Aqueous Two Phase Extraction for Purification and Concentration of Biomolecules from Pineapple (*Ananas comosus* L. Merryl)

A thesis submitted to the

University of Mysore

in fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Biotechnology

by

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Certificate

I B. Ravindra Babu, certify that this thesis is the result of research work done by me under the supervision of Dr. KSMS Raghavarao at Department of Food Engineering, Central Food Technological Research Institute, Mysore – 20. I am submitting thesis for possible award of Doctoral of Philosophy (Ph. D) degree in Biotechnology of the University of Mysore.

I further certify that this thesis has not been submitted by me for award of any other degree/diploma of this or any other University.

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CERTIFICATE

I hereby certify that this Ph.D. thesis entitled '**Aqueous two phase extraction for purification and concentration of biomolecules from pineapple (*Ananas comosus* L. Merryl)'**, submitted by **Mr. B. Raindra Babu** for the degree of **Doctor of Philosophy in Biotechnology** of the University of Mysore, is the result of the research work carried out by him in the Department of Food Engineering, Central Food Technological Research Institute, Mysore, under my guidance and supervision during the period 2004 to 2008.

(Dr. KSMS Raghavarao)
Research Supervisor

Date :

Place:

Dedicated to Lord Shirdi Sai Baba

Abstract

Thesis mainly covers the separation, purification and concentration of biomolecules by aqueous two phase extraction and membrane processes. Standardization of process parameters to obtain optimal separation during aqueous two phase extraction and membrane processes is carried out. A practical approach for simultaneous fractionation and purification of mixture of enzymes (bromelain and polyphenol oxidase) from pineapple (*Ananas comosus* L. Merryl) by aqueous two phase extraction is developed. Major emphasis is given to develop alternative/newer approaches for the concentration of biomolecules and liquid foods. Performance evaluation of direct osmosis process for the concentration of biomolecules (bromelain enzyme from pineapple waste) and liquid foods (pineapple juice) is carried out in detail. Modeling of mass and heat transfer in osmotic membrane distillation process is studied in detail. The basic study of mathematical modeling of the osmotic membrane distillation/direct osmosis enhances the understanding of the process. Also, contribution of concentration and temperature polarization effects during osmotic membrane distillation process is discussed. Integrated approaches for the purification and concentration of biomolecules are studied in detail. The proposed protocols will be helpful in developing effective and efficient processes for the purification and/or concentration of enzymes, proteins and liquid foods. The major aspects covered in the thesis are the development of improved/newer processes for the purification and concentration of biomolecules and liquid foods.

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B. Ravindra Babu

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LIST OF ABBREVIATIONS

A	activity recovery (%)
A_{Br}	activity of bromelain (CDU ml^{-1})
A_{ppor}	activity of polyphenol oxidase (U ml^{-1})
ATPE	aqueous two phase extraction
ATPSs	aqueous two phase systems
a	water activity (-)
C	concentration (mg ml^{-1})
CDU	casein digestion unit
C_P	specific heat ($Jkg^{-1}K^{-1}$)
d_p	membrane pore diameter (m)
D	diffusion coefficient ($m^2 s^{-1}$)
Da	Dalton
DO	direct osmosis
D^k_{we}	Knudsen effective diffusivities ($m^2 s^{-1}$)
$D^o_{w\text{-}air}$	molecular effective diffusivities ($m^2 s^{-1}$)
ΔH_v	water latent heat of vaporization (J mol^{-1} or J kg^{-1})
h	heat transfer coefficient in boundary layer ($Wm^{-2}K^{-1}$)
h_m	membrane heat transfer coefficient ($Wm^{-2}K^{-1}$)
J_w	flux ($m^3 m^{-2} s^{-1}$)
k	mass transfer coefficient in boundary layer (ms^{-1})
k	Norrish constant (Eq. 3A.2)
k_m	membrane mass transfer coefficient (ms^{-1})
k_{mp}	membrane permeability ($ms^{-1}Pa^{-1}$)
k^T	thermal conductivity ($Wm^{-1}K^{-1}$)
K	overall mass transfer co-efficient (ms^{-1})
K_0	dusty-gas model constant (m)
K_1	dusty-gas model constant
K_{Br}	partition coefficient of bromelain (-)
K_P	partition coefficient of protein (-)
K_{PPO}	partition coefficient of polyphenol oxidase (-)
K_S	partition coefficient of sugar (-)

L	characteristic length (m)
MF	microfiltration
M_w	molecular weights of constituents (kg mol $^{-1}$)
M_B	molecular weight of the osmotic agents (NaCl or $CaCl_2$)
m	molality (kg of solute/kg of solvent)
NF	nanofiltration
OA	osmotic agent
OMD	osmotic membrane distillation
P	total pressure (Pa)
PF	purification factor
p*	pure water vapour pressure (Pa)
PEG	polyethylene glycol
PPO	polyphenol oxidase
Pr	Prandtl number
Q	heat flux, Wm^{-2}
R	gas constant, 8314 (J mol $^{-1}K^{-1}$)
RO	reverse osmosis
Re	Reynolds number
r	membrane pore radius (m)
Sc	Schmidt number
Sh	Sherwood number
T	temperature (°K)
T_m	temperature at membrane surface (°K)
U	overall heat transfer coefficient ($Wm^{-2}K^{-1}$)
UF	ultrafiltration
u	velocity of the fluid (ms $^{-1}$)
V	volume (ml)
x	mole fraction (-)

Greek letters

Δ	difference
δ	membrane thickness (m)
ε	porosity (-)

λ	mean free path (0.047 μm)
φ	association factor for water
ϕ	vapor pressure polarization coefficient
ρ	density of the fluid (kgm $^{-3}$)
ϑ	solute molal volume (m^3/kmol)
γ	Interfacial tension (N m^{-1})
τ	tortuosity factor (-)
π	osmotic pressure (Pa)
μ	viscosity of the fluid (Pas)
μ_v	viscosity of water vapour at atmospheric pressure and ambient temperature (0.1, Pa.s)

Subscripts

1	feed side
2	osmotic agent side
b	bottom phase
i	Initial
m	Membrane
OA	osmotic agent
P	protein
S	sugar
t	top phase
w	water

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SYNOPSIS

Biotechnology has witnessed phenomenal growth in recent years. However, the growth of the downstream processing (DSP) of biological products is relatively modest. For the success of the commercial production of the enzymes and proteins, there is a need for efficient downstream processing techniques. The downstream processing of biomolecules requires purification techniques which are both economically feasible and delicate enough to preserve the biological activity. Aqueous two-phase extraction is one such method with several advantages over the conventional methods. The overall productivity increases when newer, membrane process such as direct osmosis and osmotic membrane distillation is integrated with this extraction.

The present work is aimed at the development of improved/newer methods for the extraction, purification and concentration of biomolecules/fruit juices and the objectives are given below:

1. Optimization of various process parameters during aqueous two phase extraction for the differential partitioning of target biomolecules and impurities.
2. Optimization of various process parameters for newer membrane process (osmotic membrane distillation and direct osmosis) for the concentration of biomolecules (bromelain/phycocyanin) and fruit juices (pineapple juice/sweetlime juice).

3. Modeling of mass transfer mechanism in these membrane processes.
4. Quality evaluation of the product with the help of sensory analysis.
5. Integration of ultrafiltration with direct osmosis/osmotic membrane distillation for the purification and concentration of selected biomolecules (bromelain).
6. Aqueous two-phase extraction coupled with membrane processes for the purification/concentration of the selected biomolecules (bromelain and polyphenol oxidase).

The thesis is broadly divided into six chapters, wherein fractionation and purification of biomolecules using aqueous two phase extraction and concentration of biomolecules/fruit juices by newer membrane processes and their integration are discussed in detail. Each chapter in the present investigation is systematically divided into introduction, theoretical aspects, materials and methods, results and discussion and conclusions, followed by tables and figures.

Chapter 1: General introduction: Bioseparation and downstream processing

This chapter covers various bioseparation processes and their applications in bioprocessing are explained briefly. The general introduction of aqueous two phase extraction and newer membrane processes such as membrane chromatography, direct osmosis, osmotic membrane distillation

and their applications for the purification and concentration of biomolecules are covered in this chapter.

Chapter 2: Aqueous two phase extraction for the separation and purification of biomolecules from pineapple (*Ananas comosus* L.)

Aqueous two-phase systems (ATPSs) form readily upon mixing aqueous solutions of two hydrophilic polymers, or of a polymer and a salt, above a certain threshold concentration. Extraction using ATPSs in many cases offers a better alternative to existing methods, especially with respect to scale of operation, yield, enrichment of product, and scope for continuous operation for the separation and purification of desired enzymes/proteins from a complex mixture. Hence, it is gaining importance as an efficient and versatile technique for the separation and purification of biomolecules. In this chapter, characterization of aqueous two phase systems and separation and purification of mixture of enzymes (bromelain and polyphenol oxidase) present in pineapple by aqueous two phase extraction are discussed in detail.

The physical properties of aqueous two phase systems, such as density, viscosity and interfacial tension determine the phase demixing rate and also contribute to the biomolecule partition behavior. The influence of polyethylene glycol molecular weight (PEG 1500, 4000, 6000 and 20000) and concentration of phase forming components (PEG concentration in the range of 12 - 18% w/w and potassium phosphate concentration in the range of 14 - 20% w/w) on physical properties such as density, viscosity, and interfacial tension is studied. It was observed that density difference between top and

bottom phases (from 85 ± 2.0 to 157 ± 2.0 kg m^{-3}) and the PEG rich top phase viscosity (from 7.66 ± 0.02 to 39.33 ± 2.2 mPa s) increases respectively with an increase in concentration of phase forming components. The influence of polyethylene glycol molecular weight (PEG 1500, 4000, 6000 and 20000) on interfacial tension is studied and about 3.0 fold (0.62 ± 0.03 to 1.92 ± 0.02 mN/m) increase in interfacial tension is observed. It is observed that the concentration of phase forming components has prominent effect on density, viscosity and interfacial tension.

Further, aqueous two-phase extraction is employed for the first time for simultaneous fractionation and purification of mixture of enzymes (bromelain and polyphenol oxidase) from pineapple (*Ananas comosus* L*)*. Influence of several parameters such as phase forming polyethylene glycol molecular weight (PEG 1500, 4000, 6000 and 20000), concentration of phase forming components (PEG concentration in the range of 12 - 18% w/w and potassium phosphate concentration in the range of 14 - 20% w/w) and system pH (6.0 to 9.0) on differential partitioning of bromelain and polyphenol oxidase required for the purification is studied. Bromelain preferentially partitioned to the top (polyethylene glycol) phase, while polyphenol oxidase to the bottom (potassium phosphate) phase. Partition coefficient of both the enzymes decreased with an increase in molecular weight of polyethylene glycol. Phase composition of the system was found to have significant effect on enzyme partitioning and degree of purification. The polyethylene glycol/potassium phosphate system (comprising of 18% w/w PEG 1500 and 14% w/w phosphate) resulted in about 228 ± 4.2% activity recovery and 4.0 ± 0.1 fold

increase in purity in case of bromelain and about 90 ± 1.0% activity recovery and 2.7 ± 0.1 fold increase in purity in case of polyphenol oxidase.

Chapter 3: Direct osmosis for the concentration of biomolecules and fruit juices

Direct osmosis is an emerging membrane based separation process. The process utilizes a semi-permeable dense hydrophilic membrane, which separates two aqueous solutions (feed and osmotic agent solution) having different osmotic pressures. The driving force is the difference of osmotic pressure across the membrane. Direct osmosis process offers several advantages such as lower energy consumption and achieving higher concentration.

In this chapter, direct osmosis is employed as an alternate method for concentration of biomolecules and fruit juices. Concentration of bromelain enzyme extracted from pineapple waste by direct osmosis process is explored. The effect of various process parameters, such as osmotic agent concentration (1- 6 molality) and flow rate (25 to 100 ml/min) of osmotic agent and feed on transmembrane flux and on concentration (activity) of bromelain is evaluated. Osmotic agents namely sodium chloride and sucrose at varying concentrations are employed. In comparison with sucrose, higher transmembrane flux is observed in case of sodium chloride due to higher osmotic pressure of sodium chloride. The flow rate of osmotic agent is found to have significant effect on transmembrane flux (19 ± 0.4 to 31 ± 0.5% increase in flux). Bromelain enzyme extract is concentrated up to 4 fold (from 450 ± 2 to 1800 ± 10 CDU/ml) at ambient temperature (25.0 ± 2.0°C).

Further, direct osmosis is employed for the concentration of pineapple juice at ambient temperature and atmospheric pressure. The effect of osmotic agent concentrations namely sodium chloride (6, 12 and 26 % w/w) and sucrose (20, 30, 40 % w/w) on transmembrane flux is studied. Also, combination of sucrose and sodium chloride is investigated as an alternative osmotic agent. The sucrose-sodium chloride combination is found to be overcome the drawback of sucrose (low flux) and sodium chloride (salt migration) as osmotic agents during direct osmosis process. The effect of the hydrodynamic conditions in the module and feed temperature (25.0 ± 2.0 - 45.0 ± 2.0°C) on transmembrane flux is evaluated. For the range of hydrodynamic conditions studied, it is observed that transmembrane flux increases with Reynolds number. The increase in feed temperature resulted in an increase in transmembrane flux (about 78 ± 2.0%). The pineapple juice is concentrated up to a total soluble solid content of 60.0 ± 0.2°Brix at ambient temperature (25.0 ± 2.0°C). The physicochemical characteristics of fresh and concentrated pineapple juice are also studied.

Chapter 4: Osmotic membrane distillation for the concentration of biomolecules and fruit juices

Osmotic membrane distillation process employs porous hydrophobic membrane, which separates two aqueous solutions (feed and osmotic agent solution) having different solute concentrations. The driving force in osmotic membrane distillation is the vapor pressure difference across the membrane. Water evaporates from the surface of the feed solution having higher vapor pressure, diffuses in the form of vapor through the membrane, and condenses

on the surface of the osmotic agent solution, which is at lower vapor pressure. The primary advantage of osmotic membrane distillation lies in its ability to concentrate aqueous liquid streams to very high levels at ambient temperature and pressure, with minimal product deterioration.

In the present chapter osmotic membrane distillation process is employed as an alternative method for the concentration of bromelain from pineapple waste and phycocyanin from *Spirulina platensis.* The effect of various process parameters such as osmotic agent concentration (2 to 10 molality) and flow rate of feed and osmotic agent (25 to 100 ml/min) on transmembrane flux is evaluated. The transmembrane flux is found to increase with an increase in osmotic agent flow rate (38.0 ± 2.0 to 51.0 ± 2.0%). The overall mass and heat transfer coefficients during the concentration of biomolecules are estimated. The magnitude of temperature polarization is found to be low and the maximum temperature difference across the membrane was only about 1.0 ± 0.1°K, that too at higher concentration of calcium chloride solution (10 molality).

Osmotic membrane distillation process is also employed for the concentration of fruit juices (pineapple and sweetlime juice). Effect of various process parameters such as concentration (2 to 10 molality) and flow rate (25 to 100 ml/min) of feed and osmotic agent on the transmembrane flux is evaluated in case of pineapple juice/sweet lime juice. The feed and osmotic agent side mass transfer coefficients are estimated based on classical empirical correlation of dimensionless numbers, whereas, membrane mass transfer coefficients is estimated using dusty gas model. Membrane mass and

heat transfer coefficients are estimated to be 2.5 x 10^{-10} ms^{-1} Pa^{-1} and 390 W m^{-2} k^{-1}, respectively. Concentration polarization is found to have more significant effect on flux reduction when compared to temperature polarization. The flux across the membrane during the process is predicted using resistances-in-series model. The experimental values are found to correlate well with the predicted ones.

Chapter 5: Integrated bioseparation for the purification and concentration of biomolecules

The productivity and yield of bioprocesses can be considerably improved by employing integrated processes. In the present work integration of existing membrane processes with newer membrane processes (direct osmosis/osmotic membrane distillation) and integration of aqueous two phase extraction with membrane processes for the purification and concentration of biomolecules has been attempted. The effect of transmembrane pressure on permeate flux during the purification of bromelain by ultrafiltration process is studied. The purified bromelain enzyme extract by ultrafiltration process is concentrated up to 3960 ± 5.0 CDU/ml by direct osmosis process. Integration of various unit operations such as extraction, precipitation and concentration is also attempted for the purification and concentration of biomolecules. Concentrated bromelain enzyme extract is found to be more stable (in terms of proteolytic activity) than that of dilute enzyme extract at refrigeration (5.0 ± 2.0°C) as well as at ambient (25.0 ± 2.0°C) conditions. The integration of aqueous two phase extraction with ultrafiltration process has resulted in selective removal of phase forming components from bromelain rich top

phase and PPO rich bottom phase. Further, the purified enzyme extracts are concentrated by direct osmosis/osmotic membrane distillation processes.

Chapter 6: Summary and conclusions

This chapter mainly summarizes the overall conclusions of the investigation and indicates suggestions for taking forward the research with continuous improvement of the processes employed. Aqueous two phase extraction could be used successfully employed for the fractionation and purification of bromelain and polyphenol oxidase from *Ananas comosus*. Athermal membrane processes such as direct osmosis and osmotic distillation are successfully demonstrated for the concentration of biomolecules and fruit juices. Also, two schemes of integrated bioseparation processes such as integration of existing and newer membrane processes and integration of aqueous two phase extraction with membrane processes are developed for the purification and concentration of biomolecules.

Attention needs to be paid on the development of equipments for efficient mass transfer and phase separation during the extractions and purification of biomolecules by aqueous two phase extraction. The main limitations of existing membrane processes are membrane fouling, concentration polarization and low flux rates. Nanofibrous microfiltration/ultrafiltration has a potential to overcome such drawbacks of existing membrane processes. The potential of nanofibrous microfiltration/ultrafiltration membrane in food/biotechnology applications needs to be studied in detail. Successful application of newer membrane

processes (direct osmosis/osmotic membrane distillation) for the concentration of biomolecules/fruit juices has been demonstrated in lab scale employing plate and frame module. The large scale studies of these needs to be undertaken to ensure the scale-up. Integration of aqueous two phase extraction with nanofibrous microfiltration/ultrafiltration and direct osmosis/osmotic membrane distillation needs to be explored for possible improvement in the productivity.

(Research Guide) (Candidate)

CHAPTER - 1

General Introduction

CHAPTER – 1A

Aqueous two phase extraction

1A.1 Aqueous two phase extraction

Bioseparation and downstream processing occupy a complex and important position in biotechnology and biochemical engineering. Improved bioseparation/downstream processing techniques are increasingly important for biotechnology because separation is often the limiting factor for the commercial success of biological processes. The separation of many biomolecules is still performed by batch mode in small-scale processes such as column chromatography, salt and solvent induced precipitation and electrophoresis. These unit operations have scale up problems and expensive at larger scale and low product recovery (Diamond and Hsu, 1992; Raghavarao *et al*., 1998). The downstream processing of biological materials requires purification techniques that are delicate enough to preserve the biological activity. There is, therefore, a need in the industry for efficient and economical methods of separation, purification and concentration of the biomolecules.

Liquid-liquid extraction using aqueous two phase systems (ATPSs) is one such method. Unlike conventional liquid-liquid extraction involving organic/aqueous, aqueous two phase extraction (ATPE) employs two aqueous phases. ATPE has been successful to a large extent in overcoming the drawbacks of conventional extraction processes such as low solubility and denaturation of biomolecules in organic solvents. Liquid–liquid extraction in aqueous two-phase systems (ATPSs) has proved to be a promising separation strategy for many biological products such as proteins, enzymes, viruses, cells and other biological materials (Raghavarao *et al*., 1991;

Chethana *et al*., 2006). Aqueous two-phase system was developed in Sweden during mid-1950s for the separation of macromolecules, cells and organelles. Since then, attention has been directed towards widening its application scenario (Albertsson, 1986). ATPE has the potential to produce a concentrated and purified product in one step when compared to the number of steps involved in conventional downstream processing such as clarification, filtration, concentration, recovery, and purification. ATPE offers many advantages such as a biocompatible environment, eco-friendly, low interfacial tension, low energy, easy scale-up, and continuous operation (Raghavarao *et al*., 1991; Chethana *et al*., 2007, Madhusudan *et al*., 2008). The ATPS also provides good resolution, high yield, and relatively high capacity. Furthermore, the equipment and methods of conventional organic/aqueous phase extraction can be easily adapted to ATPE. In the following sections the factors affecting the aqueous two phase systems, partitioning of biomolecules and equipments for extraction of biomolecules using ATPSs are discussed briefly.

1A.2 Formation of aqueous two phase systems

ATPS are of two types, polymer/polymer type and polymer/salt type. Aqueous two-phase systems (ATPSs) form readily upon mixing aqueous solutions of two hydrophilic polymers, or of a polymer and a salt, above a certain threshold concentration. Most commonly used phase forming components for the formation of aqueous two phase systems were shown in Table 1A.1-1A.2. Among these, aqueous two-phase systems formed by PEG–dextran–water and PEG–salt–water systems are widely used for separation and purification of biomolecules. However, PEG–salt–water two-phase

systems have certain advantages over PEG–dextran–water systems such as low viscosity and lower cost.

1A.3 Factors affecting the aqueous two phase systems

1A.3.1 Nature and concentration of the phase forming components

The hydrophobicity, molecular weight and concentration of the phase forming polymers in the case of polymer/polymer phase systems and, the type of salt and its concentration in the case of polymer/salt phase systems, influence the formation of ATPS. The lower the molecular weight, the higher is the concentration of the polymer required for phase formation and vice-versa. At low concentrations of polymers, a homogeneous solution results as indicated by point H^* in Fig. 1A.1. However, as the concentration of the components increases above critical concentration, it gives a heterogeneous system, which separates into two phases. The curve formed by joining the points of critical concentrations, which separates the homogeneous region (shaded area in Fig. 1A.1) and the two phase region, is known as a binodial curve. Pairs of points like T_1 and B_1 are called nodes and the straight line, T_1B_1 is called the tie line. Polymer molecular weight will influence the polymer concentrations required for phase separation and the phase diagram symmetry. Generally, it has been observed for the PEG/dextran system, increasing polymer molecular weight will lead to lower polymer concentration required for phase separation. In addition, as the difference in molecular size between the two polymers increases, the phase diagram becomes more asymmetric (Diamond and Hsu, 1992).

1A.3.2 Temperature

Temperature has considerable influence on the phase diagram. Decreasing the temperature of polymer/polymer type phase system will lead to lower polymer concentration required for phase separation. However, this reduction in the polymer concentration achieved by lowering the temperature is marginal. Also, the decrease in temperature causes an increase in phase viscosity, which is not desirable for industrial scale extraction (Zaslavsky, 1995).

1A.3.3 Salt type and concentration

Salt type (univalent or multivalent) and concentration have considerable effects on the phase system. Zaslavsky *et al*. (1987) have demonstrated that increasing the concentration of the univalent salts (up to 0.1 Molarity) in PEG/dextran system will alter the composition of the phases without significant effect upon the position of the binodial. However, multivalent salts, such as phosphate, sulfate and tartarate in the same system show an increasing tendency to partition to the bottom (dextran rich) phase with increasing salt concentration and distance from the critical point (Zaslavsky *et al*., 1988). These salts will significantly alter both phase composition and binodial position of the PEG/dextran system. Generally, the binodial is shifted to lower polymer concentration.

1A.4 Factors affecting the biomolecule partitioning in aqueous two phase systems

1A.4.1 Phase forming polymer molecular weight and concentration

The molecular weight has significant effect on partitioning of biomolecules because it affects the phase composition. Generally, increasing the molecular weight of a phase forming polymer will cause biomolecule to partition more towards the opposite phase. Similarly, when a phase forming polymer's molecular weight is decreased, a biomolecule will tend to partition into that polymer rich top phase. The extent of the effect of polymer molecular weight on partitioning behavior also depends on the molecular weight of the biomolecules. Albertsson *et al*. (1987) reported that the effect of polymer molecular weight was more prominent for the biomolecules of higher molecular weight (up to 250 kDa). Near the plait point (Fig.1A.1) of the binodial curve, the partition coefficient of biomolecule is one. As polymer concentration is increased (moving away from the plait point) protein partition coefficient becomes exceedingly greater or lower than one (Albertsson, 1986).

1A.4.2 System temperature

The effect of system temperature on protein partitioning has not yet been thoroughly investigated. The change in system temperature has indirect effect on partitioning of biomolecules. The change in temperature causes sharp change of binodial curve or protein denaturation which in turn affects the partition behavior of the biomolecule during aqueous two phase extraction. In general, the increase in temperature results in an increase in phase composition and decrease in tie line length (Albertsson, 1986).

1A.4.3 Biomolecule size

The size of the biomolecule has a significant role on its partitioning behavior during aqueous two phase extraction. Generally, the small molecules tend to partition themselves evenly between two phases, where as large molecules tend to distribute in an uneven manner, while very large biomolecules partition themselves to one of the phases (Albertsson, 1986).

1A.4.4 Solution pH

The partitioning of proteins/enzymes in ATPS is affected by net charge on the biomaterial which in turn depends on the pH of the solution. The net charge on the biomolecules can be varied by changing the pH of the solution. Also, the pH change may lead to denaturing of the biomolecules there by causing conformational changes which lead to change in partition behavior of the biomolecules. This is due to increased surface area of the denatured biomolecules causes more hydrophobic interactions.

1A.4.5 Protein concentration

In general, partitioning is not dependent on the concentration of the protein/enzymes. However, at very high concentration of the protein there could be a possibility of the formation of third (protein) phase by itself.

1A.4.6 Chemical modification of phase polymers

The chemical modifications to PEG, like covalent bonding of fatty acid chains, charged groups, hydrophobic derivatives and biospecific affinity ligands, have considerable effect on partition behavior of proteins in the PEG-

rich phase (Raghavarao *et al*., 1995). Charged PEG derivatives such as trimethylamino-PEG (TMA-PEG) and sulfonate-PEG (S-PEG) gives the information about the net charges and isoelectric point of proteins as well as particles. Johansson and Shanbhag (1984) observed that the increase in concentration of the derivatized PEG resulted in 10 times increase in partition coefficient compared to that of normal PEG. Shanbhag and Jensen (2000) employed hydrophobic polymer derivatives during the partitioning study of the biomolecules in PEG/dextran system. In recent years metal ion affinity partitioning was widely used to enhance the partitioning of various biomolecules such as human hemoglobin, bovine hemoglobin, whale and horse myoglobins (Wuenschell *et al*., 1990; Chung and Arnold, 1990).

1.A.5 Equipments for extraction of biomolecules

1A.5.1 Spray column

Aqueous two phase extraction of biomolecules in spray column is one of the simple and easy to operate (Fig. 1A.2). In spray columns one is made continuous phase, while the other phase is dispersed in the form of droplets with the help of sieve plate or nozzle. During operation, the heavy continuous phase and light dispersed phase will flow in countercurrent direction. The major drawbacks of spray column are flooding and backmixing. Use of spray columns for the extraction of various biomolecules such as bovine serum albumin, horse radish peroxidase have been demonstrated well in the literature (Raghavarao *et al*, 1991; Srinivas, *et al*., 2002a,b).

1A.5.2 Packed column

The packed column consists of stack of packings (such as glass spheres, raschig rings, polystyrene rings) arranged regularly or irregularly in a column over a perforated support. The selection of the packing material is markedly important. The packing should be wetted preferentially by the continuous phase, and its diameter should be less than 1/8 of the column diameter in random packing. The schematic representation of packed column was shown in Fig. 1A.3. Packed columns are more efficient than spray columns for the extraction of biomolecules by aqueous two phase extraction. The packing material used in the columns reduces backmixing and provides tortuous pathways for the two liquids, and can also cause distortions and breakup of the drops. One of the drawbacks of packed column is lower throughput compared to that of spray column. Patil *et al*. (1991) and Igarashi *et al*. (2004) employed packed columns for the extraction of biomolecules using aqueous two phase systems.

1A.5.3 Perforated rotating disc contactor

Perforated rotating disc contactor (PRDC) is well suited for aqueous systems with low interfacial tension such as ATPS formed by PEG and phosphates salts. The perforated rotating disc contactor cntains a column equipped with perforated discs mounted on a central rotating shaft as shown in Fig. 1A.4. There are a few research articles published on the use of PRDC for the extraction of various biomolecules such as cutinase (Cunha , 2003), bovine serum albumin (Porto *et al*., 2000; Sarubbo *et al*., 2003), trypsin

(Oliveira, 2002) and α-toxin (Cavalcanti *et al*., 2007). The main advantage of PRDC is greater efficiency of contacting and better operational flexibility.

1A.5.4 Graesser raining bucket contactor

The graesser raining bucket differential contactor was first developed to handle difficult settling systems encountered in the coal tar industry. In recent years, it is gaining importance for the continuous extraction of biomolecules using aqueous two phase systems. The main advantages of the graesser raining bucket contactor are simple design and low cost. The graesser contactor is composed of a horizontal cylindrical shell containing an internal rotor assembly consisting of a series of circular discs mounted on a central shaft as shown in Fig. 1A.5. Between each pair of discs, a series of buckets are supported by tie rods extending the length of the apparatus. During the operation, the interface level of the two liquids is controlled at the central line of the vessel. Gentle mixing is provided by the movement of the buckets, transporting portions of each phase to the other phase. Phase separation occurs in the end zones on the right and left sides of the cylindrical vessel. Application of graesser raining bucket differential contactor in ATPS was well reported in the literature (Coimbra *et al*., 1994; Zuniga *et al*., 2005; Zuniga *et al*., 2006).

1A.5.5 York-Sheibel column

In York-Sheibel column dispersion is achieved in the stirring zone by the impeller and the coalescence is achieved in the wire-mesh region. The main advantage of this column is higher throughputs compared to that of packed

column. Extraction of biomolecules using ATPS in York-Sheibel columns was reported by Jafarabad *et al*., (1992).

The application of ATPE for the purification of various biomolecules has been demonstrated well in the literature. However, ATPE did not realize the wide commercial adoption that it richly deserves, due to several factors including the cost of phase forming polymers, a lack of knowledge of the technique, and poor understanding of the mechanism governing phase formation and solute partition. In **Chapter 2**, the characterization of aqueous two phase systems, partitioning mechanism of biomolecules and fractionation and purification of mixture of enzymes has been explained.

Table 1A.1: Commonly used phase forming solutes for polymer-polymer two phase systems

Polymer 1	Polymer 2
Polyethylene glycol	Dextran Ficoll Polyvenyl alcohol Pullulan
Polypropylene glycol	Dextran Polyvinylpyrrolidone Polyethylene glycol Polyvinyl alcohol Hydroxypropyl dextran Methoxypolyethylene glycol
Polyvinyl alcohol	Dextran Hydroxypropyl dextran Methyl cellulose
Polyvinylpyrrolidone	Dextran Hydroxypropyl dextran Methyl cellulose
Methyl cellulose	Dextran Hydroxypropyl dextran
Ethylhydroxyethyl cellulose	Dextran
Hydroxypropyl dextran	Dextran
Ficoll	Dextran

Table 1A.2: Commonly used phase forming solutes for polymer-salt two phase systems

Polymer 1	Salt
Polyethylene glycol	Potassium phosphate
	Sodium sulfate
	Sodium formate
	Sodium potassium tartrate
	Ammonium sulfate
	Magnesium sulfate
Polypropylene glycol	Potassium phosphate
Polyvinylpyrrolidone	Potassium phosphate
Methoxypolyethylene glycol	Potassium phosphate

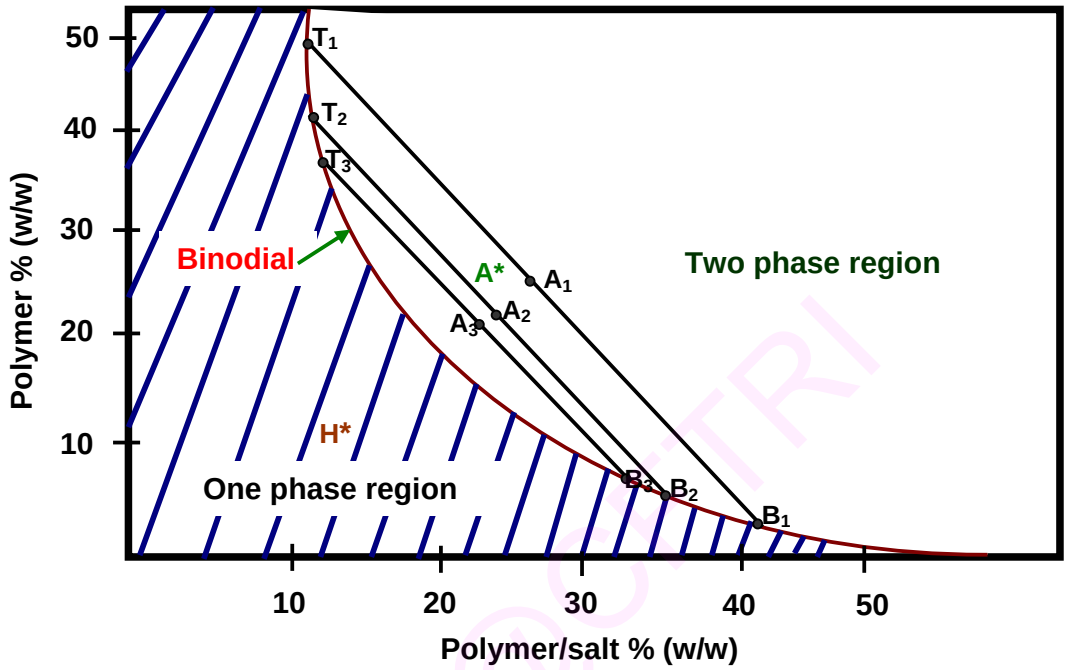


Figure 1A.1: Phase diagram of a polymer/polymer or polymer/salt system.

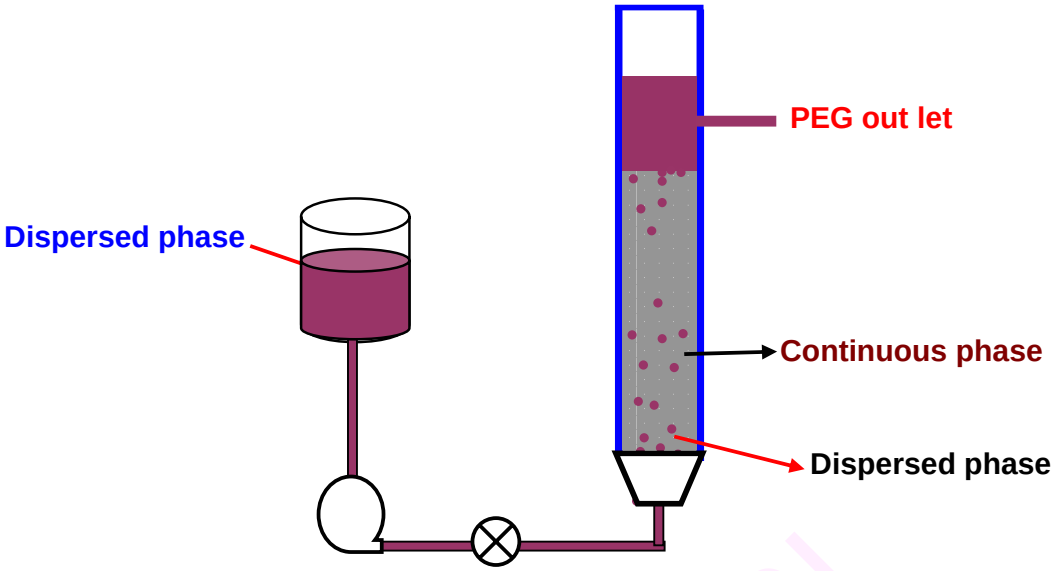


Figure 1A.2: Schematic diagram of the spray column

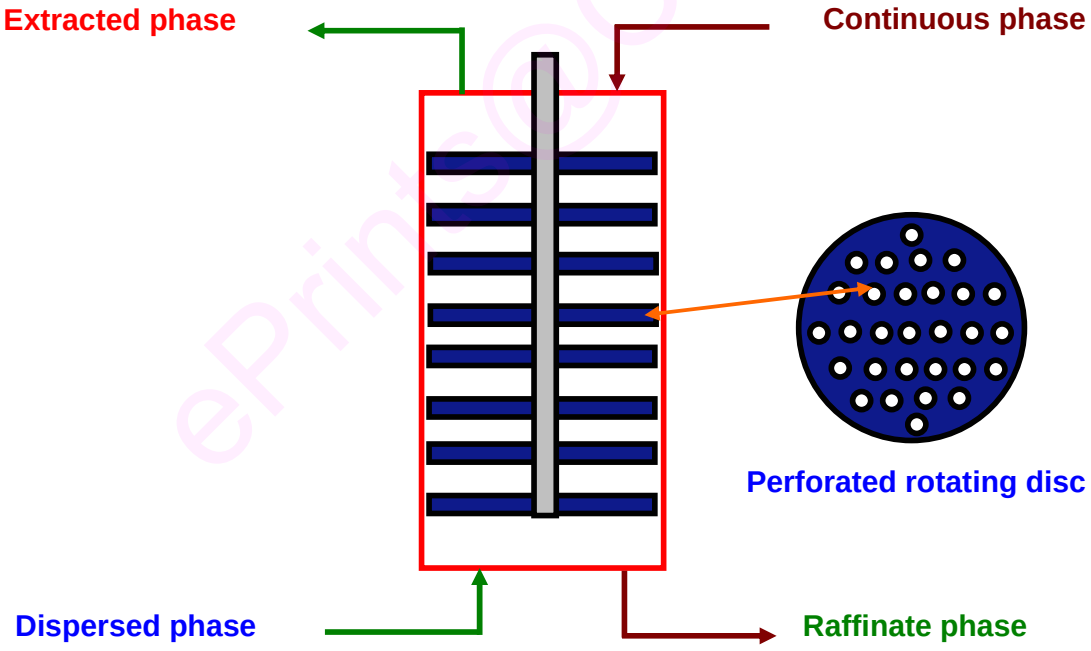


Figure 1A.3: Schematic diagram of the perforated rotating disc contactor

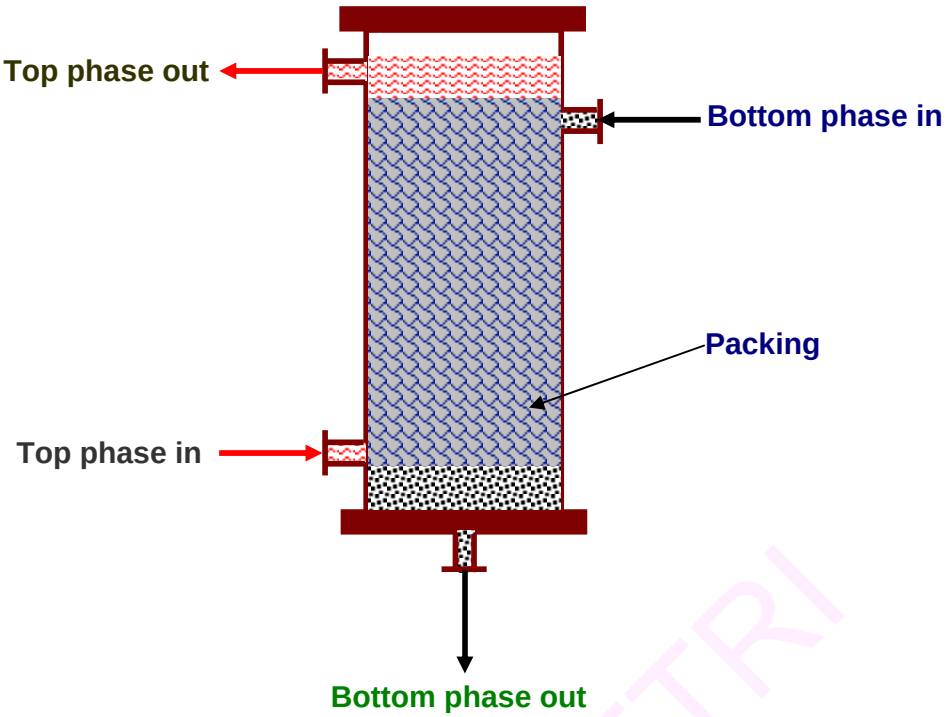


Figure 1A.4: Schematic representation of packed bed column

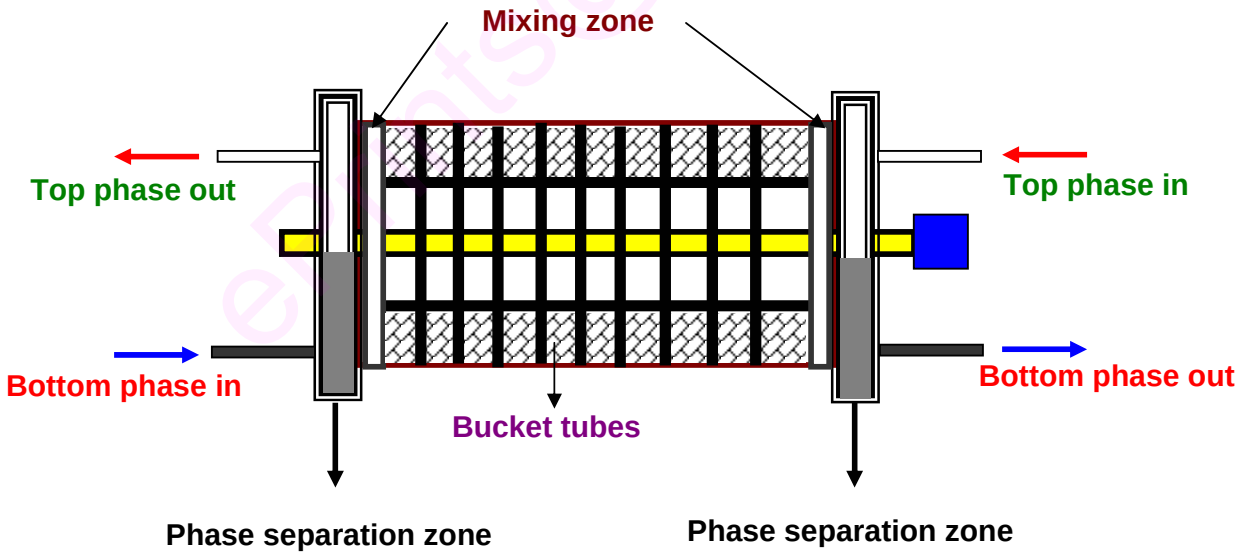


Figure 1A.5: Schematic representation of graesser raining bucket contactor

CHAPTER – 1B

Membrane bioseparation

1B.1 Introduction

Membrane is defined as “an intervening phase separating two phases and/or acting as an active or passive barrier to the transport of matter between phases". Membrane separation technologies offer advantages over existing separation processes such as high selectivity, low energy consumption, moderate cost to performance ratio, and compact and modular design. Membranes can be categorized based upon structure as either dense or porous membranes, based on material composition as organic (polymeric) or inorganic (ceramic or metallic), on the basis of their morphology as symmetric or asymmetric (Mulder, 1998). Although membrane materials vary vastly according to chemical composition and process type, the principal objectives in membrane manufacture are always the same. An ideal membrane must have the following characteristics (Cheryan, 1986):

- High hydraulic permeability towards the solvent
- High selectivity
- Good mechanical durability
- Good chemical and thermal stability
- Compatibility with substances being processed
- Excellent manufacturing reproducibility
- Ease of manufacture

An important component of a membrane separation system is the actual module or configuration, within which the membrane is housed. A good membrane module must have the characteristics such as a high membrane

area to module bulk volume ratio, a high degree of turbulence for mass transfer promotion on the feed side, a low energy expenditure per unit productivity, a low cost per unit membrane area, a design that facilitates cleaning, a design that permits modularization. The membrane configurations normally employed for bioseparation are plate and frame membrane module, tubular membrane module, spiral wound membrane module and hollow fibre membrane module (Girard, 2000; Ghosh, 2003; Raghavarao *et al*., 2005).

