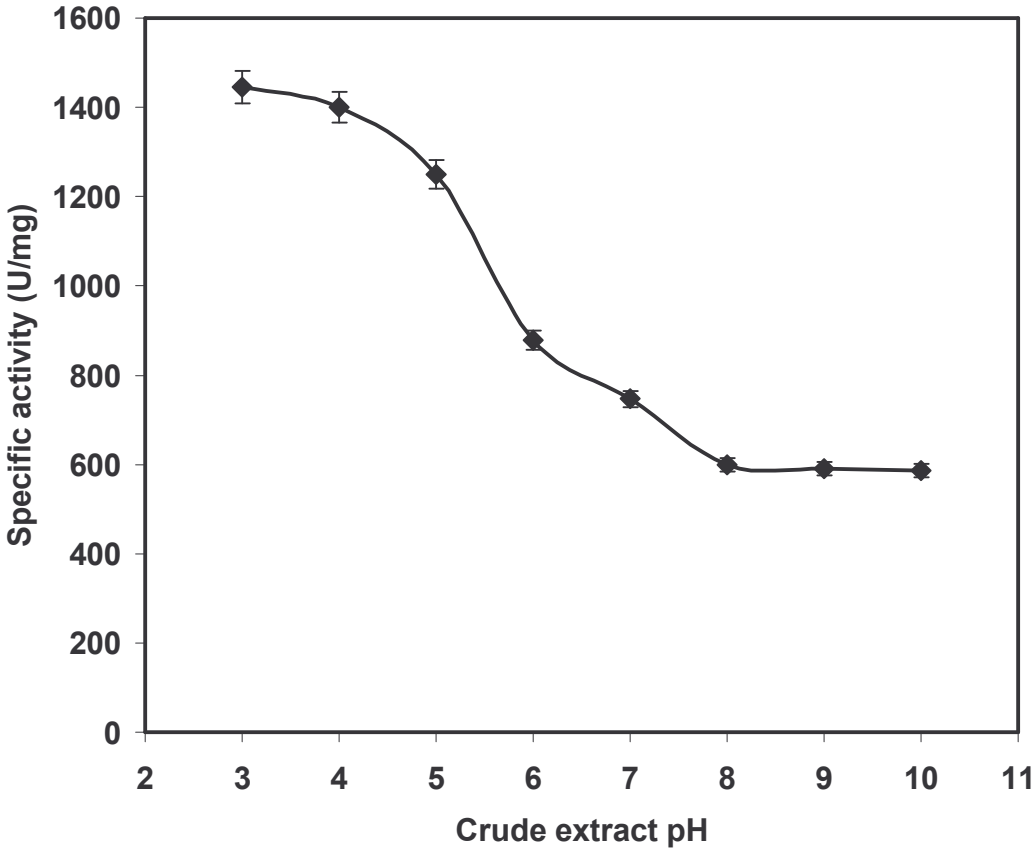


Fig. 4C.3. Stability of peroxidase at different pH of crude extract (storage temperature: 4±2°C)

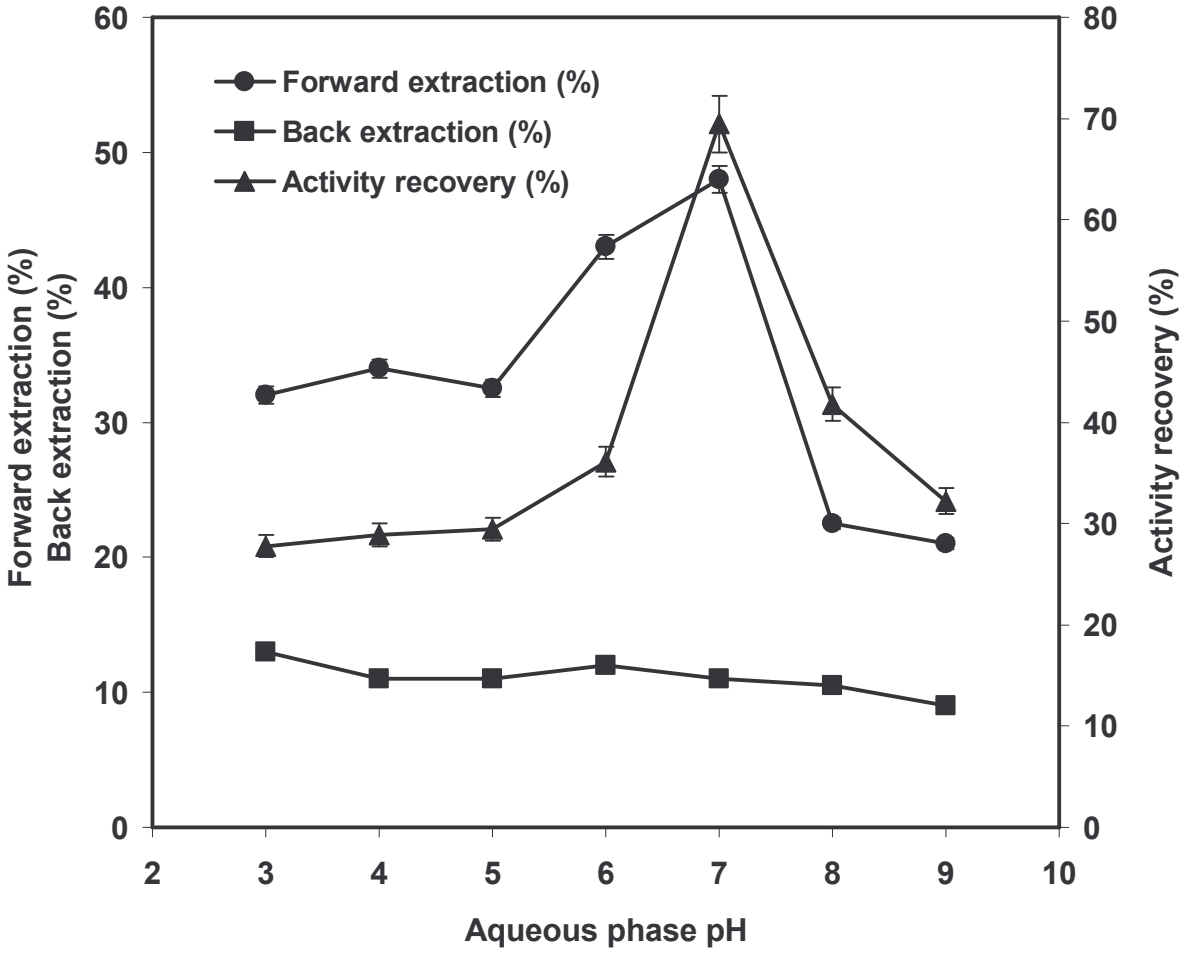


Fig. 4C.4. Effect of forward extraction aqueous phase pH on forward and back extraction efficiencies and activity recovery in Triton-X-100/toluene system

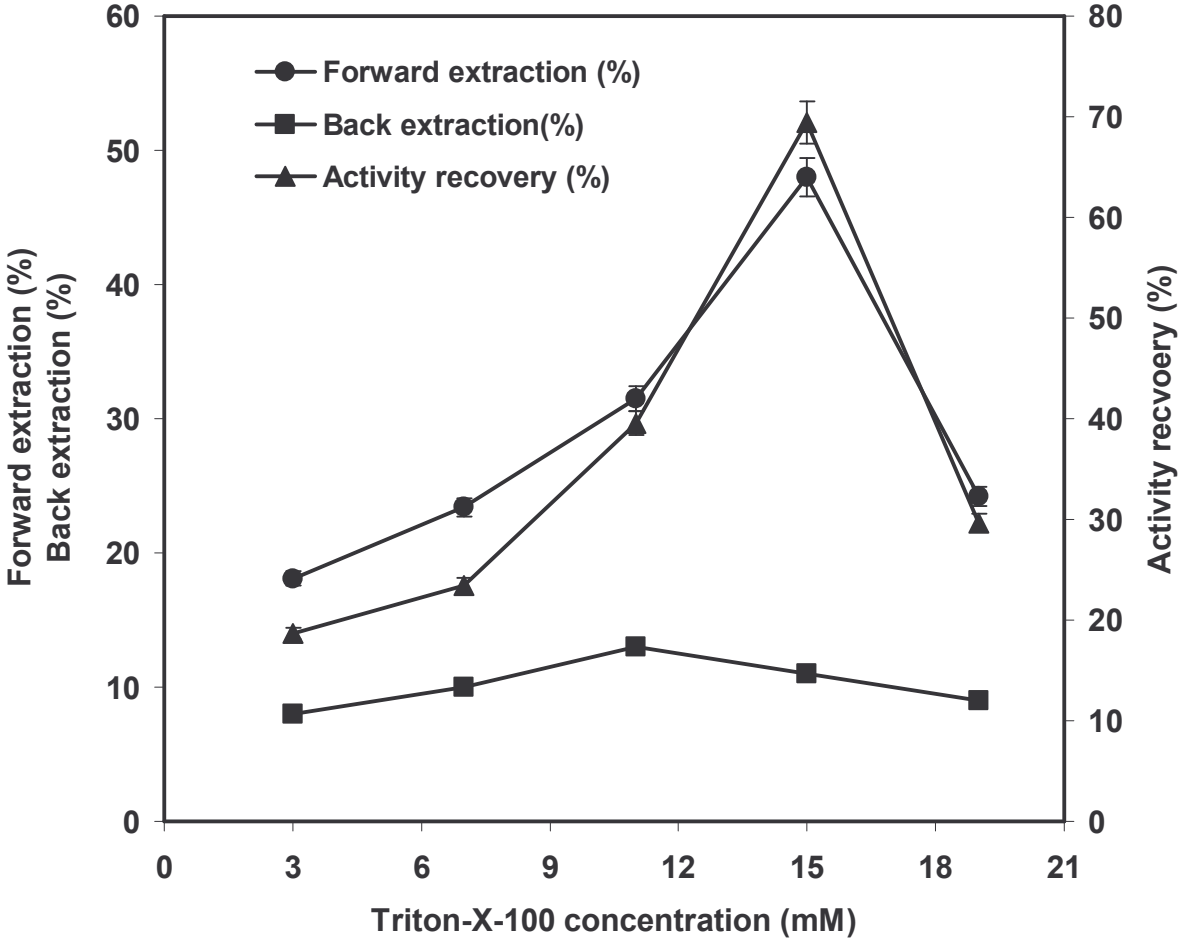


Fig. 4C.5. Effect of Triton-X-100 concentration on forward and back extraction efficiencies and activity recovery