The stirred membrane cell (Fig. 1B.1) is suitable for bench-top feasibility studies. However, the flux data obtained in stirred cells are not a good indication of the flux that can be obtained with larger modules. A majority of the laboratory scale modules are designed for use with flat sheet membranes, as these membrane modules are more versatile when compared with tubular or hollow fibre membrane modules. Flat sheet membranes are easier for examination and cleaning. As a result the same membrane module can be used to test many different types of membranes. Tubular membrane modules are usually operated under turbulent flow conditions, which help in the reduction of the concentration polarization effect. However, the main disadvantage of the tubular unit is the low surface area per unit volume of the membrane contactor and hence the requirement for high floor space. Another disadvantage is the high hold-up volume within these units.

The hollow fibre membrane configuration (Fig. 1B.2) consists of a membrane in the form of a self-supporting tube and has the advantage of a 'back-flushing' provision. However, the disadvantage of the hollow fibre

module is the cost of membrane replacement due to sensitivity to suspended solids. Thus even if one single membrane fibre ruptures, the entire membrane cartridge needs to be replaced. The spiral wound module is one of the most compact and inexpensive designs available today. These modules are basically flat sheets arranged in parallel to form a narrow slit to fluid flow. The main advantage of the spiral wound module is its surface area per unit volume of the membrane contactor, which is fairly high and results in a lower floor area. The other advantages of spiral wound module are low capital cost and low power consumption.

Membrane processes are well suited for the processing of biological molecules since they operate at relatively low temperatures and pressures and involve no phase changes or chemical additives, thereby minimizing the extent of denaturation, deactivation, and/or degradation of biological products. Membrane processes play a critical role in production, purification, and formulation of biotechnology products (Charcosset, 2006). During the last two decades, new membranes and membrane processes and modules have been developed specifically to meet the various requirements of the biotechnology industry such as membranes for sterile filtration, clarification, virus removal, protein concentration, buffer exchange, and protein purification (Ghosh, 2003; Reis and Zydney, 2007). Current research and development efforts are directed toward drastic improvements in selectivity while maintaining the inherent high-throughput characteristics of membrane. Membranes are used extensively throughout the production, purification, and formulation of biotechnology products. In the following sections the existing membrane

processes such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) and newer membrane processes such as membrane chromatography, osmotic membrane distillation (OMD) and direct osmosis (DO) processes are discussed briefly.

1B.2 Existing membrane processes

1B.2.1 Microfiltration

Microfiltration (MF) relies on the use of microporous membranes for the separation of micron sized particles from fluids. The various applications of microfiltration in biosepration includes cell harvesting from bioreactors, clarification of fruit juices and beverages, water purification, air sterilization and media sterilization in bioreactors. Microfiltration membranes have pore size between 0.05 and 10 μm and are designed to retain cells and cell debris while allowing proteins and smaller solutes to pass into the filtrate. Membrane thickness is in the range of 10–150 nm. In microfiltration the applied pressure is in the range of 0.1–2 bar. Microfiltration (MF) competes with centrifugation, depth filtration and expanded-bed chromatography for the initial harvesting of therapeutic products. In contrast to centrifugation, MF process generates a particle-free harvest solution that requires no additional clarification before subsequent purification. Microfiltration also, finds its application in clarification of fruit juice, wastewater treatment and separation of oil–water emulsions. Current MF systems are generally operated at constant flux instead of constant transmembrane pressure to improve product yield and throughput (Kwon *et al*., 2000; Li *et al*., 2000).

1B.2.2 Ultrafiltration

Ultrafiltration (UF) uses a membranes having pore size ranging form 1-100 nm. The applied pressure is in the range of 1–10 bar (Mulder, 1998). Ultrafiltration membranes are made from organic polymers (such as polysulfone, polythersulfone, cellulose acetate, regenerated cellulose acetate, polyamide, polyvinylidedefluroid) or inorganic materials (such as alumina, borosilicate glass, pyrolyzed carbon, zirconia carbon, stainless steel). Ultrafiltration is widely used for the fractionation of macromolecules (such as proteins, nucleic acids), concentration of macromolecules, removal of salts and other lower molecular compounds from solutions, removal of cells and cell debris, harvesting of biomass and clarification of fruit juices (Girard, 2000; Ghosh, 2003).

1B.2.3 Nanofiltration

Nanofiltration (NF) is a membrane separation technology classified between ultrafiltration (UF) and reverse osmosis and is used in a wide range of applications. The applied pressure is in the range of 5 to 25 bar and pore size of the membrane is between 0.5 to 2 nm. Substances with molar masses higher than ~300 g mol^{-1} and multivalent ions are (partially) retained by NF membranes. In drinking water production, NF is used to remove pesticides and organic matter. Other applications are known in the fields of waste water treatment, textile industry, biotechnology and food industry (Muldar, 1998).

1B.2.4 Reverse osmosis

Reverse osmosis (RO) uses dense membranes having pore size of <2 nm and the porosity of the membrane is about 50 per cent. The separation

mechanism is based on solution-diffusion across the membrane. Membranes are made up of cellulose triacetate, polyether urea and polyamide. RO finds application in the concentration of liquid foods such as fruit juices, milk, etc. and desalination of brackish and seawater. The low permeability of reverse osmosis membranes requires high pressures and, consequently, relatively high energy consumption (Muldar, 1998).

1B.3 Newer membrane processes

1B.3.1 Membrane chromatography

Column chromatography has several limitations such as low recovery, high pressure drop and process time. Membrane chromatography uses microfiltration (or larger) pore size membranes that contain functional ligands attached to the inner pore surface throughout the membrane structure to provide highly selective separations through adsorption/binding interactions. Although the equilibrium binding capacity in membranes tends to be low, the convective flow through the pores reduces the mass transfer resistance compared to column (bead) chromatography. Chromatographic membranes have been used in a variety of configurations, as staked membranes, hollow fibers, spiral wound membranes and a variety of adsorptive mechanisms, such as ion-exchange, hydrophobic, reversed-phase, and affinity based procedures. One of the major limitations with membrane chromatography is non-uniform flow distribution across the membrane, due to the large diameter-to-length ratio of the modules. Purification procedures using chromatographic membranes have been reported for a wide variety of compounds, such as

proteins (monoclonal antibody, serum antibody, serum albumin, enzymes, etc.), DNA and viruses (Ghosh, 2002).

1B.3.2 Osmotic membrane distillation

Osmotic membrane distillation is a novel alternate membrane process for the concentration of thermally sensitive aqueous solutions such as fruit juices or natural colors at ambient temperature and atmospheric pressure. It employs a porous hydrophobic membrane, which separates the two aqueous solutions (feed and osmotic solution) having different solute concentrations. Water evaporates from surface of the solution of higher vapor pressure (feed), the vapor passes through the pores of the membrane and condenses on surface of the solution of lower vapor pressure (osmotic agent). This migration of water in the form of vapor results in the concentration of the feed and dilution of the osmotic agent solution and the products can be concentrated up to 70°Brix without product damage. Furthermore, OMD can also be employed as a pre-concentration step prior to lypholization (freeze drying) of temperature-sensitive biological products such as vaccines, hormones, enzymes and proteins to obtain the product in powder form without product deterioration. (Hogan *et al*., 1998; Jiao *et al*., 2004, Raghavarao *et al*., 2005).

1B.3.3 Direct osmosis

Direct osmosis (DO) is another non-pressure driven membrane process capable of concentrating liquid foods, natural colours and biomolecules at ambient conditions without product deterioration. Direct osmosis employs a semi-permeable dense hydrophilic membrane, which

separates two aqueous solutions (feed and osmotic agent solution) having difference in osmotic pressures, which acts as the driving force. The transfer of water occurs from the feed side to osmotic agent side till the osmotic pressures on both the sides become equal. Direct osmosis offers several advantages such as low energy consumption, higher retention of thermolabile components and achievement of higher concentration (45-60°Brix) (Raghavarao *et al*., 2005; Cath *et al*., 2006).

Existing membrane processes suffer from the drawbacks such as membrane fouling, concentration polarization, maximum achievable concentration and shear damage to the product (in case of proteins). Even the new membrane process, membrane distillation (MD), is not without limitations as it suffers from membrane wetting, temperature polarization and loss of volatiles (Lawson and Lloyd, 1997; Girard and Fukomoto, 2000). Recently, technological advances related to the development of new membrane processes and modulus have enabled to overcome the above limitations to a large extent. Newer membrane processes such as direct osmosis (DO) and osmotic membrane distillation (OMD) have the potential to concentrate liquid foods, natural colours and biomolecules at ambient temperature and pressure without product deterioration. Theoretical aspects and effect of process parameters during the concentration of fruit juices and biomolecules by direct osmosis and osmotic membrane distillation has been discussed in detail in **Chapter 3 and 4.** Also, integration of these newer membrane processes with existing membrane process and aqueous two phase extraction has been explored for the purification and concentration of biomolecules in **Chapter 5**.

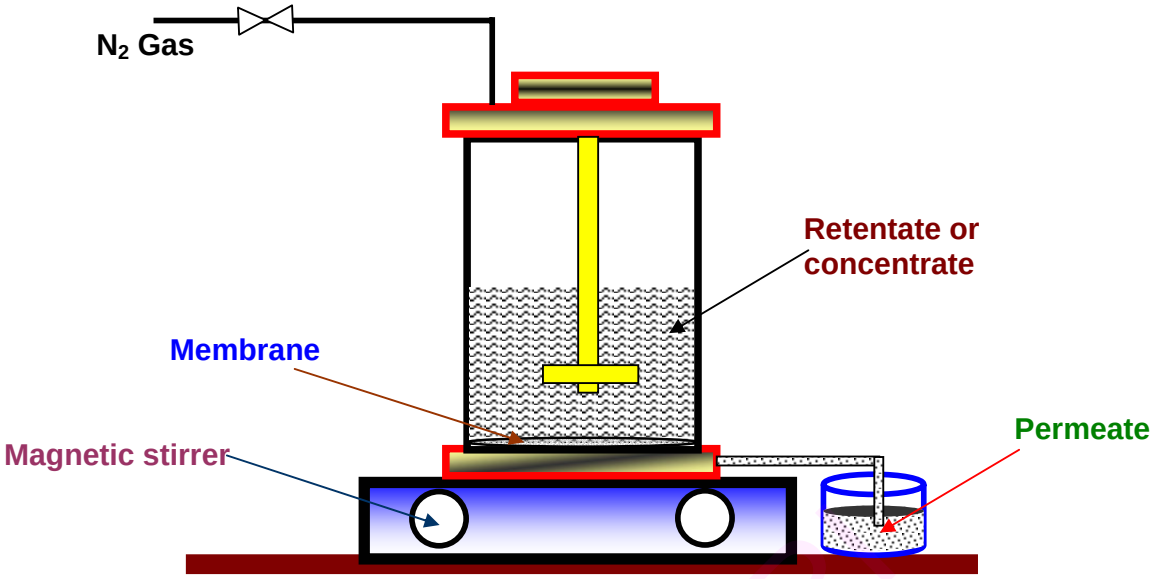


Figure 1B.1: Stirred membrane cell

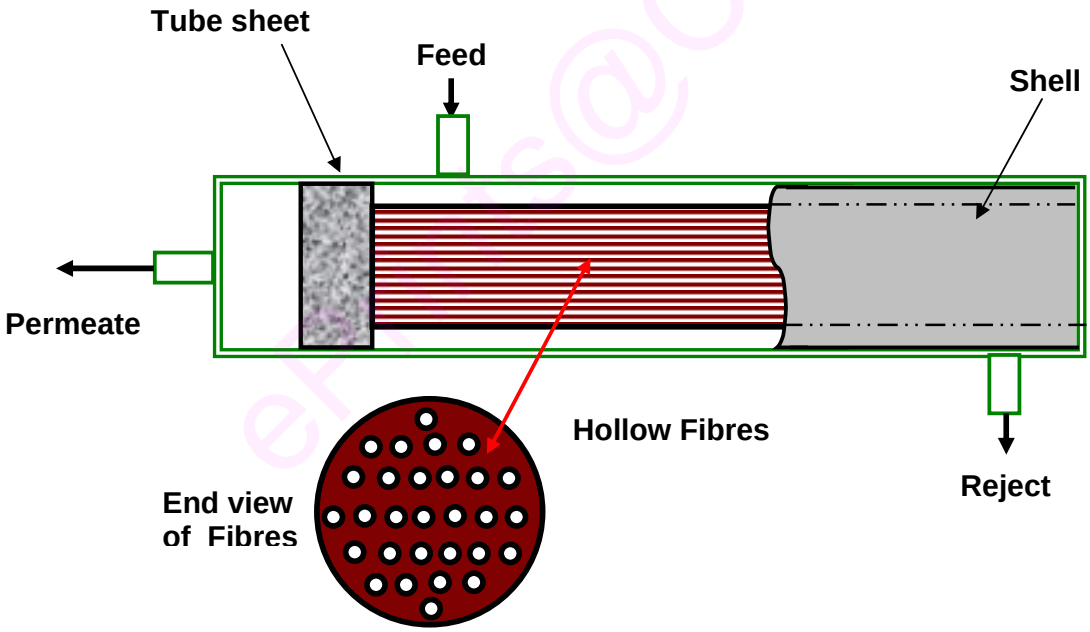


Figure 1B. 2: Hollow fibre membrane module

CHAPTER – 1C

Aim and scope of the present work

1C.1. Aim and scope of the present work

Downstream processing is an important component of Biotechnology, since the final cost of the product largely depends on the purification and concentration of methods used for the product recovery. The downstream processing of biomolecules require purification techniques which are both economically feasible and delicate enough to preserve the biological activity. Aqueous two-phase extraction is one such method with several advantages over the conventional methods. The overall productivity increases when newer, membrane process such as direct osmosis and osmotic membrane distillation is integrated with this extraction. In the present study, a practical approach for the design of ATPE process, application of membrane processes and an integrated approach of these methods have been discussed in detail.

The thesis consists of six chapters. In **Chapter 1**, introduction of aqueous two phase extraction and membrane process and their applications during the downstream processing of biomolecules has been explained.

In **Chapter 2**, the phase systems of ATPE were characterized with respect to density, viscosity and interfacial tension. Further, the optimization of process parameters for the fractionation of mixture of enzymes (bromelain and polyphenol oxidase) from the pineapple (*Ananas comosus* L. Merryl*)* by employing ATPE is presented.

Direct osmosis, a non-thermal membrane process, can concentrate biomolecules and fruit juices to very high levels at ambient temperature and

pressure, with minimal product deterioration. Very little information is reported in literature about this process. In **Chapter 3**, effect of various process parameters during the concentration of biomolecules from pineapple waste by direct osmosis process is presented. Also, concentration of pineapple juice by direct osmosis process is described.

Osmotic membrane distillation is a novel membrane process for the removal of water from dilute aqueous solutions such as liquid foods or biomolecules. In **Chapter 4,** optimization of various process parameters during the concentration of biomolecules (bromelain and phycocyanin) and fruit juices (pineapple juice/sweetlime juice) by OMD is presented. Modeling of mass and heat transfer during osmotic membrane distillation process is described. Also, contribution of concentration and temperature polarization effects during osmotic membrane distillation process is discussed

The productivity, yield and economy of bioprocesses can be considerably improved by process integration. In **Chapter 5**, integrated approaches (integration of ultrafiltration with OMD/DO and integration of ATPE with ultrafiltration followed by DO) for the purification and concentration of biomolecules are discussed. Also, integration of various unit operations such as extraction, precipitation, separation and concentration for the purification and concentration of biomolecules is presented.

Overall conclusions followed by the suggestions for future work are presented in **Chapter 6**.

CHAPTER - 2

Aqueous two phase extraction for the purification of biomolecules

CHAPTER – 2A

Characterization of aqueous two phase systems

2A.1 Introduction

Aqueous two-phase system (ATPS) forms when two polymers or one polymer and a salt are dissolved in an aqueous solvent above their critical concentrations. The mixture will then separate into two immiscible phases, where the light phase (top phase) is rich in one polymer and the heavy phase (bottom phase) is rich in the second polymer or the salt. Aqueous two-phase systems (ATPSs) have been extensively exploited for the recovery and purification of different bioproducts such as proteins, viruses and intact cells, virus-like particles, inclusion bodies, plasmid DNA, surrogate mimics for viral vectors and adenoviral vectors and even inorganic compounds produced by microorganisms (Walker *et al*., 1999; Rito-Palomares *et al*., 2001; Ribeiro *et al*., 2002; Barhate *et al*., 2004; Tinade *et al*., 2005; Platis and Labrou, 2006; Benavides *et al*., 2006). Despite the several practical applications of aqueous two phase systems (ATPSs), information regarding the characterization of physical properties of aqueous two phase systems is scanty in literature.

Forciniti *et al*. (1990) studied the effect of polymer molecular weight, polymer concentration and temperature on the interfacial tension. Wu *et al*. (1996) investigated the effect of polymer molecular weight and concentration, salt concentration and type, and total composition on interfacial tension. Mishima *et al*. (1998) determined the interfacial tension of aqueous two phase systems containing PEG and dipotassium hydrogenphosphate by drop volume method. In order to understand the demixing rate and partition behavior of biomolecules, the physical properties such as interfacial tension, density and viscosity needs to be measured at the same composition of

phase forming components used for the partitioning of biomolecules. Because the physical properties of the aqueous two-phase systems change remarkably with polymer molecular weight and concentration of the phase forming components.

The physical properties of aqueous two phase systems, such as density, viscosity and interfacial tension determine the phase demixing rate (Raghavarao *et al*., 2005). The combined effect of density difference between two phases, viscosity of individual phases and interfacial tension will influence the demixing rate. The slow demixing rate of the phase systems near the critical point is mainly due to the low density difference between the phases, whereas of those far away from the critical point; it is due to high viscosity of the phases. The phase viscosity also plays an important role in biomolecule partitioning in flow processes (Raymond and Fisher, 1980), especially in industrial scale applications in which large volumes of phases are to be handled (Hustedt *et al*., 1985). When two mutually immiscible soluble liquid phases are brought together, the interface possesses a definite amount of free energy per unit area by virtue of the unbalanced force field acting on the surface molecules (Raghavarao *et al*., 2005). Each unit area of the interface has some definite quantity of free energy and as a result, the interface tends to contract. This free energy is mathematically equivalent to interfacial tension. The interfacial tension between the phases is an important determining factor that influences the partitioning behavior of particles or cells. Because the cells or particles usually distribute themselves between the one of the bulk phases and interface, leaving the other phase depleted of particles. The degree to

which interfacial accumulation occurs increases with increasing interfacial tension due to decrease in free energy associated with loss of free liquid-liquid interfacial area between the native phases (Wu *et al*., 1996). To understand the mechanism behind the partitioning of biomolecules in ATPSs, it is important to know the properties of the liquid-liquid interface and particularly the value of the interfacial tension, which plays an important role even in the design of extraction equipment. There is a need for the systematic measurement of physical properties of aqueous two phase systems. In this chapter, characterization of ATPSs in terms of density, viscosity and interfacial tension for polyethylene glycol (PEG)/phosphate phase systems is carried out. The effect of molecular weight of PEG (PEG 1500, 4000, 6000 and 20000) and concentration of phase forming components (polyethylene glycol concentration in the range of 12 to 18 w/w% and potassium phosphate concentration in the range of 14 to 20 w/w%) on physical properties of phase systems is reported.

2A. 2 Materials and Methods

2A. 2.1 Materials

Polyethylene glycol (PEG) 1500 was purchased from Merck Chemicals, India and PEG 4000, PEG 6000 and PEG 20000 were procured from SRL Chemicals, India. Potassium hydrogen phosphate (mono-basic and di-basic, analytical grade) were procured from Ranbaxy Limited, India.

2A. 2.2 Methods

2A.2.2.1 Aqueous two-phase systems

Predetermined quantities of phase forming solutes and water were weighed to make the total weight of the system 100 w/w%. The contents were mixed thoroughly using a magnetic stirrer for equilibration and were allowed to separate for about five hours in a separating funnel. The equilibrated and separated phases were collected and used for further experiments. All experiments were conducted in triplicates at 25 ± 2°C.

2A.2.2.2 Density

The density of top and bottom phase was determined using a specific gravity bottle using water as a reference at 25 ± 2°C.

2A.2.2.3 Viscosity

The viscosity of top and bottom phase was measured using Ostwald viscometer (capillary diameter 1.0 mm) using water as a reference at 25 ± 2°C.

2A.2.2.3 Interfacial tension

Interfacial tension measurements were performed by spinning drop tensiometer (SITE 100, Kruss, Germany) as shown in Fig. 2A.1. The spinning drop tensiometer (SITE 100) is a computer controlled tensiometer for measuring small interfacial tensions (Fig. 2A.1). The spinning drop tensiometer measures the size of the rotating drop of a light phase in a heavy

phase. At high rotational speeds where the drop shape is independent from the gravitational acceleration, the interfacial tension between two phases can be calculated from rotational speed, drop size (diameter and length) and density difference between the drop and surrounding medium. Interfacial tension was calculated as mean of rotation for 2500 to 7500 rpm for 10 counts at 25 ± 2°C using the drop shape analysis software (DSA 2). The images of the bubble formed were shown in Fig. 2A.2.

2A. 3 Results and Discussion

The effect of molecular weight of PEG, concentration of phase forming components on physical properties of aqueous two phase systems such as density, viscosity and interfacial tension were discussed in the following sections.

2A. 3.1 Effect of polymer molecular weight

The density and viscosity of top and bottom phases for different molecular weights of the PEG (PEG 1500, 4000, 6000 and 20000) is presented in Table 2A.1. It can be observed from the Table 2A.1 that the viscosity of the top phase is more than that of bottom phase and viscosity is higher for the phases of higher molecular weights of PEG. The density difference between the two phases is very low for the systems studied. The variation in interfacial tension for PEG (14 w/w%) and potassium phosphate (14 w/w%) system at different molecular weights of PEG is presented in Table. 2A.1. It can be observed that the interfacial tension increased with an increase in PEG molecular weight.

2A. 3.2 Effect of polymer concentration

The variation in density and viscosity of top and bottom phases for PEG 1500 (PEG concentration in the range of 12 to 18 w/w%)/potassium phosphate (14 w/w%) and PEG 4000 (PEG concentration in the range of 12 to 18 w/w%)/potassium phosphate (14 w/w%) systems is represented in Table 2A.2. The density difference between top and bottom phases increases with an increase in PEG concentration. It can be observed from the Table 2A.2 that increase in viscosity of the top phase with an increase in polymer concentration is more prominent when compared to that of bottom phase. For example, in case of PEG 1500 (PEG concentration in the range of 12 to 18 w/w%)/potassium phosphate (14 w/w%) system the top phase viscosity increased from 7.66 ± 0.02 to 12.23 ± 1.0 mPa.s, whereas marginal increase in bottom phase viscosity was observed (1.52 ± 0.1 to 1.59 ± 0.1 mPa.s). The variation in interfacial tension for PEG 1500 (PEG concentration in the range of 12 to 18 w/w%)/potassium phosphate (14 w/w%) and PEG 4000 (PEG concentration in the range of 12 to 18 w/w%)/potassium phosphate (14 w/w%) systems is shown in Fig. 2A.3. It can be observed that the interfacial tension increased with an increase in polymer concentration.

2A. 3.3 Effect of potassium phosphate concentration

The physical properties of top and bottom phases for PEG 1500 (12 w/w %)/potassium phosphate (potassium phosphate concentration in the range of 14 to 20 w/w%) and PEG 4000 (12 w/w%)/potassium phosphate

(potassium phosphate concentration in the range of 14 to 20 w/w%) systems is represented in Table 2A.3. The density difference between the two phases increased from 83 ± 2.0 to 157 ± 2.0 kgm^{-3} with an increase in potassium phosphate concentration. The viscosity of the top phase increased with an increase in concentration of phosphate. A tendency of increase in interfacial tension (Fig. 2A.4) was observed for PEG 1500 (12 w/w%)/potassium phosphate (potassium phosphate concentration in the range of 14 to 20 w/w%) and PEG 4000 (12 w/w%)/potassium phosphate (potassium phosphate concentration in the range of 14 to 20 w/w%) phase systems, respectively.

2A. 4 Conclusions

Phase forming components concentration found to have significant effect on physical properties of phase systems. The density difference between top and bottom phases increased with an increase in phase forming polymer and salt concentrations. The interfacial tension of the PEG/potassium phosphate system increased with an increase in concentration of phase forming components and molecular weight of polymer.

Table 2A.1: Physical properties of individual phases at different molecular weights of polymer

PEG molecular weight[a]	Top phase			Bottom phase			Interfacial tension ($mN.m^{-1}$)
	Volume (ml)	Density ($kg.m^{-3}$)	Viscosity (mPa.s)	Volume (ml)	Density ($kg.m^{-3}$)	Viscosity (mPa.s)	
1500	19.8 ± 0.1	1088 ± 2.0	8.67 ± 0.6	24.2 ± 0.1	1191 ± 2.0	1.54 ± 0.1	0.62 ± 0.03
4000	17.7 ± 0.1	1084 ± 1.0	26.28 ± 1.0	26.3 ± 0.1	1182 ± 1.0	1.48 ± 0.1	1.22 ± 0.01
6000	16.8 ± 0.2	1083 ± 1.0	54.7 ± 2.0	27.2 ± 0.3	1179 ± 2.0	1.46 ± 0.1	1.61 ± 0.02
20000	15.4 ± 0.1	1089 ± 2.0	84.78 ± 2.0	28.0 ± 0.2	1178 ± 1.0	1.45 ± 0.1	1.92 ± 0.02

[a] 14 (w/w)% PEG+14 (w/w)% potassium phosphate (K_2HPO_4 and KH_2PO_4 in the ratio of 1.82:1.0)

Table 2A.2: Physical properties of individual phases at different concentration of polymer

PEG (w/w)%	Phosphate (w/w)%[a]	Top phase			Bottom phase			Interfacial tension mN m^{-1}
		Volume (ml)	Density (kg m^{-3})	Viscosity (mPa s)	Volume (ml)	Density (kg m^{-3})	Viscosity (mPa s)	
PEG 1500/Potassium phosphate system								
12	14	18.2 ± 0.1	1087 ± 2.0	7.66 ± 0.02	25.8 ± 0.2	1172 ± 2.0	1.52 ± 0.1	0.38 ± 0.02
14	14	19.8 ± 0.1	1088 ± 2.0	8.67 ± 1.0	24.2 ± 0.1	1191 ± 2.0	1.54 ± 0.1	0.62 ± 0.04
16	14	22.4 ± 0.1	1088 ± 1.0	10.60 ± 1.0	21.0 ± 0.2	1200 ± 1.0	1.57 ± 0.1	0.96 ± 0.02
18	14	22.6 ± 0.2	1088 ± 2.0	12.23 ± 1.0	20.2 ± 0.1	1216 ± 2.0	1.59 ± 0.1	1.31 ± 0.06
PEG 4000/Potassium phosphate system								
12	14	16.7 ± 0.2	1088 ± 2.0	20.54 ± 1.0	27.5 ± 0.2	1171 ± 2.0	1.46 ± 0.1	0.94 ± 0.01
14	14	17.7 ± 0.1	1084 ± 1.0	26.28 ± 1.0	26.3 ± 0.1	1182 ± 1.0	1.48 ± 0.1	1.22 ± 0.02
16	14	19.8 ± 0.1	1089 ± 1.0	28.11 ± 1.6	24.0 ± 0.2	1194 ± 1.0	1.50 ± 0.2	1.51 ± 0.04
18	14	21.2 ± 0.2	1090± 2.0	31.96 ± 2.0	21.8± 0.2	1207 ± 2.0	1.54± 0.1	1.88 ± 0.05

[a] K_2HPO_4 and KH_2PO_4 in the ratio of 1.82:1.0

Table 2A.3: Physical properties of individual phases at different concentration of phosphate

PEG (w/w)%	Phosphate (w/w)% [a]	Top phase			Bottom phase			Interfacial tension mN m^{-1}
		Volume (ml)	Density (kg m^{-3})	Viscosity (mPa s)	Volume (ml)	Density (kg m^{-3})	Viscosity (mPa s)	
PEG 1500/Potassium phosphate system								
12	14	18.2 ± 0.2	1087 ± 2.0	7.66 ± 0.02	25.8 ± 0.2	1172 ± 2.0	1.52 ± 0.1	0.38 ± 0.02
12	16	15.8 ± 0.1	1089 ± 1.0	9.52 ± 1.4	27.4 ± 0.1	1197 ± 2.0	1.57 ± 0.1	0.88 ± 0.04
12	18	14.0 ± 0.2	1090 ± 1.0	12.26 ± 1.8	28.4 ± 0.1	1223 ± 1.0	1.62 ± 0.1	1.50 ± 0.02
12	20	12.4 ± 0.1	1091 ± 2.0	15.43 ± 2.0	28.0 ± 0.2	1248 ± 2.0	1.73 ± 0.1	2.14 ± 0.02
PEG 4000/Potassium phosphate system								
12	14	16.7 ± 0.2	1088 ± 2.0	20.54 ± 1.0	27.5 ± 0.2	1171 ± 2.0	1.46 ± 0.1	0.94 ± 0.01
12	16	14.6 ± 0.2	1084 ± 1.0	30.43 ± 1.2	27.8 ± 0.1	1192 ± 1.0	1.49 ± 0.1	1.53 ± 0.02
12	18	13.4 ± 0.1	1089 ± 1.0	38.04 ± 2.0	28.6 ± 0.1	1217 ± 1.0	1.60 ± 0.1	2.19 ± 0.02
12	20	12.6 ± 0.2	1091 ± 2.0	39.33 ± 2.2	29.0 ± 0.2	1243 ± 2.0	1.65 ± 0.1	2.96 ± 0.02

[a] K_2HPO_4 and KH_2PO_4 in the ratio of 1.82:1.0

Fig. 2A.1: Photograph of spinning drop tensiometer

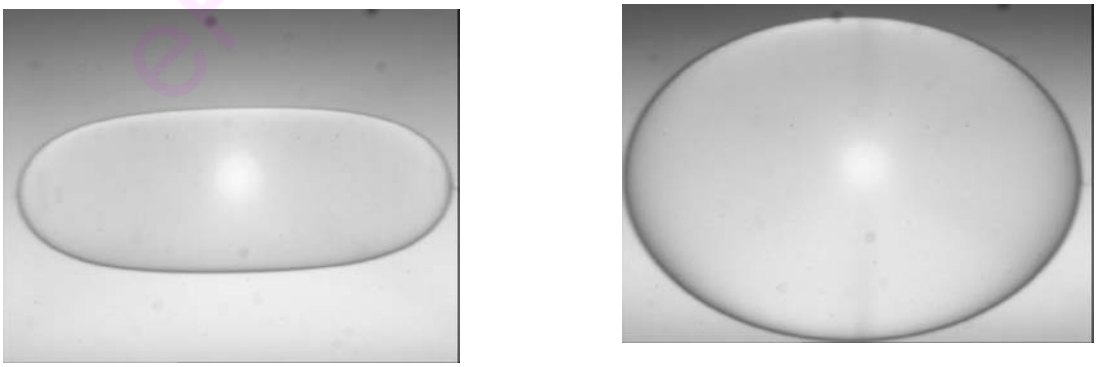


Fig. 2A.2: Images of the bubble formed during the measurement of interfacial tension

CHAPTER – 2B

Fractionation and purification of bromelain and polyphenol oxidase

2B.1. Introduction

Pineapple (*Ananas comosus* L.) is widely grown in tropical regions. Bromelain and polyphenol oxidase (PPO) are the commercially important enzymes present in pineapple. Bromelain is a mixture of proteolytic enzymes present in stem and fruit of the pineapple. The enzyme extracted from stem is called stem bromelain (EC 3.4.22.32) and from fruit is known as fruit bromelain (EC 3.4.22.33). Bromelain is known for its clinical and therapeutic applications, particularly for modulation of tumor growth, third degree burns, improvement of antibiotic action and as a drug for the oral systemic treatment of inflammatory, blood-coagulation-related and malignant diseases (Maurer, 2001). The bromelain enzyme inhibits the action of the cholera toxin. Bioavailability of the heparin, which is a negatively charged water-soluble polymer, used for the treatment of several diseases, enhanced by complexing it with the positively charged proteolytic enzyme bromelain across the gastrointestinal epithelial barrier (Grabovac and Bernkop-Schnurch, 2006). It also used in food processing for meat tenderization and as a dietary supplement (Maurer, 2001). Polyphenol oxidase (PPO; EC 1.14.18.1 and EC 1.10.3.1) is an enzyme that catalyzes the oxidation of phenolic compounds to quinines, which further polymerize to red, brown or black pigments (Lee, 1991). It is widely distributed in nature and responsible for melanin production in mammals (Vaidya *et al*., 2006). PPO finds its application in the production of black tea, cocoa, flavonoid-derived colorants and antioxidants (Ridgway and Tucker, 1999). PPO can be used as an efficient reagent for cleaning waste water containing polyphenols and for the removal of oestrogenic substances from aquatic environment (Ridgway and Tucker, 1999; Shi *et al*.,

2001). PPO can be used during the synthesis of value-added products such as the substituted catechol, l-DOPA for the treatment of Parkinson's disease (Pialis and Saville, 1998). PPO-based amperometric biosensors can be used for the determination of phenols and substituted phenols at low levels (Ghindilis *et al*., 1992). Conventional methods for purification of enzymes/proteins have their own limitations such as scale up, high production cost etc (Albertsson, 1986; Raghavarao *et al*., 1995).

The extraction and purification of bromelain or PPO from the pineapple fruit was investigated by many researchers (Doko *et al*., 1991; Das *et al*., 1997; Rabelo *et al*., 2004). Doko *et al*. (1991) developed a process for the extraction and purification of bromelain from pineapple juice involving membrane processes such as microfiltration and ultrafiltration/diafiltration followed by ammonium sulfate precipitation, centrifugation and finally freeze drying. The authors reported about 97 and 62% bromelain activity recovery for control and pretreated pineapple juice, respectively. The decrease in bromelain activity recovery was due to the adsorption of enzyme on the membrane. Das *et al*. (1997) reported the extraction and purification of the PPO from the pineapple. The process involved dialysis, ammonium sulphate fractionation and column chromatography. PPO activity recovery and purity were reported to be about 45% with 25 fold, respectively. The main drawback of the above method is high processing cost and scale-up. Rabelo *et al*. (2004) demonstrated the extraction and purification of the bromelain in aqueous two-phase system using PEO-PPO-PEO block copolymers. Bromelain activity recovery and purification factor was found in the range 7.5 to 79.5% and 0.1

to 1.25 fold, respectively. The extent of enzyme activity recovery and purification factor could not be further increased due to hydrophobic interactions between bromelain and copolymer molecules.