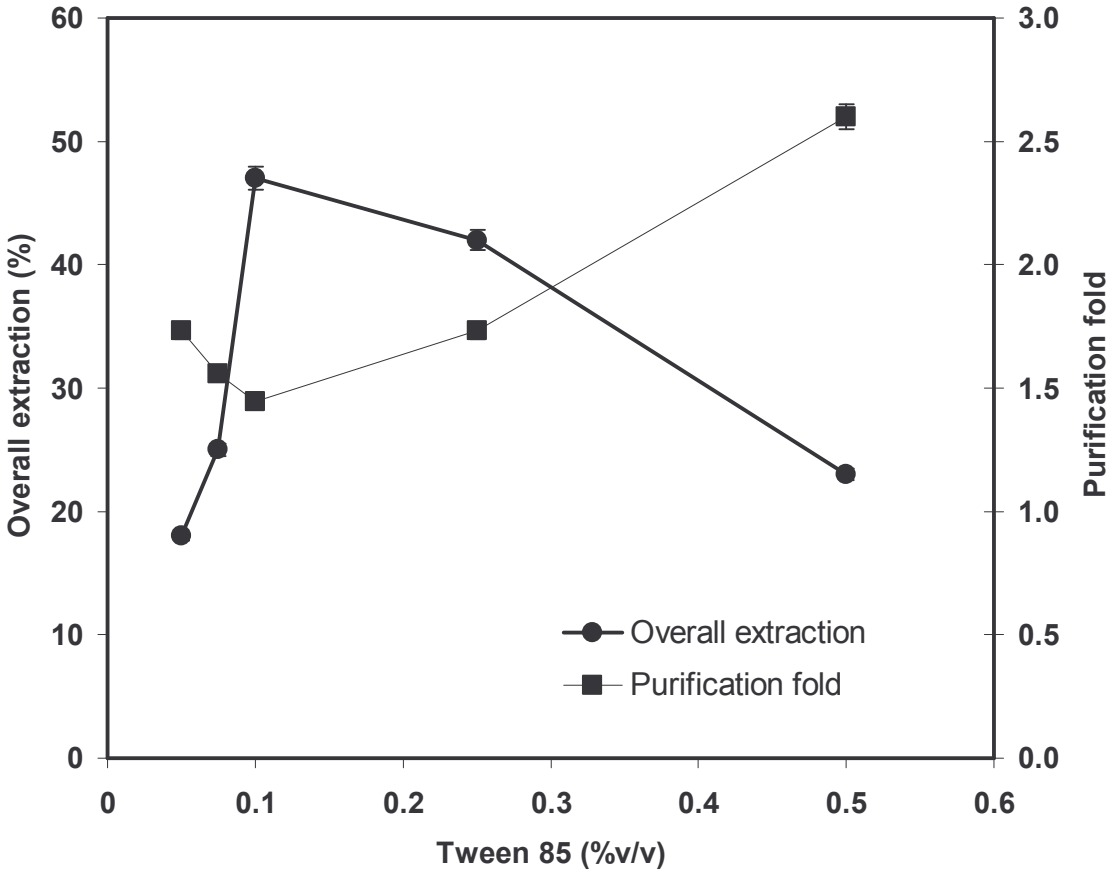


Fig. 4C.6. Effect of Tween 85 concentration on overall extraction efficiency and purification fold

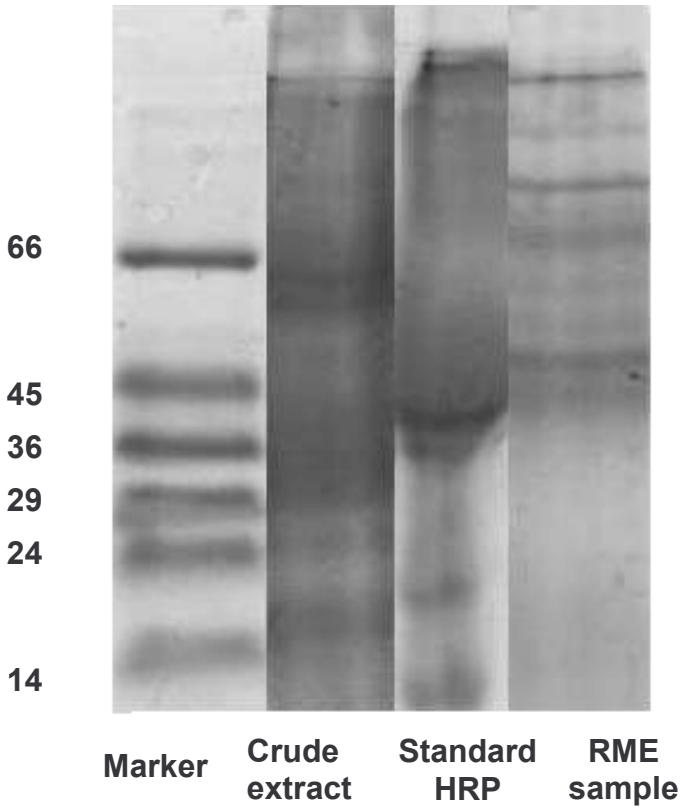


Fig. 4C.7. SDS-PAGE analysis of peroxidase extracted using AOT/isooctane reverse micelles

CHAPTER 5

MATHEMATICAL MODELING

SECTION A

ESTIMATION OF REVERSE MICELLAR SIZE

5A.1 INTRODUCTION

Mathematical modeling of the extraction of biomolecules in reverse micelles is essential for an in-depth understanding and effective use of RME in downstream processing. Models proposed in the literature range from simple geometric models [Bonner *et al.,* 1980; Levashov *et al.,* 1982; Sheu *et al.,* 1986; Zampieri *et al.,* 1986] to more rigorous molecular thermodynamic models [Bratko *et al.,*1988; Sheu *et al.,*1986; Woll and Hatton, 1989; Caselli *et al.*,1988a&b].

Bonner *et al*. (1980) were the first to propose the shell and core model or the "water shell" model to determine the radii of protein filled and unfilled reverse micelles assuming that the protein was in the water pool of the reverse micelle. Ultracentrifugation technique was used to substantiate the claims of the model. The model predicted the change in the dimensions of the reverse micelle upon uptake of proteins. It was observed that the protein containing reverse micelles were generally larger than the unfilled ones, especially at low W_0. The model proposed that the W_0 value of filled reverse micelle does not change and is same as that of unfilled reverse micelle. However, later studies by researchers [Levashov *et al.,* 1982; Zampieri *et al.,* 1986] revealed that the above assumption was not correct. Levashov *et al.* (1982) provided an alternative description of protein containing reverse micelles from ultracentrifugation. The model predicted that the diameter of the reverse micelle changed very little upon protein solubilization. It was proposed that the reverse micellar water boundary shifted outward upon inclusion of protein and contended that their proposed scenario was consistent with deeper penetration of the surfactant head group region by water, owing to the presence of the protein in the micellar core. However, the model received stern opposition from Luisi's group [Zampieri *et al.,* 1986] and Hatton's group [Sheu *et al.,1*986]. According to the above two groups, the model proposed by Levashov *et al,* violated the area and volume constraints imposed on the system by the predetermined water, protein and surfactant concentrations. Zampieri *et al.* (1986) reported that both filled and unfilled reverse micelles

increased in their size with increase in W_0 and, the size of the empty reverse micelle increased after protein uptake. These conclusions were made based on the studies carried out with two different dyes (one water soluble and the other strong interfacially active) and an assumption that the two dyes distributed between reverse micelles in proportion to water and surfactant, respectively. Sheu *et al*. (1986), however, disagreed with the above conclusions and suggested that the distribution of solutes over the micelle population may not be uniform as assumed by Zamprieri *et al*. (1986). The shell and core model combined with Poisson-Boltzmann approximation was developed by Bratko *et al*. (1988), which described the thermodynamics of protein solubilization in reverse micelles, assuming the electrostatic interactions and ideal mixing of the proteins into the micellar solutions as the important model components. A good agreement between the predictions of the model and the known salting out effect of cyctochrome-c in AOT/isooctane system was observed. Caselli *et al*. (1988b) proposed a simplified model for the prediction of filled and unfilled reverse micelles, which was based on the minimization of an approximate expression for the system free energy, incorporating contributions from the electrostatic interactions between the protein and charged reverse micellar head groups. Woll and Hatton (1989) proposed a model to relate the protein-reverse micelle complex size to protein size and charge, and surfactant concentration. Rahaman and Hatton (1991) developed a thermodynamic model to predict the sizes of protein filled and unfilled reverse micelles made both by injection and phase transfer methods. This model assumed a bidisperse population of reverse micelles consisting of empty and filled reverse micelles. These results differed from those of Caselli *et al. (*1988b).

Several experimental methods and empirical models have been employed to determine the size and shape of reverse micellar aggregates. The hydraulic core radius (Fig. 5A.1), which is the distance between the centre of the core and the inner surface of surfactant head is measured using instruments such as Dynamic light scattering (DLS), Small angle neutron scattering (SANS), Quasi-

elastic light scattering (QELS), Small angle X-ray scattering (SAXS), ultracentrifugation [Assih *et al.,* 1982; Harikrishna *et al*., 2002] or Fluorescence recovery after fringe pattern photobleaching (Chatenay *et al.,* 1987),. Several empirical equations, which use W_0 value for the estimation of the hydraulic core radius, have been reported. The various empirical models proposed mainly for ionic surfactants (AOT in particular) are provided in Table 2B.1. In all these models, the size of the reverse micelle has been directly related to the water content (W_o) of the reverse micelle. Many geometric models have also been derived (Table 2B.1) assuming the reverse micelles to be spherical in shape. A few of the models propose different equations for filled and unfilled reverse micelles, whereas the others employ the same equation for both.

The objectives of the present study are i) to study the effect of surfactant (CTAB) concentration, salt concentration and aqueous phase pH on the size of the reverse micelle (radius of the core) and W_0 value for both filled and unfilled reverse micelles, ii) to estimate the size of the reverse micelles using different empirical and geometric models, iii) to check the suitability of the empirical model derived (using geometric model) for the estimation of reverse micellar radius, and iv) to estimate the number of reverse micelles and aggregation number of surfactants.

5A.2 MATERIALS AND METHODS

5A.2.1. Materials

All chemicals and reagents used in the study are same as that described in Materials and Methods section of Chapter 4.

5A.2.2. Methods

The unfilled reverse micelles (without solute) were formed by mixing the aqueous phase (buffer of known pH and NaCl concentration) with organic phase (isooctane) containing known concentration of CTAB for 15 min at 500 rpm. In case of filled revere micelles (with solute), the crude extract of pineapple core was

used as the aqueous phase. The surfactant concentration was varied from 40 to 200 mM. The phases were separated by centrifugation and the organic phase obtained was used for the estimation of amount of water present. All other conditions of extraction were maintained same as that described in Chapter 4. Experiments were also carried out as above for different NaCl concentrations (0-0.3 M) and aqueous pH variation (5-10), while maintaining all other conditions the same.

5A.3.3.Analyses

i) Water Content (W_0) and radius of the reverse micellar core (R_m)

The amount of water present (%) in the organic phase of forward extraction was measured using Karl Fischer (KF) auto titrator (DL 32, Mettler Toledo, Germany). The amount of water (percent) was converted into molar value and used for the estimation of W_0. Readings were taken in triplicate and average values are reported.

The radius of the reverse micellar core (R_m) was estimated using the empirical and geometric models, reported in the literature (Table 5A.1). The W_0 value obtained from KF titration was used for the estimation of R_m and values were expressed in nanometers. The physical properties of CTAB and water used in the estimation of R_m were taken from literature and are presented in Table 5A.2.

ii) Estimation of number of reverse micelles

The number of reverse micelles formed for a given concentration of CTAB was estimated using the following equations proposed by Bru *et al.* (1989) and Jolivalt *et al.* (1993), respectively

$$N_m = \frac{3V_t}{4\pi R_m{}^3} \qquad (5A.1)$$

$$N_m = \frac{1}{36\pi} \frac{(A_S[S])^3}{(V_w[H_2O])^2} NV_m \qquad (5A.2)$$

iii) Estimation of surfactant aggregation number

The surfactant aggregation number was estimated by three methods indicated below.

a) Ratio of CTAB molecules to reverse micelles

$$N_{ag} = \frac{\text{Number of CTAB molecules in the organic phase}}{\text{Number of reverse micelles present}} \qquad (5A.3)$$

b) Equations proposed by Levashov *et al.* (1982) for the estimation of unfilled and filled reverse micelles, respectively

$$N_e^s = \frac{M_0}{(M_{CTAB} + M_w W_0)} \qquad (5A.4)$$

$$N_f^s = \frac{M_f}{(M_{CTAB} + M_w W_0)} \qquad (5A.5)$$

c) Equation proposed by Matzke *et al*., (1992)

$$N_{ag} = 32.1 - 1.25W_0 + 0.873W_o^2 \qquad (5A.6)$$

iv) Area occupied by surfactant molecule on the surface of reverse micelle

The area occupied by CTAB molecule on the surface of reverse micelle was estimated using the following equation proposed by Levashov *et al.* (1982).

$$f_{CTAB} = \frac{4\pi R_m^2}{N_{ag}} \qquad (5A.7)$$

5A.3 RESULTS AND DISCUSSION

5A.3.1 *Determination of water content (W_0)*

The amount of water present in the organic phase was estimated by KF method. Figure 5A.2 shows the amount (%) of water present at different concentrations of CTAB. The amount of water in the organic phase increased linearly with the surfactant concentration for unfilled reverse micelles. In case of filled reverse micelles, a similar trend was observed, except above CTAB concentration of 150 mM. The increase in amount of water present in the organic phase could be either due to increase in number of reverse micelles or an increase in the size of the reverse micelles. The study on the effect of surfactant concentration had earlier shown a reduction in the forward extraction efficiency at higher CTAB concentration (above 150 mM) and it was attributed to the inter micellar collision and collapse of reverse micellar structure. Decreased water percentage in the organic phase above 150 mM CTAB concentration substantiated the observation made earlier.

The molar ratio of surfactant to water termed as water content (W_0) was estimated based on the amount of water present in the organic phase and CTAB concentration. For the estimation of W_0, it was assumed that all the surfactant added to the organic phase was available in the reverse micellar form and reverse micelles are uniformly distributed in the organic phase. Figure 5A.3 shows the change in W_0 values with CTAB concentration. For unfilled micelles, the W_0 value almost remained constant (51-55) at different CTAB concentrations. In case of filled micelles, the concentration did not change appreciably, except for concentration above 150 mM. This indicated that with the increase in surfactant concentration the number of surfactant molecule per reverse micelle will remain almost the same and additional molecules of surfactant added would contribute to the formation of new reverse micelles. Except at CTAB concentration of 200 mM the W_0 values for the filled reverse micelles (64-68) were higher as compared to that of unfilled ones (51-55). This indicated the probable higher size of filled reverse micelles as compared to unfilled reverse micelles. Rabie and Vera (1996)

reported the effect of surfactant (AOT) concentration on W_0. With an increase in AOT concentration, the water uptake also increased, although the increase was not linear.

5A.3.2 Estimation of the size of the reverse micelles

The water content (W_0) values obtained at different concentrations of CTAB were used for the estimation of the size of the reverse micelles employing the equations listed in Table 5A.1. The size of the reverse micelles estimated for unfilled and filled micelles using empirical and geometric models are shown in Tables 5A.3 and 5A.4, respectively. All the empirical models predicted bigger size for filled reverse micelles as compared to the unfilled ones, except at concentration 200 mM. An increase of nearly 15-21% was observed for filled reverse micelles. Amongst the empirical models, the model proposed by Bru *et al.* (1989) resulted in relatively higher values for both filled (8.9-9.6 nm) and unfilled (8.4-11.9 nm) reverse micelles, which is obviously due to the higher multiplication factor of W_0. The change in surfactant concentration did not significantly change the size of the unfilled reverse micelle, which indicated that increase in surfactant concentration only increases the number of micelles and does not affect the size of reverse micelle. The same trend was observed with filled reverse micelles, except for concentrations above 150 mM. Like in the case of empirical models, the size of filled reverse micelles estimated using geometric models was bigger as compared to unfilled reverse micelles. The degree of increase was in the range of 15-20%. Further, the geometric models proposed by Leavashov *et al.* (1982) and Sheu *et al.* (1986) resulted in relatively higher values (7.8-10.9 nm) as compared to other geometric models (7.1-9.5 nm) and were closer to the values obtained from empirical models. The values were almost similar for the other three geometric models for both filled and unfilled reverse micelles. In general, the empirical models gave slightly higher values as compared that obtained from geometric models. It has been reported [Bonner *et al.,* 1980; Caselli *et al.,* 1988; Sheu *et al.,* 1986; Zampieri *et al.,* 1986; Chang *et al.,* 1994] that incorporation of solute into micelles increases the size of the microstructure to some extent. The

results obtained based on the instrumental analysis during some of the above study have also indicated the increase in size of reverse micelle with the inclusion of solute. However, a few studies [Levashov *et al.,* 1982; Matzke *et al.,* 1992] have reported that reverse micelles adjusts the size depending upon the W_0 value in such a manner that it remains more or less the same for both filled and unfilled reverse micelles.

To derive an empirical relationship between W_0 and size of the reverse micelle (R_m) a plot of R_m (estimated using Krie and Hustdet model) at different W_0 values was drawn (Fig. 5A.4) for filled and unfilled reverse micelles. The geometric model reported by Krie and Hustdet (1992) was selected for the estimation of reverse micellar size as this model was tested by the above authors for CTAB system. The data gave a good fit with very high coefficient of regression (R^2) value of 0.92 and 0.99, respectively for unfilled and filled reverse micelles. The correlations obtained are given below and was found to be almost the same for filled and unfilled reverse micelles.

$R_m = 0.138\ W_0$, for unfilled reverse micelles (5A.8)

$R_m = 0.137\ W_0$, for filled reverse micelles (5A.9)

The empirical equation obtained above was found to be closer to that ($R_m = 0.15W_0$) reported by Motlekar and Bhagwat (2001).

Similarly, the concentration of NaCl (0-0.3 M) and aqueous phase pH (5-10) was varied, while maintaining the other conditions the same. The experiments were carried out for both blank (buffer of known pH and NaCl concentration) as well as with crude extract containing bromelain. The W_0 value for the organic phase was estimated using Karl Fischer titration method. The W_0 values obtained are presented in Table 5A.5. The size of the reverse micellar core was very small when salt was not added during RME. When the NaCl concentration was

increased beyond 0.1 M, the size of the reverse micelles decreased in both filled and unfilled reverse micelles. The reduction in size with an increase in salt concentration may be attributed to the lowered repulsion between the surfactant heads caused by the ions, which in turn reduces the extraction efficiency. It was observed that the unfilled reverse micelles were bigger as compared to the filled reverse micelles when salt concentration was more than 0.1 M. Variation in aqueous phase pH did not show any definite trend in size variation although the size marginally decreased above pH 6.0. The results indicated that the effect of pH on the size is not as predominant as that of salt and surfactant concentration. A plot of R_m (estimated using Krie and Hustdet model) versus W_0 values was drawn for variation in NaCl concentration (Fig. 5A.5) and aqueous phase pH (Fig. 5A.6). Both the plots showed an excellent fit (R^2 = 0.99-1.0) for unfilled as well filled reverse micelles. The correlation obtained (shown below) was found to be same for filled and unfilled reverse micelles in both the cases.

$R_m = 0.138\ W_0$ for unfilled reverse micelles (5A.10)

The correlation obtained was also found to be almost same as that obtained for CTAB concentration variation.

Validation of the model

To check the goodness of the empirical equation obtained, the experiments were carried out as detailed earlier at different CTAB concentrations (20, 60 and 80 mM). The W_0 and reverse micellar size were estimated as detailed earlier. A parity plot of estimated (Krie and Hustdet model) and predicted (using the empirical equation) values of reverse micellar size was drawn (Fig. 5A.7). The predicted values gave an excellent fit (R^2 = 0.99) for both unfilled and filled reverse micelles and indicated the goodness of the fit.

5A.3.3 *Estimation of number of reverse micelles*

Bru *et al.* (1989) and Jolivalt *et al.* (1993) have proposed equations (eqns. 5A.1 and 5A.2) for the estimation of number of reverse micelles (N_m) at a known concentration of surfactant. Both these equations have been derived based on the geometric conditions i.e. assuming the reverse micelles to be of spherical in shape. The equation proposed by Jolivalt *et al.* (1993) resulted in slightly higher value as compared to Bru *et al*. (1989) equation for both unfilled and filled reverse micelles. As could be seen from Table 5A.6, the number of reverse micelles increased almost linearly with an increase in CTAB concentration for both filled and unfilled reverse micelles. Except at 200 mM, the number was found to be less in filled as compared to that of unfilled reverse micelles at any given CTAB concentration. The reason that could be attributed to the above observation is the formation of more compact reverse micelles with higher number of surfactant molecules to host the enzyme in the water core, rather than forming more number of reverse micelles. The above observation was substantiated by studies on the estimation of surfactant aggregation number, which showed an increase in surfactant aggregation number for filled micelles as compared to empty micelles (discussed in Section 5A.3.4).

5A.3.4 *Estimation of surfactant aggregation number*

In addition to the size of the reverse micelles, the number of surfactant molecules per micelle (N_{ag}, which is also called as aggregation number) is an important structural parameter. The aggregation number indicates the number of micelles in a system for a known concentration of surfactant. Levashov *et al*. (1982) proposed two different equations for the estimation of the aggregation number (using W_0 value) for unfilled and unfilled reverse micelles. Matzke *et al.* (1992) measured the aggregation number of surfactants using Low Angle Laser Light Scattering (LALLS). The data was correlated with W_0 using the second order polynomial (eq. 5A.6). This study assumed that only solute and water were included in the revere micellar core and were evenly distributed among the reverse micelles. It was also reported that the equation developed gave higher values (for

the same data) as compared to values estimated by other researchers using different methods. The aggregation number was also estimated as a ratio of surfactant molecule to number of reverse micelles for a given CTAB concentration. The results of the study are shown in Table 5A.7. The aggregation number was higher for the filled as compared to unfilled reverse micelles up to 150 mM concentration in all the three methods used for the estimation. The aggregation number estimated using Matzke *et al*. (1992) equation was relatively higher as compared to the other two methods and it was nearly 2.5 times higher compared to the values obtained from equation 5A.3. Although, the number did not vary significantly with CTAB concentration, a gradual decrease was observed in case of filled reverse micelles. It was assumed that the increase in surfactant concentration increases the number of micelles, which in turn leads to the distribution of surfactant molecules to form more stable reverse micelles. Higher number of surfactant molecules with filled reverse micelles may also be attributed to the formation of stable reverse micelles around the solute. Chang *et al*. (1994) also compared the experimental values of aggregation with the theoretical value obtained using the equation proposed by Matzke *et al.* (1992). It was found that the error between the calculated values and experimental values of N_{ag} was small at lower water content values (<10), but was remarkable at higher values of water content. The aggregation number was also estimated using the equation 5A.3 (Lye *et al.,* 1995). Although, the trend observed was similar, the aggregation number was found to be lower as compared to the other two equations.