In view of the potential applications of bromelain and PPO in various fields and earlier attempts for separation and purification, an effective and economically viable method needs to be developed for the separation and purification of these enzymes. Partitioning in aqueous two-phase systems (ATPSs) has been shown to provide a powerful method for separation and purification of mixture of proteins/enzymes. These systems are composed of aqueous solutions of either two water-soluble polymers or a polymer and a salt and suitable for purification of biological materials as both the phases contain 70-90% water (Albertsson, 1986; Raghavarao *et al*., 1995). Furthermore, ATPSs can remove undesirable byproducts present in the system such as unidentified polysaccharides, pigments and interfering proteins that lower the activity of enzyme. Compared to other separation and purification methods, extraction using ATPS has many advantages such as ease of scale-up, low cost, scope for continuous operation and environment friendly thus, making it an attractive alternative for separation and purification of biomolecules (Tanuja *et al*., 1997; Srinivas *et al*., 1999; Raghavarao *et al*., 2003; Giraldo-Zuniga *et al*., 2005).

In this chapter differential partitioning and purification of bromelain and PPO from pineapple fruit (*Ananas comosus* L.) in aqueous two-phase extraction (ATPE) is discussed. Influence of various parameters such as

polymer molecular weight (PEG 1500, 4000, 6000 and 20000), concentration of polymer (PEG concentration in the range of 12 - 18% w/w) and phase forming salt (potassium phosphate concentration in the range of 14 - 20% w/w) and system pH (pH in the range of 6.0 to 9.0) on differential partitioning of bromelain and PPO in ATPE is evaluated.

2B.2 Materials and methods

2B.2.1 Materials

2B.2.1.1 Plant material

The pineapple fruits (*Ananas comosus* L.) used in this study was obtained from the local market. The fruits were washed and peeled. After removal of core, the fruit was cut into small pieces and stored at 4°C for further experiments.

2B.2.1.2 Chemicals

Polyethylene glycol (PEG) 1500 was purchased from Merck Chemicals, India and PEG 4000, PEG 6000 and PEG 20000 were procured from SRL Chemicals, India. Potassium hydrogen phosphate (mono-basic and di-basic), trichloroacetic acid, sodium acetate were procured from Ranbaxy Limited, India. Casein (Hammarsten grade), catechol were procured from Loba Chemicals, India. All other chemicals were of analytical grade.

2B.2.2 Methods

2B.2.2.1 Enzyme Extraction

The frozen pieces of pineapple were homogenized in cold extraction buffer (0.01 M sodium phosphate buffer, pH 7, containing 1% polyvinyl pyrolydine) in 1:1.5 ratio and homogenate was filtered. The filtrate was centrifuged at 10,000 rpm (4°C, 20 min) and the supernatant (crude enzyme extract) was used for further experiments.

2B.2.2.2 Aqueous two-phase extraction

Predetermined quantities of phase forming solutes were weighed and added to crude enzyme extract to make the total weight of the system 100 w/w%. During the experiment the weight percentage of crude enzyme extract was maintained at 20 w/w%. The contents were mixed thoroughly using a magnetic stirrer for equilibration and were allowed to separate for about five hours in a separating funnel. After clear separation of two phases, the volumes of top and bottom phases of the system were noted and analyzed for bromelain and PPO enzyme activities, total protein and sugar. All partition experiments were conducted in triplicates at 25 ± 2°C.

2B.2.2.3 Enzyme assays

The bromelain activity was determined according to the casein digestion unit (CDU) method using casein (0.6%) as a substrate in the presence of cysteine and EDTA at 37 ± 2°C and pH 7.0 for 10 min (Murachi, 1976). The assay was based on proteolytic hydrolysis of the casein substrate.

The unhydrolyzed casein substrate is precipitated with trichloroacetic acid and precipitated proteins were removed using Whatman filter paper (No. 1). The absorbance of the clear filtrate (solubilized casein) was compared with that of tyrosine using water as a blank at 275 nm. One unit of bromelain activity is defined as 1 µg of tyrosine released in 1 min per ml of enzyme extract when casein is hydrolyzed under the standard conditions of 37 ± 2°C and pH 7.0 for 10 min (Murachi, 1976).

Polyphenol oxidase was assayed according to the spectrophotometric procedure reported in literature (Das *et al*., 1997). The assay mixture consisted of 2.6 ml of sodium phosphate buffer (0.01 molarity, pH 6.5), 0.3 ml of catechol (0.5 molarity), and 0.1 ml of enzyme extract. The increase in absorbance was measured at 420 nm. One unit of PPO activity is defined as the amount of the enzyme that causes an increase in absorbance of 0.001/min at 25 ± 2°C.

2B. 2.2.4 Protein determination

Concentration of the protein was determined by dye binding method (Bradford, 1976) using Coomassie Blue G250. The reagent was prepared by dissolving 100 mg of Coomassie Blue G250 in a mixture of 50 ml of 95% ethanol and 100 ml 85 (w/v)% phosphoric acid. After the dye was completely dissolved, the final volume was made up to 1000 ml using double distilled water. Protein was quantified by adding known quantity of the sample to 2.0 ml of the reagent and making up the volume to 3.0 ml with double distilled water. Optical density was measured at 595 nm. The concentration of the

protein was determined from the standard graph. Bovine Serum Albumin (BSA, SRL Chemicals, India) was used as a standard.

2B.2.2.5 Sugar content

The total sugar present in pineapple juice was estimated by Dubois method (Dubois *et al*., 1956). Simple sugars, oligosaccarides or polyglycons and their derivative including the methyl ethers with free reducing groups gives yellow to orange color when treated with phenol and concentrated sulphuric acid. According to this method a known quantity of aliquots of the phase was made up to 500 µl by adding distilled water and 300 µl of 5% phenol was added and mixed thoroughly. To the above mixture 1.8 ml of concentrated sulphuric acid was added and allowed to cool to 25 ± 2°C. Yellow to orange color was developed in the reaction mixture. The intensity of the color in the mixture was measured at 480 nm and compared with standard graph.

2B.2.3 Estimation of partition coefficient

The activity partition coefficients of bromelain (K_{Br}) and PPO (K_{PPO}) were calculated as the ratio of the respective enzyme activity in the top phase to that of the bottom phase (Albertsson, 1986).

$$K_{Br} = \frac{(A_{Br})_t}{(A_{Br})_b} \quad \ldots. \quad (2B.1)$$

$$K_{PPO} = \frac{(A_{PPO})_t}{(A_{PPO})_b} \quad \text{....} \quad (2B.2)$$

where A_{Br} and A_{PPO} were the bromelain and PPO activities in units per ml, respectively. The subscripts 't' and 'b' represent the top and bottom phase, respectively. Similarly partitioning coefficients for protein (K_P) and sugar (K_S) were given by

$$K_P = \frac{(C_P)_t}{(C_P)_b} \quad \text{....} \quad (2B.3)$$

$$K_S = \frac{(C_S)_t}{(C_S)_b} \quad \text{....} \quad (2B.4)$$

where C_P and C_S are the protein and sugar concentration in microgram per ml, respectively.

2B.2.4 Estimation of purification factor

The purification factor of each enzyme in its respective extracting phase (top phase for bromelain and bottom phase for PPO) can be calculated by the following equations (Rabelo *et al*., 2004)

$$PF_{Br} = \frac{(A_{Br})_t/(C_P)_t}{(A_{Br})_i/(C_P)_i} \quad \text{....} \quad (2B.5)$$

$$PF_{PPO} = \frac{(A_{PPO})_b/(C_P)_b}{(A_{PPO})_i/(C_P)_i} \quad \text{....} \quad (2B.6)$$

where $(A_{Br})_i$ and $(A_{PPO})_i$ are the bromelain and PPO activities in units per ml in the initial extract (before partition), respectively; $(C_P)_i$ is the total protein concentration in microgram per ml in the initial extract (before partition).

2B.2.5 Estimation of enzyme activity recovery

The bromelain and PPO enzyme activity recovery after partition was defined as (Schmidt et al., 1994)

$$R_{Br} = \frac{(A_{Br})_t . V_t}{(A_{Br})_i . V_i} .100 \qquad \text{.... (2B.7)}$$

$$R_{PPO} = \frac{(A_{PPO})_b . V_b}{(A_{PPO})_i . V_i} .100 \qquad \text{.... (2B.8)}$$

where V_i, V_t and V_b are the initial, top and bottom phase volumes, respectively.

2B.3 Results and discussion

The influence of various parameters such as molecular weight of polyethylene glycol (PEG 1500, 4000, 6000 and 20000), concentration of polyethylene glycol (PEG concentration in the range of 12 - 18% w/w) and potassium phosphate (potassium phosphate concentration in the range of 14 - 20% w/w) and pH (pH in the range of 6.0 to 9.0) on differential partitioning during aqueous two-phase extraction of bromelain and PPO, which has direct bearing on the purity as well as enzyme (activity) recovery are discussed in the following sections.

2B.3.1 Partitioning behavior of biomolecules in aqueous two phase extraction

The schematic representation of the basic mechanism of partitioning of biomolecules during ATPE is shown in Fig. 2B.1. Typical partitioning of biomolecules in a polymer (PEG)/salt system is shown in Fig. 2B.1a. In PEG-salt systems partitioning of biomolecules depends on volume exclusion effect of the polymer in the polymer rich (top) phase and salting out in the salt rich (bottom) phase. The volume occupied by the polymer increases with an increase in polymer concentration (Fig. 2B.1b) and with an increase in polymer chain length or molecular weight of the polymer (Fig. 2B.1c), which resulted in decrease in space available for biomolecules in the top phase and as a result the biomolecules tend to partition to the bottom phase, which is called "volume exclusion effect" (Almedia *et al*., 1998; Rabelo *et al*., 2004). The solubility of biomolecules in the salt rich (bottom) phase decreases with an increase in salt concentration, which results in increased partitioning of biomolecules to the top phase and is called "salting out effect" (Fig. 2B.1d) (Almedia *et al*., 1998; Trinade *et al*., 2005). The system comprising of polymer with high concentration or high molecular weight polymer with high salt concentration will result in partitioning of biomolecules at the inter phase due to the influence of both volume exclusion and salting out effect as shown in Fig. 2B.1e. It is worth to notify that the partition coefficient of any biomolecule during ATPE depends on combined volume exclusion and salting out effect (Almedia *et al*., 1998; Rabelo *et al*., 2004; Cavalcanti *et al*., 2006).

2B.3.2 Effect of PEG molecular weight

Influence of PEG molecular weight (PEG 1500, 4000, 6000 and 20000) on differential partitioning of bromelain (K_{Br}), PPO (K_{PPO}), total proteins (K_P) and sugars (K_S) was studied. The compositions of PEG and phosphate (Table 2B.1) were selected from the respective phase diagrams available in literature (Albertsson, 1986). The partition coefficient of bromelain was higher than 1 whereas for PPO it was much less than 1 (Fig. 2B.2), which indicates that bromelain preferentially partitioned to the top phase while PPO to the bottom phase. Partition coefficient of both bromelain and PPO decreased with an increase in PEG molecular weight. About 31 ± 2% decrease in partition coefficient of bromelain and 33 ± 2% decrease in partition coefficient of PPO was observed. The increase in concentration of bromealin and PPO (decrease in K_{Br} and K_{PPO}) in the bottom phase is due to more dominant effect of volume exclusion over salting out.

The partitioning behavior of total proteins (K_P) and sugar (K_S) and activity recovery of enzymes (bromelain and PPO) with change in polymer molecular weight is reported in Table 2B.1. It can be observed that K_P, K_S decreased with an increase in PEG molecular weight. The decrease in partition coefficient of total proteins and sugar is due to volume exclusion effect of the polymer in the top phase. The bromelain activity recovery was very high (RBr >>100%) for the conditions studied and it is probably due to the structural alteration of the enzyme active sites in the presence of PEG. Higher enzyme activity recovery was reported during purification of α-toxin, cutinase, α-amylase and proteases during aqueous two phase extraction

(Schmidt *et al*., 1994; Almedia *et al*., 1998; Cavalcanti *et al*., 2006; Porto *et al*., 2008). The enzyme activity recovery of bromelain decreased with an increase in PEG molecular weight and it is due to increased partitioning of bromelain to the bottom phase. Similarly, the decrease in purification factor of bromelain is due to relatively more partitioning of bromelain to the bottom phase when compared to that of other proteins.

In case of PPO the enzyme activity recovery in the bottom phase increased, but its purity decreased due to increased partitioning of total proteins to the bottom phase (Table 2B.1). In order to maintain a balance between purification factor and enzyme activity recovery of bromelain and PPO, further experiments were conducted with PEG 1500 and PEG 4000 and potassium phosphate system.

2B.3.3 Effect of PEG concentration

The variation of partition coefficient of bromelain and PPO with an increase in the concentration of PEG (from 12 to 18% w/w) for the two different molecular weights 1500 and 4000 is shown in Fig. 2B.3. The variation in partition coefficient of bromelain (Fig. 2B.3) was not much significant and the purity increased marginally with an increase in PEG concentration (Table 2B.2). For example, increase in PEG 1500 concentration from 12 to 18w/w% resulted in 3.6 ± 0.1 to 4.0 ± 0.1 fold increase in purity due to increased partitioning of total proteins to the bottom phase (Table 2B.2).

It can be observed (from the Fig. 2B.3 and Table 2B.2) that, in all the cases an increase in PEG concentration resulted in decrease in partition coefficient of PPO and total proteins. For example, increasing the concentration of PEG 1500 from 12 to 18 w/w% resulted in decrease in partition coefficient of PPO and total proteins from 0.29 ± 0.04 to 0.19 ± 0.02 and 3.6 ± 0.1 to 2.1 ± 0.1, respectively. This decrease in partition coefficient of PPO and total proteins is due to the influence of volume exclusion, which increases with an increase in polymer concentration. The observed influence of PEG concentration on partition coefficient of bromelain is low probably due to its low molecular weight (molecular weight of fruit bromelain 23 kDa) when compared to that of PPO (molecular weight of PPO ≈ 104 kDa).

2B.3.4 Effect of potassium phosphate concentration

ATPE experiments were conducted by increasing potassium phosphate concentration from 14 w/w% to 20 w/w%. The effect of potassium phosphate concentration on the partitioning coefficient of bromelain and PPO is shown in Fig. 2B.4. The increase in potassium phosphate concentration from 14 to 20 w/w% resulted in an increase in partition coefficients of bromelain, PPO and total proteins (Fig. 2B.4 and Table 2B.3). For instance, in case of PEG 1500 the partition coefficient of bromelain and PPO (Fig. 2B.4) increased from 5.7 ± 0.1 to 17.3 ± 0.1 and 0.31 ± 0.05 to 0.70 ± 0.04, respectively. This could be due to “salting out effect” (Fig. 2B.1d), which resulted in increased partitioning of the both the enzymes to the top phase. However, the enzyme activity recovery of bromelain in the top phase was low at higher potassium phosphate concentration (Table 2B.3) due to precipitation

of enzyme at the interface (as per visual observation). The decrease in purity of bromelain at the top phase is due to increased partitioning of proteins (Table 2B.3). Significant change in partition coefficient of sugar was not observed. From the Table 2B.3, it can be observed that the purity of PPO increased with an increase in potassium phosphate concentration even though there is a decrease in activity recovery in the bottom phase. This is due to relatively more partitioning of proteins to the top phase when compared to that of PPO.

2B.3.5 Effect of system pH

The pH of the system will influence the ionizable groups of a protein and alters the protein surface charges. Proteins/enzymes above their isoelectric point will partition more to the top phase as the pH increases. The effect of pH over the range of 6.0 to 9.0 on the partition coefficient of bromelain and PPO is shown in Figure 2B.5. The pH of the system was adjusted by the addition of acid (1 N HCl) or alkali (1 N NaOH). In case of bromelain, an increase in pH resulted in an increase in partition coefficient (Figure 2B.5), since, the pH used in the experiment was above the isoelectric point of the bromelain (isoelectricpoint of bromelain is 4.6). Where as in case of PPO, an increase in pH of the system did not show any significant effect on the partition behavior. This could be due to the presence of isoenzymes and variation in PPO isoelelctic point over a range.

In summary, optimal results were observed for PEG 1500 (18%) and potassium phosphate (14%) system at pH 7.0 resulting in bromelain and PPO

purity of 4.0 ± 0.1 and 2.7 ± 0.1 fold, respectively. The corresponding partition coefficients were 4.2 ± 0.1 and 0.12 ± 0.02, respectively. This ensured differential partitioning of bromelain and PPO in top and bottom phases.

2B.4. Conclusions

Differential partitioning of bromelain to the top phase and PPO to the bottom phase could be achieved by ATPE. PEG molecular weight and potassium phosphate concentration were found to have prominent effect on partition coefficient and purity of these enzymes. PEG 1500 and potassium phosphate system shows selective partitioning of bromelain and PPO with highest enzyme activity recovery and purity.

Table 2B.1: Variation in phase volume ratio, activity recovery and purification factor at different molecular weights of polyethylene glycol

PEG molecular weight[a]	Volume ratio	K_P	K_S	R_{Br}	R_{PPO}	PF_{Br}	PF_{PPO}
1500	0.82 ± 0.02	3.2 ± 0.1	0.53 ± 0.01	218.9 ± 3.0	79.8 ± 1.0	3.7 ± 0.1	2.9 ± 0.12
4000	0.67 ± 0.02	2.1 ± 0.1	0.61 ± 0.02	160.7 ± 3.0	85.6 ± 1.2	3.1 ± 0.1	2.1 ± 0.10
6000	0.62 ± 0.02	1.9 ± 0.1	0.51 ± 0.01	143.6 ± 2.2	90.0 ± 1.2	2.7 ± 0.1	1.9 ± 0.10
20000	0.55 ± 0.01	1.6 ± 0.1	0.49 ± 0.01	117.6 ± 2.2	94.4 ± 1.0	2.4 ± 0.1	1.7 ± 0.10

[a] 14 (w/w)% PEG+14 (w/w)% potassium phosphate (K_2HPO_4 and KH_2PO_4 in the ratio of 1.82:1.0)

Table 2B.2: Variation in phase volume ratio, activity recovery and purification factor at different concentrations of polyethylene glycol

PEG (w/w)%	Phosphate (w/w)% [a]	Volume ratio	K_P	K_S	R_{Br}	R_{PPO}	PF_{Br}	PF_{PPO}
PEG 1500/Potassium phosphate system								
12	14	0.71 ± 0.01	3.6 ± 0.10	0.62 ± 0.02	211.2 ± 2.2	78.0 ± 1.0	3.6 ± 0.10	2.9 ± 0.10
14	14	0.82 ± 0.02	3.2 ± 0.10	0.53 ± 0.01	218.9 ± 3.0	79.8 ± 1.2	3.7 ± 0.10	2.9 ± 0.10
16	14	1.07 ± 0.01	2.5 ± 0.08	0.59 ± 0.03	226.6 ± 3.2	85.1 ± 1.2	3.9 ± 0.10	2.8 ± 0.10
18	14	1.12 ± 0.02	2.1 ± 0.06	0.58 ± 0.03	228.4 ± 4.2	89.9 ± 1.0	4.0 ± 0.10	2.7 ± 0.10
PEG 4000/Potassium phosphate system								
12	14	0.61 ± 0.01	2.7 ± 0.10	0.63 ± 0.01	159.2 ± 2.0	83.1 ± 1.0	2.9 ± 0.10	2.2 ± 0.10
14	14	0.67 ± 0.02	2.1 ± 0.10	0.61 ± 0.02	160.7 ± 3.0	85.6 ± 1.2	3.1 ± 0.10	2.1 ± 0.10
16	14	0.83 ± 0.01	1.5 ± 0.08	0.60 ± 0.01	163.2 ± 2.0	89.3 ± 1.0	3.3 ± 0.10	2.0 ± 0.10
18	14	0.97 ± 0.01	1.3 ± 0.08	0.60 ± 0.02	169.0 ± 3.0	91.3 ± 1.2	3.5 ± 0.10	2.0 ± 0.10

[a] K_2HPO_4 and KH_2PO_4 in the ratio of 1.82:1.0

Table 2B.3: Variation in phase volume ratio, activity recovery and purification factor at different concentrations of phosphate

PEG (w/w)%	Phosphate (w/w)%[a]	Volume ratio	K_P	K_S	R_{Br}	R_{PPO}	PF_{Br}	PF_{PPO}
PEG 1500/Potassium phosphate system								
12	14	0.71 ± 0.01	3.6 ± 0.10	0.62 ± 0.02	211.2 ± 2.2	78.0 ± 1.0	3.6 ± 0.08	2.9 ± 0.10
12	16	0.58 ± 0.02	4.6 ± 0.20	0.64 ± 0.02	223.6 ± 3.0	76.5 ± 1.2	3.3 ± 0.12	2.9 ± 0.10
12	18	0.49 ± 0.01	5.9 ± 0.10	0.65 ± 0.01	194.8 ± 3.2	72.7 ± 0.8	3.1 ± 0.10	3.0 ± 0.14
12	20	0.44 ± 0.02	7.6 ± 0.20	0.66 ± 0.01	147.5 ± 2.0	65.1 ± 1.2	2.4 ± 0.10	3.1 ± 0.10
PEG 4000/Potassium phosphate system								
12	14	0.61 ± 0.01	2.7 ± 0.10	0.63 ± 0.01	159.2 ± 2.0	83.1 ± 1.0	2.9 ± 0.08	2.2 ± 0.08
12	16	0.53 ± 0.02	4.0 ± 0.20	0.64 ± 0.02	152.3 ± 2.3	77.6 ± 1.2	2.7 ± 0.10	2.6 ± 0.12
12	18	0.47 ± 0.01	4.9 ± 0.10	0.65 ± 0.01	134.6 ± 2.2	73.2 ± 1.0	2.7 ± 0.10	2.7 ± 0.10
12	20	0.43 ± 0.02	5.7 ± 0.10	0.66 ± 0.02	121.6 ± 1.4	67.4 ± 1.2	2.5 ± 0.12	2.8 ± 0.10

[a] K_2HPO_4 and KH_2PO_4 in the ratio of 1.82:1.0

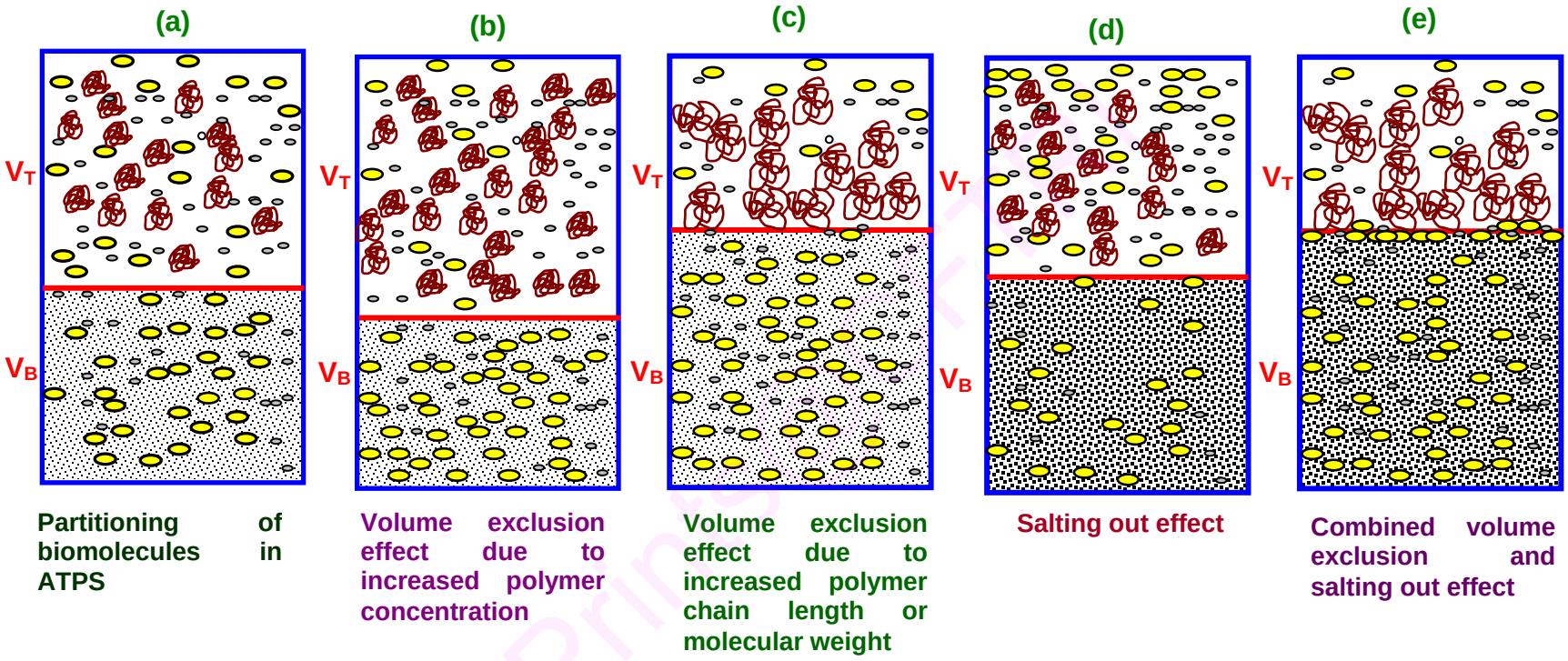


Figure 2B.1: Schematic representation of partitioning behavior of biomolecules in ATPE (a) Typical polymer/salt system; (b) Effect of increase in polymer concentration; (c) Effect of increase in chain length or molecular weight; (d) Effect of increase in salt concentration; (e) Combined volume exclusion and salting out effect (- Polymer; - Enzymes/Proteins; -Salt; V_T – volume of top phase; V_B – volume of bottom phase)

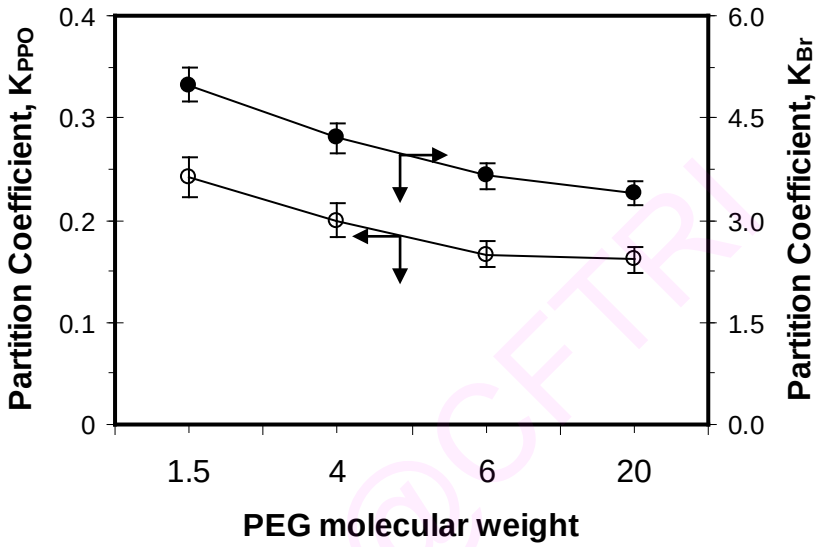


Figure 2B.2: Effect of PEG molecular weight on the partition coefficient (open symbols represent partition coefficient of PPO and closed symbols represent partition coefficient of bromelain)

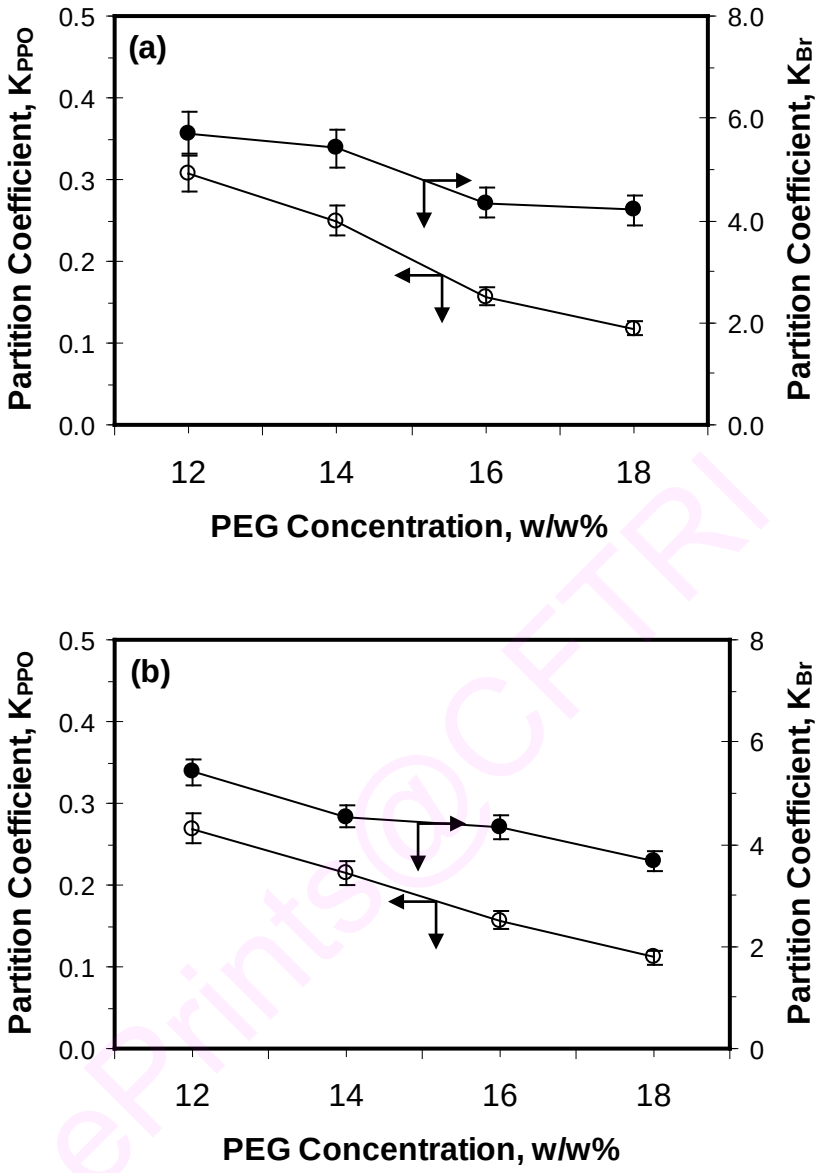


Figure 2B.3: Effect of PEG concentration on partition coefficient (a). PEG 1500 (12 to 18 w/w%)/potassium phosphate (14 w/w%) system; (b). PEG 4000 (12 to 18 w/w%)/potassium phosphate (14 w/w%) system (open symbols represent partition coefficient of PPO and closed symbols represent partition coefficient of bromelain)

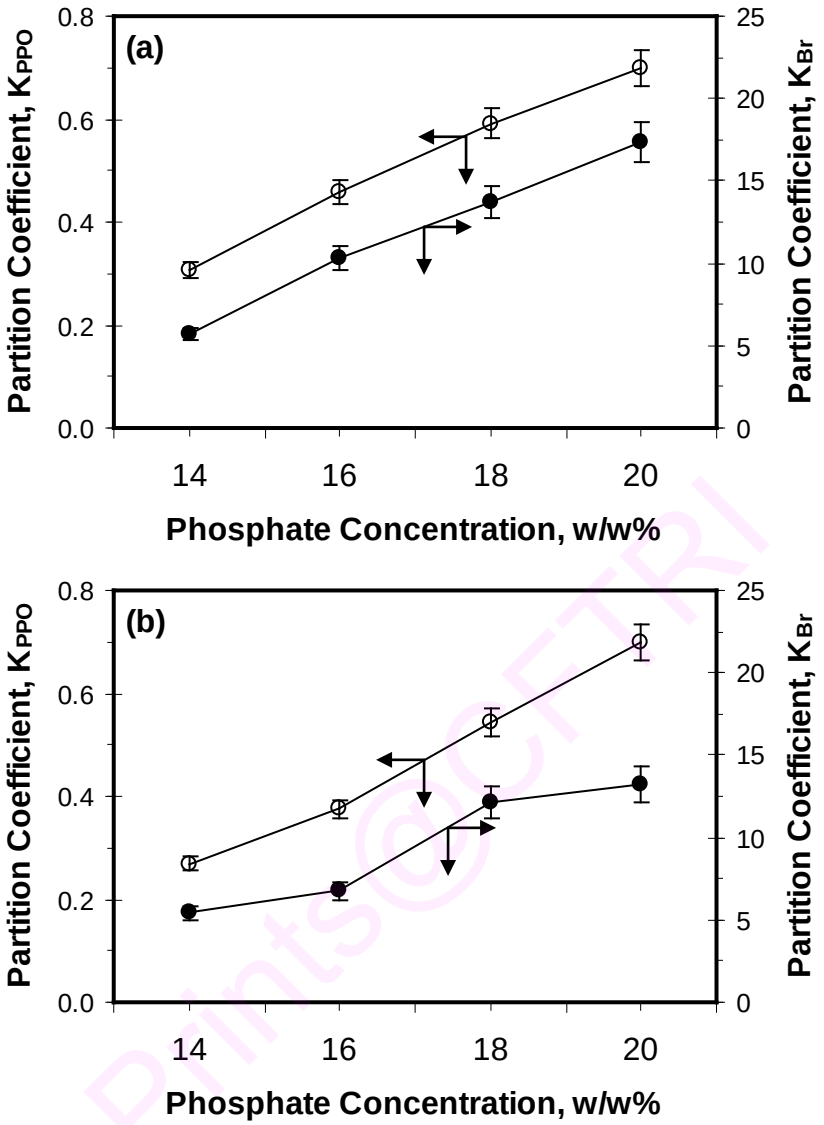


Figure 2B.4: Effect of Potassium phosphate concentration on partition coefficient (a). PEG 1500 (12 w/w%)/potassium phosphate (14-20 w/w%) system; (b). PEG 4000 (12 w/w%)/potassium phosphate (14-20 w/w%) system (open symbols represent partition coefficient of PPO and closed symbols represent partition coefficient of bromelain)

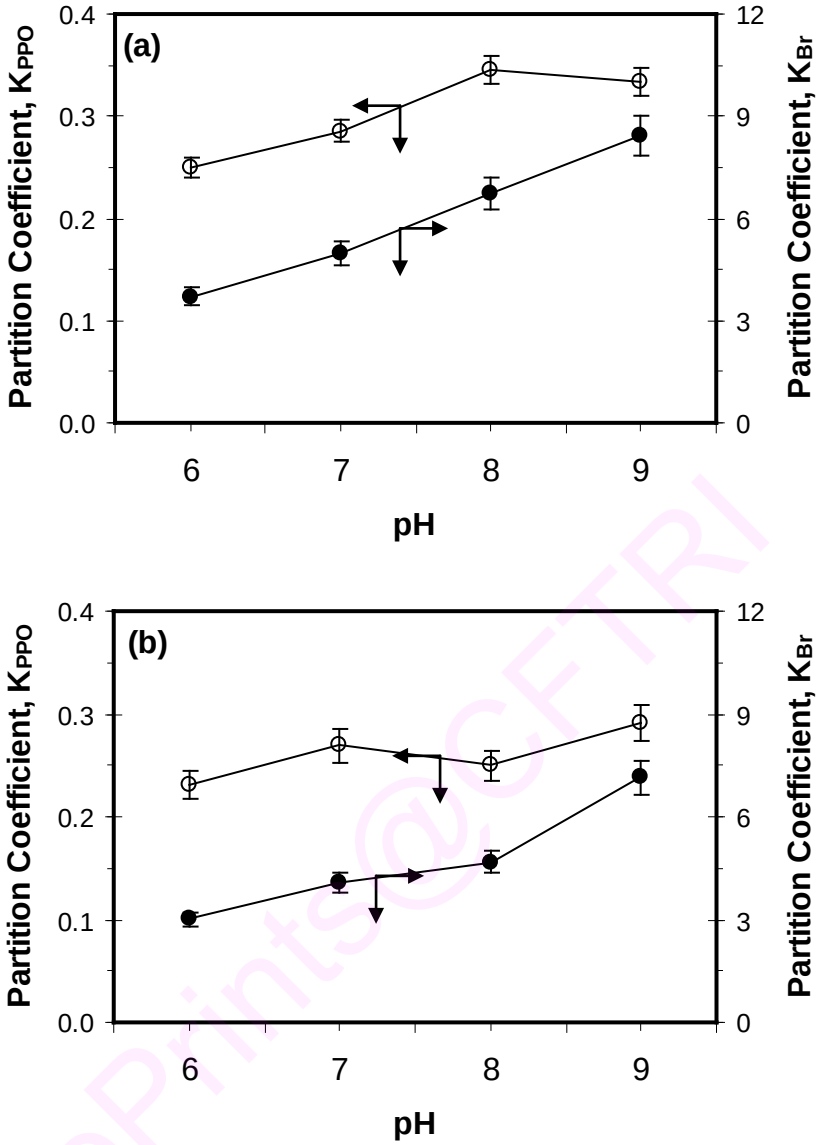


Figure 2B.5: Effect of pH on partition coefficient (a). PEG 1500 (14 w/w%)/potassium phosphate (14 w/w%) system; (b). PEG 4000 (14 w/w%)/potassium phosphate (14 w/w%) system (open symbols represent partition coefficient of PPO and closed symbols represent partition coefficient of bromelain)

CHAPTER - 3

Direct Osmosis

CHAPTER – 3A

Direct osmosis for the concentration of biomolecules

3A.1 Introduction

Most of the biomolecules extracted from their source are present in dilute form. Concentration of dilute enzyme extract is required in order to improve the stability and for easy storage, transportation and to reduce the water load on further final purification steps such as freeze drying and crystallization (Ghosh, 2003; Raghavarao *et al*., 2005). At laboratory scale, biomolecules are concentrated by dialysis against polyethylene glycol and centrifugal ultrafiltration. The main drawback of these existing processes is scale up. In recent years, ultrafiltration is gaining importance for the concentration of biomolecules (Ghosh, 2003). It is also associated with several drawbacks such as membrane fouling and concentration polarization. Hence, there is a need to develop an alternative membrane process for the concentration of biomolecules. Direct osmosis appears to be feasible method for the concentration of biomolecules, since it operates at ambient temperature and atmospheric pressure without causing any heat or shear damage to the product and more importantly it offers easy scale up (Rahgavarao *et al.*, 2005).

When miscible solutions of different concentration are separated by a membrane that is permeable to the solvent but nearly impermeable to the solute, diffusion of solvent occurs from the less concentrated to the more concentrated solution, where the solvent activity is lower. The diffusion of solvent is called osmosis and it can be stopped by increasing the pressure of the concentrated solution until the activity of the solution of the solvent is the same on both sides of the membrane (Mccabe et al., 1993). During direct

osmosis process transfer of water occurs from the feed side to osmotic agent side till the osmotic pressures on both the sides become equal (Figure 3A.1). Any solution having osmotic pressure greater than feed can be used as an osmotic agent solution. Generally concentrated solutions of sodium chloride, sucrose, fructose or high fructose corn syrup are used as osmotic agents during direct osmosis process (Wong and Winger, 1999). The parameters, which affect the direct osmosis process performance, are osmotic pressure difference between feed and osmotic agent, physical properties of feed as well as osmotic agent and membrane characteristics such as thickness and water permeability (Raghavarao *et al*., 2005). Direct osmosis offers several advantages such as low energy consumption and achievement of higher concentration (45-60°Brix) without product deterioration (Wong and Winger, 1999).