5A.3.5 Estimation of surfactant area at the surface of the reverse micelle

The area occupied by the surfactant molecule at the reverse micellar surface is an important parameter that is used in some of the models reported for the estimation of reverse micellar radius. The equation provided by Levashov *et al*. (1982) was used for the estimation of the above parameter. The area was estimated for both unfilled and filled reverse micelles and results are shown in Table 5A.8. The values did not differ appreciably for filled and unfilled and also it did not change with the surfactant concentration. The value obtained in the

present study (0.55 to 0.59 nm^2) is close to the value (0.65 nm^2) reported by Krie and Hustdet (1992) for CTAB and also lies in the range (0.50-0.70 nm^2) reported by Evans and Ninham, (1983) for ionic surfactants.

5A. 4 Conclusions

The amount of water in the organic phase increased linearly with the surfactant concentration. W_0 values for the filled reverse micelles (64-68) were higher as compared to that of unfilled ones (51-55), indicating a higher size of filled reverse micelles as compared to unfilled reverse micelles with variation in CTAB concentration. However, no definite trend was observed with change in salt concentration and aqueous phase pH. All the empirical models predicted filled reverse micelles of bigger size as compared to that of the unfilled ones, except at concentration 200 mM. The geometric models proposed by Leavashov *et al.* and Sheu *et al.* resulted in relatively higher values (7.8-10.9 nm) as compared to other geometric models (7.1-9.5 nm) and were closer to the values obtained from empirical models. The correlation obtained between W_0 and estimated reverse micellar size was found to be almost same for variation in CTAB and NaCl concentrations, and aqueous phase pH with filled and unfilled reverse micelles. The empirical model derived showed a good fit for different CTAB concentrations. The number of reverse micelles in organic phase increased almost linearly with an increase in CTAB concentration for both filled and unfilled reverse micelles. The aggregation number was higher for the filled as compared to unfilled reverse micelles up to 150 mM concentration in all the three methods used for the estimation. Higher number of surfactant molecules with filled reverse micelles may also be attributed to the formation of stable reverse micelles around the solute. The estimated value of surfactant area at the surface of the reverse micelle was close to that reported for ionic surfactants.

Table 5A.1. Models used for the estimation of radius of reverse micellar core (R_m in nm)

Geometric Models	
Levashov *et al.,* (1982)	$R_m = \{\frac{3}{4\pi}(N_{ag}\, W_0 V_w)\}^{1/3}$
Sheu *et al.,* (1986)	For empty micelle $R_m^e = \left\{\frac{3}{4\pi}\left(N_e{}^w V_w + N_e{}^s V_S\right)\right\}^{1/3}$ For filled micelle $R_m^f = \left\{\frac{3}{4\pi}\left(N_f{}^w V_w + N_f{}^s V_S + V_p\right)\right\}^{1/3}$
Krei and Hustedt (1992)	$R_m = \frac{3W_0 M_w}{A_s N \rho_w}$
Jolivalt *et al.,* (1993)	$R_m = \frac{3V_w W_0}{A_s}$
Regalado *et al.,* (1994)	$R_m = \frac{3V_t W_0}{A_{sm}}$
Empirical Models	
Bru *et al.,* (1989)	$R_m = 0.175W_0$
Gaikar and Kulkarni, (2001)	$R_m = 0.164W_0$
Motlekar and Bhagwat, (2001)	$R_m = 0.15W_0$
Kinugassa *et al,* (2003)	$R_m = 0.145W_0 + 0.57$

Table 5A.2. Physical properties of CTAB, water and bromelain used in the estimation of R_m

Symbol	Property	Value	Reference
V_s	Head group volume of surfactant (CTAB)	394 $Å^3$	Estimated based on surfactant head group area
A_{sm}	Molar area of surfactant (CTAB) head group	3×10^5 m^2	Estimated based on molecular area
A_s	Area per surfactant (CTAB) head group	0.65 nm^2	Krei and Hustdet, (1992)
M_{CTAB}	Molecular weight of CTAB	355g/mol	MERCK Product Catalogue
M_w	Molecular weight of water	18g/mol	Perry and Green (1996)
ρ_w	Density of water	1000 kg/m^3	
V_w	Water molecule volume	30.15 $Å^3$	Sheu *et al.,* (1986)
V_m	Molar volume of water	18×10^{-6} m^3/mol	Regalado *et al.,* (1994)
V_p	Volume of bromelain molecule	9202 $Å^3$	Estimated assuming the molecule to be spherical in shape

Table 5A.3. Size of the reverse micellar core estimated using empirical equations

CTAB conc. (mM)	*W_0*		Radius of the reverse micelle (R_m), nm							
			Gaikar and Kulkarni (2001)		*Kinugassa (2003)*		*Motlekar and Bhagwat (2001)*		*Bru et al.,(1989)*	
	UF	F	UF	F	UF	F	UF	F	UF	F
50	53	68	8.7	11.1	8.3	10.4	7.9	10.2	9.3	11.9
100	55	66	9.0	10.8	8.5	10.1	8.2	9.9	9.6	11.5
150	52	64	8.5	10.5	8.1	9.8	7.8	9.6	9.1	11.2
200	51	48	8.4	7.9	8.0	7.5	7.6	7.2	8.9	8.4

UF: Unfilled; F: Filled

Table 5A.4. Size of the reverse micellar core estimated using equations based on geometric consideration												
CTAB conc. (mM)	W_0		R_m, Radius of the reverse micellar core (nm)									
			Levashov et al., (1982)		Sheu *et al., (1986)*		*Krei and Hustedt (1992)*		*Jolivalt (1993)*		Regaldo *et al.*, (1994)	
	UF	F	UF	F	UF	F	UF	F	UF	F	UF	F
50	53	68	8.6	10.9	8.0	9.8	7.3	9.2	7.4	9.5	7.3	9.4
100	55	66	8.9	10.6	8.2	9.2	7.7	9.1	7.6	9.2	7.6	9.1
150	52	64	8.5	10.3	7.8	9.3	7.1	8.7	7.2	8.9	7.2	8.9
200	51	48	8.6	7.9	7.7	6.8	7.1	6.7	7.1	6.7	7.1	6.6

UF: Unfilled; F: Filled

Table 5A.5. Water content (W_0) and reverse micellar core radius (R_m) estimated for different NaCl concentrations and aqueous phase pH

Parameter varied	W_0		R_m (nm)	
	UF	F	UF	F
NaCl concentration (M)				
Without salt	1	19	0.2	2.6
0.1	54	67	7.4	9.3
0.2	46	34	6.3	4.7
0.3	36	34	5.0	4.7
Aqueous phase pH				
5.0	70	61	9.7	8.4
6.0	63	55	8.7	7.6
7.0	59	59	8.1	8.1
8.0	54	57	7.5	7.9
9.0	62	53	8.5	7.4
10.0	56	54	7.7	7.5

UF: Unfilled; F: Filled

Table 5A.6. Estimation of number of unfilled and filled reverse micelles

CTAB concentration (mM)	W_0		Number of reverse micelles (x 10^{17})			
			Bru et al., (1989)		Jolivalt et al., (1993)	
	UF	F	UF	F	UF	F
50	53	68	2.2	0.7	2.9	1.8
100	55	66	4.3	2.9	5.3	3.7
150	52	64	7.1	4.6	9.1	6.1
200	51	48	9.9	11.0	12.2	13.8

UF: Unfilled; F: Filled

Table 5A.7. Estimation of surfactant aggregation number in unfilled and filled reverse micelles

CTAB concentration (mM)	W_0		Surfactant aggregation number (N_{ag})					
			Levashov et al., (1982)		Estimated using eq. No 5A.3		Matzke et al., (1992)	
	UF	F	UF	F	UF	F	UF	F
50	53	68	1690	2700	1071	1650	2418	3984
100	55	66	1809	2555	1108	1364	2604	3752
150	52	64	1632	2413	1015	1460	2328	3528
200	51	48	1575	1410	985	708	2239	1667

Table 5A.8. Area occupied by the surfactant molecule on the surface of reverse micelle

CTAB Concentration (mM)	Area (nm^2)	
	UF	F
50	0.55	0.56
100	0.55	0.56
150	0.56	0.55
200	0.59	0.55

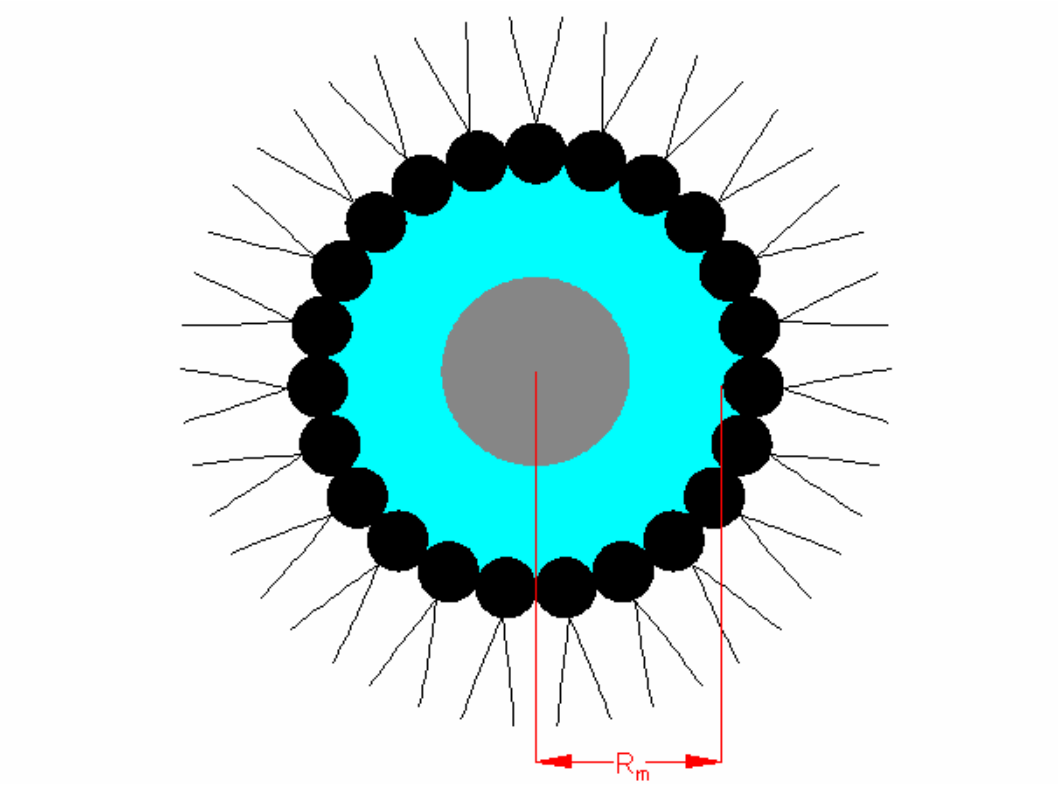


Fig. 5A.1 Schematic representation of a reverse micelle with solute in the hydraulic core

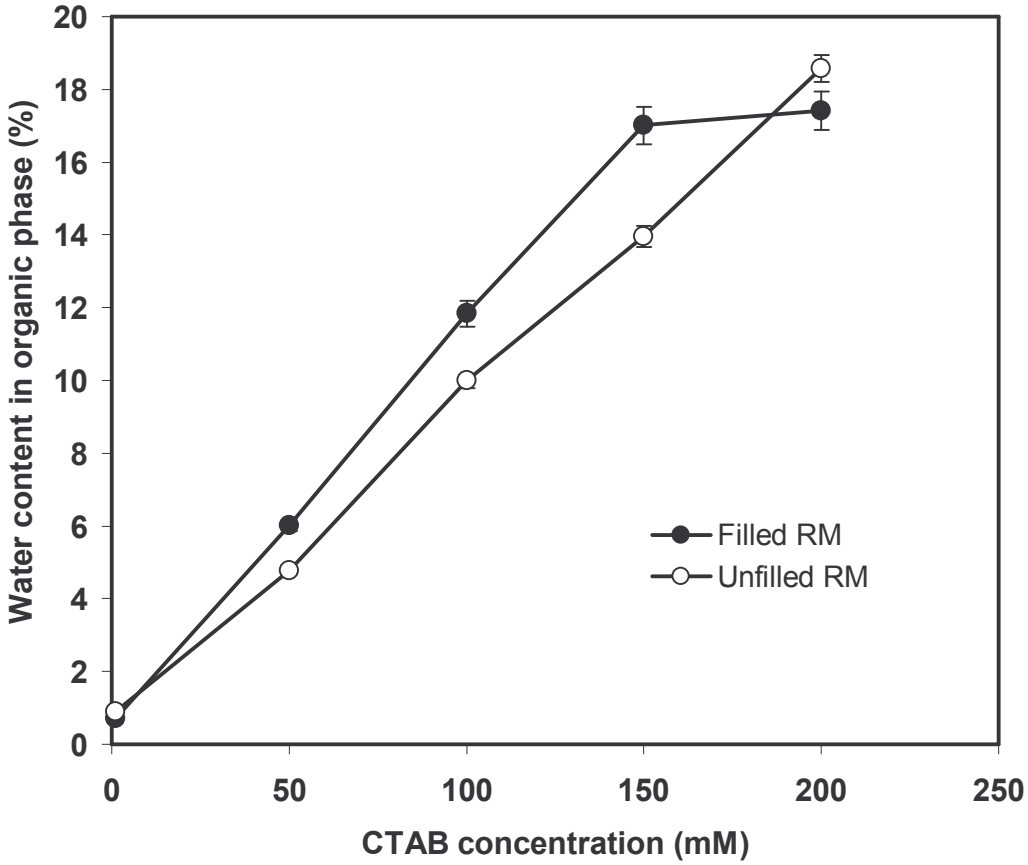


Fig.5A.2. Variation in amount of water present (%) at different CTAB concentrations

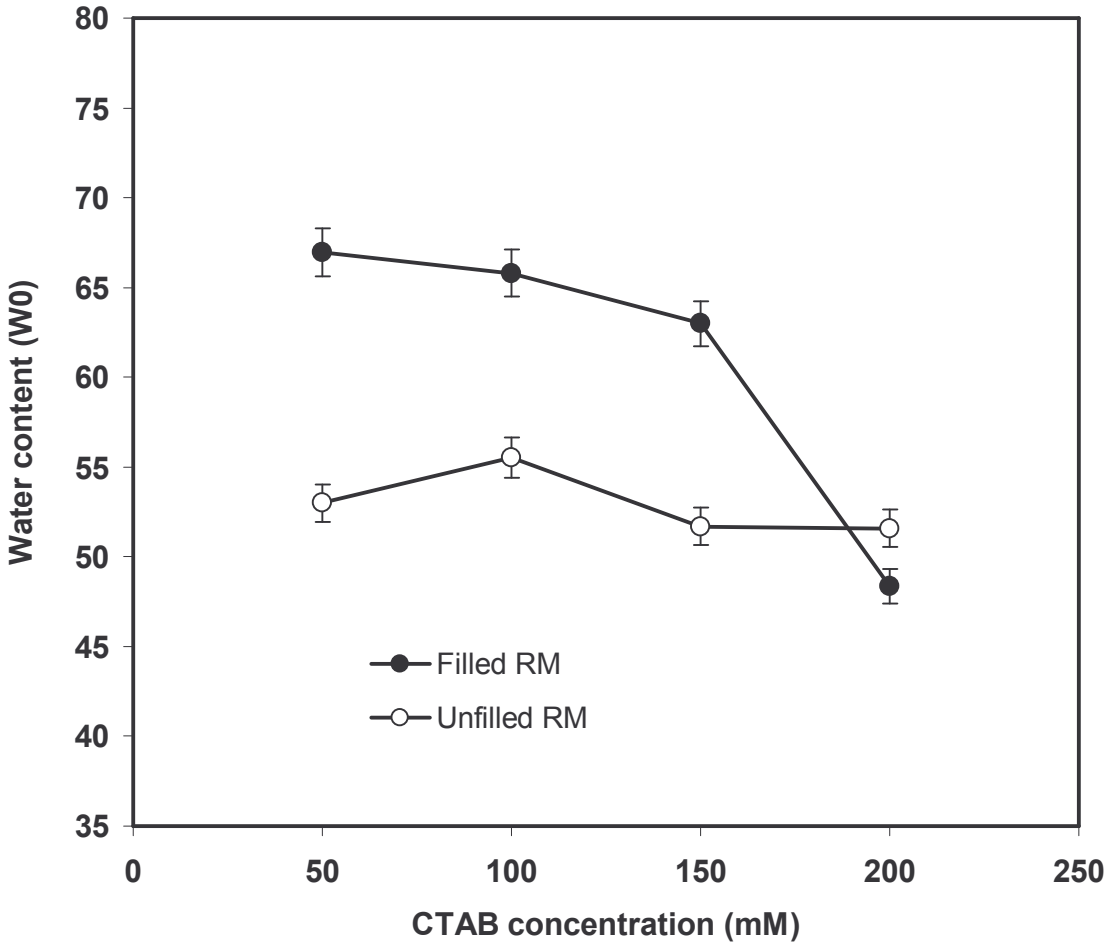


Fig.5A.3. Water content (W_0) values at different CTAB concentrations

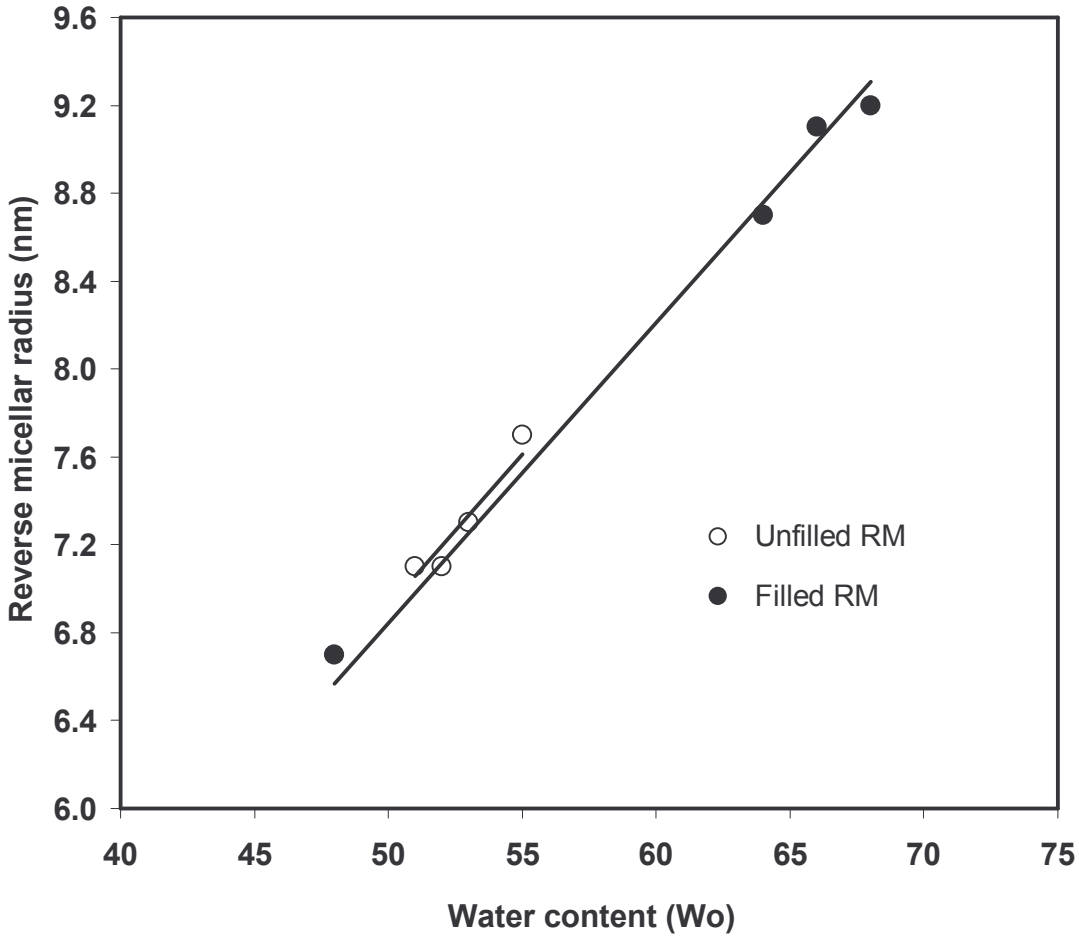


Fig. 5A.4. Radius of reverse micelle estimated using Krie and Hustdet model at different CTAB concentrations