Direct osmosis finds its application in the concentration of fruit juices, vegetable juices, skim coconut milk, skim milk and coffee extract etc. Direct osmosis process can also be used for the concentration of pharmaceutical products and dealcoholization of wine or beer (Wong and Winger, 1999; Herron *et al*., 2001). Eastern European farmers used to concentrate fruit juices by similar process by immersing a bag filled with fruit juice in a brine solution (Cussler, 1984). In earlier years, direct osmosis process could not be exploited commercially because of low flux due to higher thickness of membranes (Wong and Winger, 1999). With the advent of thin membranes in recent years, increased flux are realized and hence the direct osmosis process has been gaining due importance for the concentration of heat

sensitive liquid foods/natural colors (Wrolstad *et al*., 1993; Petrotos *et al*., 1998; Rodriguez-Saona *et al*., 2001).

Pineapple (*Ananas cosmosus*) is one of the most important tropical fruit of India. The fruit can be consumed fresh or in various forms and out of which pineapple juice is one of the popular product. According to FAO (2001), more than 80 countries produce about 14 million tonnes of pineapple annually. It is estimated that only two thirds of pineapple produced is traded in international market as a fresh fruit or processed product, while small amount of fruits can be sold at low prices in the domestic market. Pineapple core and peel are the process wastes after the juice extraction from pineapple. In recent years, considerable research has been carried out to produce value-added products from pineapple waste (Bardiya *et al*., 1996; Imandi *et al*., 2007). Bromelain is one of the commercially important enzyme present in pineapple waste (peel and core of the pineapple). It is used for various applications such as brewing, meat tenderization, prevention of diarrheora, digestive aids and treatment of edema (Maurer, 2001). In this chapter, concentration of bromelain from pineapple waste (peel and core of the pineapple) by direct osmosis process is discussed. The effect of various process parameters such as osmotic agent concentration and flow rate of feed and osmotic agent on transmembrane flux is studied.

3A.2 Materials and Methods

3A.2.1 Materials

3A.2.1.1 Membranes

Direct osmosis membranes from Osmotek, Inc., Corvallis, OR, were used for all the experiments. The direct osmosis membranes are asymmetric, which have a very thin semi-permeable non-porous active skin layer and porous support layer. A nylon mesh was incorporated into the porous support layer for increased strength.

3A.2.1.2 Raw material

The pineapple fruits (*Ananas comosus* L. Merryl) used in this study was obtained from the local market. The peel and core portion of the fruits were separated and stored at 4°C for enzyme extraction.

3A.2.1.3 Chemicals

Sodium chloride, sucrose, trichloroacetic acid and sodium acetate were procured from Ranbaxy Ltd., India. Casein (Hammarsten grade) was procured from Loba Chemicals, India. Bovine Serum Albumin (BSA) was procured from SRL Ltd., India.

3A.2.2 Methods

3A.2.2.1 Enzyme Extract

The frozen pieces of pineapple peel and core were homogenized in cold extraction buffer (0.01 M sodium phosphate buffer, pH 7, containing 1% polyvinyl pyrolodine) in 1:1.5 ratio and homogenate was filtered. The filtrate was centrifuged at 10,000 rpm (4°C, 20 min) and the supernatant (crude enzyme extract) was used for further experiments.

3A.2.2.2 Osmotic agent solution

Osmotic agent solutions were prepared by dissolving known quantity of sucrose and sodium chloride in distilled water.

3A. 2.2.3 Estimation of Osmotic pressure

The osmotic pressure (π) of the concentrated solution is related to water activity of the solution as given by the following equation (Toledo, 1991; Rastogi and Raghavarao, 1994).

$$\pi = -2.3026\,(RT/V)\log a_w \qquad \ldots. (3A.\ 1)$$

where R is gas constant (8.314 J/(K.mol), T the temperature in °K, V the molar volume (18 ml/mol of water) and a_w the water activity.

The water activity can be expressed in terms of mole fraction of water by the following equation (Norrish, 1966; Toledo, 1991).

$$\log a_w = \log x_w - k(1 - x_w)^2 \quad \text{.... (3A. 2)}$$

where x_w is the mole fraction of water and k is a constant which depends upon the type of osmotic agent.

In a multi component system involving two solutes, the water activity can be expressed as (Ross, 1975)

$$a_w = (a_{w1})^0 (a_{w2})^0 \quad \text{.... (3A.3 3)}$$

where $(a_{w1})^0$ and $(a_{w2})^0$ are the water activity (from Equ. 3A. 2) of the each component in the solution.

The osmotic pressure of feed solution (enzyme extract) was taken from the literature based on the initial total soluble solids present in the enzyme extract (Perry *et al*., 1963; Girard *et al*., 2000).

3A.2.2.4 Experimental set up

The experimental setup and membrane module used in this study are shown in Fig. 3A.2 (a & b). The membrane having an area of 0.012 m^2 was placed over a polyester mesh (0.25 mm), supported between Viton gasket (3.0 mm) and two stainless steel (SS316) frames. Feed solution (enzyme extract) and osmotic agent solution were circulated on either side of the membrane in co-current mode using peristaltic pumps (Monostat, Model 72-

315-230). The transmembrane flux was calculated, by measuring the increase in volume of osmotic agent once in every hour. The temperature of feed solution and osmotic agent solution were measured using calibrated thermometer. All the experiments were conducted for the period of 4 hours and average fluxes were reported. All the experiments, unless otherwise mentioned were carried out at the temperature of 25 ± 2ºC.

3A.2.2.5 Bromelain assay

The bromelain activity was determined according to the casein digestion unit (CDU) method using casein as a substrate. One unit of bromelain activity is defined as 1 µg of tyrosine released in 1 min per ml of enzyme extract when casein is hydrolyzed under the standard conditions of 37 ± 2°C and pH 7.0 for 10 min (Murachi, 1976).

3A.2.2.6 Protein determination

Concentration of the protein was determined by dye binding method (Bradford, 1976) using Coomassie Blue G250. Optical density was measured at 595 nm. The concentration of the protein was determined from the standard graph. Bovine Serum Albumin (BSA, SRL Chemicals, India) was used as a standard.

3A. 2.2.7 Overall mass transfer coefficient

The transmembrane flux during direct osmosis process can be given as per the following equation (Petrotos, 1998; Raghavarao *et al*., 2005)

$$J = K(\pi_{OA} - \pi_{feed}) = K(\Delta\pi) \quad \ldots. \text{ (3A. 4)}$$

where K is the overall mass transfer coefficient and $\Delta\pi$ is the driving force, which is the difference between the osmotic pressure of the osmotic solution (π_{OA}) and that of pineapple juice (π_{feed}).

3A. 2.2.8 Membrane resistivity

The resistivity of the membrane support layer to solute transfer during direct osmosis process was proposed and given by Loeb *et al*., (1997)

$$k_m = (1/J)\, ln(\pi_{OA} / \pi_{feed}) \quad \text{.... (3A. 5)}$$

where k_m is the resistance to solute diffusion within the membrane porous support layer (m^2h/l), J is the water flux through the membrane (l/m^2h).

3A.3 Results and Discussion

The influence of various parameters such as concentration of osmotic agent (sodium chloride and sucrose) solution and flow rate of feed and osmotic agent on transmembrane flux which in turn affects the extent to which the enzyme extract gets concentrated (reflecting the final bromelain activity) was discussed in the following sections.

3A.3.1 Concentration of osmotic agent solution

Effect of sodium chloride (concentration in the range of 2 to 6 molality) and sucrose (concentration in the range of 1 to 4 molality) concentration on transmembrane flux during concentration of bromelain enzyme extract from the pineapple waste is presented in Fig. 3A.3. During the experiments, the

feed side and osmotic agent side flow rates were maintained at 100 ml/min. The transmembrane flux increases with an increase in concentration of the osmotic agents (both sodium chloride and sucrose). The increase in flux is attributed to an increase in osmotic pressure difference across the membrane due to the increase in the concentration of osmotic agent solution, which results in an increased driving force for water transport through the membrane. It may be noted that the sodium chloride solution shows higher transmembrane flux as compared to that of sucrose solution, due to the higher osmotic pressure of sodium chloride solution.

During direct osmosis process back transfer of sodium chloride to the extent of 1.0 ± 0.2% to 2.0 ± 0.2% was observed. In order to know the effect of sodium chloride on bromelain activity about 0 to 4% sodium chloride was added to bromelain enzyme extract from pineapple waste. From the Fig. 3A.4, it can be observed that the addition of sodium chloride in the above range did not show significant effect on bromelain activity. The increase in bromealin activity as the feed solution gets concentrated with an increase in osmotic agent (sodium chloride and sucrose) solution concentration is shown in Fig. 3A.5. An increase in osmotic agent solution concentration increased the transmembrane flux, which in turn resulted in an increase in concentration bromelain in the feed solution. It can be seen from the Fig. 3A.5 that the extent to which enzyme was concentrated was more at higher concentrations of sodium chloride (6 molality) and sucrose (4 molality). The overall mass transfer coefficient (from Equ. 3A.4) and membrane resistivity (from Equ. 3A.5) during the concentration of bromelain enzyme extract from pineapple

waste were shown in Table 3A.1. The overall mass transfer coefficient decreased with an increase in osmotic agent concentration. However, significant change in membrane resistivtiy to solute transfer was not observed.

3A.3.2 Flow rate of osmotic agent

In order to study the effect of osmotic agent flow rate, experiments were performed by varying the osmotic agent flow rate from 25 to 100 ml/min. During the study, the feed (bromelain enzyme extract) flow rate and concentration of osmotic agent solutions (sodium chloride at 6 molality and sucrose at 4 molality) were maintained constant. The variation in transmembrane flux with osmotic agent side flow rate is shown in Fig. 3A.6. Increase in osmotic agent flow rate from 25 to 100 ml/min resulted in about 19.0 ± 0.4% and 31.0 ± 0.5% increase in flux was observed for sodium chloride and sucrose, respectively. The increase in flux with an increase in flow rate can be attributed to reduction in concentration polarization layer.

The increase in osmotic agent flow rate resulted in an increase in water transport through the membrane which in turn resulted in an increase in concentration (activity) of bromelain extract (Fig. 3A.6). About 21.0 ± 0.4% increase in concentration of bromelain was observed with an increase in osmotic agent flow rate. From the above result, it can be observed that the osmotic agent flow rate has prominent effect on transmembrane flux which is indirectly affects the concentration of bromelain in feed.

3A.3.3 Flow rate of feed

Experiments were performed for by varying feed (bromelain enzyme extract) flow rate from 25 to 100 ml/min. During the study osmotic agent side flow rate (100 ml/min) and concentration (sodium chloride at 6 molality and sucrose at 4 molality) were maintained constant. It can be observed that transmembrane flux (indirectly concentration of bromelain in feed) is not much affected by feed flow rate (Fig. 3A.7). For example, in case of sodium chloride (6 molality), increasing feed (bromelain enzyme extract) flow rate from 25 to 100 ml/min resulted in about 5.7 ± 0.1% increase in transmembrane flux which in turn resulted in about 8.0 ± 0.2% increase in bromelain concentration (activity). This result is expected due to the lower feed concentration which resulted in less concentration polarization effect, and consequently the flux is less dependent on the feed flow rate.

3A.3.4 Concentration of bromelain extract

Based upon best operating conditions obtained, the concentration of bromelain extract was carried out using sodium chloride (6 molality) as osmotic agent solution. The concentration of osmotic agent solution was maintained constant during the course of the experiment. The transmembrane flux decreased as feed concentration increased as shown in Fig. 3A.8. An increase in feed concentration decreased the driving force across the membrane, which in turn decreased the transmembrane flux. Bromelain extract was concentrated from 450 ± 2 to 1800 ± 10 CDU/ml.

3A.4 Conclusions

The feasibility of direct osmosis as an alternative method for the concentration of biomolecules (such as bromelain from pineapple waste) was demonstrated. Sodium chloride solution shows higher transmembrane flux as compared to that of sucrose solution. The osmotic agent concentration and flow rate were found to be significant effect on transmembrane flux. Bromelain enzyme extract was concentrated up to 4 fold (from 450 ± 2 to 1800 ± 10 CDU/ml) at 25 ± 2˚C.

Table 3A.1: Values of overall mass transfer coefficient and membrane resistivity at various osmotic agent concentrations

S.No.	Concentration (molality)	Driving force ($\Delta\pi$) kPa	Protein concentration (mg/ml)	K X 10^4 (l/(m^2h. kPa)	K_m (m^2h/l)
Sodium chloride					
1	2	10762	0.48 ± 0.06	1.85 ± 0.10	1.94 ± 0.10
2	3	20297	0.52 ± 0.04	1.11 ± 0.10	1.99 ± 0.12
3	4	32156	0.57 ± 0.06	0.79 ± 0.10	1.94 ± 0.10
4	5	46096	0.63 ± 0.04	0.62 ± 0.14	1.86 ± 0.10
5	6	61898	0.80 ± 0.08	0.52 ± 0.12	1.75 ± 0.12
Sucrose					
6	1	2512	0.34 ± 0.06	4.48 ± 0.12	2.20 ± 0.10
7	2	5713	0.36 ± 0.08	2.35 ± 0.10	2.42 ± 0.12
8	3	9322	0.40 ± 0.06	1.77 ± 0.12	2.26 ± 0.10
9	4	13295	0.48 ± 0.04	1.56 ± 0.10	1.96 ± 0.10

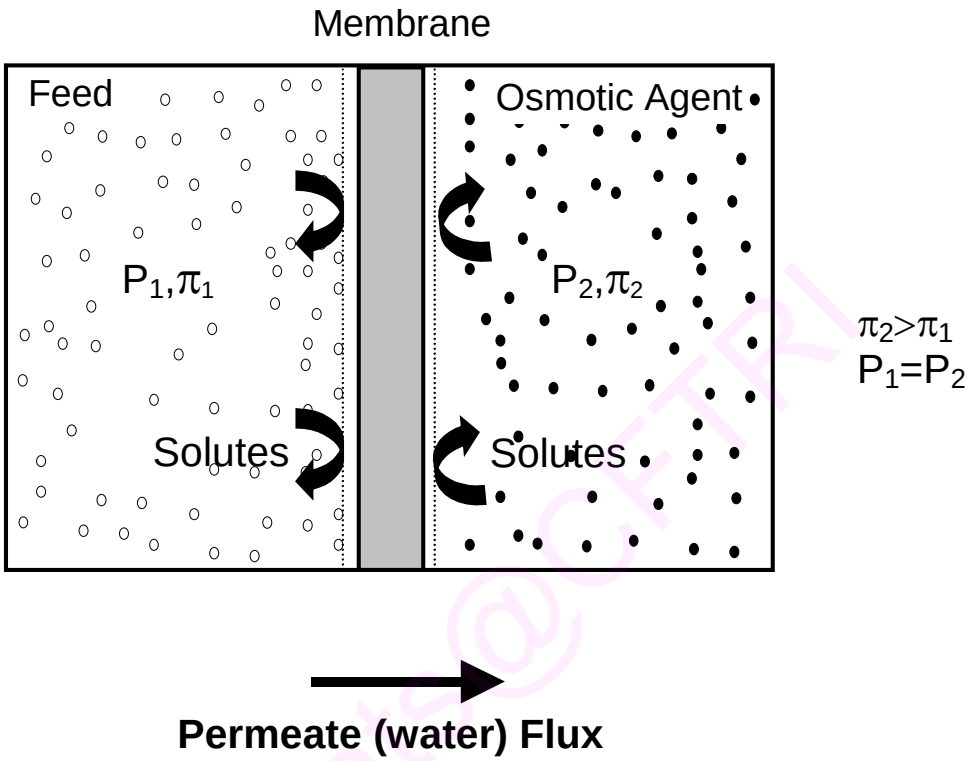


Figure 3A.1: Mechanism of direct osmosis (water transport occurs from the solution of low osmotic pressure to the solution of high osmotic pressure. π_1, π_2: Osmotic pressure of feed and osmotic agent solution; P_1, P_2: Feed side and osmotic agent side hydraulic pressure)

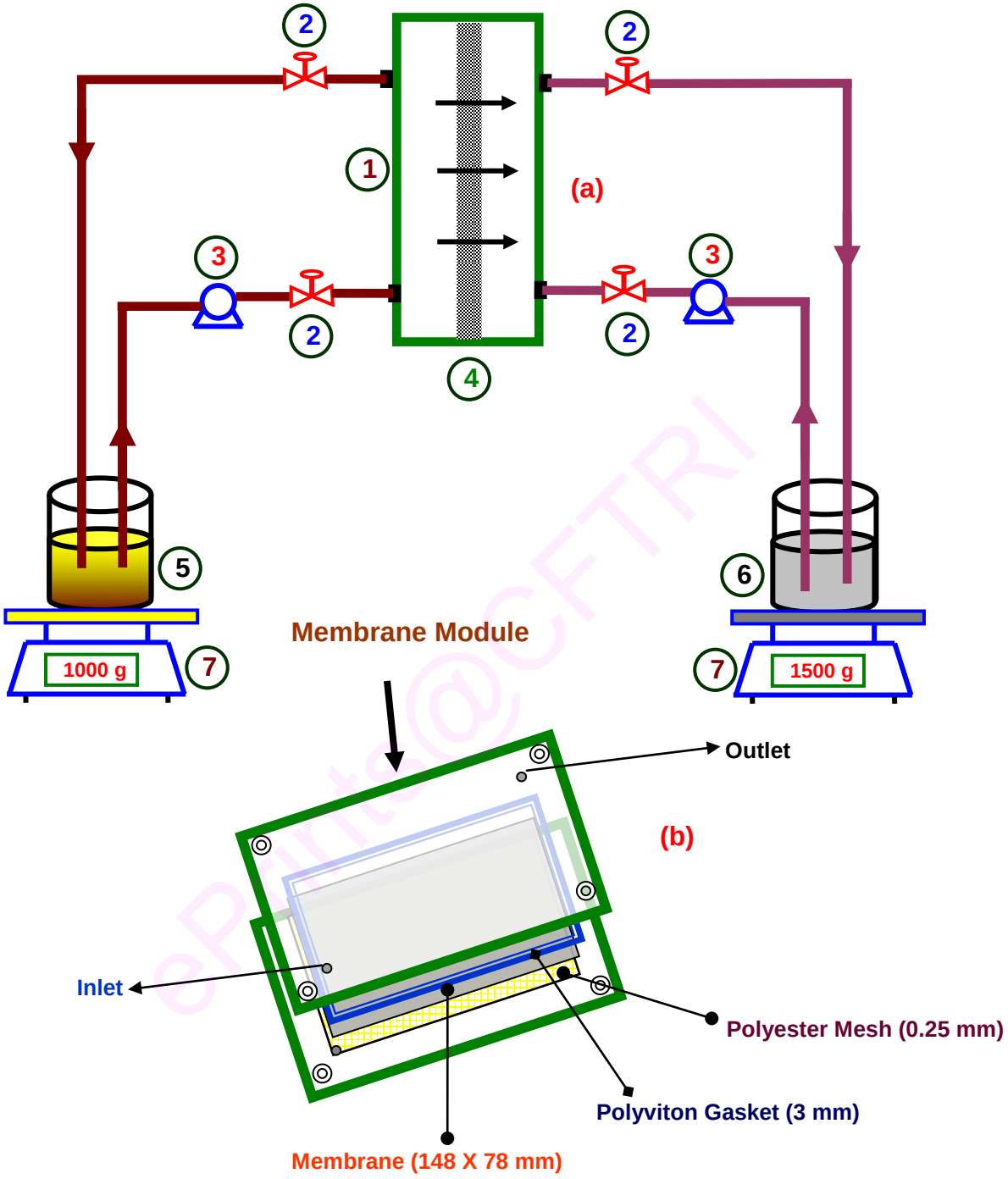


Figure 3A.2: (a) Apparatus for direct osmosis process. (1. DO flat module; 2. ball valve; 3. peristaltic pump; 4. feed reservoir; 5. osmotic agent reservoir; 6. balance); (b) DO membrane module

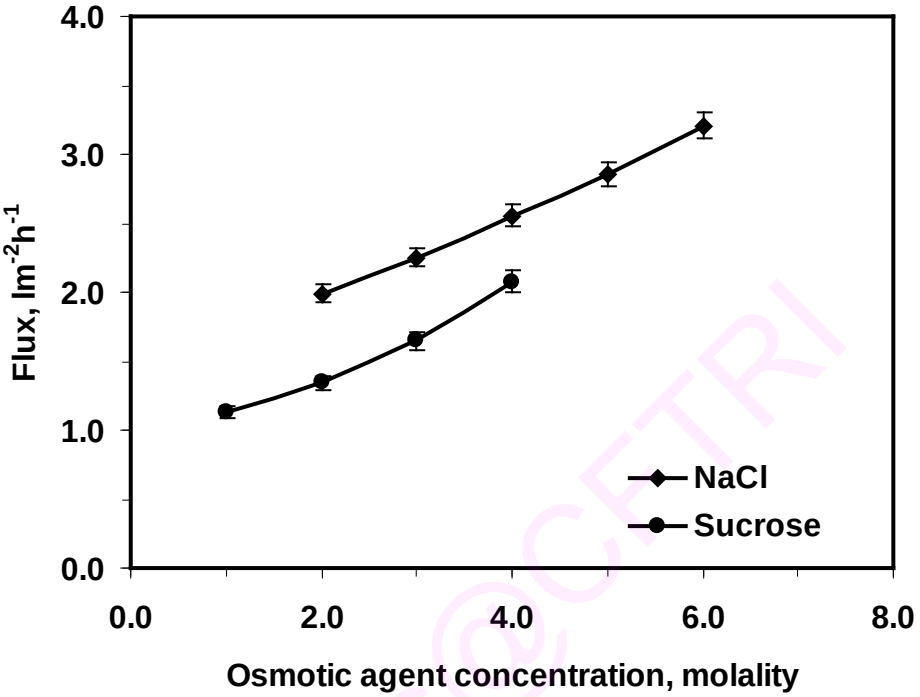


Figure 3A.3: Effect of osmotic agent concentration on transmembrane flux (feed flow rate = 100 ml min^{-1}; temperature = 25 ± 2°C; osmotic agent flow rate = 100 ml min^{-1})

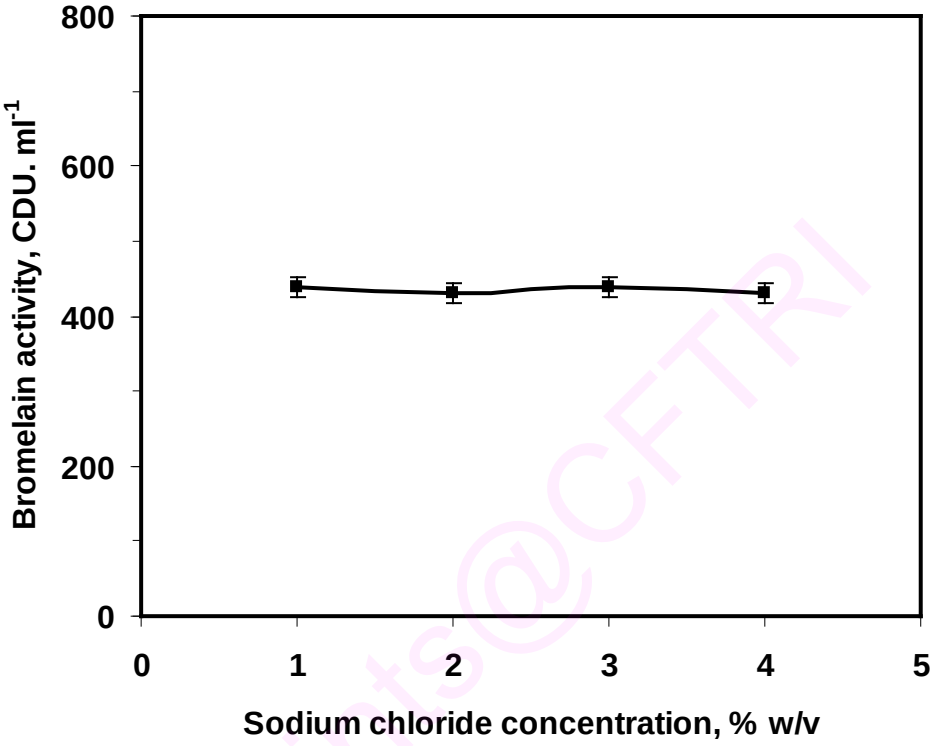


Figure 3A.4: Effect of sodium chloride addition on bromelain activity

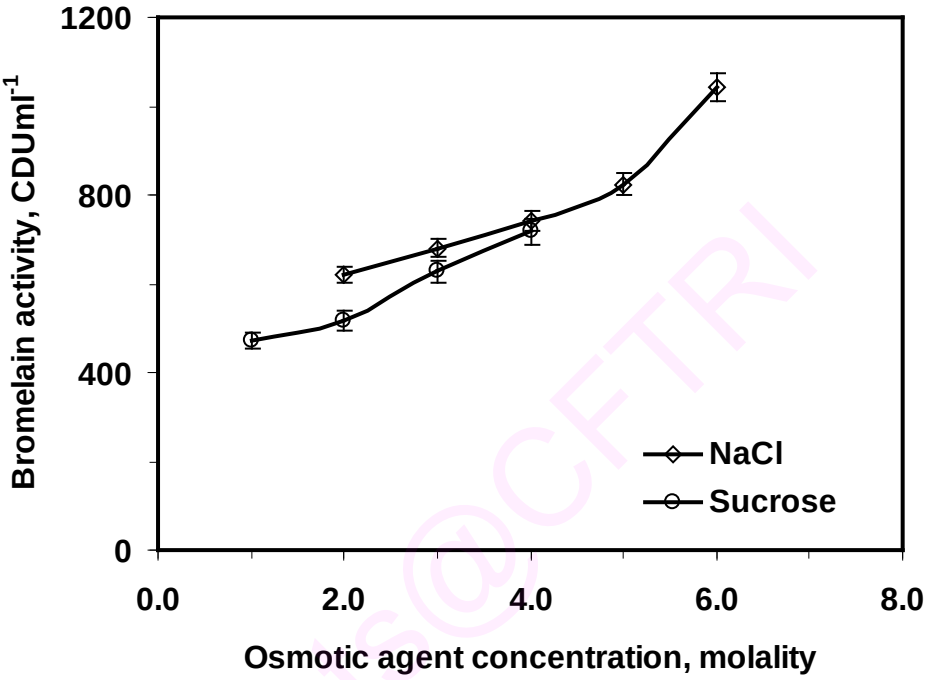


Figure 3A.5: Concentration (activity) of bromelain in feed with an increase in osmotic agent concentration (feed flow rate = 100 ml min^{-1}; temperature = 25 ± 2°C; osmotic agent flow rate = 100 ml min^{-1})

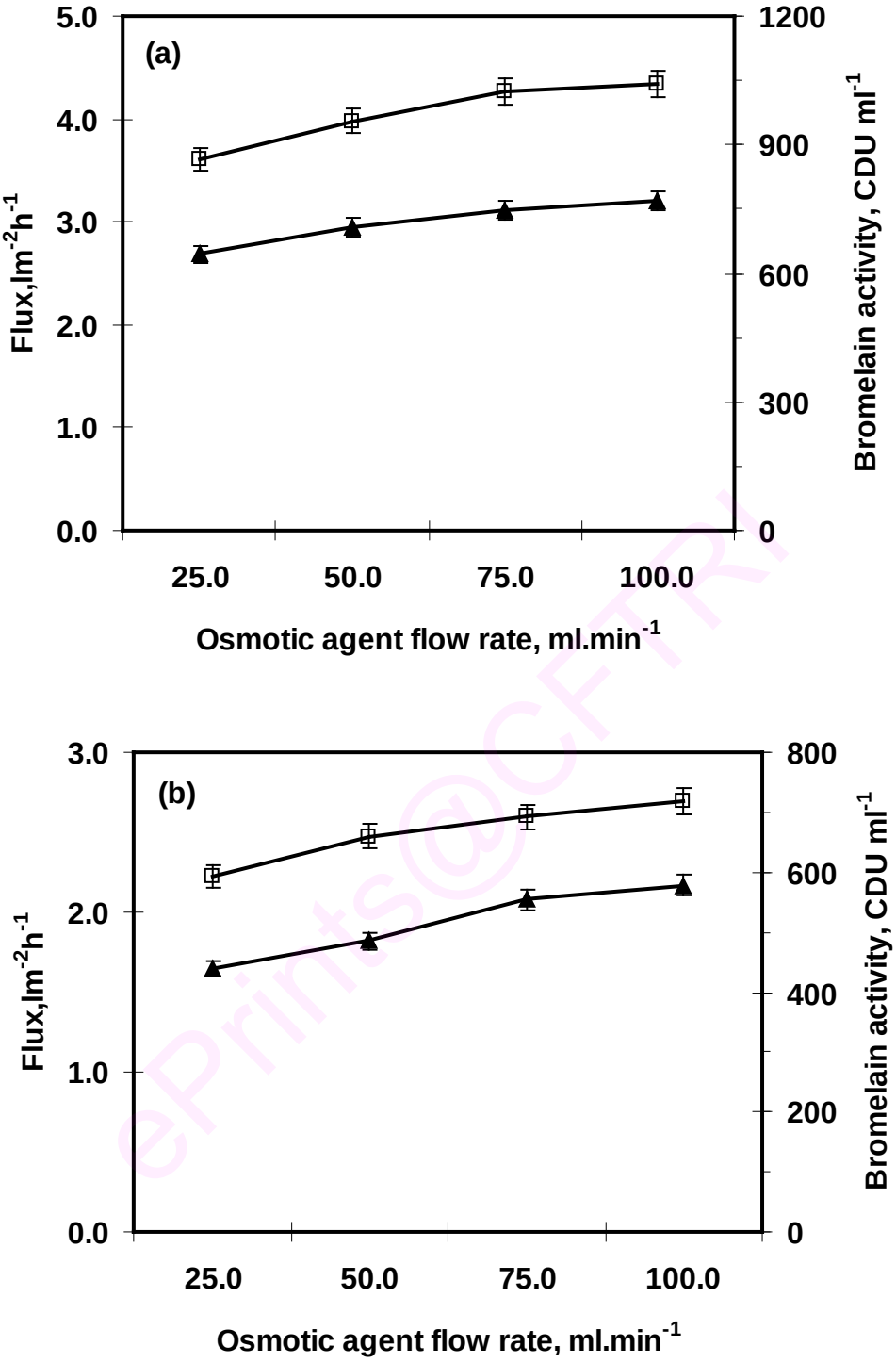


Figure 3A.6: Effect of osmotic agent flow rate on transmembrane flux and concentration (activity) of bromelain a) sodium chloride; b) sucrose (closed symbols represents flux and open symbols represents bromelain activity)

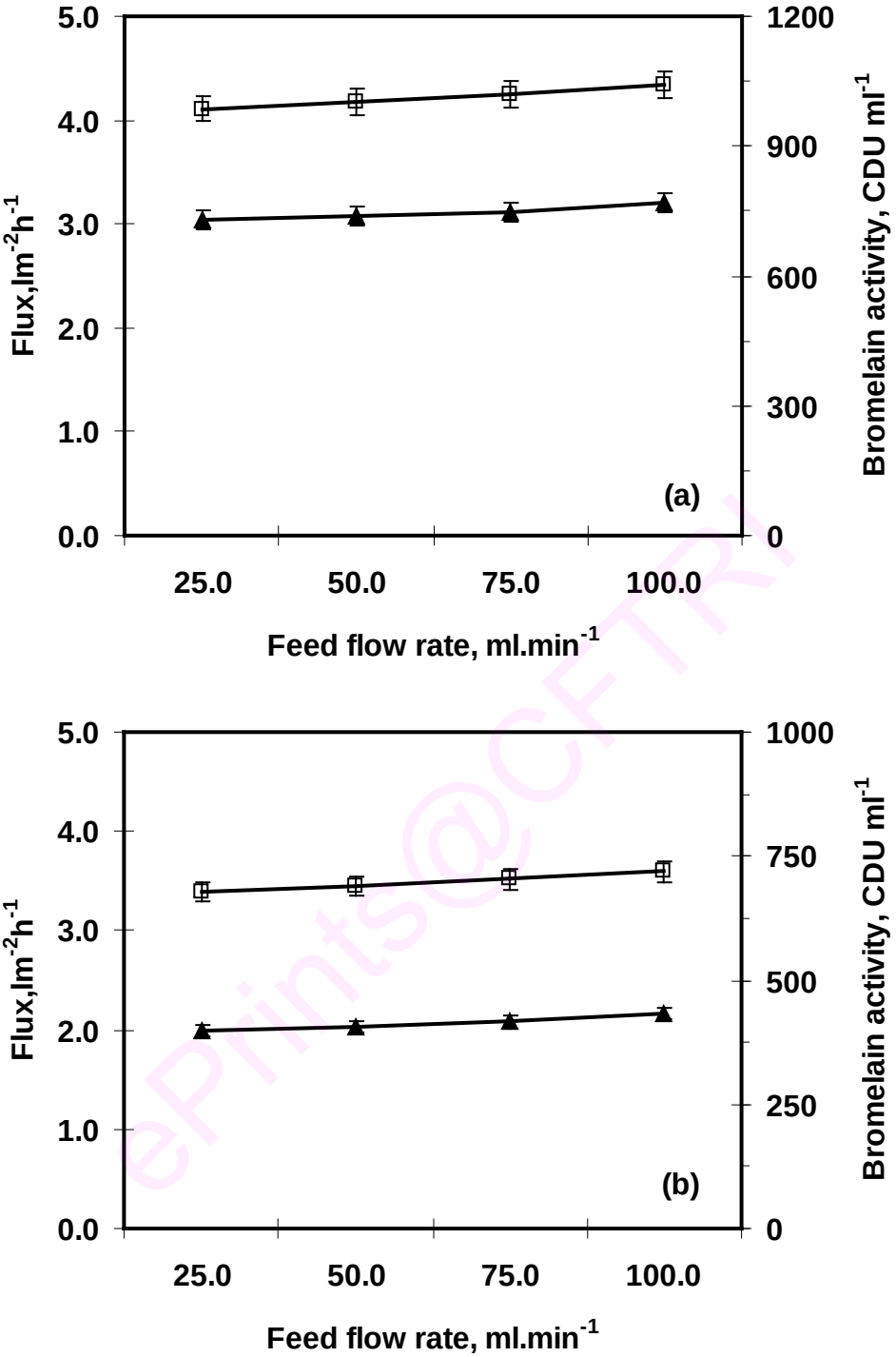


Figure 3A.7: Effect of feed flow rate on transmembrane flux and concentration (activity) of bromelain a) sodium chloride; b) sucrose (closed symbols represents flux and open symbols represents bromelain activity)

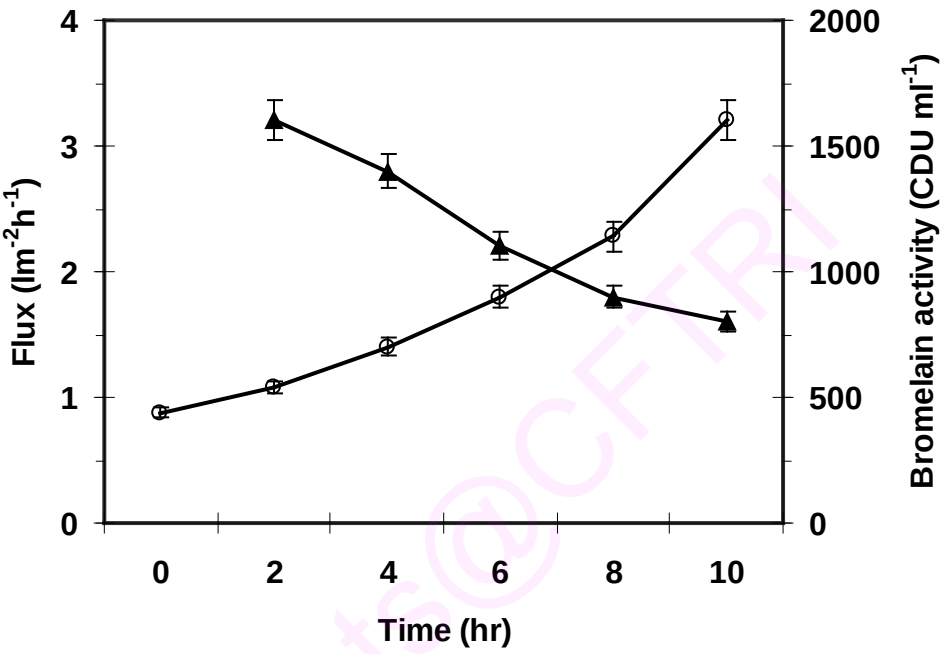


Figure 3A.8: Evolution of the transmembrane flux and bromelain concentration (activity) during direct osmosis process (closed symbols represents flux and open symbols represents activity).

CHAPTER – 3B

Direct osmosis for the concentration of fruit juices

3B.1 Introduction

Liquid foods such as fruit juices are of high nutritive value as they are naturally enriched with minerals, vitamins and other beneficial components required for human health. Removal of water helps in reducing the water activity, thereby facilitating the increase in the shelf life of these liquid foods. Hence, it is desirable to concentrate these liquid foods to improve shelf life, stability, and to reduce storage/transportation costs (Petrotos and Lazarides, 2001). Concentration of liquid foods by conventional method such as evaporation results in product deterioration with respect to loss of flavors, taste and nutritive components resulting in low quality product, besides being energy intensive (Petrotos and Lazarides, 2001). Freeze concentration is another technique employed for the concentration of fruit juices. However, the main drawback of the freeze concentration is the maximum achievable concentration (40-45 °Brix) and high investment cost (Raghavarao *et al*., 2005). Hence, in recent years, membrane processes such as microfiltration, ultrafiltration and reverse osmosis are being employed for clarification and concentration of fruit juices (Girard *et al*., 2000). The limitations of these membrane processes are maximum attainable concentration (only up to 25-30^{0}Brix), concentration polarization and membrane fouling (Narayan *et al*., 2002). Interest in newer nonthermal membrane processes for concentrating fruit juices has increased significantly in the last few years and direct osmosis is one such process (Jiao *et al*., 2004; Nagaraj *et al*., 2006). It is a non-pressure driven membrane process capable of concentrating liquid foods at ambient conditions without product deterioration.

Direct osmosis membranes cause migration of osmotic agent (salt or sugar) to the product (Popper *et al*., 1966; Wong and Winger, 1999, Girard *et al*., 2000). Most of the earlier studies reported in literature on direct osmosis dealt with either vegetable juice (Petrotos *et al*., 1998) or natural colour (Rodriguez-Saona *et al*., 2001) using concentrated salt solution as an osmotic agent. In such applications, the migration of osmotic agent into the product was not considered because migration of salt resulted in improved quality of the product. In case of concentration of fruit juices using direct osmosis by utilizing concentrated sugar solution (Girard *et al*., 2000), due to lower osmotic pressure and higher viscosity of sugar solution the transmembrane flux was low. Use of concentrated salt solution as osmotic agent is expected to result in salty taste in the fruit juices due to migration of salt. Hence, it was thought desirable to study the effect of mixed osmotic agent (salt and sucrose) on transmembrane flux. The effect of various process parameters such as osmotic agent concentration, flow rate and feed temperature on transmembrane flux during concentration of pineapple juice by direct osmosis process has been studied. The physicochemical characteristics of fresh and concentrated pineapple juice such as pH, titratable acidity, ascorbic acid content, color, density and viscosity were also studied. Also, sensory evaluation of pineapple juice concentrate by direct osmosis process was studied.