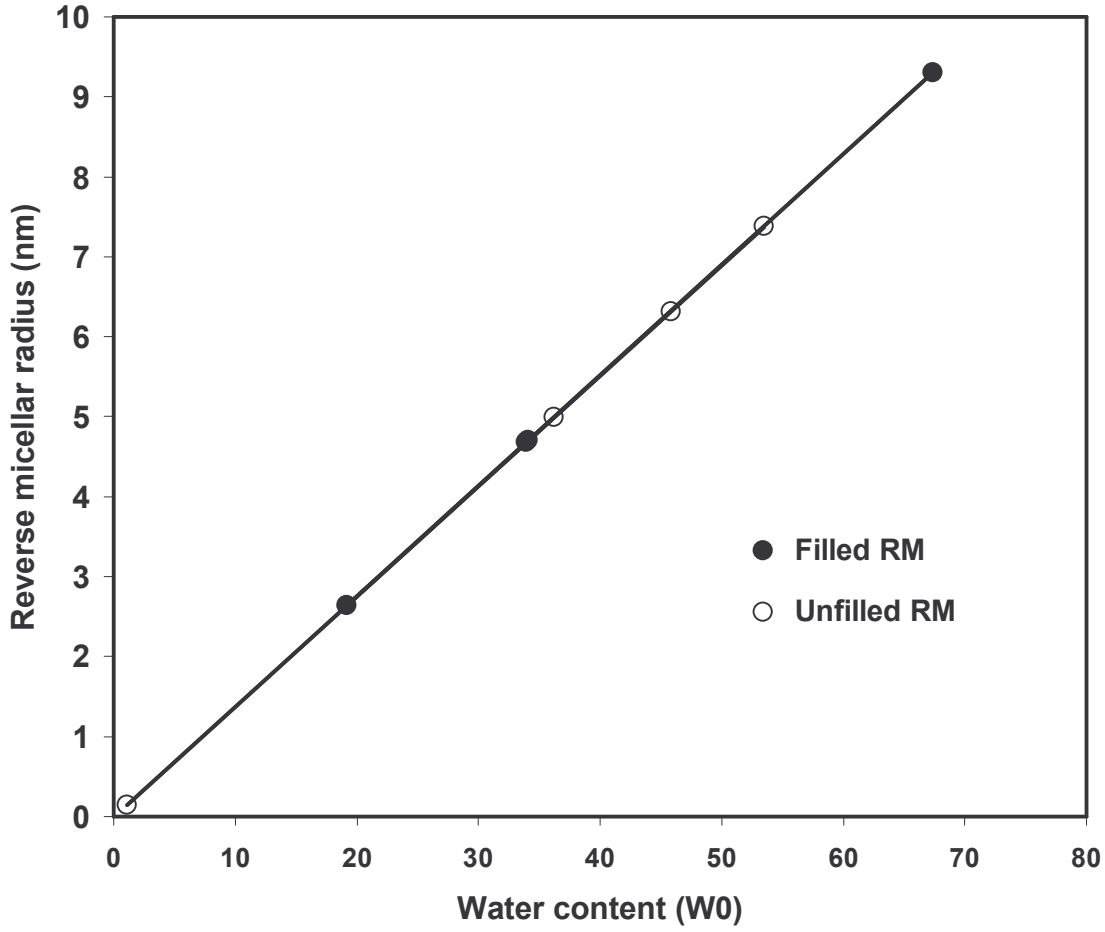


Fig. 5A.5. Radius of reverse micelle estimated using Krie and Hustdet model at different NaCl concentrations

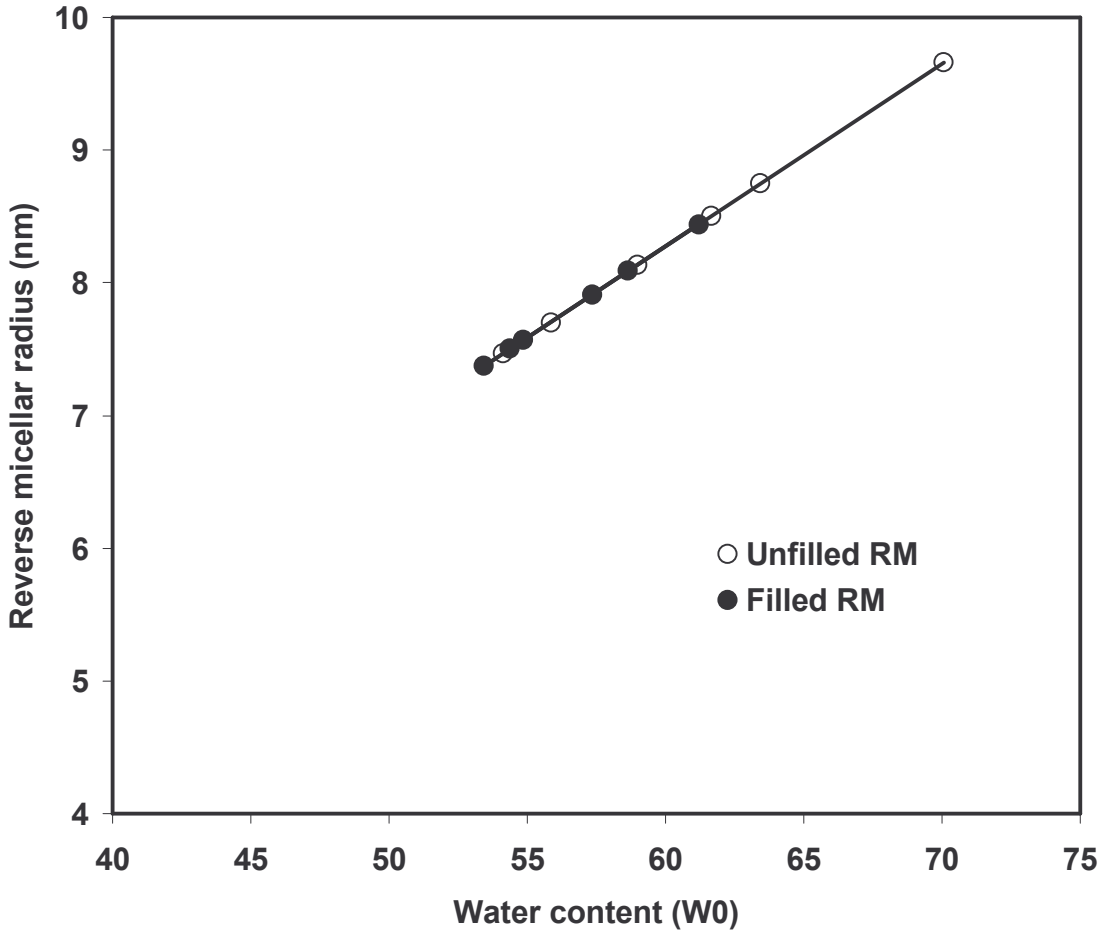


Fig. 5A.6. Radius of reverse micelle estimated using Krie and Hustdet model at different pH of aqueous phase

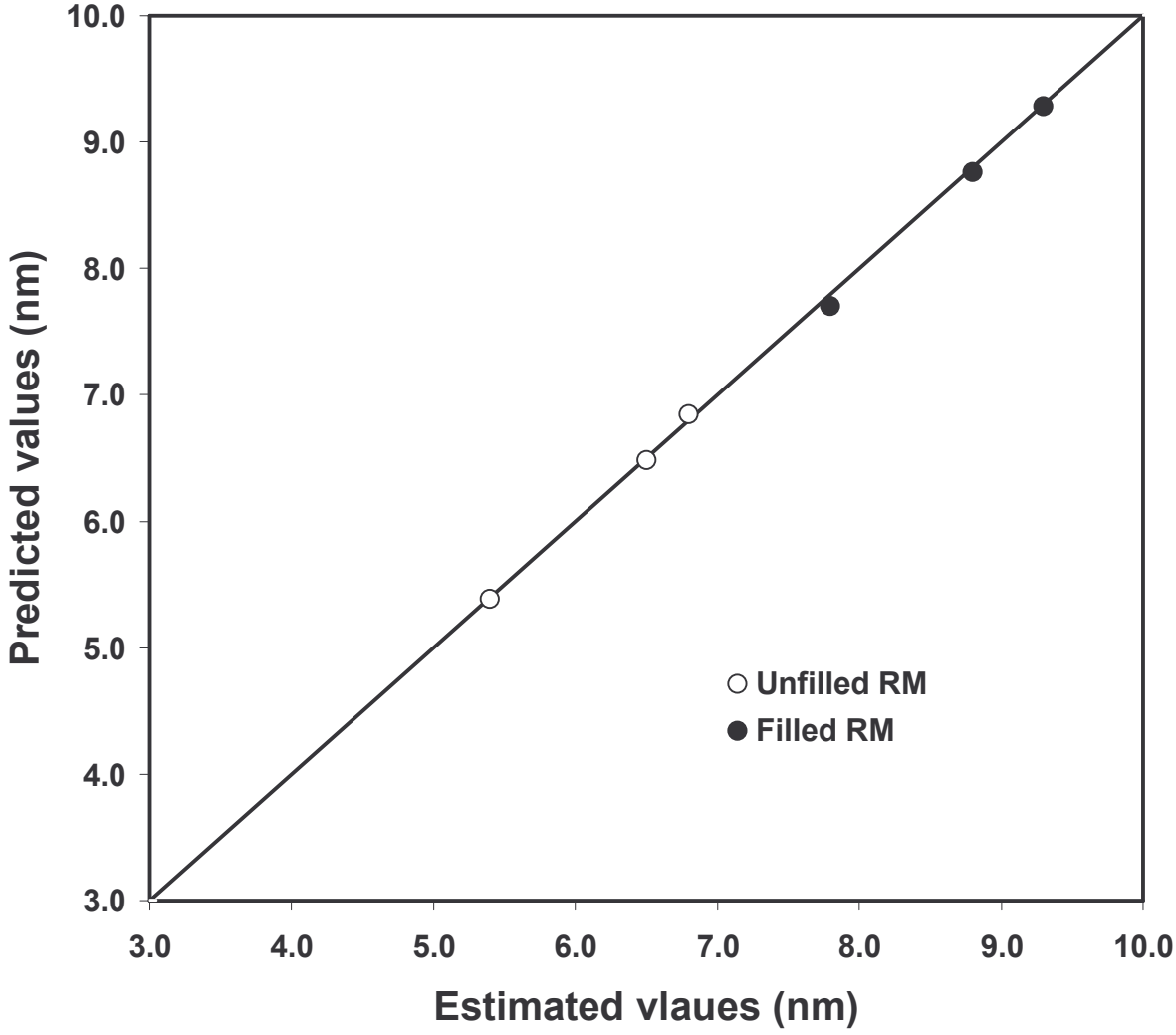


Fig. 5A.7 Parity plot showing the estimated and predicted radii of reverse micelle at different surfactant concentrations

SECTION B

MASS TRANSFER KINETICS

5B. 1 INTRODUCTION

Rate of protein transfer to or from a reverse micellar phase and factors affecting the rate are important for the practical application of RME for extraction and purification of proteins/enzymes and for the scale-up of the process. The mechanism of solute transfer during forward extraction in RME is divided into three steps, namely, diffusion of protein from the bulk aqueous phase to the interface, the formation of a protein-containing reverse micelle at the interface, diffusion of protein containing reverse micelle into the organic phase. During back extraction the protein filled reverse micelles coalesce with the interface to release the protein. Generally, the overall mass transfer rate during an extraction depends on the step that is rate limiting [Harikrishna *et al.,* 2002].