3B. 2 Materials and Methods

3B. 2.1 Materials

3B. 2.1.1 Membranes

The direct osmosis membranes (Osmotek, Inc., Corvallis, OR) were used for all the experiments. The direct osmosis membranes are asymmetric, which have a very thin semi-permeable non-porous active skin layer and porous support layer. A nylon mesh was incorporated into the porous support layer for increased strength. The active layer effectively excludes the passage of solutes with a nominal molecular weight cut-off of 100 Da. The thickness of the membrane as determined by scanning electron microscope (SEM) was found to vary between 50 to 100 μm.

3B. 2.1.2 Chemicals

Sodium chloride, sucrose and potassium chromate were procured from Ranbaxy Ltd., India, silver nitrate was procured from HiMedia Laboratories Pvt. Ltd., India.

3B. 2.2 Methods

3B. 2.2.1 Feed solution (Fruit juice)

Fresh pineapple (*Annana Comosus* L.) fruits were procured from a local market. After rinsing the fruit in tap water, the shell and core were removed using a stainless steel knife. The flesh was cut into small pieces and

the juice was extracted using a screw type juice extractor (1.00 mm sieve, Process Machinery and Equipments, Calcutta) at room temperature. The pineapple juice (12.4° Brix) was filtered using muslin cloth (600 μm) and stored at 4-5°C and used for the studies.

3B. 2.2.2 Osmotic agent solution

Osmotic agent solutions were prepared by dissolving sucrose and sodium chloride in distilled water in the proportions as mentioned in Table 3B.1. The osmotic pressure (π) of the sucrose and sodium chloride solutions are calculated by using Equ. 3A.1. The osmotic pressure of feed solution (pineapple juice) was taken from the literature based on the initial total soluble solids present in the fruit juice (Perry *et al*., 1963; Girard *et al*., 2000).

3B. 2.2.3 Experimental set up

The detail of direct osmosis experimental setup used for the concentration of pineapple juice was explained in Chapter 3A (Fig. 3A.2). Feed solution (pineapple) and osmotic agent solution were circulated on either side of the membrane in co-current mode using peristaltic pumps (Monostat, Model 72-315-230). The ratio of feed solution to osmotic agent solution was maintained at 1:10 for all the experiments conducted. The transmembrane flux was calculated, by measuring the increase in volume of osmotic agent once in every hour. The temperature of feed solution and osmotic agent solution were measured using calibrated thermometer. The feed flow rate was maintained at 100 ml/min during the study. All the experiments were conducted for the period of 18 hours, however the average

flux was calculated based on first 5 hours of the process time. All the experiments, unless otherwise mentioned, were carried out at the temperature of 25 ± 2ºC.

3B. 2.2.4 Physico-chemical characteristics

Concentration of fresh and concentrated pineapple juices, in terms of soluble solids was measured using Erma's handheld refractometer at 25 ± 2°C. Results were reported as degree Brix. The specific gravity of feed and osmotic agent solution were measured using specific gravity bottle, whereas viscosity was measured with the help of Ostwald viscometer (capillary diameter 1.0 mm) using water as a standard at 25 ± 2°C (Lees, 1975). Colour of pineapple juice was measured using Hunter Lab colorimeter (Hunter Associates Laboratory Inc., Reston, VA, USA). The data were expressed in terms of L*, a*, b* parameters; Hue angle (H^0, which gives the dominant wavelength) and color purity (C^0, which gives the depth or strength of the hue or the extent to which pure hue is admixed with white) from the tristimulus parameter L^* (lightness), a^* ($+a^*$ = red, $-a^*$ = green) and b^* ($+b^*$ = yellow, $-b^*$ = blue) as $H^0 = \tan^{-1}(b^*/a^*)$ and $C^0 = (a^{*2} + b^{*2})^{1/2}$, respectively (Cisse *et al*., 2005; Rattanathanalerk *et al*., 2005).

Around 20 ml of juice was taken and its pH was determined at room temperature (25 ± 2 °C) using pH meter (Control dynamics, Model: APX175 E/C). After determination of the pH, a known amount of fruit juice was titrated against 0.10 N sodium hydroxide using phenolphthalein as an indicator until the end point was pale pink (AOAC, 1990). Results were expressed as w/w%

of citric acid. Amount of ascorbic acid present in the fruit juice was determined by titrating known amount of fruit juice and metaphosphoric acid-acetic acid mixture against 2,6-dichlorophenol indophenol dye solution. The results are expressed as mg of ascorbic acid per 100 ml of fruit juice (AOAC, 1990; James, 1995). The sodium chloride content in the sample was estimated by the procedure given by (Ranganna, 1986).

3B. 2.2.5 Sensory Aspects:

Quantitative Descriptive Analysis (QDA) was used for evaluation of sensory properties of pineapple juice concentrate, where 15 cm scale is used anchoring at 1.25 cm as low or detection threshold and 13.75 cm as high or saturation threshold (Stone *et al*., 1974). The intensity of the attributes was quantified on the structured scale. The concentrated pineapple juice was reconstituted to single strength juice at the same concentration as the initial juice prior to sensory evaluation. A group of 8-10 panelists were trained in the preliminary sessions for descriptive sensory analysis. The members of the panel were drawn from the scientific staff, familiar with sensory analysis techniques. The panel was asked to evaluate the juice characteristics like pineapple flavor, sweetness, saltiness and overall quality. The samples were coded as shown in Table 3B. 2.

3B.3 Results and Discussion

3B.3.1 Effect of osmotic agent solution concentration on transmembrane flux

The effect of osmotic agent concentrations namely sodium chloride (6, 12 and 26 % w/w) and sucrose (20, 30, 40 % w/w) on transmembrane flux for pineapple juice was presented in Fig. 3B.1. During the experiments, the feed side and osmotic agent side flow rates were maintained at 100 ml/min. The transmembrane flux increased from 0.44 ± 0.04 to 1.39 ± 0.06 l/m^{2}h with an increase in sodium chloride solution concentration from 6 to 26 w/w% and the sodium chloride transfer through the membrane was increased from 1.0 ± 0.04 to 2.0 ± 0.1%. Whereas in case of using sucrose as osmotic agent, the transmembrane flux was increased from 0.28 ± 0.02 to 0.58 ± 0.04 l/m^{2}h with an increase in sucrose solution concentration from 30 to 50 w/w%. The increase in flux is attributed to an increase in osmotic pressure difference across the membrane due to the increase in the concentration of osmotic agent solution, which results in an increased driving force for water transport through the membrane. It may be noted that the use of sodium chloride solution as osmotic agent showed higher transmembrane flux as compared to sucrose solution, due to the higher osmotic pressure of sodium chloride solution.

3B.3.2 Effect of mixed osmotic agent concentration on transmembrane flux

In order to overcome the drawbacks associated with sucrose solution (low flux) and sodium chloride solution (saltiness in juice) as osmotic agents,

an attempt was made to explore the use of mixed osmotic agent (sodium chloride and sucrose) solutions during direct osmosis experiments. The effect of mixed osmotic agent on transmembrane flux is shown in Fig. 3B.2. In the first set of experiments, the sucrose concentration was fixed at 30 w/w% and sodium chloride concentration was varied from 0 to 16 w/w%. The addition of sodium chloride to the sucrose solution resulted in significant increase in osmotic pressure from 3511 to 52065 kPa (Table 3B.1) due to lower molecular weight of sodium chloride, which in turn increased the driving force for transmembrane flux. The addition of sodium chloride resulted in increased transmembrane flux from 0.28 ± 0.02 to 1.15 ± 0.06 l/m^{2}h, however, it led to increased sodium chloride migration, up to 1.28 ± 0.02%, to the feed side (Fig. 3B.2a).

In another set of experiments, the sodium chloride concentration was fixed at 12 w/w% and sucrose concentration was varied from 0 to 40 w/w%, which resulted in an increase in osmotic pressure from 6035 to 43532 kPa (Table 3B.1). The addition of sucrose resulted in an increased transmembrane flux (from 0.89 ± 0.04 to 1.18 ± 0.06 l/m^{2}h) and decreased sodium chloride transfer to the feed side (from 1.87 ± 0.03 to 0.58 ± 0.02%, Fig. 3B.2b). Lower sodium chloride migration to product may probably be due to the increased solution viscosity from 1.10 ± 0.06 to 9.40 ± 0.4 mPa.s (Table 3B.1) due to increasing concentration of sucrose from 0 to 40% at constant salt concentration (12%) of osmotic agent solution.

3B.3.3 Effect of osmotic agent concentration on overall mass transfer coefficient and membrane resistivity

The variation of transmembrane flux with osmotic pressure difference ($\Delta\pi$) or driving force is presented in Fig. 3B.3. It is indicated that the flux increases sharply in the beginning and later at a slower rate, with an increase in driving force. The use of sodium chloride as osmotic agent resulted in higher flux as compared to sucrose as well as mixed osmotic solute due to lower viscosity of the sodium chloride solution (Table 3B.1). The overall mass transfer coefficient (K) was estimated using Eq. (3A.1). The effect of pure (sodium chloride or sucrose) and mixed osmotic agent (combination of sucrose and sodium chloride) concentration on overall mass transfer coefficient is shown in Fig. 3B.4 and 3B.5. For all the cases the overall mass transfer coefficient (K) decreases with increase in osmotic agent concentration. Since in all the cases the concentration of pineapple juice and hydrodynamic flow conditions are same on both sides (feed side and osmotic agent solution side), the reduction may be due to change in physical properties of osmotic solution which affects the osmotic agent side film coefficient and in turn diffusion through the membrane. The physical properties such as density, viscosity and diffusivity are likely to be changed with concentration. The density of the osmotic solution varies slightly with concentration and diffusivity practically remains constant, thus the viscosity of the osmotic agent solution (Table 3B.1) is responsible for reduction in overall mass transfer coefficient. The membrane resistivity (k_m) of support layer to solute transfer was estimated using Eq. (3A.5) and presented in Table 3B.2,

which indicated that the membrane resistivity is almost constant irrespective of concentration of osmotic agent.

3B.3.4 Effect of osmotic solution combination/concentration on final feed concentration

The effect of osmotic solution combination/concentration on final feed concentration is shown in Table 3B.2. An increase in concentration of sodium chloride from 6 to 26 w/w% resulted in an increase in the concentration of pineapple juice from 14.8 ± 0.2 to 59.0 ± 0.4°Brix. Whereas, increase in concentration of sucrose solution, from 30 to 50 w/w% resulted in lower increase in the feed concentration, from 13.2 ± 0.2 to 15.8 ± 0.1°Brix. It may be attributed to low osmotic pressure and high viscosity of sucrose solution. The use of sodium chloride as osmotic agent resulted in 1.0 ± 0.2 to 2.0 ± 0.2% migration of sodium chloride towards the feed side, which rendered the product less acceptable. The use of combination of sucrose (40 w/w%) and sodium chloride (12 w/w%) yielded final product concentration of 60.0 ± 0.2°Brix with higher overall acceptability due to lower salt migration. The pineapple juice could not be concentrated further due to high viscosity.

3B.3.5 Effect of hydrodynamic conditions on transmembrane flux

In order to study the effect of hydrodynamic conditions on transmembrane flux, two sets of experiments were conducted. In the first set, the feed (pineapple juice) side Reynolds number was maintained at 8.5 (corresponding flow rate of 100 ml/min), while varying the osmotic agent solution Reynolds number from 0.2 to 1.6 (corresponding flow rate varied from

12 to 100 ml/min). In case of mixed osmotic agent solution (sucrose, 40 w/w% and sodium chloride, 12 w/w%), the transmembrane flux was found to increase from 0.92 ± 0.04 to 1.22 ± 0.06 l/m^{2}h when the Reynolds number (flow rate) was increased from 0.2 to 1.6 (Fig. 3B.6). This could be attributed to the reduction in hydrodynamic boundary layer thickness with an increase in Reynolds number (flow rate), which in turn increased the transmembrane flux.

In second set, the effect of feed side hydrodynamic conditions on transmembrane flux was studied for the pineapple juice over a range of concentration from 12.4 ± 0.2 to 50.0 ± 0.2°Brix. Experiments were performed by varying the feed (pineapple juice) side Reynolds number from 0.2 to 8.5 (corresponding feed flow rate from 25 to 100 ml/min), while maintaining the mixed osmotic agent solution (sucrose, 40 w/w% and sodium chloride, 12 w/w%) Reynolds number at 1.6 (corresponding osmotic agent flow rate is 100 ml/min). The transmembrane flux was found to increase with an increase in Reynolds number for different feed concentration from 12.4 ± 0.2 to 50.0 ± 0.2°Brix (Fig. 3B.7 and 3B.8). The concentration polarization effect was more prominent at higher concentrations of the pineapple juice due to the higher viscosity at that concentration, which results in greater concentration polarization effect or increased hydrodynamic boundary layer thickness.

3B.3.6 Effect of feed temperature on transmembrane flux

Experiments were carried out by varying the feed temperature over the range of 25.0 ± 2.0 - 45.0 ± 2.0°C. During the course of experiments both feed and mixed osmotic agent solution (sucrose (40 w/w%) - sodium chloride (12

w/w%)) were circulated at constant flow rate of 100 ml/min. The effect of feed temperature on transmembrane flux is as shown in Fig. 3B.9. About 78.0 ± 2.0 % increase in transmembrane flux was observed with an increase in temperature from 25.0 ± 2.0 – 45.0 ± 2.0°C. This may be due to the increase in temperature lowers the viscosity of solution and increases the diffusion coefficients, which results in an increase in transmembrane flux (Wrolstad *et al*., 1993; Petrotos *et al*., 1998).

3B.3.7 Sensory evaluation

The result of the sensory analysis of pineapple juice concentrate (is shown in Fig. 3B.10) indicated that the sample E was having lower saltiness, higher sweetness and overall acceptability. The physico-chemical properties indicated the higher retention of ascorbic acid content and lower salt migration (0.58%) during the concentration process (Table 3A.3). The presence of high level of sugar in the product can reduce the saltiness threshold, and also presence of salt has enhancing effect on sucrose sweetness (Amerine, *et al*., 1965; Sacchetti, 2001; Ade-Omowaye, 2002). The use of combined solutes is expected to result in better sensory quality of the product by controlling the diffusion of lower molecular weight solute (NaCl) into the product during direct osmosis process.

3B.4 Conclusions

In case of direct osmosis, the use of mixed osmotic agent (sucrose 40% w/w% and sodium chloride 12 w/w%) for the concentration of pineapple juice up to 60.0 ± 0.2°Brix resulted in higher fluxes with lower salt migration

(0.58 ± 0.02 %). The hydrodynamic conditions on osmotic agent and feed side were found to have significant effect on transmembrane flux and the effect was more prominent at higher feed concentration. The transmembrane flux was found to increase with an increase in feed temperature. The sucrose-sodium chloride combination was able to overcome the drawback of sucrose (low flux) and sodium chloride (salt migration) as osmotic agents during direct osmosis process. The sensory analysis indicated that the concentrated pineapple juice using mixed osmotic agent was found to be most preferable.

Table 3B.1: Values of concentration of sucrose and sodium chloride used in the preparation of osmotic solutions

Osmotic agent solution No.	Sucrose (w/w%)	Sodium chloride (w/w%)	Osmotic Pressure (π), kPa	Density Kg/m^3	Viscosity (mPa.s)
1	30	0	3511	1129 ± 3	2.54 ± 0.10
2	40	0	5770	1198 ± 4	4.77 ± 0.20
3	50	0	9264	1245 ± 5	8.98 ± 0.40
4	0	6	4573	1062 ± 2	1.10 ± 0.06
5	0	12	13896	1086 ± 3	1.30 ± 0.08
6	0	26	62331	1214 ± 3	1.85 ± 0.10
7	10	12	17710	1130 ± 4	1.44 ± 0.10
8	20	12	23068	1180 ± 3	2.16 ± 0.20
9	30	12	30990	1231 ± 2	4.02 ± 0.25
10	40	12	43532	1291 ± 4	9.40 ± 0.40
11	30	4	8004	1165 ± 5	2.89 ± 0.10
12	30	8	16787	1197 ± 4	3.28 ± 0.20
13	30	16	52065	1269 ± 5	5.29 ± 0.30

Table 3B.2: Values of k_m and final concentration of the pineapple juice at various osmotic agent concentrations

S.No.	Concentration (w/w%)		Flux $l\ m^{-2}h^{-1}$	Driving force ($\Delta\pi$), kPa	k_m $m^2h^1\ l^{-1}$	Final feed conc. °Brix
	Sucrose	NaCl				
Pure osmotic agent						
1	0	6	0.44 ± 0.04	2781	2.11 ± 0.10	14.8 ± 0.2
2	0	12	0.89 ± 0.04	12104	2.30 ± 0.10	46.4 ± 0.2
3	0	26	1.39 ± 0.06	60539	2.55 ± 0.12	59.0 ± 0.4
4	30	0	0.28 ± 0.02	1719	2.40 ± 0.10	13.2 ± 0.2
5	40	0	0.44 ± 0.02	3978	2.66 ± 0.12	14.4 ± 0.1
6	50	0	0.58 ± 0.04	7472	2.83 ± 0.10	15.8 ± 0.1
Mixed osmotic agent						
7	10	12	0.98 ± 0.02	15918	2.34 ± 0.10	49.4 ± 0.2
8	20	12	1.07 ± 0.02	21276	2.39 ± 0.12	49.8 ± 0.2
9	30	12	1.13 ± 0.06	29198	2.52 ± 0.11	57.0 ± 0.1
10	40	12	1.18 ± 0.06	41740	2.70 ± 0.12	60.0 ± 0.2
11	30	4	0.64 ± 0.02	6212	2.34 ± 0.10	16.4 ± 0.2
12	30	8	0.87 ± 0.03	14995	2.57 ± 0.12	48.6 ± 0.1
13	30	16	1.15 ± 0.06	50273	2.93 ± 0.10	58.0 ± 0.2

Table 3B.3: Comparison of physico-chemical characteristics of pineapple juices

Characteristic	Fresh juice	DO juice concentrate	Reconstituted juice
pH	3.62 ± 0.10	3.85 ± 0.10	3.70 ± 0.10
Titratable acidity (w/w% citric acid)	0.80 ± 0.10	2.50 ± 0.20	0.75 ± 0.10
Ascorbic acid (mg/100ml)	12.50 ± 1.0	45.0 ± 2.0	12.10 ± 1.0
°Brix	12.40 ± .10	60.0 ± 0.20	12.4 ± 0.20
Density (kg/m^3)	1060 ± 2.0	1260 ± 4.0	1070 ± 4.0
Viscosity (mPa.s)	1.40 ± 0.20	35.0 ± 2.0	1.60 ± 0.20
NaCl concentration (%)	-	1.70 ± 0.02	0.58 ± 0.02
L*	40.18 ± 1.0	25.34 ± 1.0	39.14 ± 1.0
a*	3.64 ± 0.6	7.84 ± 0.6	3.87 ± 0.3
b*	19.83 ± 1.0	13.49 ± 1.0	19.44 ± 1.0
Hue angle (H^0)	79.60 ± 1.2	59.84 ± 0.06	78.74 ± 0.3
Color purity (C^0)	20.17 ± 1.1	15.60 ± 1.2	19.82 ± 1.1

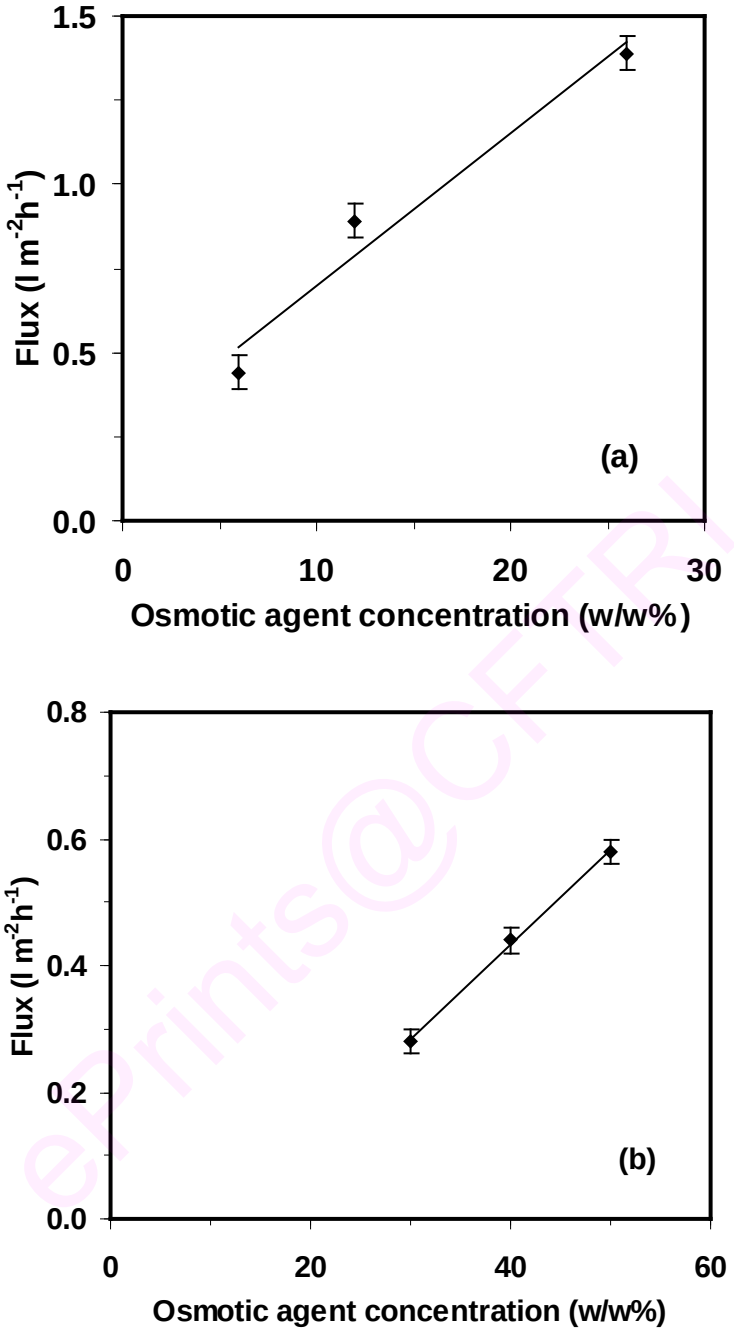


Figure 3B.1: Effect of osmotic agent solution concentration on transmembrane flux during the concentration of pineapple juice (a) sodium chloride; (b) sucrose

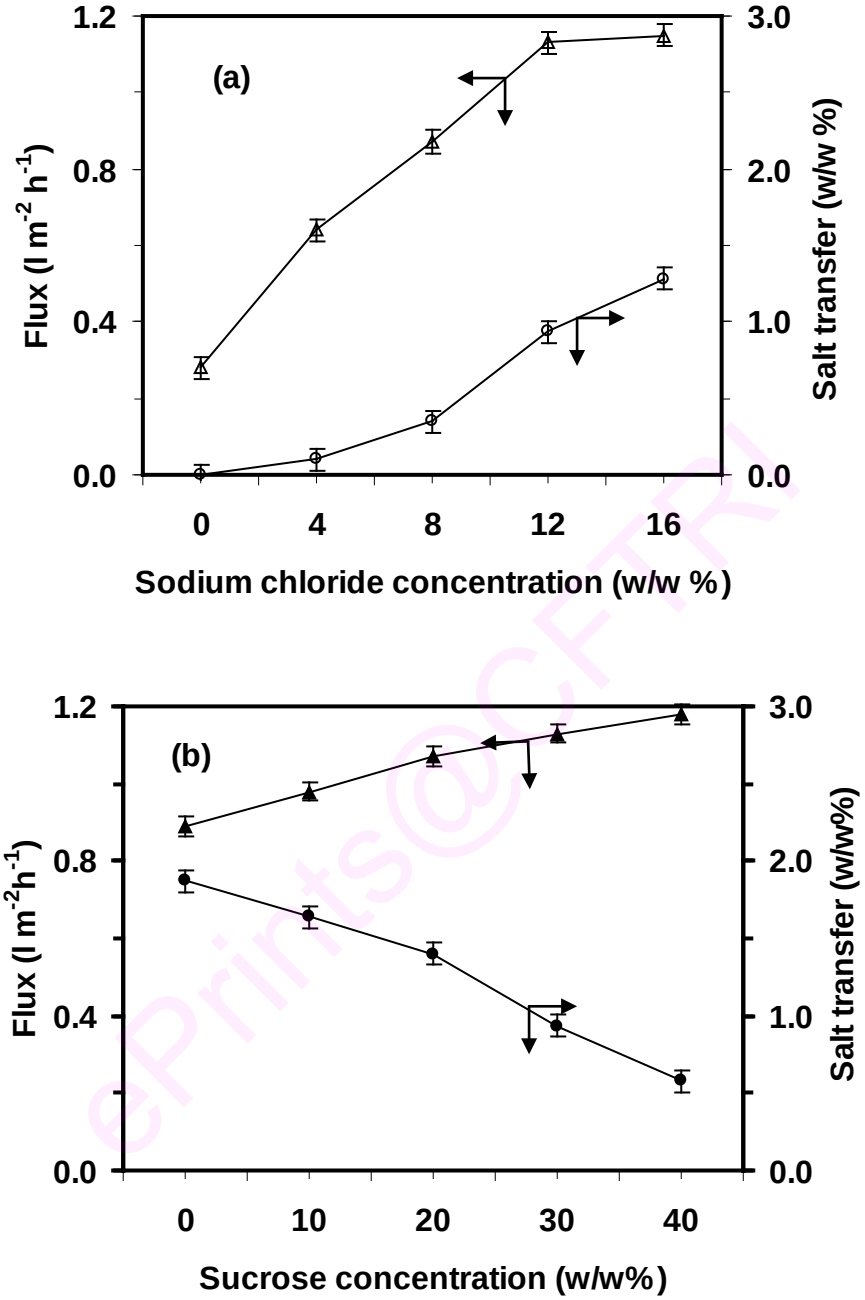


Figure 3B.2: Effect of mixed osmotic agent on flux (-△-; -▲-) and salt transfer (-○-; -●-) during direct osmosis (a) addition of sodium chloride (0 to 16 w/w%) to sucrose (30 w/w%); (b) addition of sucrose (0 to 40 w/w%) to sodium chloride (12 w/w%)

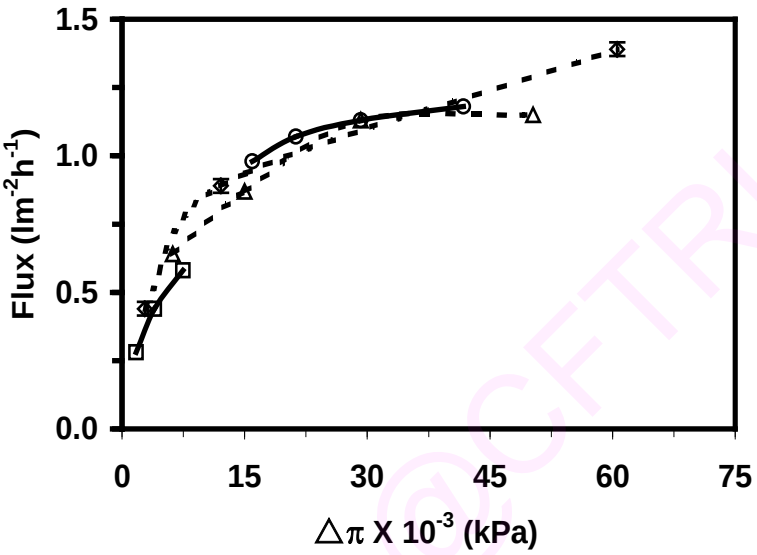


Figure 3B.3: Direct osmosis flux as a function of osmotic pressure difference (sodium chloride (6-26 w/w%, -◇-); sucrose (30-50 w/w%, -□-); sodium chloride (12 w/w%)-sucrose (10-40 w/w%, -△-); sucrose (30 w/w%) –sodium chloride (4-16 w/w%, -○-).

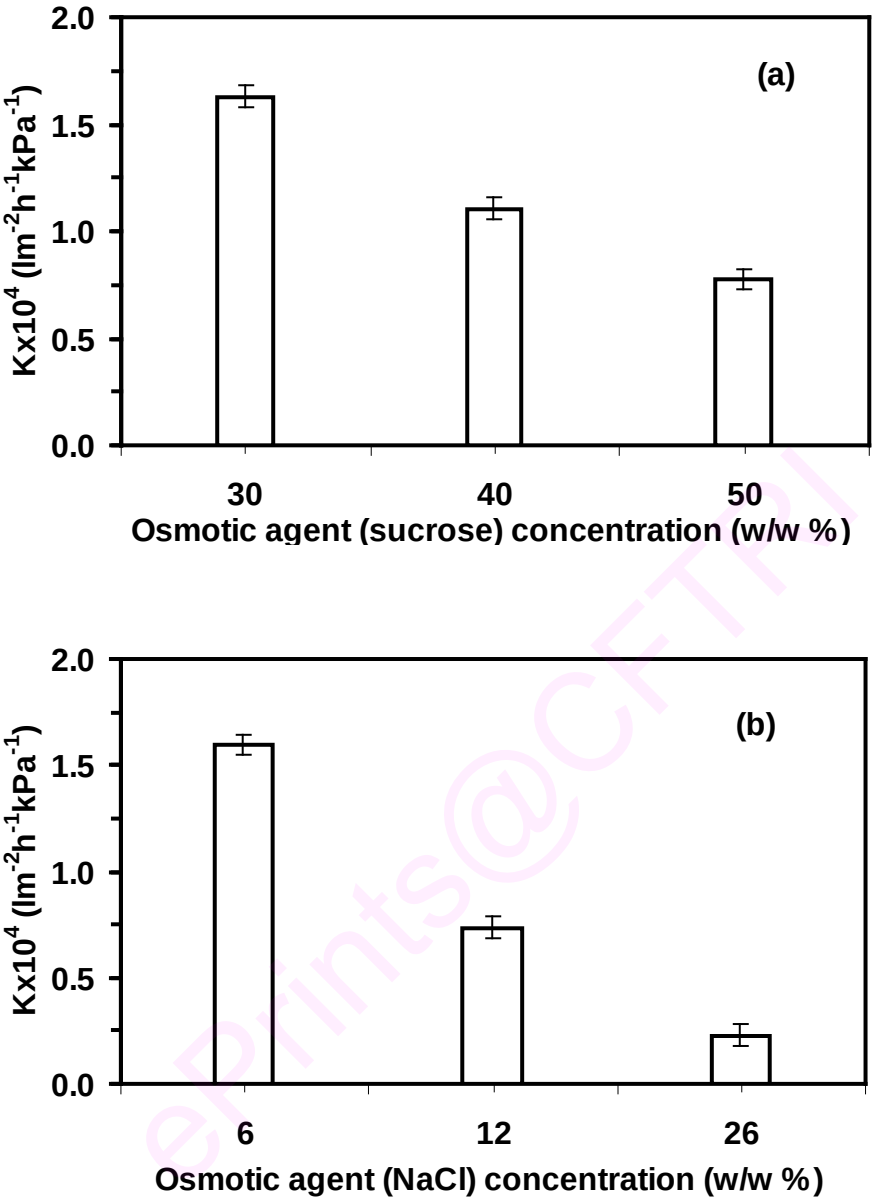


Figure 3B.4: Effect of osmotic agent concentration on overall mass transfer coefficient during the concentration of pineapple juice (a) sucrose (30 to 50 w/w%); (b) sodium chloride (6 to 26 w/w%)

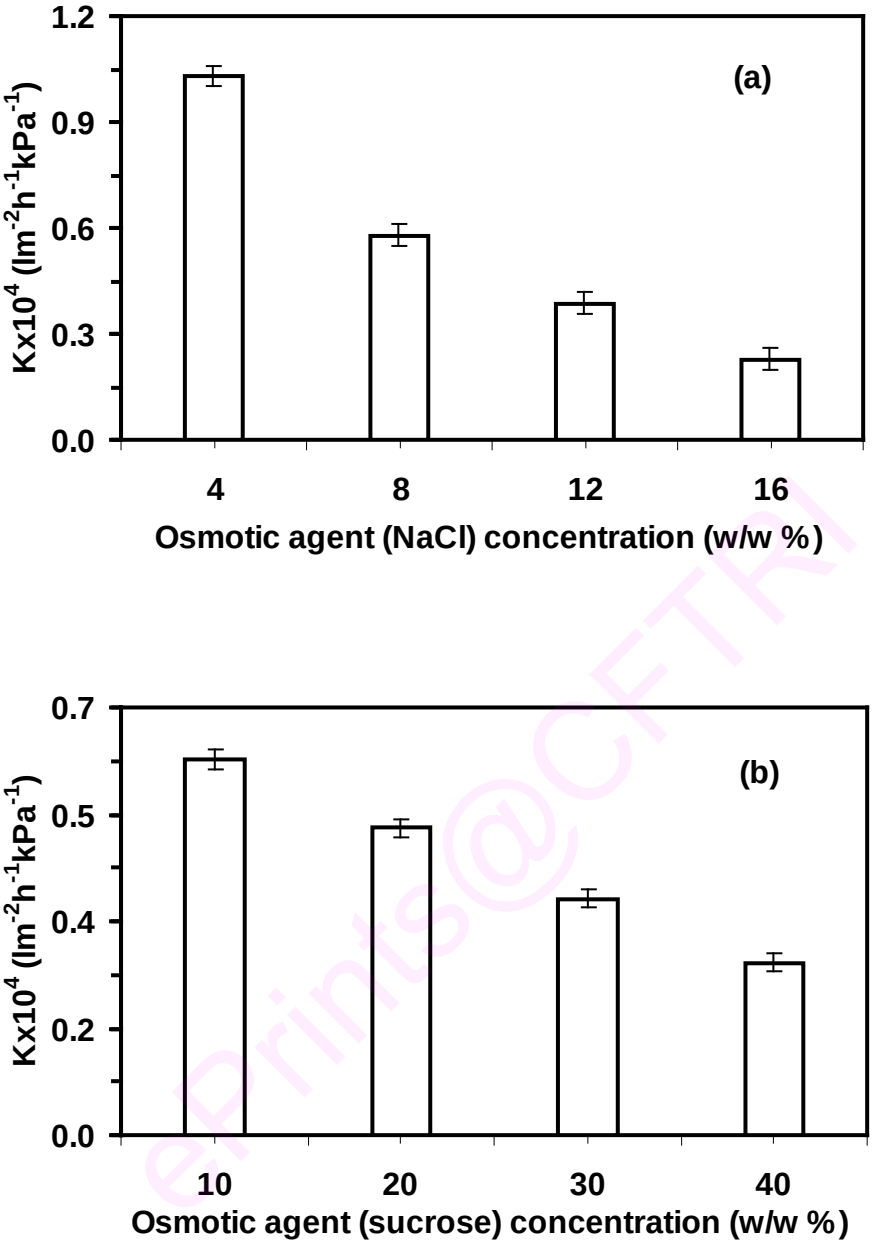


Figure 3B.5: Effect of mixed osmotic agent concentration on overall mass transfer coefficient during the concentration of pineapple juice (a) sucrose (30 w/w%) – sodium chloride (4 to 16 w/w%); (b) sodium chloride (12 w/w%) – sucrose (10 to 40 w/w%)

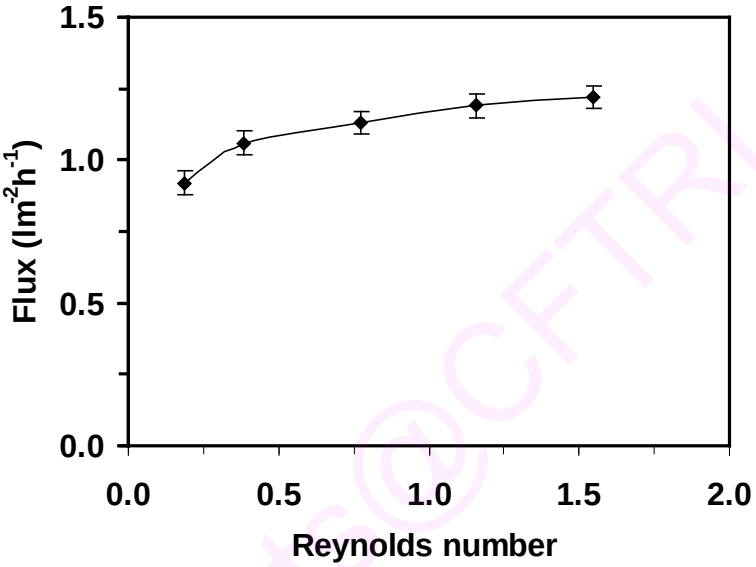


Figure 3B.6: Effect of osmotic agent side hydrodynamic condition on transmembrane flux during the concentration of pineapple juice

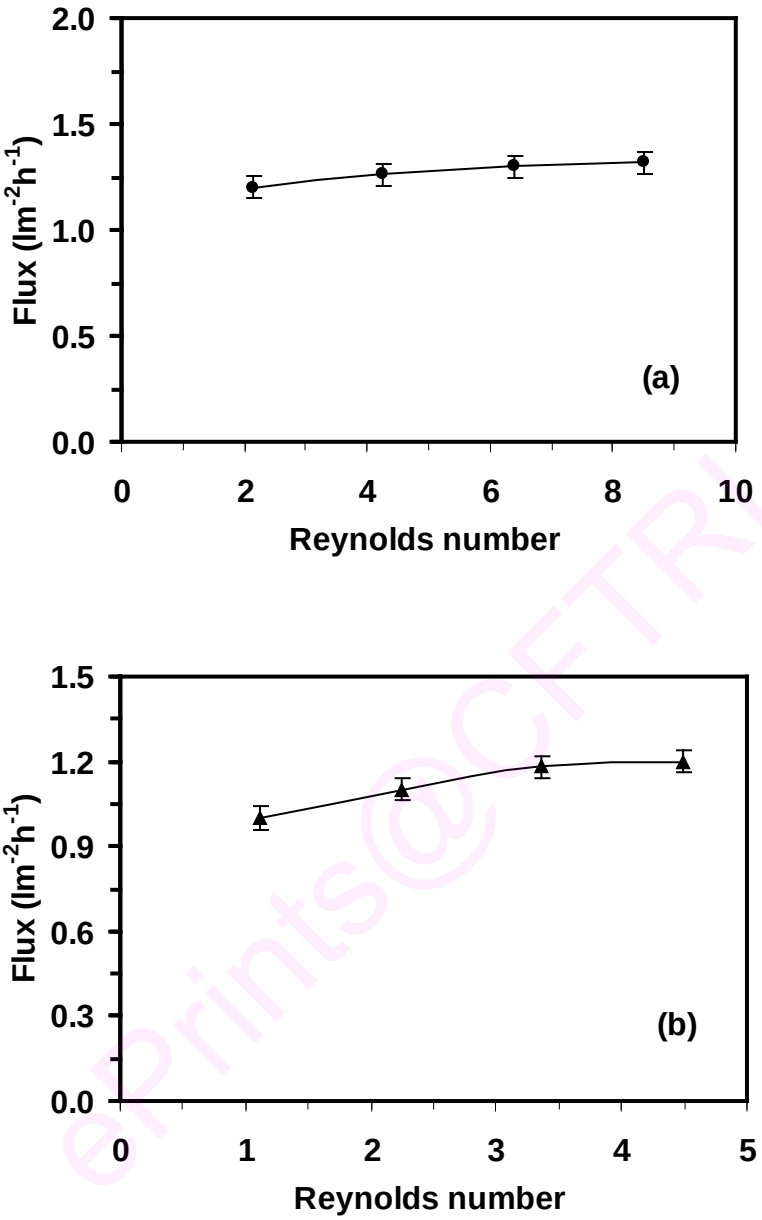


Figure 3B.7: Effect of feed side hydrodynamic condition on transmembrane flux using sucrose (40 w/w%) - sodium chloride (12 w/w%) solution. The feed side concentration was (a) 12.4 ± 0.2°Brix; and (b) 25 ± 0.2°Brix.

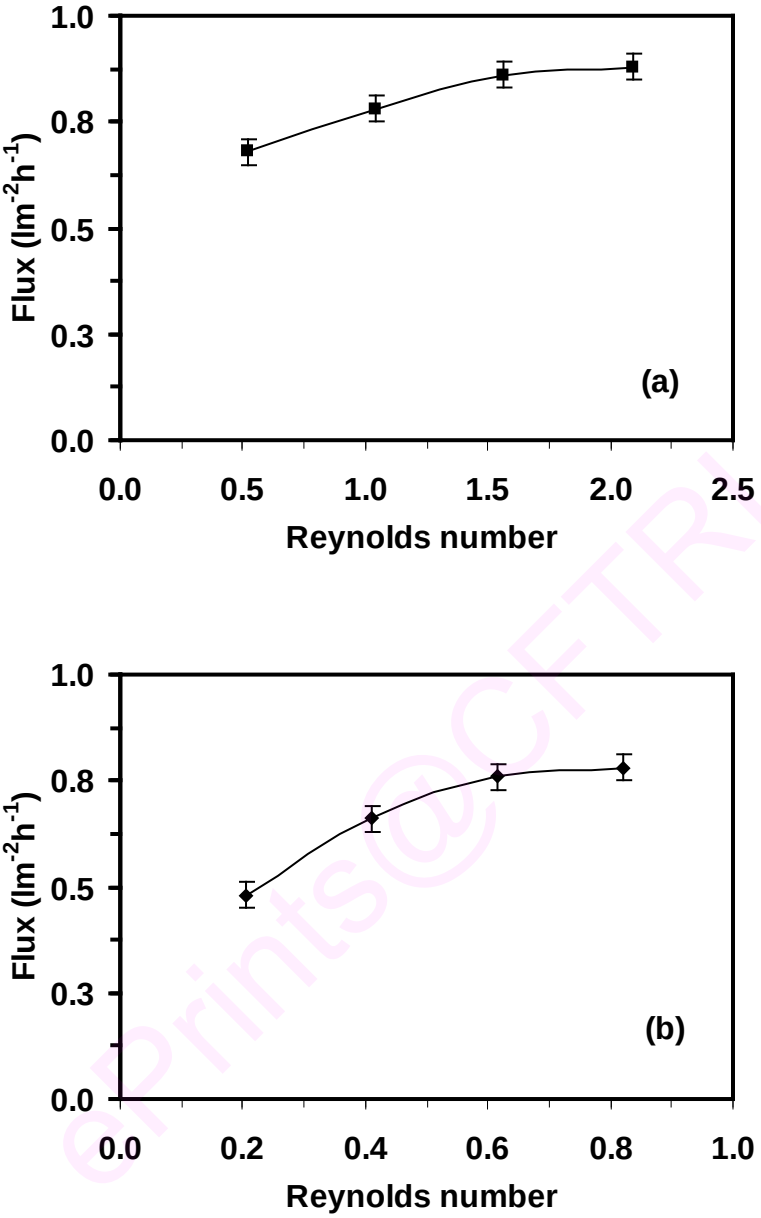


Figure 3B.8: Effect of feed side hydrodynamic condition on transmembrane flux using sucrose (40 w/w%) - sodium chloride (12 w/w%) solution. The feed side concentration was (a) 37.5 ± 0.2°Brix; and (b) 50 ± 0.2°Brix.

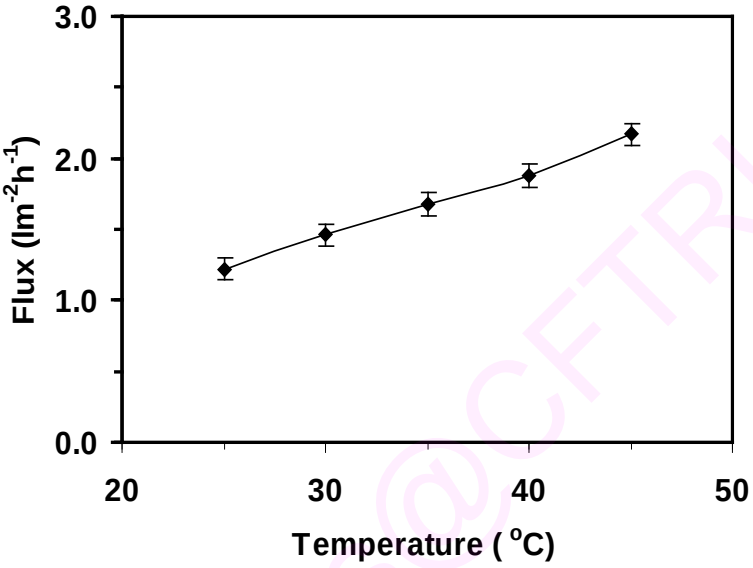


Figure 3B.9: Effect of feed temperature on transmembrane flux using sucrose (40 w/w%)–sodium chloride (12 w/w%) solution during the concentration of pineapple juice

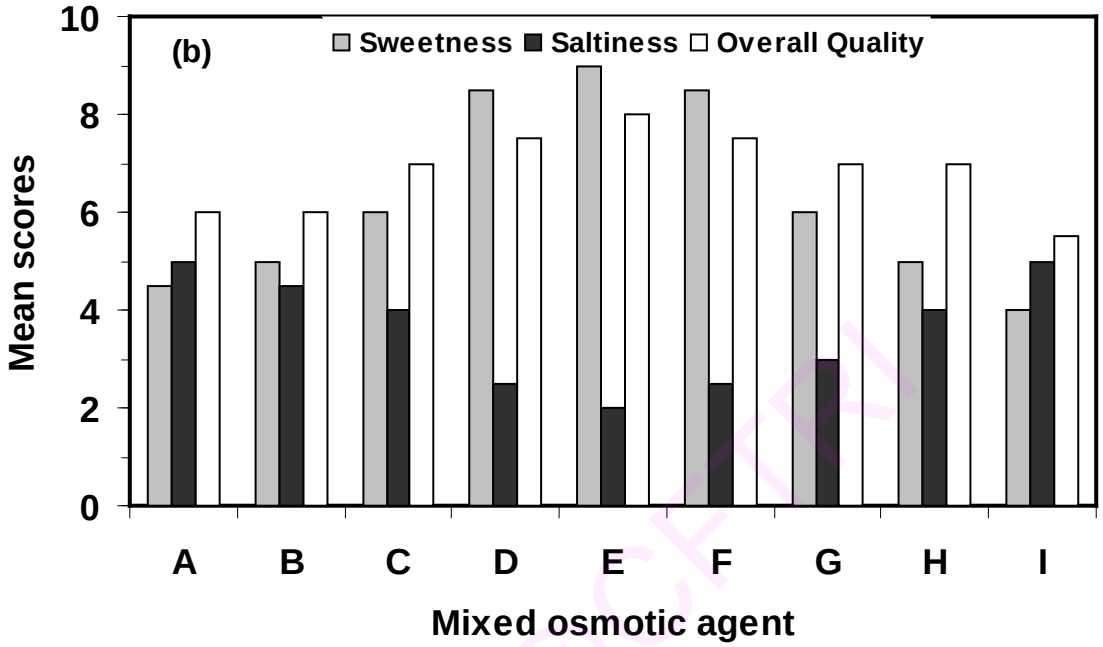


Figure 3B.10: Sensory profile of pineapple juice concentrates by direct osmosis (A: sodium chloride (12 w/w%)- sucrose (0 w/w%); B: sodium chloride (12 %w/w)- sucrose (10 w/w%); C: sodium chloride (12 w/w%)- sucrose (20 w/w%); D: sodium chloride (12 w/w%)- sucrose (30 w/w%); E: sodium chloride (12 w/w%)- sucrose (40 w/w%); F: sodium chloride (0 w/w%)- sucrose (30 w/w%); G: sodium chloride (4 w/w%)- sucrose (30 w/w%); H: sodium chloride (8 w/w%)- sucrose (30 w/w%); I: sodium chloride (16 w/w%)- sucrose (30 w/w%)).

CHAPTER - 4

Osmotic Membrane Distillation

CHAPTER – 4A

Osmotic membrane distillations for the concentration of biomolecules

4A.1 Introduction

Concentration of biomolecules and natural colors by existing membrane processes such as ultrafiltration (UF) and nanofiltration (NF) processes are associated certain drawbacks such as membrane fouling and concentration polarization (Raghavarao *et al*., 2005). Recently, technological advances related to the development of new membrane processes and improvements in process engineering have enabled the above limitations to be overcome to a large extent. Newer membrane processes such as osmotic membrane distillation (OMD) and direct osmosis (DO) have the potential to concentrate biomolecules and natural colors at ambient temperature and atmospheric pressure without product deterioration (Jiao *et al*., 2004). The application of direct osmosis for the concentration of biomolecules and fruit juices has been discussed in Chapter 3. In this chapter osmotic membrane distillation process is employed as an alternative method for the concentration of biomolecules, an enzyme (bromelain from pineapple waste) and a protein (phycocyanin from *Spirulina platensis*).

In osmotic membrane distillation process, the feed (biomolecule) to be concentrated and the osmotic agent solution are circulated on either side of the porous hydrophobic membrane. The driving force for water vapor transport through the membrane pores is the water vapor pressure difference across the membrane, induced by the water activity difference between feed (enzyme/protein) and osmotic agent solution. Mass transfer during OMD process occurs due to difference in water activity (solute concentration), which in turn induces vapor pressure difference at vapor-liquid interface. This results

in water vapor transfer from dilute feed side to osmotic agent side. Osmotic membrane distillation process is a simultaneous heat and mass transfer operation. During OMD process, water evaporates from the surface of the solution having higher vapour pressure (feed), the vapour diffuses through the pores of the membrane and condenses on the surface of the solution having lower vapour pressure (osmotic agent, OA). Evaporation cools the feed and condensation warms up the OA solution. Simultaneous mass and heat transfer during water transport in OMD process causes difference in concentration and temperature near the membrane surface compared to bulk stream. This resultant concentration and temperature gradients across the membrane translates into a lower vapour pressure gradient, which in turn results in a reduction of the driving force (Hogan *et al*., 1998; Raghavarao *et al*., 2005).

The pineapple (*Ananas comosus*) is one of the most popular tropical fruits. The origin of the pineapple is the American continent, probably Brazil and Paraguay. It has spread throughout tropical and subtropical regions as a commercial fruit crop. In India, it is estimated that about 0.23 million tonnes of solid waste is generated per annum from pineapple process industry (Bardiya *et al*., 1996). The pineapple waste is either used as an animal feed or disposed as a waste. In recent years, considerable research has been carried out to produce value-added products from pineapple waste (Bardiya *et al*., 1996; Imandi *et al*., 2007). Bromelain is one of the commercially important enzyme present in pineapple waste (peel and core of the pineapple). In this

study concentration of bromelain from pineapple waste (peel and core of the pineapple) is also attempted by osmotic membrane distillation.

Phycocyanin is a protein and also a natural food colorant, present in a *Spirulina platensis* (a blue green algae). Phycocyanin solution obtained after separation and extraction from spirulina is in dilute form and needs to be concentrated to reduce the water load during final processing steps of freeze drying, which increases its stability and shelf life. Phycocyanin is used in various pharmaceutical applications such as phycofluor probes in immunodiagnostics, prevention or inhibition of cancer and also in coloring of many food products such as fermented milk products, ice creams, chewing gum, soft drinks, alcoholic drinks, desserts, sweet cake decoration, milk shakes, cosmetics. Since phycocyanin is a heat sensitive product (Sarada *et al*., 1999), it is required to concentrate dilute phycocyanin solution using concentration methods, which operate, at ambient temperature. Jaoquen *et al*. (1999) showed that the use of these pressure driven membrane processes (such as ultrafiltration (UF), Nanofiltration (NF) and Reverse Osmosis (RO)) during the concentration of phycocyanin resulted in shear damage apart from membrane fouling. However, NF process exhibited relatively better performance in terms of pigment recovery, concentration factor as compared to other membrane processes such as RO and UF (Jaoquen *et al*. 1999). Osmotic membrane distillation process appears to be feasible method for the concentration of dilute phycocyanin solution, since it operates at ambient temperature and atmospheric pressure without causing any heat or shear damage to the product. It also reduces the amount of water load on

subsequent processing step such as freeze-drying, to obtain the final product in powder form.

The main objective of this chapter is study of effect of parameters such as osmotic agent concentration and flow rate on transmembrane flux during the concentration of phycocyanin solution and bromelain enzyme extract. An attempt has been made to calculate the mass and heat transfer coefficients on feed side and osmotic agent solution side using classical mass and heat transfer correlations. The concentration polarization and temperature polarization phenomena during concentration of phycocyanin solution and bromelain enzyme extract by osmotic membrane distillation have been evaluated.

4A.2 Theoretical Consideratins

4A.2.1 Mass transfer

The transmembrane flux in case of osmotic membrane distillation is given by Alves and Coelhoso (2004)

$$J_w = k_{mp}\Delta p_{wm} = k_{mp}\left(p_{wm1} - p_{wm2}\right) \quad \ldots. \quad (4A.1)$$

where p_{wm1} and p_{wm2} are the water vapor pressures near the membrane surface on feed and osmotic agent side, respectively and k_{mp} is the membrane mass transfer coefficient.

The water vapor pressures p_{wm1} and p_{wm2} can be related to the water activity at the membrane surface by

$$p_{wmi} = P^{*}_{w,Tmi}\, a_{wmi} \quad \text{.... (4A.2)}$$

where p_{wmi} is the water vapor pressure, $P^{*}_{w,Tmi}$ is the vapor pressure of pure water at temperature T_{mi}, and a_{wmi} represents the water activity.

A boundary layer will be formed on each side of the membrane during osmotic membrane distillation process also, and the water activity at the membrane surface will be different from the water activity in the bulk, resulting in a reduction of the driving force and in turn flux. The flux in the boundary layer can be defined in terms of respective individual mass transfer coefficient by the following equation (Alves and Coelhoso, 2004).

$$J_w = k_1 (a_1 - a_{m1}) = k_2 (a_{m2} - a_2) \quad \text{.... (4A.3)}$$

where a_1 and a_2 are the bulk water activities and k_1 and k_2 are the individual mass transfer coefficients in the feed and osmotic agent side boundary layers, respectively.

4A.2.2 Mass transfer through the membrane

The term 'Dusty gas model' is relatively unfamiliar in the membrane separation field. Only recently the vapor transport through porous membrane has been described in the literature using the dusty gas model, which is

based on the well-developed kinetic theory of gases (Lawson and Lloyd, 1997; Alves and Coelhoso, 2004). In this model, the porous medium is visualized as a group of uniformly distributed dust particles held stationary in space. The presence of gas-surface interaction is taken into account by considering the dust particles as giant molecules. According to the dusty gas model, the membrane mass transfer coefficient when both molecular and Knudsen mechanisms exist simultaneously is given by Alves and Coelhoso (2004),

$$k_m = \frac{1.8\times10^{-5}P^*}{RT\delta}\left(\frac{1}{D^k_{we}} + \frac{P_{air}}{D^o_{w-air}}\right)^{-1} \quad \text{.... (4A.4)}$$

where P_{air} is the air partial pressure, R the gas constant, T absolute temperature and D^k_{we} and $D^o_{w\text{-}air}$ are the Knudsen and molecular effective diffusivities expressed as

$$D^k_{we} = K_0\left(\frac{8RT}{\pi M_w}\right)^{0.5} \quad \text{where} \quad K_0 = \frac{\varepsilon d_p}{3\tau} \quad \text{.... (4A.5)}$$

$$D^0_{w-air} = K_1 P D_{w-air} \quad \text{where} \quad K_1 = \frac{\varepsilon}{\tau} \quad \text{.... (4A.6)}$$

In above equations, M_w is the water molar mass, P the total pressure, $D_{w\text{-}air}$ the ordinary water diffusion coefficient, ε the membrane porosity, δ the membrane thickness, τ the membrane tortuosity, d_p the membrane pore diameter, and K_0 and K_1 are the constants depending on the membrane pore geometry. For the water-air system, $PD_{w\text{-}air}$ can be calculated using following equation (Bird *et al*., 1960),

$$PD_{w-air} = 4.46\times10^{-6}T^{2.334} \quad \text{.... (4A.7)}$$

4A.2.3 Mass transfer in boundary layers

The boundary layers of concentrated feed and diluted osmotic agent solution present on either side of the membrane significantly affect the resistance to mass transfer. The liquid mass transfer coefficients depend on the properties of the solutions and on hydrodynamic conditions of the systems. These coefficients can be estimated with the help of empirical correlation of dimensionless numbers, namely, Sherwood (Sh), Reynolds (Re) and Schmidt (Sc) numbers. In case of a flat plate membrane cell, the liquid mass transfer coefficient in the feed and osmotic agent side boundary layer were estimated using the following correlation (Geankoplis, 1997; Nagaraj *et al*., 2006).

$$Sh = 0.66\,Re^{0.5}\,Sc^{0.33} \qquad \text{.... (4A.8)}$$

where $Sh = \frac{kL}{D_w}$, $Re = \frac{uL\rho}{\mu}$, $Sc = \frac{\mu}{\rho D_w}$, L the characteristic length, D_w the water diffusion coefficient, ρ the solution density, μ the solution dynamic viscosity, k the feed or osmotic agent side liquid mass transfer coefficient and u the velocity of the fluid, flowing over the membrane surface.

The water diffusion coefficient (D_w) estimated by the following empirical equation (McCabe *et al*., 1993),

$$Dw = \frac{(117.3\times10^{-18})(\varphi M_B)^{0.5}\,T}{\mu\,\vartheta_A^{\,0.6}} \qquad \text{.... (4A.9)}$$

where φ is association factor for water; ϑ is the solute molal volume, m^3/kmol; T is the temperature, °K; M_B is the molecular weight of the solute.

4A.2.4 Overall mass transfer coefficient

The relation between mass flux and overall mass transfer coefficient during osmotic membrane distillation process (which accounts both concentration and temperature polarization effects) can be obtained by substituting a_{m1} and a_{m2} (in terms of water vapor pressure, from Eqs. (4A.2) and (4A.3)) values in Eq. (4A.1)

$$J_w = \frac{1}{\frac{p^*_{w,Tm1}}{k_1} + \frac{1}{k_{mp}} + \frac{p^*_{w,Tm2}}{k_2}} (a_1 p^*_{w,Tm1} - a_2 p^*_{w,Tm2}) \quad \text{.... (4A.10)}$$

The overall mass transfer coefficient K is given by

$$K = \frac{1}{\frac{p^*_{w,Tm1}}{k_1} + \frac{1}{k_{mp}} + \frac{p^*_{w,Tm2}}{k_2}} \quad \text{.... (4A.11)}$$

where $p^*_{w,Tm1}$ and $p^*_{w,Tm2}$ are the pure water vapor pressures near the membrane surface (by considering the concentration and temperature near the membrane surface) on feed and osmotic agent sides, respectively.

4A.2.5 Heat transfer

The water transport in osmotic membrane distillation is a simultaneous heat and mass transfer process. Even though osmotic membrane distillation is carried out with the same bulk solution temperature, a temperature

difference is created at membrane interfaces due to the water evaporation in one side and further condensation in the other side. Evaporation cools the feed side and condensation warms up the osmotic agent side. The resultant temperature gradient across the membrane, translates into a lower vapor pressure gradient, which in turn, decreases water transport across the membrane. Heat transfer also occurs between bulk solutions and membrane surface. At steady state conditions, the heat transfer equations are as follows (Gostoli, 1999; Courel *et al*., 2000)

$$Q = h_1(T_1 - T_{m1}) = h_2(T_{m2} - T_2) \quad \text{.... (4A.12)}$$

$$Q = N_w \Delta H_v - h_m(T_{m2} - T_{m1}) \quad \text{.... (4A.13)}$$

where Q is the heat flux, h_1, h_2 and h_m are the heat transfer coefficients of the feed side, osmotic agent side and membrane heat transfer coefficients, respectively, T_1 and T_2 are the bulk temperatures of feed and osmotic agent, respectively, T_{m1} and T_{m2} are the temperatures at the membrane interfaces of feed and osmotic agent sides, respectively, ΔH_v is the water latent heat of vaporization and N_w is the mass flux.

4A.2.6 Heat transfer through the membrane

The membrane heat transfer coefficient is given by the ratio of conductivity of the solid barrier to its thickness as given by the following equation (Lawson, 1997).

$$h_m = \left(\frac{\varepsilon k^T_{gas} + (1-\varepsilon) k^T_{Polymer}}{\delta} \right) \quad \text{.... (4A.14)}$$

where k^T_{gas} and $k^T_{polymer}$ are the thermal conductivities of the air and of the membrane material, respectively.

4A.2.7 Heat transfer in boundary layers

Heat transfer across the boundary layer influences the rate of mass transfer and which is mainly dependent on the physical properties and hydrodynamic conditions of the feed and osmotic agent solutions. The boundary layer heat transfer coefficients can be estimated from empirical correlations involving dimensionless numbers, like Nusselt (Nu), Reynolds (Re) and Prandtl (Pr) numbers given by (Nagaraj *et al*., 2006).

$$Nu = 0.66 \cdot Re^{0.50} \cdot Pr^{0.33} \quad \text{.... (4A.15)}$$

where $Nu = \frac{hL}{k^T}$ and $Pr = \frac{\mu C_P}{k^T}$; K^T the thermal conductivity of feed or osmotic agent solution, C_p the specific heat feed or osmotic agent solution, L the characteristic length, h the boundary layer heat transfer coefficient of feed or osmotic agent side; and μ the dynamic viscosity of the feed or osmotic solution.

4A.2.8 Overall heat transfer coefficient

The relation between heat flux and overall heat transfer coefficient during osmotic membrane distillation process can be obtained by substituting T_{m1} and T_{m2} (from Eq. (4A.12)) values in Eq. (4A.13) and is given by (Celere and Gostoli, 2006)

$$Q = \frac{1}{\frac{1}{h_1} + \frac{1}{h_m} + \frac{1}{h_2}} \left(\frac{N_w \, \Delta H_v}{h_m} - (T_2 - T_1) \right) \quad \text{.... (4A.16)}$$

The overall heat transfer coefficient U is given by

$$U = \frac{1}{\frac{1}{h_1} + \frac{1}{h_m} + \frac{1}{h_2}} \quad \text{.... (4A.17)}$$

Further, assuming bulk temperatures are equal and maintained constant (T_1 = T_2, near-isothermal condition for all practical purposes), an expression that related the heat flux to the water flux and to individual heat transfer coefficients is obtained and is given by

$$Q = \frac{N_w \, \Delta H_v}{1 + \left(\frac{h_m}{h_1} + \frac{h_m}{h_2} \right)} \quad \text{.... (4A.18)}$$

The temperature near the membrane surface during osmotic membrane distillation process can be calculated [rearranging the Eqs. (4A.12) and (4A.13)] by the following equation

$$T_{m1} = \left(\frac{h_m (T_2 + (h_1/h_2)T_1) + h_1 T_1 - N_w \Delta H_v}{h_m + h_1 (1 + h_m/h_2)} \right) \quad \text{.... (4A.19)}$$

$$T_{m2} = \left(\frac{h_m (T_1 + (h_2/h_1)T_2) + h_2 T_2 + N_w \Delta H_v}{h_m + h_2 (1 + h_m/h_1)} \right) \quad \text{.... (4A.20)}$$

4A.2.9 Polarization effects during osmotic membrane distillation process

4A.2.9.1 Concentration polarization effect

The vapor pressure and temperature profiles during osmotic membrane distillation have been presented schematically in Fig. 4A.1. During osmotic membrane distillation process as mass transfer proceeds, a polarization (boundary) layer is created due to increased solute concentration on one (feed) side of the membrane surface and decreased solute concentration on opposite (osmotic agent) side. The concentration polarization (CP) layer reduces the transmembrane flux by depressing the driving force for water transport. The reduction in driving force due to CP effect (by neglecting temperature polarization effect or assuming the temperature near the respective membrane surfaces was same as that of bulk) is given by (Bui *et al.*, 2005)

$$\text{Reduction in driving force due to CP} = \Delta P_b - \Delta P_{m,T}$$

$$= (a_1 p^*_{w,T1} - a_2 p^*_{w,T2}) \_ (a_{m1} p^*_{w,T1} - a_{m2} p^*_{w,T2}) \quad \text{..... (4A.21)}$$

where ΔP_b is the water vapor pressure difference evaluated at the bulk conditions, and $\Delta P_{m,T}$ is the water vapor pressure difference across the membrane surfaces.

4A.2.9.2 Total polarization effect

It is the reduction in driving force due to both concentration and temperature polarization effects on feed as well as osmotic agent side and is given by

$$\text{Reduction in driving force by PE} = \Delta P_b - \Delta P_m$$

$$= (a_1 p^*_{w,T1} - a_2 p^*_{w,T2}) - (a_{m1} p^*_{w,Tm1} - a_{m2} p^*_{w,Tm2}) \quad \ldots\ldots (4A.22)$$

$$= (a_1 p^*_{w,T1} - a_{m1} p^*_{w,Tm1}) + (a_{m2} p^*_{w,Tm2} - a_2 p^*_{w,T2}) \quad \ldots\ldots (4A.23)$$

$$= \Delta P_f + \Delta P_{OA}$$

where ΔP_m is the water vapor pressure difference across the membrane surfaces evaluated at concentration and temperature at the membrane surfaces. The above equation indicates that the overall polarization effect is the sum of the polarization effect on feed side and osmotic agent side. The driving force reduction due to temperature polarization can be calculated by the difference in driving force reduction due to concentration polarization effect and due to overall polarization effect.

4A.3 Materials and Methods

4A.3.1 Materials

Freshly harvested biomass was washed thoroughly using deionised water to remove all the nutrients from the culture broth (Sarada *et al*., 1999; Naidu *et al*., 1999). The algal cells were homogenized (Vander Ploeg, USA; Pressure range 100 - 400 kg/m^2), acidified (pH 4 - 6) and centrifuged (Westfalia Separator, Model TA-05-00-105) at 25 ± 2°C to get crude phycocyanin solution. The yield of the phycocyanin solution was about 45 - 50%. The crude phycocyanin sample (0.5 mg/ml) was stored at 4°C and subjected to osmotic membrane distillation process.

Bromelain extract from pineapple waste is prepared as per the procedure given in Chapter 3. The bromelain enzyme extract was stored at 4°C for further experiments. Calcium chloride ($CaCl_2.2H_2O$) and Sodium chloride (NaCl) procured from Ranbaxy Limited, India, were used as osmotic agents.

4A.3.2 Methods

4A.*3.2.1 Experimental set up and procedure*

The experimental setup used in study is shown in Fig. 4A.2. Hydrophobic polypropylene membranes (Accurel Enka, Germany) having pore size 0.2 μm were used for all the experiments. The membrane having membrane area 0.012 m^2 was placed over a polyester mesh (0.25 mm),

supported between Viton gasket (3.0 mm) and two stainless steel (SS316) frames. The length and breadth of the mass transfer zone were 148 mm and 78 mm, respectively. Feed solution (phycocyanin solution or bromelain enzyme extract) and osmotic agent solution were circulated on either side of the membrane in co-current mode using peristaltic pumps (Fig. 4A.2). The transmembrane flux was calculated by measuring the increase in volume of osmotic agent every hour. All the experiments were performed for a period of 5 hours and the average values of the flux were reported. All the experiments, unless otherwise mentioned, were carried out at the temperature of 25 ± 2ºC. The feed flow rates were varied from 25 to 100 ml/min and the corresponding feed velocities were 0.94×10^{-3} to 3.75×10^{-3} ms^{-1}. For the same flow rates of the osmotic agent, the corresponding feed velocities were 1.12×10^{-2} to 4.50×10^{-2} ms^{-1}. The difference in the feed and osmotic agent feed velocities was due to difference in the flow area as created by the thickness of gasket and polyester mesh in the feed and osmotic agent side, respectively.

4A.3.2.2 Determination of phycocyanin concentration

The concentration of phycocyanin was determined using UV-spectrophotometer, model Shimadzu UV1601, Japan, by measuring the optical density at 620nm for phycocyanin and 650nm for allophycocyanin (Tandaeu and Hounard, 1988). The concentration was calculated by the following formulae

$$\text{Concentration (mg/ml)} = \frac{A_{620} - 0.7(A_{650})}{7.38} \qquad \text{..... (4A.24)}$$

where A_{620} and A_{650} are the optical density at 620 nm at 650 nm, for phycocyanin and allophycocyanin, respectively.

4A.3.2.3 Determination of bromelain activity and protein concentration

The bromelain activity was determined according to the casein digestion unit (CDU) method using casein as a substrate and protein concentration by Bradford method. The detailed estimation procedures are given in Chapter 2.

4A.3.2.4 Determination of physical properties

The specific gravity of feed and osmotic agent solution were measured using specific gravity bottle, and viscosity was measured using Oswald viscometer (Capillary diameter 1.0 mm) using water as standard at 25 ± 2 °C. The physical properties and water activity of osmotic agent solutions are shown in Table 4A.1. The thermal conductivity and specific heat of phycocyanin solution and bromelain enzyme extract were determined as per the equation provided by Toledo (1991) and Sweat (1995) for proteins. The thermal conductivity and specific heat of osmotic solutions were taken from the literature (Colin et al., 1985; Lewis 1987; Washburn, 2000). The value of thermal conductivity of gas (k_{gas}^{T} =0.025 $Wm^{-1}K^{-1}$) and membrane material (k_{PP}^{T} =0.20 $Wm^{-1}K^{-1}$) were taken from the literature (Lawson and Lloyd, 1997).

4A.4 Results and Discussion

4A.4.1 Effect of osmotic agent solution concentration on flux

The effect of osmotic agent concentrations namely calcium chloride (2, 4, 6, 8 and 10 molality) and sodium chloride (2, 3, 4, 5 and 6 molality) on transmembrane flux during the concentration of phycocyanin solution and bromelain enzyme extract is presented in Fig. 4A.3. During the experiments, the feed side and osmotic agent side flow rates were maintained at 100 ml/min. The transmembrane flux in both cases increased with an increase in concentration of osmotic agent solution. This is expected and can attributed to the increase in vapour pressure difference across the membrane with an increase in the concentration of osmotic agent solution, which is then resulted in an increased driving force for water transport through the membrane. It may be noted that the calcium chloride showed higher transmembrane flux as compared to sodium chloride, due to its higher osmotic activity.

4A.4.2 Effect of osmotic agent flow rate on flux

Experiments were performed for phycocyanin solution and bromelain extract by varying osmotic agent flow rate from 25 ml/min to 100 ml/min (the corresponding velocity varies from 1.12×10^{-2} ms^{-1} to 4.50×10^{-2} ms^{-1}), and maintaining the concentration of osmotic agent solution at 10 molality (calcium chloride) and 6 molality (sodium chloride). The variation of transmembrane flux with osmotic agent side flow rate is shown in Fig. 4A.4. During the experiments the concentration of osmotic agent and physical properties (density and viscosity) were maintained constant. The increase in flux is

mainly due to the increase in flow rate of osmotic agent. The increase in flow rate resulted in an increase in flux up to a certain extent beyond which it did not change. For instance, the increasing the flow rate of osmotic agent from 25 to 100 ml/min resulted in 38 - 43% and 43 - 51% increase in flux in case of sodium chloride and calcium chloride, respectively for phycocyain solution and bromelain enzyme extract. The increase in flux with an increase in flow rate can be attributed to the reduction in concentration polarization layer.

4A.4.3 Effect of feed flow rate on flux

Experiments were performed by varying feed flow rate (velocity) from 25 ml/min (0.94×10^{-3} ms^{-1}) to 100 ml/min (3.75×10^{-3} ms^{-1}), while flow rate (100 ml/min) and concentration of calcium chloride (10 molality) solution was maintained. The increase in feed flow rate did not show much significant increase in transmembrane flux (only about 2-4 %) as can be seen from Fig. 4A.5. This result is expected due to the low feed concentration (viscosity) which resulted in less concentration polarization effect, and consequently the flux is less dependent on the feed flow rate.

4A.4.4 Concentration of phycocyanin solution and bromelain extract

Based on best operating conditions obtained, the concentration of phycocyanin solution (0.5 mg/ml) and bromelain extract (462 CDU/ml) was carried out using calcium chloride (10 molality) as osmotic agent solution. The concentration of osmotic agent solution was maintained constant during the course of the experiment. The transmembrane flux decreased as feed concentration increased for both phycocyanin and bromelain enzyme extract

as shown in Fig. 4A.6. The increase in feed concentration resulted in decrease in a driving force across the membrane, which in turn decreases the transmembrane flux. In case of phycocyanin about 2.2 (concentrated from 0.52 ± 0.02 mg/ml to 1.14 ± 0.10mg/ml) fold concentration was achieved, where as in case of bromelain extract it was concentrated up to 912 ± 4.0 CDU/ml (1.97 fold).

4A.4.5 Heat transfer

The boundary layer heat transfer coefficients of feed side as well as osmotic agent side were estimated using empirical correlation comprising of dimensionless numbers (Eq. 4A.15) for feed (h_1) as well as osmotic agent (h_2) for different combinations of feed/osmotic agent were calculated as given in Table 4A.2. The membrane heat transfer coefficient was estimated using the Eq. (4A.14) (Table 4A.2). The temperatures at the feed and osmotic agent side membrane interfaces (T_{m1} and T_{m2}) were estimated using Eqs. (4A.19) and (4A.20). The temperature difference across the membrane (T_{m2}-T_{m1}) increases with an increase in driving force (difference in vapor pressure) across the membrane as shown in Fig. 4A.7. The maximum temperature difference was about 1.0°K for the calcium chloride solution of 10 molality concentration.

Temperature polarization causes reduction in driving force across the membrane, which in turn decreases the flux through the membrane. The water flux through the membrane while considering the temperature polarization effect is given by (Alves andCoelhoso, 2004)

$$J_w = k_{mp}\phi\Delta p_{wb} = k_{mp}\phi\left[a_{m1}\left(p^*_{w1}\right)_{T_1} - a_{m2}\left(p^*_{w2}\right)_{T2}\right] \quad \ldots (4A.25)$$

where Δp_{wb} is the bulk water vapor pressure difference estimated using the water activity at the membrane surface. The value of vapor pressure polarization coefficient (ϕ) can be estimated by dividing the slopes of the Eqs. (4A.25) and (4A.1), as indicated in Fig. 4A.8. The value of vapor pressure polarization coefficient (ϕ) was close to 1 for all the cases studied (Table 4A.2), which indicates low temperature polarization effect in osmotic membrane distillation process of the present work, which may be considered as insignificant**.**

4A.4.6 Mass trasfer

The boundary layer mass transfer coefficient of feed side as well as osmotic agent side were estimated using empirical correlation comprising of dimensionless numbers (Eq. 4A.8). The values of boundary layer mass transfer coefficient for feed (k_1) as well as osmotic agent (k_2) for different combinations of feed and osmotic agent were calculated and given in Table 4A.3.

4A.7 Conclusions

The influence of the process parameters such as osmotic agent concentration and flow rate of feed and osmotic agent on transmembrane flux during the concentration of real systems (phycocyanin and bromelain extracts) was evaluated. The concentration and flow rate of osmotic agent was found to have prominent effect on transmembrane flux. Phycocyanin solution was

concentrated from 0.52 ± 0.02 to 1.14 ± 0.10 mg/ml and bromelain enzyme extract was concentrated from 462 ± 2.0 to 912 ± 4.0 CDU/ml at ambient temperature (25.0 ± 2.0°C). In the range of operating conditions studied, the magnitude of temperature polarization was found to be low and maximum temperature difference was only about 1.0°K.