Model for estimation of mass transfer coefficient and concentration of protein in hollow fibers was proposed by Dahuron and Cussler (1988) for both co-current and counter current flows. Woll and Hatton (1989) developed a phenomenological thermodynamic model for the partitioning of proteins between aqueous and reverse micellar phases. The model attempted to quantify the relationship between the protein solubilization characteristics of reverse micellar systems and the operating conditions such as aqueous pH and surfactant concentration. Dekker *et al.* (1989) proposed mathematical model to satisfactorily describe the time dependency of the concentration of active enzyme in all the phases, based on the flow, mass transfer and first order inactivation kinetics. The steady state experimental data of the extraction and the observed dynamic behavior of the extraction were in good agreement with the model predictions. Fraaije *et al.* (1990) reported the thermodynamic model for protein partitioning and the co-partitioning of electrolytes. Dekker *et al.,* (1990) reported different mass transfer resistances for forward and back extraction of enzyme α-amylase between an aqueous phase and reverse micellar phase. While the forward extraction rate was controlled by the diffusion of the protein in the aqueous phase boundary layer, the back extraction rate, which was much slower, was controlled by the interfacial resistances. Nitsch and Plucinski (1990) estimated the rate of

solubilization of methylene blue, water and different electrolytes in AOT reverse micelles using two-phase stirred cell and based on the kinetic behavior of mixed electrolytes, a mechanism for the interfacial solubilization was proposed. In a separate study these authors [Plucinski and Nitsch, 1992] estimated the kinetics of ion exchange between aqueous and reverse micellar phase. Model for prediction of interfacial mass transfer coefficient for transfer of proteins between aqueous and organic phase was proposed by Dungan *et al.* (1991). Brandani *et al.,* (1994) improved upon this model and proposed a thermodynamic model, which is an improvisation of model proposed by Woll and Hatton (1988). The model considered the protein concentration also as a function in addition to the factors selected by Woll and Hatton (1988) for the estimation of distribution coefficient. Brandani *et al.,* (1996b) studied the effect of nonlinear equilibrium on the mass transfer rate of α-amylase during RME. Liu *et al.* (2006) studied the partitioning equilibria and the kinetics of lysozyme and bovine serum albumin. Kinetic analysis on membrane based reverse micellar extraction of lysozyme from aqueous solutions was reported by Juang *et al.* (2006). Interfacial mass transfer co-efficients were estimated by Dovyap *et al.* (2006) using two film models for the extraction of L-isoleucine.

When it is assumed that solute extraction in RME takes place by the forward and back extraction mechanisms mentioned above, the transfer rate of solute is expressed as [Nishiki *et al.,* 1998],

$$J_P = \frac{V}{A}\left(-dc_{aq}/dt\right) = k_{aq}(C_{aq} - C_{aqi}) \qquad (5B.1)$$

$$= \frac{V}{A}\left(-dc_{org}/dt\right) = k_{org}(C_{orgi} - C_{org}) \qquad (5B.2)$$

$$= k_{f0}(C_{aq} - (1/m_f)\, C_{org}) \qquad (5B.3)$$

$$1/m_f = 1/k_{aq} + 1/k_s + 1/mk_{org} \quad (5B.4)$$

where $m_f = C^*_{org}/C^*_{aq}$ is the equilibrium constant of protein.

By integrating equation (5B.3), the time-dependent concentration of solute in the reverse micellar phase is given by

$$\ln\left\{\left(1-(1+1/m_f)C_{org}\right)/C^0_{aq}\right\} = -\left(A/V\right)\left(1+1/m_f\right)k_{f0}\,t \quad (5B.5)$$

Similarly, for back extraction equation could be written as below

$$\ln\{1-(1+m_b)(C_{aq}/C^0_{org})\} = (A/V)(1+m_b)k_{bo}t \quad (5B.6)$$

By plotting a graph of LHS of equations 5B.5 and 5B.6 versus time 't', overall mass transfer coefficients could be obtained.

Nishiki *et al,* (2000) also provided two equations for forward and back extractions in the following form

$$\ln\left\{\left(1-(1+1/m_f)(C_{org}/C^0_{aq})\right)\right\} = -\left(A/V\right)\left(1+1/m\right)k_{f0}\,t \quad (5B.7)$$

$$\ln\left\{\left(1-(1+m_b)(C_{aq}/C^0_{org})\right)\right\} = -\left(A/V\right)(1+m_b)k_{b0}\,t \quad (5B.8)$$

Dungan *et al,* (1991) derived the following equations to estimate the overall mass transfer coefficient

$$\ln\left\{\left(1-(C_{org}/C^0_{aq})\right)\right\} = -\left(A/V\right)k_{f0}\,t = k_{fo}a\,t \quad (5B.9)$$

$$\ln\left\{\left(1-(C_{aq}/C^0_{org})\right)\right\} = -\left(A/V\right)k_{b0}\,t = k_{bo}a\,t \quad (5B.10)$$

The main objective of the study was to estimate the overall mass transfer coefficient during forward and back extractions of bromelain from crude extract of pineapple core.

5B.2 MATERIALS AND METHODS

5B.2.1.Materials

All chemicals and reagents used in the study are same as that described in Materials and Methods section (4B.2) of Chapter 4.

5B.2.2. Methods

i) RME of bromelain with conventional agitation

Forward extraction was carried out by mixing 10 ml of the organic phase (solvent/co-solvents/surfactant) with equal volume of aqueous phase (enzyme extract/salt). The phases were mixed thoroughly for a known duration (15, 30, 45 and 60 min) and centrifuged at 4000 *g* for 15 min (MP 400 R, Eltek, India). Back extraction was carried out by mixing the organic phase obtained from forward extraction (mixed for 60 min) with a fresh aqueous phase (buffer/salt). The phases were mixed thoroughly for a known duration (15, 30, 45 and 60 min) and centrifuged at 4000 *g* for 15 min (MP 400 R, Eltek, India). The other processing conditions optimized for forward and back extractions (detailed in Chapter 4) were employed. All the phase mixing experiments were carried out at controlled temperature (25 ± 2^0C).

ii) Back extraction with the application of acoustic field

Back extraction with acoustic field was carried out using the ultrasonic bath described in Chapter 4. The unit was operated at 'Low' mode and organic phase obtained from the forward extraction was used for the study. The duration of exposure was varied (15, 30, 45 and 60 min) and protein content in the aqueous phase was estimated.

5B.3. Analyses

i) Protein content

Aqueous phase obtained from forward and back extractions was analyzed for total protein content by absorption at 280 nm by UV [Layne, 1957].

ii) Estimation of mass transfer coefficient

The overall mass transfer coefficient (ka, s^{-1}) for forward and back extraction was estimated using the equations 5B.9 and 5B.10, respectively.

5B.3 RESULTS AND DISCUSSION

5B.3.1 Estimation of overall mass transfer coefficients

To study the mass transfer kinetics during forward extraction, phase mixing was carried out for different time intervals and protein concentrations were estimated in both the phases. The concentration of bromelain in the organic phase at different time intervals of mixing is shown in Table 5B.1. The transfer of bromelain from aqueous phase to organic phase was high in the first 15 min of mixing with nearly 69% of the total extraction taking place during the above period. Further, a gradual decrease in the enzyme transfer was observed with the increase in mixing time. To estimate the forward extraction mass transfer coefficient, a plot of $\ln\left\{\left(1-(C_{org}/C^{0}_{aq})\right)\right\}$ against mixing time 't' was drawn (Fig. 5B.1). The mass transfer coefficient K_{fo} was estimated from the slope of the line (trend line) drawn for the points. Since, it is difficult to estimate the interfacial area or contact area under turbulent mixing conditions, the mass transfer coefficient was estimated in terms of $K_{fo}a$ (s^{-1}). The estimated mass transfer coefficient was found to be 0.22 s^{-1}.

Back transfer experiments were carried using the organic phase obtained from forward extraction. For all the experiments, forward extraction mixing time was maintained the same (1 h). Back extraction was carried out for different time intervals (15, 30, 45, 60 min) and the corresponding protein concentration in the

aqueous phase was measured. Like in the case of forward extraction, nearly 65% of the extraction had taken place in the first 15 min. Practically, there was no extraction of solute was observed after 30 min of agitation. The mass transfer coefficient for the back transfer estimated from the plot (Fig. 5B.2) of $\ln\left\{\left(1-(C_{aq}/C^{0}_{org}\right)\right\}$ against time 't' was found to be 0.14 s^{-1}, which is slightly lower than that of forward extraction. The overall protein transfer in case of back extraction was also found to be less (~50%) as compared to 69% during forward extraction.

5B.3.2 Mass transfer kinetics with the application of acoustic

Application of acoustic filed in MHz range was found to be efficient in releasing the solute from reverse micelles as compared to the conventional agitation followed during back transfer. In order to study the mass transfer kinetics during back transfer by applying acoustic filed, experiments were carried out for different durations. The results of the study are shown in Table 5B. 1. The solute transfer was found to be higher than that of conventional agitation with the application of acoustic. Nearly 78% of protein transfer was achieved at the end of 60 min with acoustic as compared to 50% with the conventional agitation. The mass transfer rate obtained from the Figure 5B.3 was nearly 2.4 times higher than that of conventional agitation employed for back extraction.

Conclusions

The transfer of bromelain across the phases was high in the initial period of mixing with approximately 65% of the total extraction taking place during the above period. The estimated mass transfer coefficient for forward extraction was higher than back extraction. The solute transfer was found to be higher than that of conventional agitation with the application of acoustic. The mass transfer rate obtained with the application of acoustic was nearly 2.4 times higher than that of conventional agitation employed for back extraction.

Table 5B. 1. Total protein concentration at different time intervals during forward and back extractions

Duration (min)	Protein Concentration (mg/ml)		
	Forward extraction (organic phase)	Back extraction (aqueous phase)	Back extraction with acoustic filed (aqueous phase)
15	2.33	1.12	1.89
30	2.97	1.59	2.61
45	3.09	1.60	2.64
60	3.39	1.62	2.66

(Error: ±2% max.)