Table 4A.1: Physical properties and water activity data for sodium chloride and calcium chloride at different concentrations

Concentration molality	Density × 10^{-3} (Kg .m^{-3})	Viscosity (mPa.s)	Water activity (-)
Sodium chloride			(Collin et al., 1985)
2	1.08 ± 0.01	1.22 ± 0.10	0.93
3	1.11 ± 0.01	1.28 ± 0.10	0.89
4	1.14 ± 0.01	1.35 ± 0.08	0.85
5	1.17 ± 0.01	1.44 ± 0.10	0.81
6	1.20 ± 0.01	1.60 ± 0.10	0.76
Calcium chloride			(Patil et al.,1991)
2	1.15 ± 0.04	1.50 ± 0.10	0.86
4	1.26 ± 0.05	2.17 ± 0.20	0.63
6	1.34 ± 0.05	3.94 ± 0.20	0.40
8	1.40 ± 0.01	6.19 ± 0.40	0.26
10	1.44 ± 0.01	9.91 ± 0.80	0.22

Table 4A.2: Estimated heat transfer coefficients

System studied	Heat transfer coefficients, Wm^2K^{-1}				Vapor pressure polarization coefficient
Feed/Osmotic agent	Feed side h_1	Membrane h_m	Osmotic agent side h_2		
			Minimum	Maximum	ϕ
Phycocyanin/$CaCl_2$	738 ± 1.0	390	4611 ± 12.0 (10 m)	8033 ± 6.0 (2 m)	0.93 ± 0.02
Phycocyanin/NaCl	738 ± 1.0	390	9126 ± 10.0 (6 m)	9785 ± 9.0 (2 m)	0.89 ± 0.01
Bromelain enzyme extract/$CaCl_2$	729 ± 2.0	390	4454 ± 10.0 (10 m)	7995 ± 4.0 (2 m)	0.97 ± 0.02
Bromelain enzyme extract/NaCl	729 ± 2.0	390	9080 ± 12.0 (6 m)	9778 ± 6.0 (2 m)	0.97 ± 0.01

h_1 = Heat transfer coefficient in feed side boundary layer

h_m = Membrane heat transfer coefficient

h_2 = Heat transfer coefficient in osmotic agent side boundary layer

Table 4A.3: Estimated mass transfer coefficients

System studied	Mass transfer coefficients, $\times 10^5$ ms^{-1}			
Feed/Osmotic agent	Feed side k_1	Membrane k_m	Osmotic agent side k_2	
			Minimum	Maximum
Phycocyanin/$CaCl_2$	5.20 ± 0.2	0.079	2.17 ± 0.2 (10 m)	10.21 ± 0.1 (2 m)
Phycocyanin/NaCl	5.22 ± 0.2	0.079	7.14 ± 0.1 (6 m)	8.82 ± 0.1 (2 m)
Bromelain enzyme extract/$CaCl_2$	5.48 ± 0.2	0.079	2.15 ± 0.2 (10 m)	10.20 ± 0.1 (2 m)
Bromelain enzyme extract /NaCl	5.48 ± 0.2	0.079	7.13 ± 0.1 (6 m)	8.82 ± 0.1 (2 m)

k_1 = Mass transfer coefficient in feed side boundary layer

k_m = Membrane mass transfer coefficient

k_2 = Mass transfer coefficient in osmotic agent side boundary layer

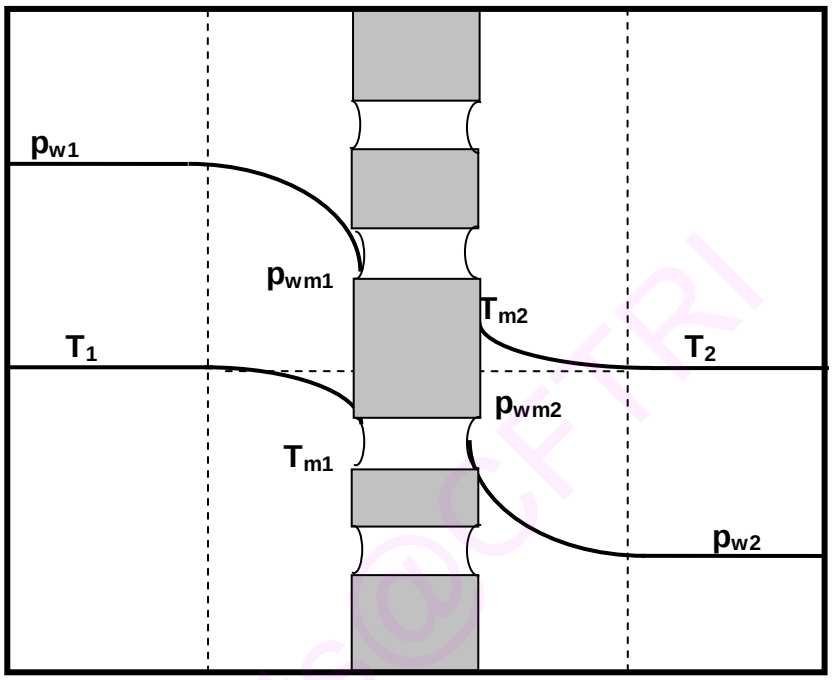


Figure 4A.1: Vapor pressure and temperature profiles during osmotic membrane distillation process (where $p_{w1} = a_1 p^*_{w,T1}$; $p_{w2} = a_2 p^*_{w,T2}$; $p_{wm1} = a_{m1} p^*_{w,Tm1}$; $p_{wm2} = a_{m2} p^*_{w,Tm2}$)

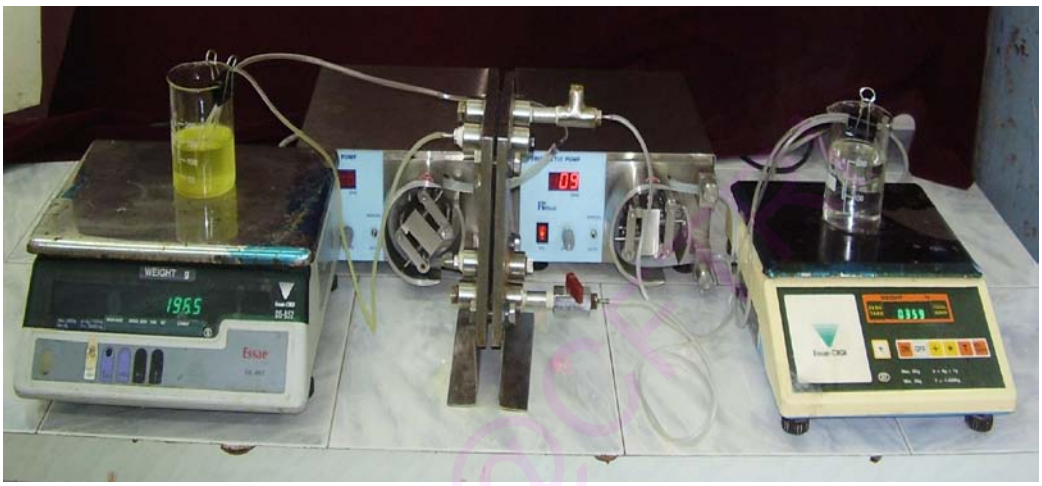


Figure 4A.2: Photograph of osmotic membrane distillation plate and frame membrane module

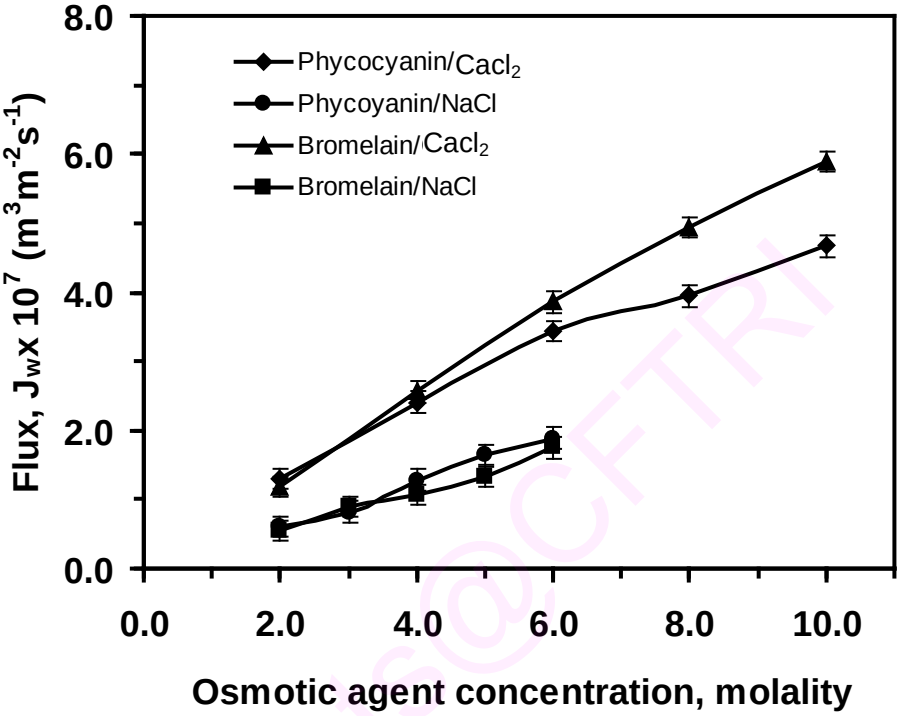


Figure 4A.3: Effect of osmotic agent concentration ($CaCl_2$ and NaCl) on transmembrane flux using Polypropylene membrane. (Feed flow rate = 100 ml/min; Temperature = 25°C; Membrane pore size = 0.2 μm; Membrane area = 0.012 m^2)

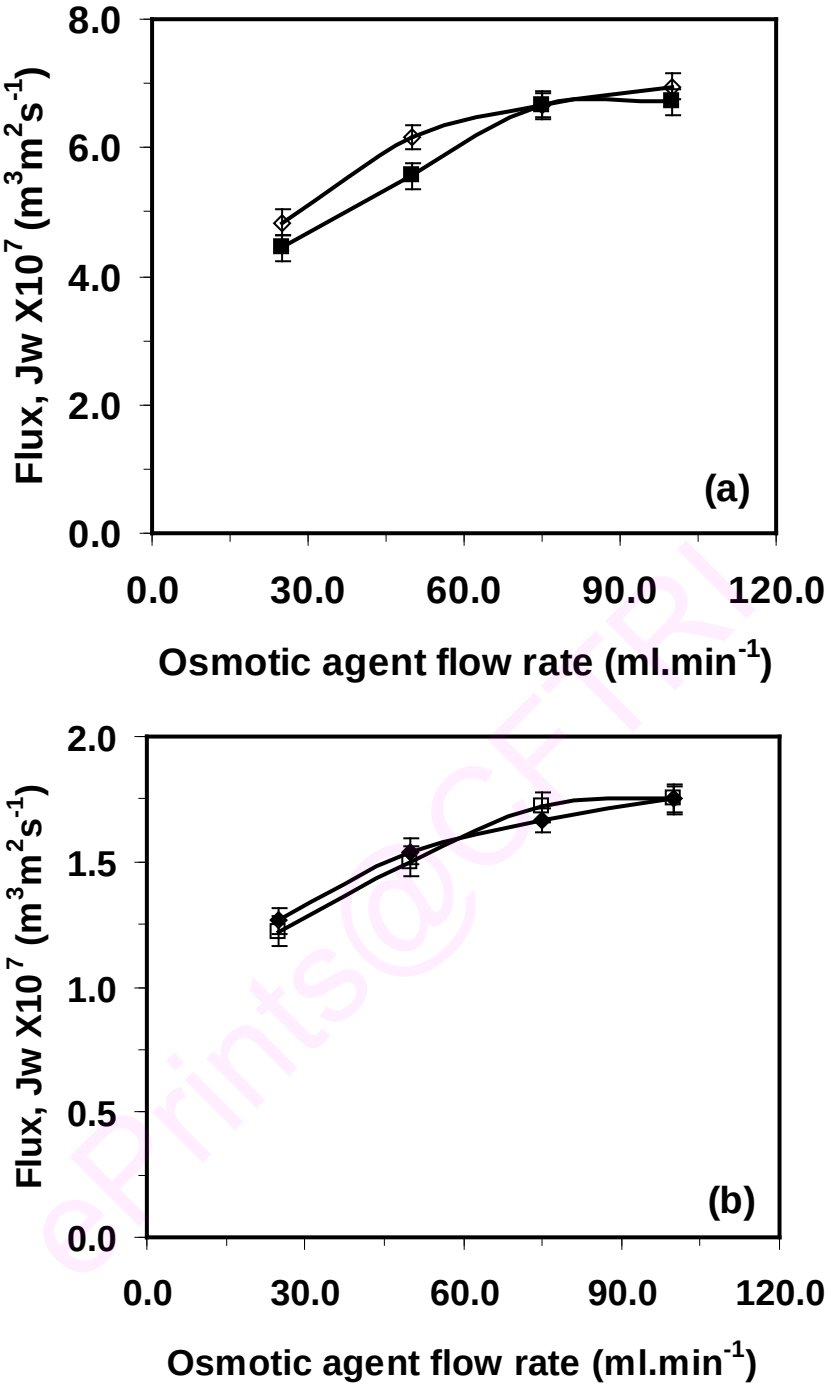


Figure 4A.4: Transmembrane flux as a function of osmotic agent flow rate (a). Phycocyanin/$CaCl_2$ (-◇-) and Bromelain extract/$CaCl_2$ (-■-); (b). Phycocyanin/ NaCl (- ◆ -) and Bromelain extract/ NaCl (-□-).

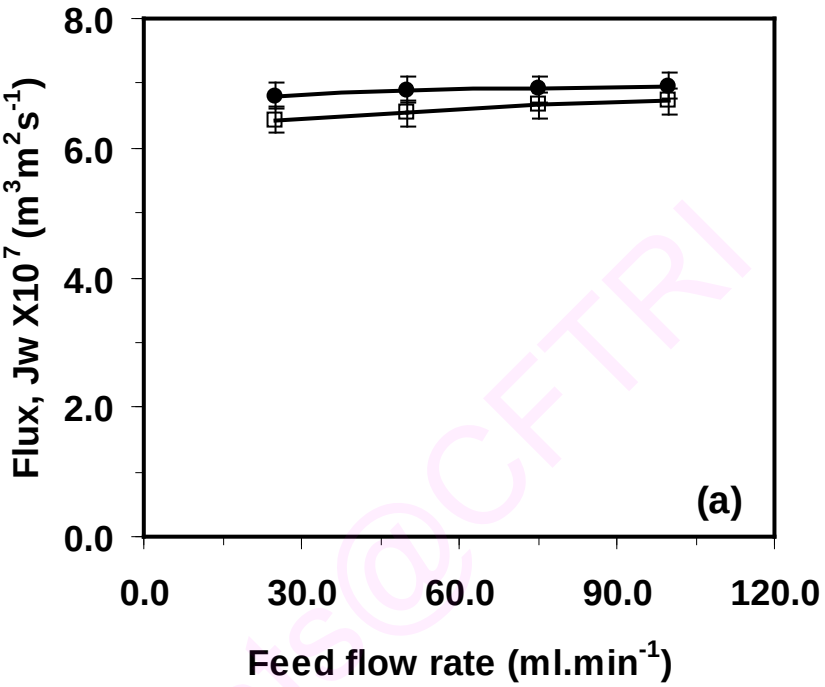


Figure 4A.5: Transmembrane flux as a function of feed flow rate (a) Phycocyanin/$CaCl_2$ (-●-) and Bromelain extract/$CaCl_2$ (-□-).

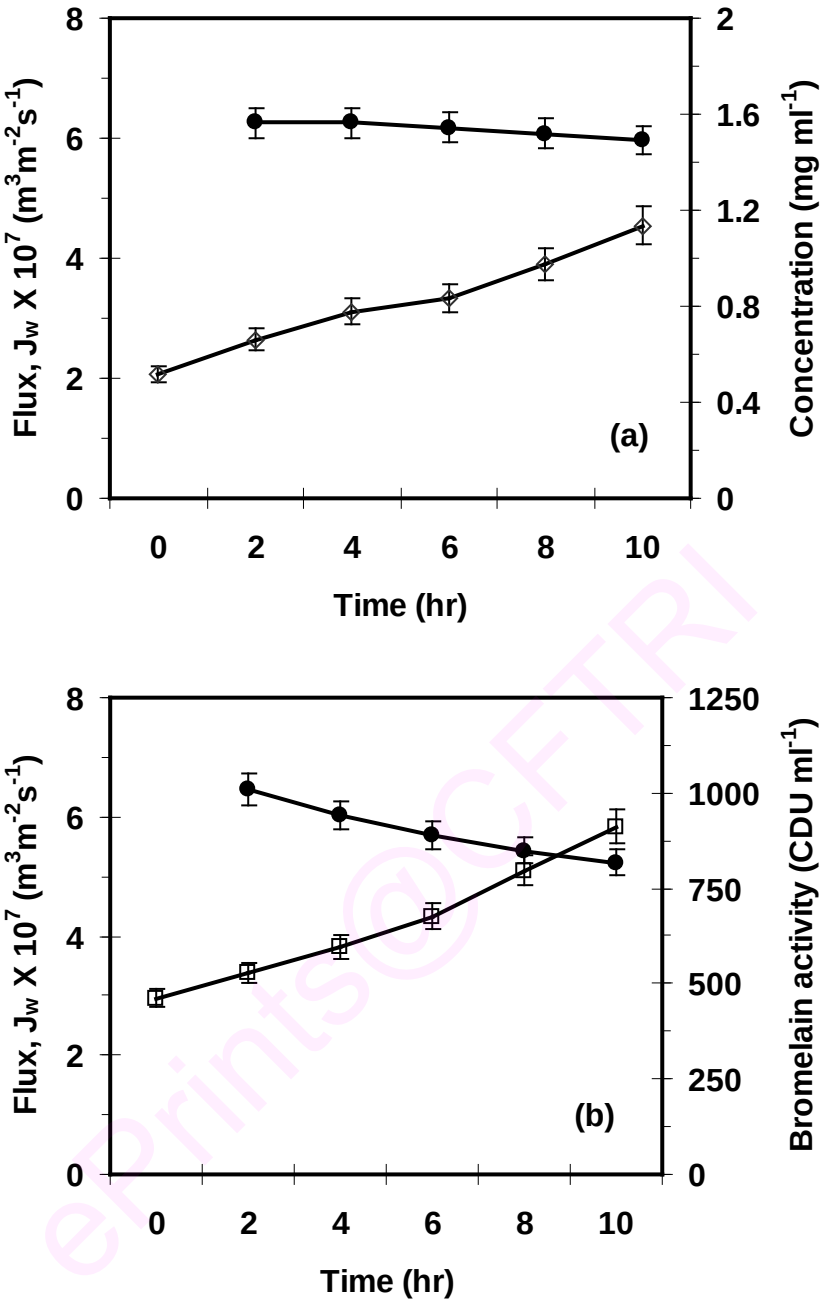


Figure 4A.6: Evolution of the transmembrane flux and concentration during osmotic membrane distillation process (a). Phycocyanin solution; (b). Bromelain extract (closed symbols represents flux and open symbols represents concentration)

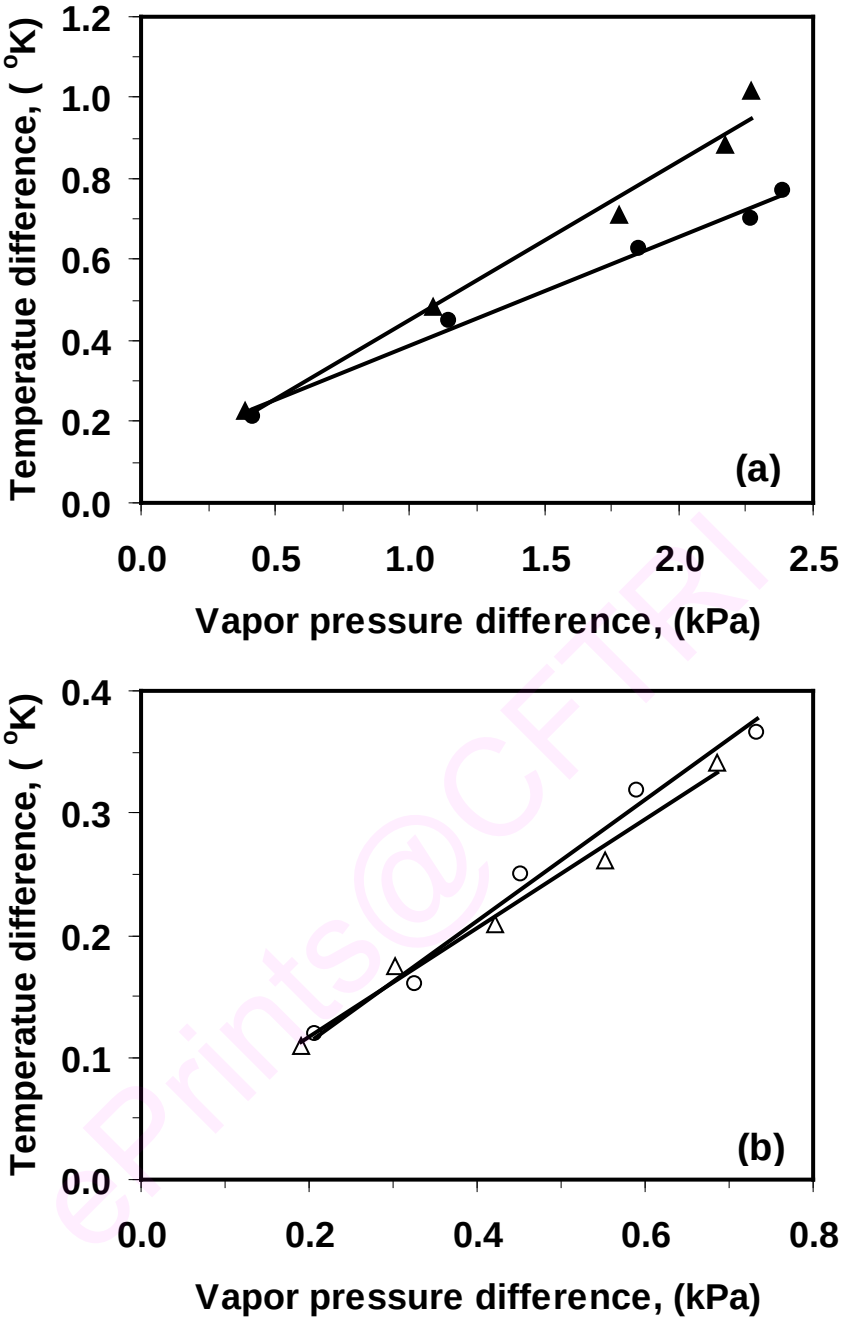
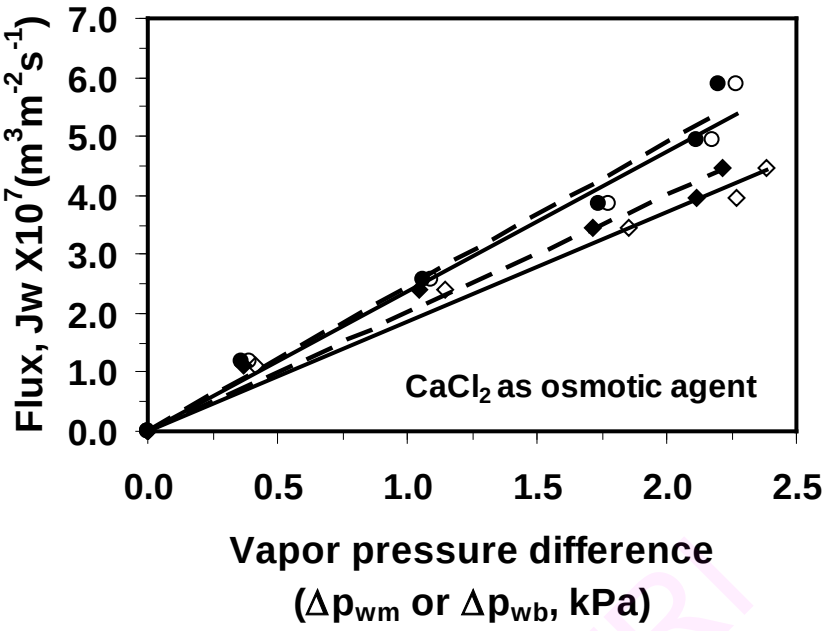


Figure 4A.7: Temperature difference at the membrane interface on both sides of the membrane as a function of water vapor pressure difference (Phycocyanin/$CaCl_2$ system (-●-); Bromelain extract/$CaCl_2$ system (-▲-); Phycocyanin/NaCl system (-○-); Bromelain extract /NaCl system (-△-)).



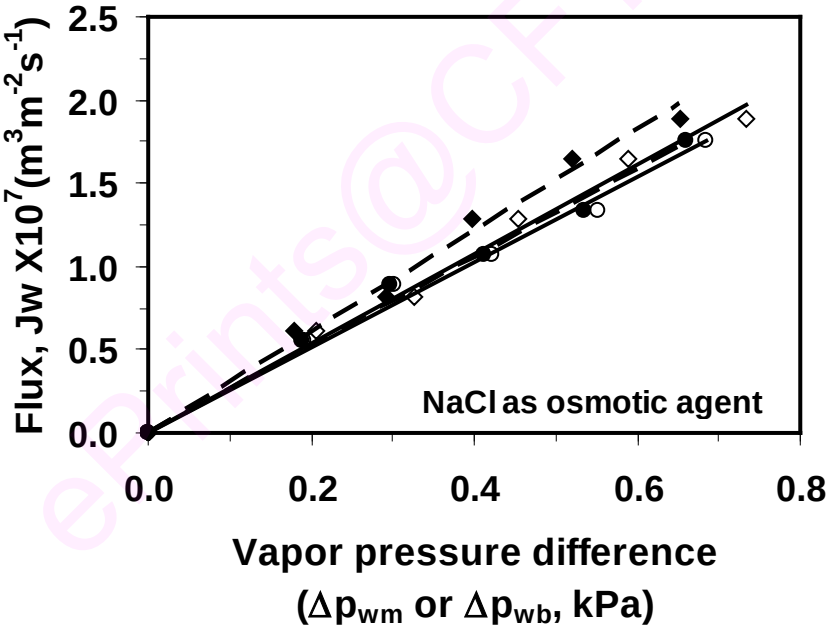


Figure 4A.8: Variation of flux with interface (Δp_{wm}) and bulk (Δp_{wb}) water vapor pressure difference. Closed and open symbols refers to the plot of flux against interface vapor pressure difference and bulk vapor pressure difference respectively (-◆- , -◇- for Phycocyanin solution; -●- ,-○- for Bromelain extract)

CHAPTER – 4B

Osmotic membrane distillation for the concentration of fruit juices

4B.1 Introduction

Concentration of liquid foods by conventional methods such as evaporation results in product deterioration with respect to loss of flavors, taste and nutritive components resulting in low quality end product (Petrotos and Lazarides, 2001). Further, evaporative concentration is energy intensive since it involves phase change of water. Hence, in recent years, membrane processes such as microfiltration (MF) ultrafiltration (UF) and reverse osmosis (RO) are being employed for clarification and concentration of fruit juices (Girard and Fukomoto, 2000). The limitations of these membrane processes are maximum attainable concentration (only up to 25–30°Brix), concentration polarization, and membrane fouling (Narayan *et al*., 2002). Even, the newer membrane process such as membrane distillation (MD, is not without limitations as it suffers from membrane wetting, temperature polarization and loss of volatiles (Lawson and Lloyd, 1997). Hence, there is a need to develop an alternate/complementary membrane process. Osmotic membrane distillation (OMD) process appears to be an attractive alternate process for the concentration of aqueous solutions such as fruit juices since it operates at ambient temperature and atmospheric pressure without causing any heat or shear damage to the product.

Pineapple (*Ananas cosmosus*) juice is a popular product due to its very pleasant aroma and flavor. It contains suspended solids, colloids, fibers and enzymes, which may affect the flux rate and product quality during membrane processing. Bromelain, a proteolytic enzyme present in pineapple affects the quality of the pineapple juice. The presence of this enzyme causes soreness

and discomfort of the mouth on excessive consumption of pineapple juice. This enzyme needs to be inactivated in order to improve the product quality and consumer acceptance (Sriwatagapongse et al., 2000; http://www.nlm.nih.gov/ medlineplus/druginflo/natural/patient-bromelain.html). Conventionally the enzymes present in the pineapple juice were inactivated by thermal treatment. Pineapple juice contains many flavor components which may be lost during heat treatment. Sriwatanapongse *et al*., (2000) reported that bromelain present in the pineapple juice can be inactivated by heating the juice up to 62 ± 1.0 to 67 ± 1.0°C for 10 min. The main drawback of this process lies in heat treatment, which invariably causes deterioration in product quality.

The objectives of the work carried out in this chapter is development of an alternative pretreatment method for the removal of bromelain from pineapple juice, study of heat and mass transfer aspects and analysis of the role of concentration and temperature polarization effects on driving force at different experimental conditions. Further, the values of theoretical flux were estimated by considering polarization effects and compared with the experimental values.

4B.2 Materials and Methods

4B.2.1 Materials

Fresh pineapples (*Ananas comosus* L.) were procured from the local market. Polypropylene membrane (0.2 μm) from Enka, Germany was used for all the experiments. Calcium chloride dehydrate ($CaCl_2.2H_2O$), trichloracetic acid, sodium acetate were procured from Ranbaxy Limited, India. Casein (Hammarsten grade), was procured from Loba Chemicals, India. All other chemicals were of analytical grade.

4B.2.2 Methods

4B.2.2.1 Pineapple juice

After rinsing the pineapple fruit in tap water, the skin was removed using a stainless steel knife. The fruit was cut into small pieces and the juice was extracted in a Food Processor (Singer FP 450) at room temperature (25 ± 2°C).

4B.2.2.2 Pineapple juice pretreatment

The pH of the pineapple juice (initial pH 3.8) was adjusted from 2.5 to 5.0 using citric acid or sodium hydroxide, whenever it is required. After pH adjustment, an adsorbent such as bentonite (0 to 5000 ppm) was added to the pineapple juice and mixed for 15 min using magnetic stirrer and centrifuged at 10000 rpm to remove bentonite- protein complex as well as the

suspended solids from the pineapple juice. This pretreated juice was stored at 4°C for further experiments and analysis.

4B.2.2.3 Osmotic agent solution

Osmotic agent solution was prepared by dissolving calcium chloride dihydrate in distilled water. The solution was made and kept overnight at room temperature before use to ensure complete dissolution of calcium chloride dihydrate. The density, viscosity, specific heat and thermal conductivity of osmotic agent solution were taken from the literature (Lewis, 1987; Sweat, 1995; Washburn, 2000).

4B.2.2.4 Experimental set up and procedure

The details of osmotic membrane distillation experimental setup used for the concentration of bromelain extract were explained in Chapter 4A (Fig. 4A.2). Feed solution (pineapple juice) and osmotic agent solution were circulated on either side of the membrane in co-current mode using peristaltic pumps. The transmembrane flux was calculated by measuring the increase in volume of osmotic agent every hour. All the experiments were performed for a period of 5 hours and the average values of the flux were reported. All the experiments, unless otherwise mentioned, were carried out at the temperature of 25 $\pm$ 2ºC.

4B.2.2.5 Analysis of pineapple juice

The pineapple juice was analyzed for soluble solids, pH, titrable acidity, color, viscosity, density, bromelain activity and protein concentration.

Concentration of pineapple juice, in terms of soluble solids, was measured using refractometer at 25 ± 2°C. The density of juice was determined using a specific gravity bottle and viscosity was measured using Ostwald viscometer (capillary diameter 1.0 mm) using water as a reference at 25 ± 2°C (Lee, 1975). The pH of the juice was determined at room temperature (25 ± 2°C) using pH meter (Control dynamics, Model: APX175 E/C). The acidity of the fruit juice was estimated by titration method using 0.10 N sodium hydroxide and phenolphthalein (AOAC, 1990). Results were expressed as % w/w of citric acid. Amount of ascorbic acid present in the fruit juice was determined by titrating known amount of fruit juice and metaphosphoric acid-acetic acid mixture against 2,6-dichlorophenol indophenol dye solution. The results are expressed in mg of ascorbic acid per 100 ml of fruit juice (Ranganna, 1986; James, 1995). The bromelain activity in pineapple juice was measured by casein digestion units (CDU) method (Murachi, 1976) and protein concentration was estimated by bradford method using BSA as a standard (Bradford, 1976).

4B.3 Results and Discussion

4B.3.1 Removal of bromelain

In the following section, removal of bromelain from the pineapple juice by adsorption processes was discussed. The effect of pH of the pineapple juice (pH 2.5 to 5.0) and bentonite concentration (1000 to 5000 ppm at pH 3) on bromelain activity and protein concentration was studied.

4B.3.1.1 Effect of bentonite concentration and pH on bromelain activity

The removal of bromelain from pineapple juice depends on the interaction between bromelain and bentonite at any given pH. Bentonite is most widely used for the clarification of fruit juices and wine (Kean and Marsh, 1956; Rai *et al*., 2006). Bentonite being negatively charged attracts positively charged bromelain (pI of the bromelain is 3.4) present in the pineapple juice. The effect of juice pH (2.5 to 5.0, at fixed bentonite concentration of 4000 ppm) on bromelain activity and protein concentration is presented in Fig. 4B.1. It can be observed from the figure that decrease in juice pH resulted in decrease in bromelain activity and protein concentration in the juice. The decrease in juice pH resulted in an increase in net negative charge of bromelain, which in turn resulted in higher attraction of enzyme towards the positively charged adsorbent.

The effect of bentonite concentration (1000 to 5000 ppm at juice pH 3.0) on bromelain activity and protein concentration is shown in Fig. 4B.2. It can be observed (Fig. 4B.2) that the bromelain activity and protein concentration in the juice decreased with an increase in bentonite concentration, mainly due to increased extent of adsorption of bromelain. From, the Figs 4B.1 & 4B.2, it can be concluded that the bromelain present in the pineapple juice is completely removed at bentonite concentration of 4000 ppm at pineapple juice pH 3.0. Such pretreatment of the juice not only removes the bromelain but also clarifies the pineapple juice. The main advantage of present process is removal of bromelain without application of heat treatment, which helps in retention of thermolabile components present in the pineapple juice. The

pretreated pineapple juice was stored at 4°C and used as a feed during osmotic membrane distillation process.

4B.3.2 Osmotic membrane distillation for the concentration of pineapple juice

In the following sections, the effect of various process parameters such as osmotic agent concentration, flow rate of feed and osmotic agent on transmembrane flux was discussed. Mass and heat transfer coefficients and contribution of concentration as well as temperature polarization effects on flux reduction at different process conditions were estimated. Further, theoretical mass as well as heat fluxes during osmotic membrane distillation process were modeled and compared with experimental results.

4B.3.2.1 Effect of osmotic agent solution concentration on transmembrane flux

The effect of osmotic agent (calcium chloride dihydrate, 2, 4, 6, 8 and 10 molality) concentration on transmembrane flux (initial concentration of pineapple juice was 12.6 ± 0.2°Brix) is presented in Fig. 4B.3. During these experiments, the feed side and osmotic agent side flow rates were maintained at 100 ml/min. It can be observed from the figure that the increase in osmotic agent concentration (2 to 10 molality) resulted in an increase in transmembrane flux. The increase in flux can be attributed to an increase in vapor pressure difference across the membrane. This is due to the increase in the concentration of osmotic agent solution, which resulted in a decrease in

vapor pressure (or water activity) on osmotic agent side, which in turn resulted in an increase in driving force for water transport across the membrane.

4B.3.2.2 Effect of osmotic agent and feed side flow rate on transmembrane flux

Experiments were carried out by maintaining feed side flow rate at 100 ml/min and varying osmotic agent side flow rate from 25 to 100 ml/min, which resulted in increase in transmembrane flux from 4.9×10^{-7} to 5.9×10^{-7} $m^3m^{-2}s^{-1}$ (Fig. 4B.4). This could be attributed to the reduction in concentration polarization layer, which in turn increases the transmembrane flux. This confirms the detrimental effect of concentration polarization in reducing the driving force across the membrane and the effect decreases with an increase in flow rate.

In order to study the effect of feed flow rate, experiments were performed by varying feed flow rate from 25 to 100 ml/min while maintaining the osmotic agent side flow rate at 100 ml/min and concentration at 10 molality. The effect of feed flow rate on transmembrane flux is shown in Fig. 4B.5. The increase in feed flow rate resulted in about 8 ± 0.2% increase in transmembrane flux. The increase in transmembrane flux is due to reduction in concentration polarization effect on feed side. The effect osmotic agent flow rate was more prominent on transmembrane flux (about 20 ± 0.4% increase flux) when compared to that of feed side flow rate. This could be due to lower concentration polarization on the feed side when compared to that of osmotic agent side, and consequently flux is more dependent on the osmotic agent

velocity. Also, at low OA flow rate, the difference in values of vapor pressure near the membrane surface to bulk stream is high and decreases with an increase in osmotic agent flow rate.

4B.3.2.3 Concentration of pineapple juice/sweetlime juice

Using the best experimental conditions (that is feed and OA flow rate of 100 ml/min and calcium chloride dehydrate concentration at 10 molality) obtained previously, the concentration of pretreated pineapple juice (12.6 ± 0.2°Brix) and sweetlime juice (5.0 ± 0.1°Brix) was carried out. The water flux, density, viscosity and concentration were measured at different time intervals during the concentration of pineapple juice. The pineapple juice was concentrated up to 62 ± 0.2°Brix where as sweetlime juice was concentrated up to 55 ± 0.2°Brix. The transmembrane flux decreased with time due to increased feed concentration as shown in Fig. 4B.6. The increase in feed concentration resulted in decrease in driving force across the membrane, which in turn decreases the transmembrane flux. The physicochemical properties of untreated, clarified, concentrated and reconstituted pineapple juice are presented in Table 4B.1. The ascorbic acid content was well preserved in the pineapple juice concentrate produced by osmotic membrane distillation process. The photograph of untreated, clarified and concentrated pineapple juice is shown in Fig. 4B.7.

4B.3.2.4 Membrane mass and heat transfer coefficients

Membrane mass (k_m) and heat (h_m) transfer coefficients during OMD process were calculated using the Eqs. (4A.4) and (4A.14), respectively. The

main characteristics of the membrane are 175 μm thickness and pore diameter 0.2 μm, as specified by the supplier. For the calculation of membrane mass and heat transfer coefficients, properties of membranes such as porosity (ε, 0.75), tortuisity (τ, 2), thermal conductivity of gas (k^T_{gas}, 0.025 W/m. K) and thermal conductivity ($k^T_{polymer}$, 0.2 W/m. K) of membrane material were taken from the literature (Lawson and Lloyd, 1997). Membrane heat and mass transfer coefficients were determined to be 390 W m^{-2} k^{-1} and 2.5 x 10^{-10} ms^{-1} Pa^{-1}, respectively.

4B.3.2.5 Boundary layer mass and heat transfer coefficients

Boundary layer mass (k) and heat (h) transfer coefficients were estimated using Eqs. (4A.8) and (4A.15), respectively. The estimated values of these coefficients at different process conditions are presented in Table 4B.2. From the table, it can be observed that an increase in osmotic agent concentration resulted in decrease in boundary layer mass and heat transfer coefficients. This can be attributed to the increase in concentration of osmotic agent, which in turn increases the thickness of boundary layer. An increase in feed flow rate from 25 to 100 ml/min at a constant osmotic agent flow rate (100 ml/min) resulted in an increase in feed side heat and mass transfer coefficients. Similarly an increase in osmotic agent flow rate from 25 to 100 ml/min at a constant feed flow rate (1 100 ml/min) was found to result in an increase in osmotic agent side heat and mass transfer coefficients (Table 4B.2). This increase could be attributed to reduction of thickness of boundary layer due to increase in feed or osmotic agent flow rate. From the Table 4B.2, it can be observed that the osmotic agent side mass transfer coefficients were

always larger than that of feed side. This may be due to the variation in the thickness of the channels on either side of the membrane (feed side -3 mm, osmotic agent side - 0.25 mm).

4B.3.2.6 Overall mass and heat transfer coefficients

Overall mass (K) and heat (U) transfer coefficients are estimated using the Eqs. (4A.11) and (4A.16) and the values for different process conditions are presented in Table 4B.2. It may be noted that even though the feed or osmotic agent side mass/heat transfer coefficients are varying with feed or osmotic agent process parameters (concentration or flow rate), the overall heat or mass transfer coefficients did not markedly change. This can be attributed to the fact that the membrane heat or mass transfer resistance is the major transport resistance (Table 4B.2).

4B.3.2.7 Polarization effects during osmotic membrane distillation process

The polarization phenomenon during osmotic membrane distillation process depends on various process parameters such as osmotic agent concentration, flow rate of feed as well as osmotic agent solution. The reduction in driving force due to concentration polarization and total polarization (combined concentration and temperature) was calculated from the Eqs.(4A.21) and (4A.22), respectively. The values of vapor pressure difference across the membrane during osmotic membrane distillation process (ΔPb) are presented in Table 4B.3. The amount of reduction in driving force (compared to that of actual driving force, Table 4B.3) due to polarization effects at various process conditions are presented in Fig. 4B.8. It can be

observed that the driving force reduction (which in turn reduces the flux) due to concentration polarization increases with an increase in OA concentration. The driving force reduction due to temperature polarization effect was low (about 3.0 ± 0.1%) and it is about 16.0 ± 0.8 to 80.0 ± 2.0 Pa only (Fig. 4B.8a). The reduction in driving force due to total polarization effect increases from 52.0 ± 2.0 to 357.0 ± 4.0 Pa (9.8 ± 0.1 to 13.4 ± 0.1%) with an increase in osmotic agent concentration. From these results, it can be inferred that the polarization phenomenon has significant effect on driving force reduction during concentration of aqueous solutions such as pineapple juice by osmotic membrane distillation process. It can also be observed that, at any concentration of osmotic agent, the driving reduction due to concentration polarization effect was more when compared to that of temperature polarization effect. For example, at calcium chloride concentration of 8 molality, the reduction in driving force due to concentration polarization was about 225.0 ± 3.0 Pa whereas the reduction due to temperature polarization effect was only about 75.0 ± 2.0 Pa.

It can be observed that with an increase in OA flow rate, reduction in driving force due to temperature polarization did not change significantly (Fig. 4B.8b). It is interesting to note that, the contribution of total polarization effect remained constant (Fig. 4B.8b) with an increase in OA flow rate. This can be attributed to the fact that at low OA flow rate the difference in vapor pressure from the membrane surface to bulk stream is high, which in turn decreases the flux resulting in low vapor pressure depression at feed side. At high flow rate, the difference in vapor pressure from bulk to membrane surface can be minimized, which indirectly helps to obtain high flux resulting in increase vapor

pressure depression near the membrane surface at feed side. Overall reduction in driving force at both the flow conditions might be the same which resulted in less significant effect of OA flow rate on reduction in driving force.