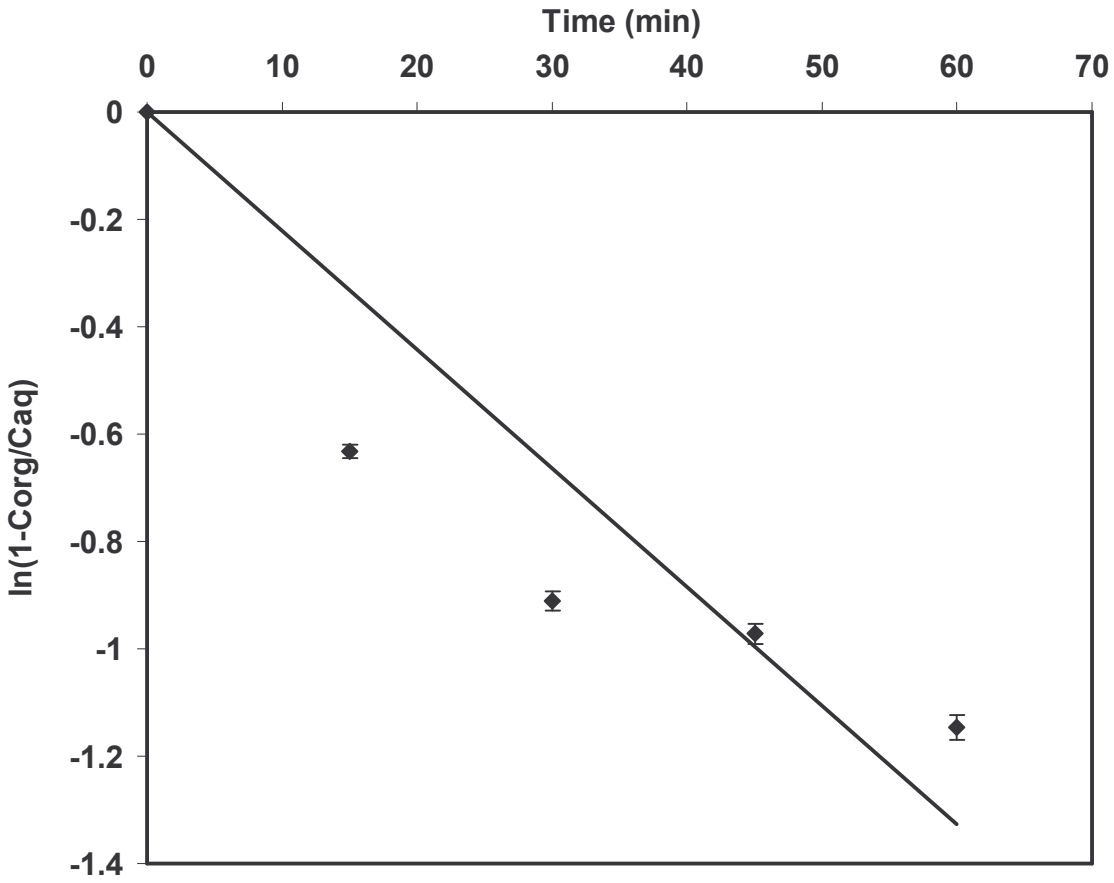


Fig. 5B.1. Mass transfer kinetics during forward extraction of bromelain

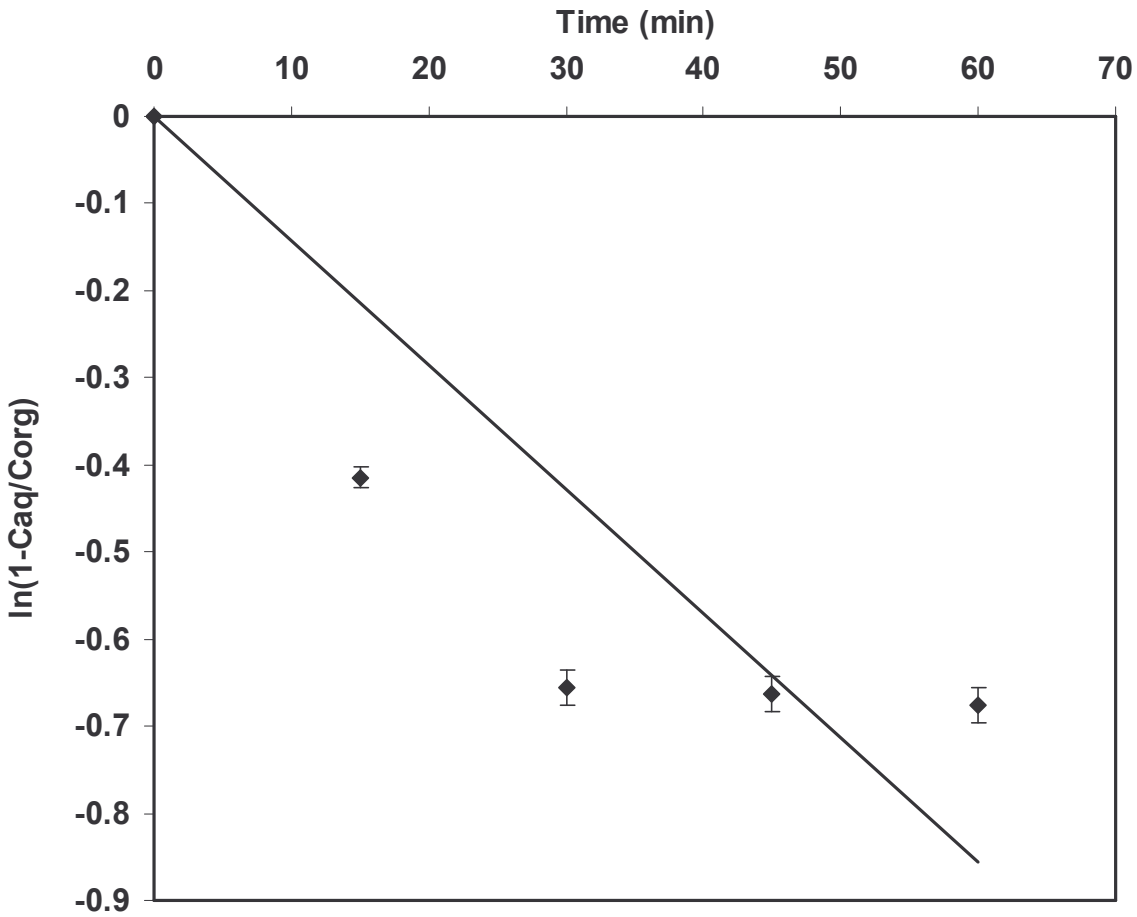


Fig. 5B.2. Mass transfer kinetics during back extraction of bromelain

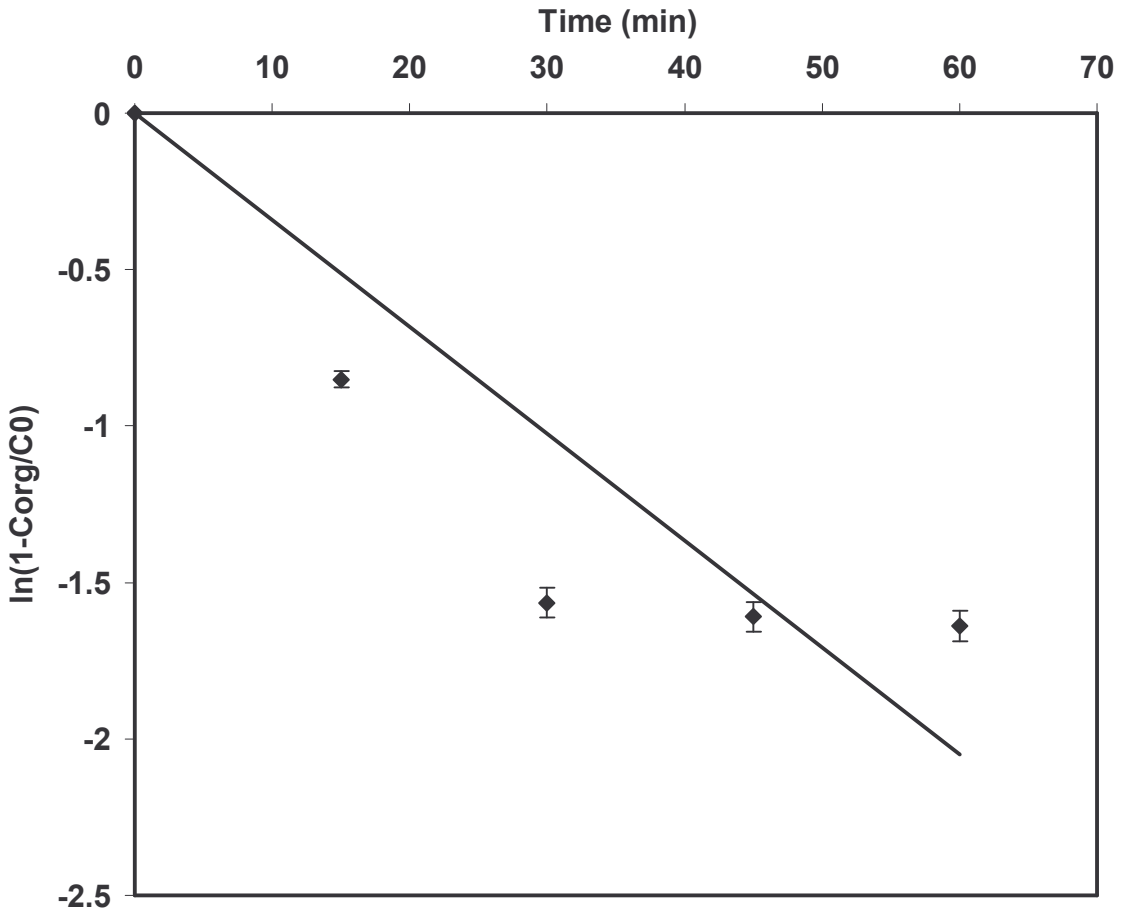


Fig. 5B.3. Mass transfer kinetics during back extraction of bromelain with the application of acoustic field

CHAPTER 6

SUGGESTIONS FOR FUTURE WORK

Suggestions for future work

The recent developments in the area of reverse micellar extraction have shown that RME could be used as an efficient and promising bioseparation technique. Many applications such as recovery of extrcellualr enzymes and other biomolecules from fermentation broths and tissue cultures, extraction of intracellualr products after cell lysis or from whole cells, recovery of products from natural and genetically engineered cells are being envisaged. Although, many research groups are working in the area of RME, still a lot of issues that are hindering the scale up and commercialization of the technique are not addressed satisfactorily. Some of the important areas having scope for further research are discussed below.

⇒ **Identification and use of new surfactants for RME**

There is a need for identification of surfactants that can readily form stable reverse micelles in many of the commonly used organic solvents. A good RME process requires surfactants that are mild, non toxic, non interactive/biocompatible and a low CMC value. The surfactants should form reverse micelles of fairly good hydraulic core so that they can accommodate solutes of bigger size. The cost effectiveness of surfactant used in RME is another parameter that needs to be taken into consideration as it has a direct bearing on the process economy.

⇒ **Studies on enhancing the rate of mass transfer across the phases**

The increase in transfer rate of solute across the phases during RME reduces the overall processing time. Most of the reports have indicated that the rate of transfer during back extraction is less as compared to forward extraction. Although, many alternative techniques have been reported for the enhancement of back extraction rate, no concrete solution has been reported so far to address this issue. Application of novel techniques such as acoustic field, electromagnetic radiation needs to be explored for deassembling the

reverse micelles, which will enhance the rate of solute transfer, without affecting the functionality of solutes.

⇒ **Scale up studies and identification of suitable equipment for RME**

RME studies are essential for assessing the performance of the technique at higher scale. However, only a very few reports are available on this aspect. Identification of suitable equipment for carrying out extraction at large scale and process optimization for higher scale operation are the two areas that need immediate attention. The equipment selected for RME should facilitate the continuous operation which would help in scaling up the process.

⇒ **Integration of RME with other downstream processes**

As RME could be employed as a primary processing step in many of the applications, there is a need for integrating RME with other conventional downstream processes to enhance the overall efficiency of the system. Practically, no reports are available on integration of processes, although it is an important aspect for the industrial adaptation of the process.

⇒ **Studies on recycling of reverse micellar components**

Process economics is one of the crucial factors, which determines the success of a process at commercial level. In this context, recycling of reverse micellar components becomes very essential, particularly when reverse micellar components are expensive. Detailed studies are needed to establish the efficacy of recycling process.

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