The contribution of polarization effects towards driving force reduction at varying feed side flow rates is shown in Fig. 4B.8c. It can be observed that an increase in feed flow rate resulted in a decrease in driving force reduction and significant change in driving force reduction was not observed due to temperature polarization effect.

4B.3.2.8 Prediction of mass and heat flux

To validate the model for a real system (pineapple juice), theoretical fluxes were estimated by accounting the concentration and temperature polarization effects. Boundary layer and membrane mass and heat transfer coefficients values were used to estimate the theoretical mass and heat fluxes. The water activity data for calcium chloride dihydrate was taken from the literature (Patil *et al*., 1991). The theoretical mass (by considering total polarization effect) and heat fluxes during osmotic membrane distillation were calculated using Eqs. 4A.10 and 4A.16. The feed and OA boundary layer resistances could not be ignored even though they were low. It may be noted that the theoretical values of the transmembrane flux could be estimated after calculating the overall mass transfer resistance (membrane plus boundary layers). The values of theoretical mass and heat fluxes are presented in Table 4B.3. Comparison of theoretical fluxes with experimental fluxes at different experimental conditions was shown in Fig. 4B.9. The model could predict the

transmembrane flux and also the effect of the parameters on transmembrane flux. The experimental fluxes were in good agreement with theoretical fluxes at all the process conditions studied.

4B.4 Conclusions

The bromelain present in pineapple juice could be completely removed by bentonite adsorption. The influence of the process parameters, such as osmotic agent concentration and flow rate of feed and osmotic agent on transmembrane flux was evaluated for clarified pineapple juice. The osmotic agent concentration and flow rate were found to have significant effect on transmembrane flux. Boundary layer and overall mass and heat transfer coefficients were estimated during the concentration of clarified pineapple juice. The effect of concentration and temperature polarization on driving force (flux) reduction at various experimental conditions was examined. It was observed that the contribution of concentration polarization on driving force (in turn flux) reduction was more prominent when compared to that of temperature polarization. Under best experimental conditions, pretreated pineapple juice was concentrated up to 62.0 ± 0.2°Brix while preserving the ascorbic acid content of the pineapple juice.

Table 4B.1: Physicochemical characteristics of pineapple juice

Characteristic	Untreated	Treated	Concentrated	Reconstituted
pH	3.80 ± 0.20	3.00 ± 0.20	3.18 ± 0.20	3.10 ± 0.20
Titarable acidity (g citric acid/100 g)	0.85 ± 0.10	2.00 ± 0.20	5.80 ± 0.20	1.94 ± 0.30
Ascorbic acid (mg/100ml)	13.50 ± 1.0	13.5 ± 0.50	52.5 ± 0.50	13.30 ± 1.0
°Brix	12.60 ± 0.20	12.60 ± 0.10	62 ± 0.20	12.60 ± 0.10
Density (kg/m^3)	1092 ± 2.0	1090 ± 2.0	1310 ± 2.0	1090 ± 2.0
Viscosity (mPas)	1.40 ± 0.20	1.24 ± 0.2	36.0 ± 2.0	1.34 ± 0.20
Bromelain activity (CDU/ml)	850 ± 20	0.0	0.0	0.0
Protein concentration (mg/ml)	0.48 ± 0.10	0.18 ± 0.20	0.49 ± 0.20	0.18 ± 0.10

Table 4B.2: Estimated mass and heat transfer coefficients

Process condition	Mass transfer coefficients			Heat transfer coefficients		
	Feed side ($k_1 \times 10^5$ ms^{-1})	OA side ($k_2 \times 10^5$ ms^{-1})	Overall ($K \times 10^{10}$ ms^{-1} Pa^{-1})	Feed side, h_1 $Wm^{-2}K^{-1}$	OA side, h_2 $Wm^{-2}K^{-1}$	Overall, U $Wm^{-2}K^{-1}$
Osmotic agent (OA) solution concentration (molality)[a]						
2	1.31 ± 0.10	10.8 ± 0.20	2.31 ± 0.10	740 ± 0.2	8090 ± 12.0	248.8 ± 0.10
4	1.31 ± 0.10	7.59 ± 0.10	2.30 ± 0.10	740 ± 0.2	7035 ± 3.0	247.7 ± 0.10
6	1.31 ± 0.10	4.72 ± 0.10	2.29 ± 0.10	740 ± 0.2	6029 ± 9.0	246.2 ± 0.10
8	1.31 ± 0.10	3.19 ± 0.10	2.27 ± 0.10	740 ± 0.2	5190 ± 6.0	244.6 ± 0.10
10	1.31 ± 0.10	2.18 ± 0.20	2.24 ± 0.10	740 ± 0.2	4460 ± 9.0	242.8 ± 0.12
Osmotic agent Flow rate (ml/min)[b]						
25	1.31 ± 0.10	1.09 ± 0.10	2.15 ± 0.10	740 ± 0.2	2234 ± 4.0	230.3 ± 0.10
50	1.31 ± 0.10	1.54 ± 0.10	2.20 ± 0.10	740 ± 0.2	3160 ± 7.0	237.4 ± 0.12
75	1.31 ± 0.10	1.89 ± 0.20	2.22 ± 0.10	740 ± 0.2	3870 ± 8.0	240.8 ± 0.10
100	1.31 ± 0.10	2.18 ± 0.20	2.24 ± 0.10	740 ± 0.2	4469 ± 1.0	242.8 ± 0.10
Feed Flow rate (ml/min)[c]						
25	0.66 ± 0.10	2.18 ± 0.20	2.11 ± 0.10	370 ± 0.2	4460 ± 9.0	182.9 ± 0.10
50	0.93 ± 0.10	2.18 ± 0.20	2.18 ± 0.10	523 ± 0.2	4460 ± 9.0	213.8 ± 0.10
75	1.14 ± 0.20	2.18 ± 0.20	2.22 ± 0.10	641 ± 0.3	4460 ± 9.0	231.1 ± 0.12
100	1.31 ± 0.10	2.18 ± 0.20	2.24 ± 0.10	740 ± 0.2	4460 ± 9.0	242.8 ± 0.12

[a]OA calcium chloride dihydrate; Feed flow rate 100 ml/min; OA flow rate 100 ml/min; [b]OA: Calcium chloride dihydrate (10 molality), Feed flow rate 100 ml/min; [c]OA: Calcium chloride dihydrate (10 molality), OA flow rate 100 ml/min; k_1 = Mass transfer coefficient in feed side boundary layer; k_2 = Mass transfer coefficient in osmotic agent side, K = Overall mass transfer coefficient; boundary layer; h_1 = Heat transfer coefficient in feed side boundary layer; h_2 = Heat transfer coefficient in osmotic agent side boundary layer; U = Overall heat transfer coefficient

Table 4B.3: Estimated mass and heat flux during osmotic membrane distillation process as well as actualdriving force

Process condition	Mass flux x 10^7 ($m^3m^{-2}s^{-1}$)	Heat flux (Wm^{-2})	Actual driving force (Pa)
Osmotic agent concentration (molality)			
2	1.20	172	538
4	2.71	394	1216
6	4.64	707	2094
8	5.79	837	2629
10	6.05	899	2782
Osmotic agent (OA) Flow rate (ml/min)			
25	5.63	626	2782
50	5.87	739	2782
75	5.97	839	2782
100	6.05	899	2782
Feed Flow rate (ml/min)			
25	5.85	709	2782
50	5.97	796	2782
75	6.02	866	2782
100	6.05	899	2782

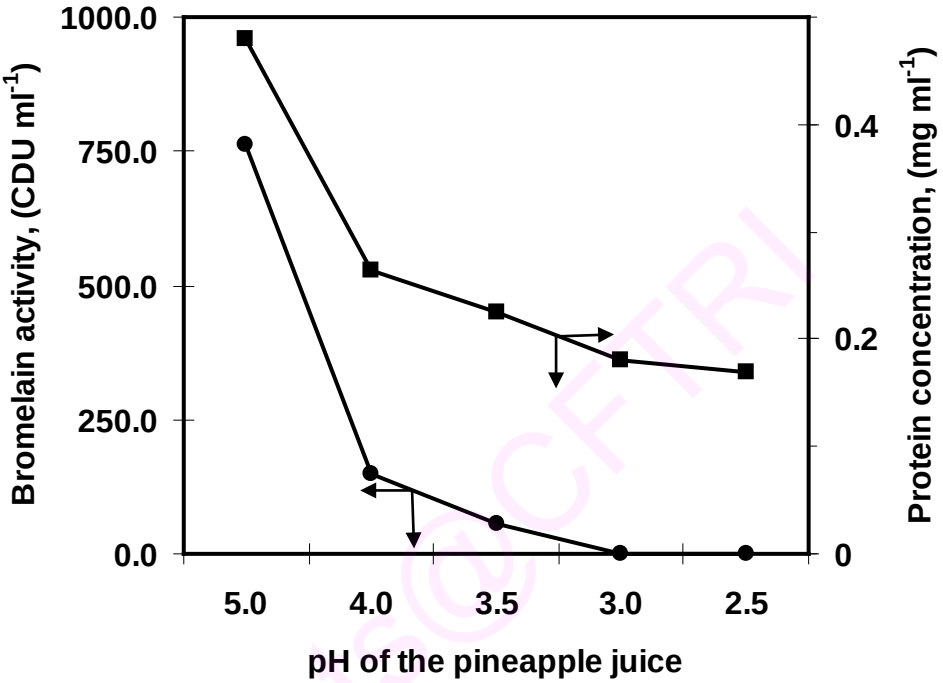


Figure 4B.1: Effect of pineapple juice pH on bromelain activity and protein concentration at bentonite concentration of 4000 ppm

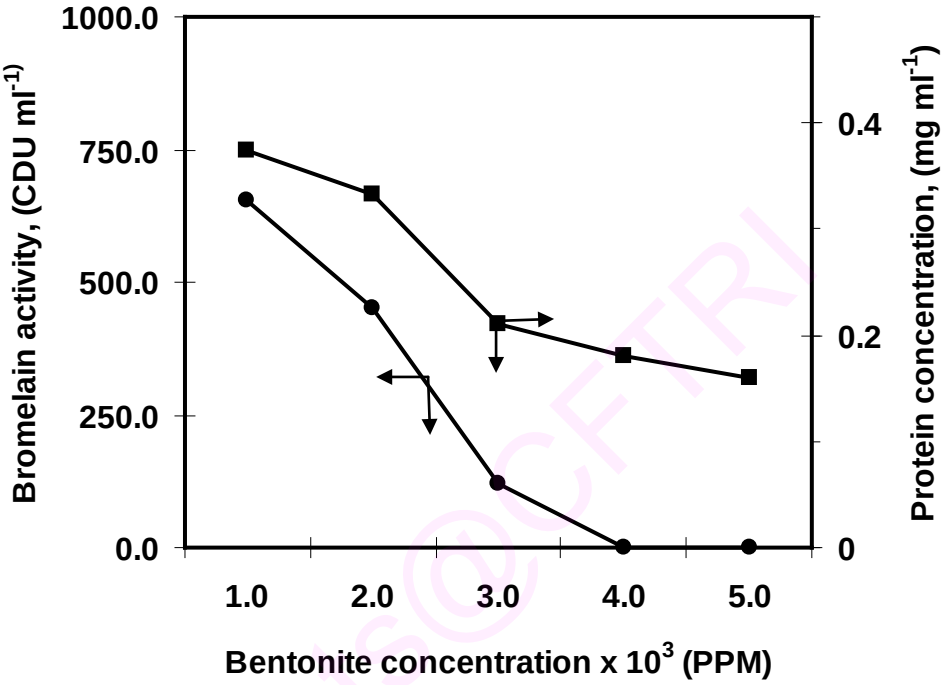


Figure 4B.2: Effect of bentonite concentration on bromelain activity and protein concentration at pineapple juice pH 3.0

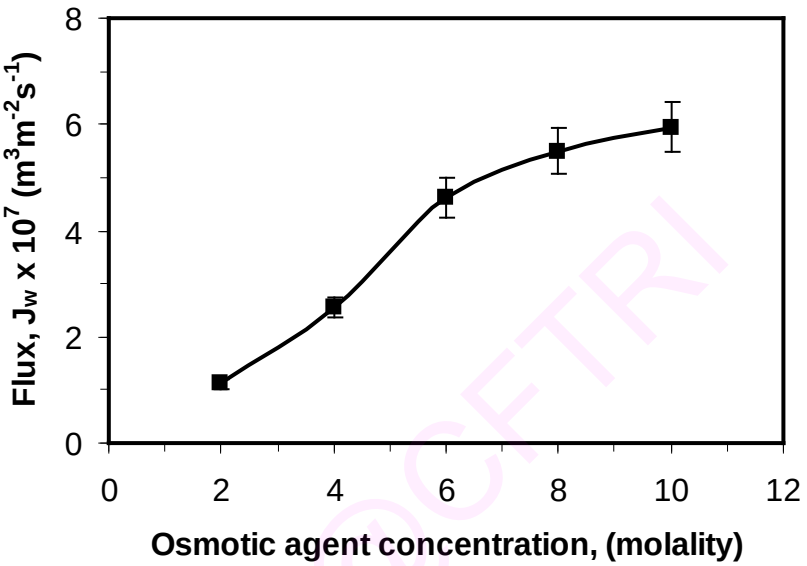


Figure 4B.3: Effect of osmotic agent concentration on transmemrbane flux (Feed flow rate 100 ml/min; OA flow rater 100 ml/min)

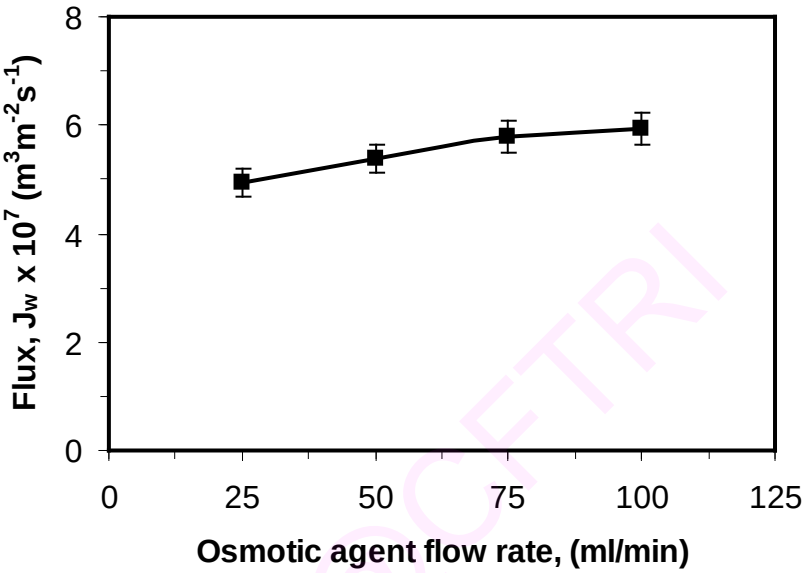


Figure 4B.4: Effect of osmotic agent flow rate on transmembrane flux (OA: Calcium chloride dihydarate (10 molality); Feed flow rate 100 ml/min)

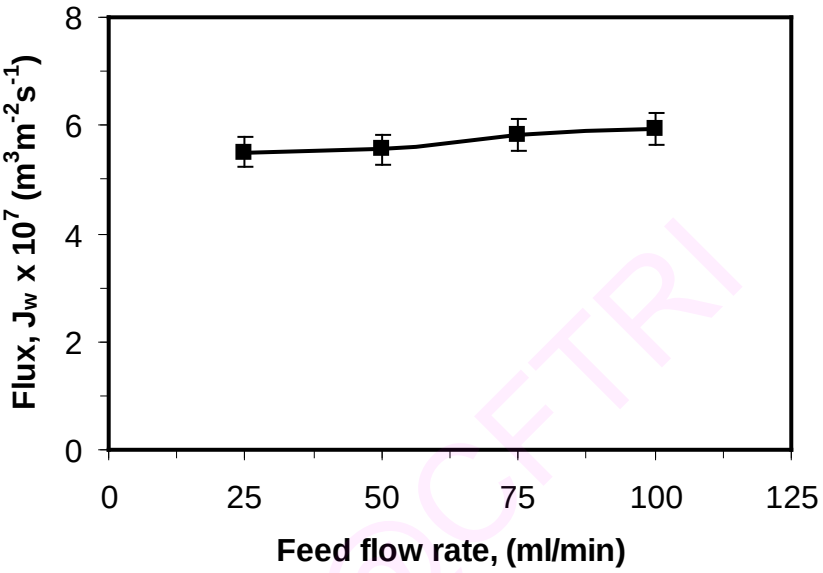


Figure 4B.5: Effect of feed flow rate on transmembrane flux (OA: Calcium chloride dihydarate (10 molality); OA flow rate 100 ml/min)

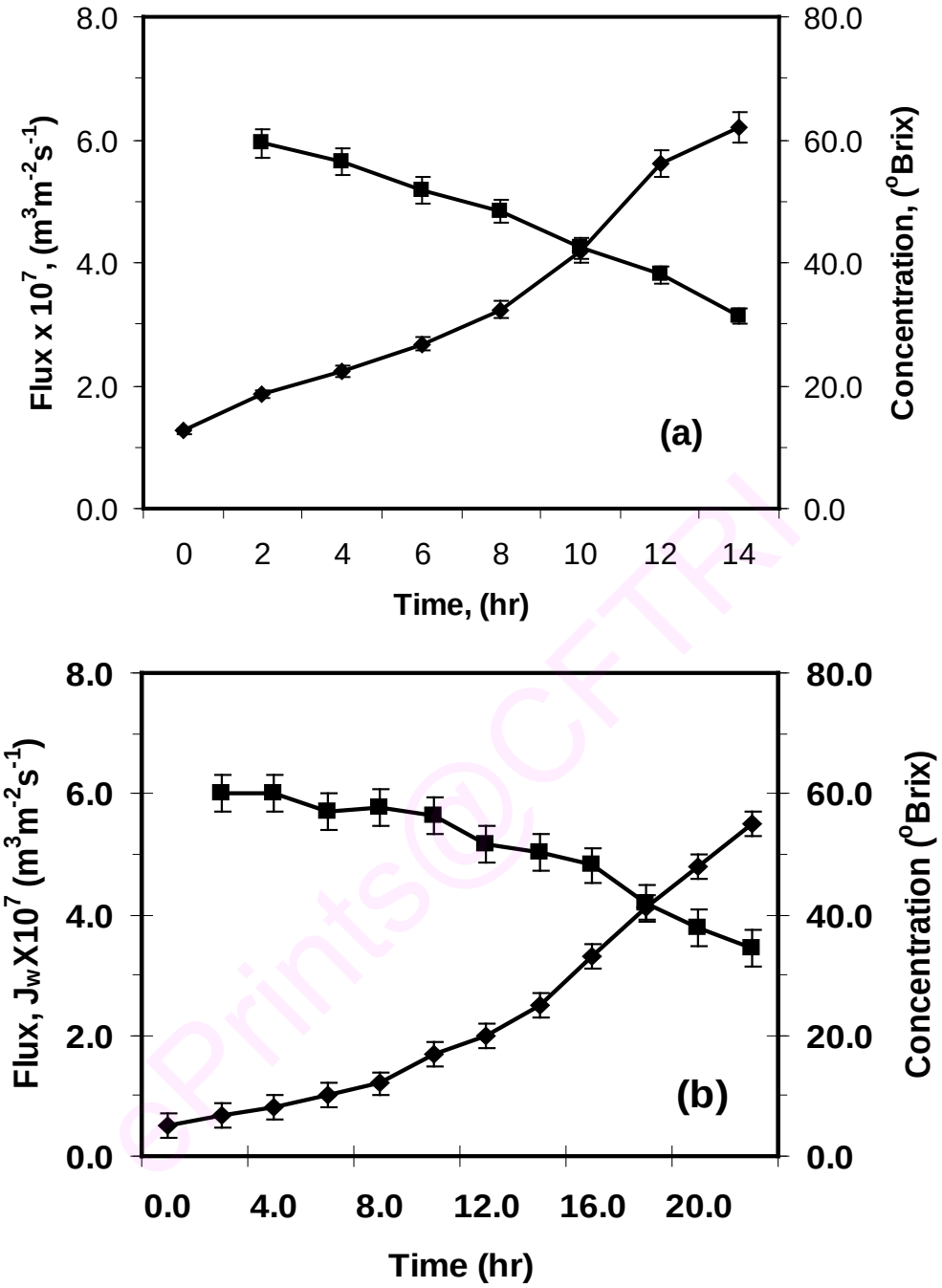


Figure 4B.6: Variation in transmembrane flux and feed concentration during the concentration of (a). Pineapple juice; (b). Sweetlime juice by osmotic membrane distillation process (-■- flux; -◆-concentration; (OA: Calcium chloride dihydarate (10 molality); Feed flow rate 100 ml/min; OA flow rate 100 ml/min))

Crude Clarified Concentrated

Figure 4B.7: Photograph of crude (untreated), clarified and concentrated pineapple juice

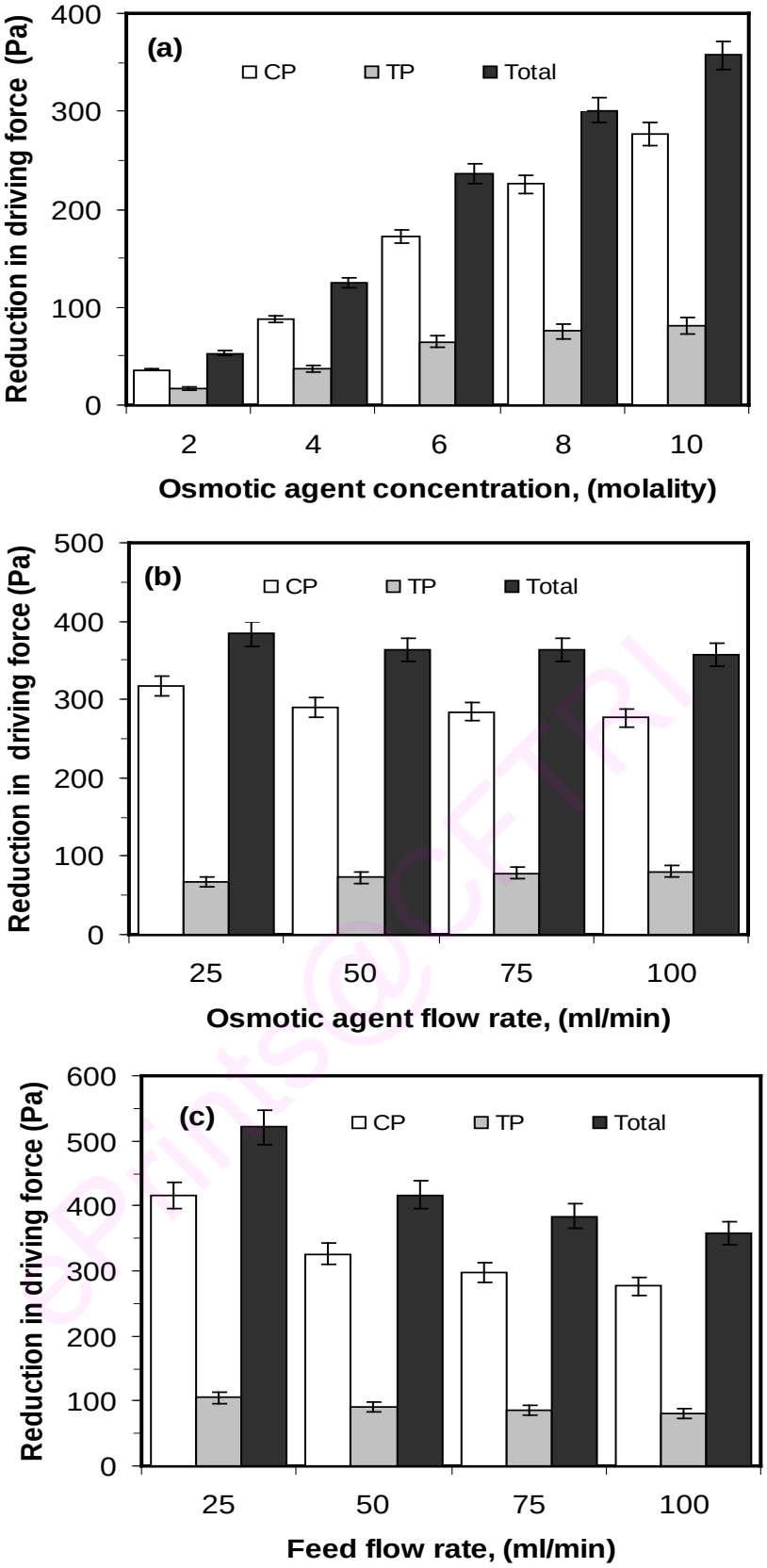


Figure 4B.8: Contribution of polarization effects on driving force reduction during OMD at various process conditions (a). OA concentration; (b). Feed flow rate; (c). OA flow rate

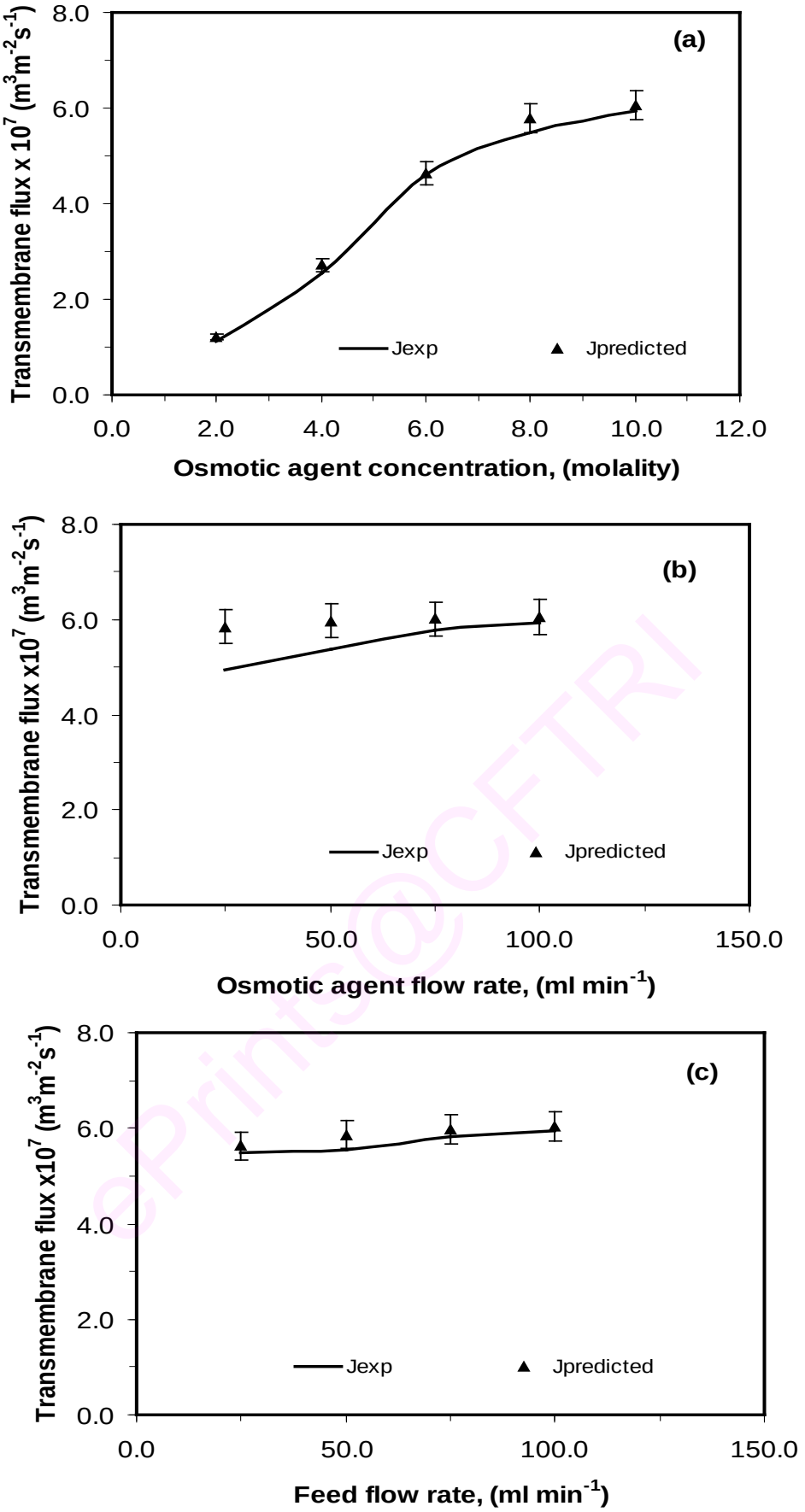


Figure 4B.9: Model fitting between experimental and theoretical flux at different experimental conditions (a). osmotic agent concentration; (b). feed flow rate; (c). osmotic agent flow rate

CHAPTER - 5

Integrated Bioseparation

CHAPTER – 5A

Integrated membrane bioseparation

5A.1 Introduction

Biomolecule purification or concentration is a crucial process in biotechnology due to its wide range of applications in biomedical and food industries. The techniques used for protein separation and purification such as chromatography, electrophoresis, and affinity chromatography are generally used for producing small quantities of proteins in research laboratories. However, these techniques are rather difficult to scale-up, which limit production levels. Besides, some methods like chromatography and electrophoresis require complex instrumentation support to run efficiently and usually yield low throughput of the products at an extremely high process cost. Hence, the separation techniques that can yield high throughput of the products at a low cost are highly desired in biotechnological industries. Membrane separation is one such technique which is gaining considerable amount of attention in recent years for the separation, purification and concentration of biomolecules (Ghosh, 2003). A large number of membrane separation processes are currently being practiced in various sectors of bioprocess industries. Despite the advantages, membrane processes often suffer from shortcomings when used individually. For example, ultrafiltration process which is widely used for the purification and concentration of biomolecules suffers from the several drawbacks such as membrane fouling and concentration polarization. Even newer membrane processes such as direct osmosis/osmotic membrane distillation have the problem of low flux. In order to overcome such limitations and to improve the overall productivity of process, purification and concentration of biomolecules, by hybrid (integrated) membrane processes appears to be very attractive (Raghavarao *et al*., 2005;

Suk and Matsuura, 2006; Ganapathi and Raghavarao, 2007b). Membrane based hybrid processes are classified as two types; one that combines a membrane process with another membrane process (for example integration of MF/UF/NF for purification /concentration of biomolecules) and other that combines a membrane process with a conventional separation process (for example integration of enzymatic reactor with pervaporation). In this chapter integration of ultrafiltration with newer membrane processes (direct osmosis/osmotic membrane distillation) and precipitation, centrifugation, dialysis followed by direct osmosis for the purification and concentration of bromelain from pineapple waste has discussed.

5A.2 Materials and Methods

5A.2.1 Materials

5A.2.1.1 Membranes

Hydrophilic direct osmosis membranes (Osmotek, Inc., Corvallis, OR), hydrophobic polypropylene membranes (0.2 μm, Accurel, Enka, Germany), hydrophilic polysulphone membranes (MWCO 10,000 Da, M/S, Permionics, Baroda, India) were used in the present study.

5A.2.1.2 Raw material

The pineapple fruits (*Ananas comosus* L) used in this study was obtained from the local market. The peel and core portion of the fruits were separated and used for enzyme extraction.

5A.2.1.3 Chemicals

Calcium chloride dihydrate, sodium chloride, trichloracetic acid and sodium acetate were procured from Ranbaxy Ltd., India. Casein (Hammarsten grade) was procured from Loba Chemicals, India. Bovine Serum Albumin (BSA) was procured from SRL Ltd., India.

5A.2.2 Methods

5A.2.2.1 Enzyme Extraction

The enzyme was extracted from the frozen pieces of pineapple peel and core as per the procedure given in Chapter 3A.

5A.2.2.2 Osmotic agent solution

Osmotic agent solutions were prepared by dissolving known quantity of calcium chloride dihydrate and sodium chloride in distilled water.

5A.2.2.3 Experimental procedure

5*A*.2.2.3.1 Ultrafiltration

Ultrafiltration experiments were conducted in a stirred cell (Amicon 8060) having working volume of 50 ml (Fig. 5A.1). The module is fitted with membrane discs, which were cut out from flat sheets of polysulphone membrane of molecular weight cut-off of 10,000 Da (M/S, Permionics, Baroda, India). The effective diameter of the membrane within the module was 45 mm.

The operating pressure was varied from 1 to 4 bars and magnetic stirrer speed was maintained at ~300 rpm. Permeate and retentate samples were collected at different time intervals and analyzed for bromelain activity, protein concentration and total sugar content. All experiments were carried out in triplicates and averaged values were reported. The purified bromelain enzyme extract (retentate) was stored at 4°C and further it was concentrated by DO/OMD process.

5A.2.2.3.2 Direct osmosis/osmotic membrane distillation (DO/OMD)

The details DO/OMD plate and frame membrane module used for the concentration of biomolecules was already described in Chapter 3 and 4. The purified bromelain extract and osmotic agent solution were circulated on either side of the membrane in co-current mode using peristaltic pumps (Monostat, Model 72-315-230). The transmembrane flux was calculated, by measuring the increase in volume of osmotic agent once in every hour. The temperature of feed solution and osmotic agent solution were measured using calibrated thermometer. All the experiments, unless otherwise mentioned, were carried out at the temperature of 25.0 ± 2.0ºC.

5A.2.2.3 Estimation of bromelain activity, protein and sugar concentration

The bromelain activity was determined according to the casein digestion unit (CDU) method using casein as a substrate (Murachi, 1976) and concentration of the protein was determined by dye binding method (Bradford, 1976) using Coomassie Blue G250. The total sugar present in bromelain

extract was estimated by Dubois method (1956). The detailed procedure for the estimation or bromelain, proteins and sugars were given in Chapter 2B.

5A.2.2.3 Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as per procedure given in Methods in Enzymology (Deutsher, 1990) using 12% polyacrylamide slab gel. In lane 1 molecular marker was loaded, in lane 2, 3 and 4 crude bromelain extract, dialyzed bromelain extract after ammonium sulphate precipitation and concentrated bromelain extract after direct osmosis processes were loaded. In all the lanes equal volumes of sample were loaded. Electrophoresis was run at 50 V, 12.5 mA for about 4-6 h. the gel was stained with a Coomassie Brilliant Blue (CBB) R250 of 0.05 % (w/v), 50 % (v/v) methanol and 12 % (v/v) of acetic acid. The gel was destained using same buffer without Coomassie Brilliant Blue.

5A.3 Results and Discussion

5A.3.1 Integrated membrane process

The integrated membrane process employed for the purification and concentration of bromelain from pineapple waste is shown in Fig. 5A.2. The ultrafiltration process is mainly employed for the elimination of sugar and impurities present in the crude bromelain enzyme extract. The variation in permeate flux with time at different pressures during ultrafiltration of enzyme extract is shown in Fig. 5A.3a. The permeate flux decreases rapidly in the initial stages and gradually thereafter. The rapid flux decline can be attributed

to the formation of concentration polarization layer. The average permeate flux was higher at 4 bar and hence further experiments were conducted at this pressure. The percentage of sugar removed and bromelain activity at various time intervals during ultrafiltration experiments (at 4 bar pressure and ~300 rpm) was shown in Fig. 5A.3b. The ultrafiltration process is able to remove 74.0 ± 2.0% of sugars present in the crude bromelain extract with 1.2 ± 0.1 fold increase in purity.

Further, the purified bromelain extract (ultrafiltration retentate) was concentrated using direct osmosis/osmotic membrane distillation process. The feed and osmotic agent flow rates were maintained at 100 ml/min. Based on best operating conditions obtained earlier (Chapter 3 and Chapter 4), sodium chloride solution (6 molality) was selected as an osmotic agent during direct osmosis process and calcium chloride dihydrate (10 molality) as an osmotic agent solution during osmotic membrane distillation process. The variation in transmembrane flux and bromelain activity during the concentration of purified bromelain extract by direct osmosis and osmotic membrane distillation are presented in Fig. 5A.4 and 5A.5, respectively. The purified bromelain extract is concentrated up to 3960 ± 5.0 CDU/ml by direct osmosis and concentrated up to 2500 ± 4.0 CDU/ml by osmotic membrane distillation.

5A.3.2 Integration of precipitation, centrifugation, dialysis and direct osmosis process

Purification of crude bromelain enzyme extract from pineapple waste was carried out using ammonium sulphate precipitation method followed by centrifugation and dialysis. Ammonium sulphate salt (31.4g/100mL) was added to the crude extract to obtain 50% saturation and then sample was centrifuged at 10000 rpm at 4°C for 20 min. The resulting pellet was dissolved in 0.01 M phosphate buffer, pH 7 and desalted by dialyzing against the same buffer. The dialyzed bromelain extract was further concentrated by direct osmosis using sodium chloride (6 molality) as an osmotic agent. During the study the feed and osmotic agent side flow rates were maintained at 100 ml/min. The bromelain activity and protein concentration of crude, dialyzed extract and concentrated extract are presented in Table 5A.1. The PAGE profiles of crude, dialyzed and concentrated enzyme extracts are given in Fig. 5A.6. During SDS-PAGE run, the quantity of the sample loaded into the lanes was kept constant (40 μl). From the PAGE, profile, it can be observed that intensity of the bands showing the increased concentration of bromelain after each step.

5A.3.3 Stability of the crude and concentrated bromelain enzyme

The stability of the crude bromelain extract (439.0 ± 2.0 CDU/ml) and concentrated bromelain extract (3960 ± 5.0 CDU/ml) at ambient temperature (25.0 ± 2.0°C) and refrigeration temperature (5.0 ± 2.0°C) is shown in Fig. 7a & b. At ambient temperature (Fig. 7a), the proteolytic activity of bromelain decreased by ~44% and ~7% of its original activity over first 24 h for crude

and concentrated extracts, respectively. At refrigeration temperature (Fig. 7b) the dilute bromelain extract was stable for ~4 days whereas concentrated enzyme extract was stable for ~7 days. Thus the concentrated bromelain extract was more stable in terms of proteolytic activity of bromelain enzyme when compared to dilute crude enzyme extract.

5A.4 Conclusions

Integrated approaches, namely (1). ultrafiltration coupled with direct osmosis/osmotic membrane distillation, and (2). precipitation, centrifugation, dialysis followed by direct osmosis process were evaluated for the purification and concentration of bromelain from pineapple waste. The bromelain enzyme could be concentrated up to 3960 ± 5.0 CDU/ml by an integrated approach involving ultrafiltration (at 4 bar pressure and 300 rpm) and direct osmosis process using sodium chloride (6 molality) as an osmotic agent. The concentrated bromelain extract was stable both at ambient (25.0 ± 2.0°C) and refrigeration (5.0 ± 2.0°C) temperature in terms of proteolytic activity of bromelain extract when compared to dilute crude extract.

Table 5A.1: Bromalin activity and protein concentration of crude, dialyzed and concentrated enzyme extracts

Type	Bromelain activity (CDU/ml)	Protein concentration (mg/ml)	Specific activity (CDU/mg)
Crude	468 ± 2.0	0.340 ± 0.005	1376 ± 9.0
Dialyzed	665 ± 5.0	0.380 ± 0.004	1750 ± 8.0
Concentrated	1628 ± 10	0.935 ± 0.006	1741 ± 4.0

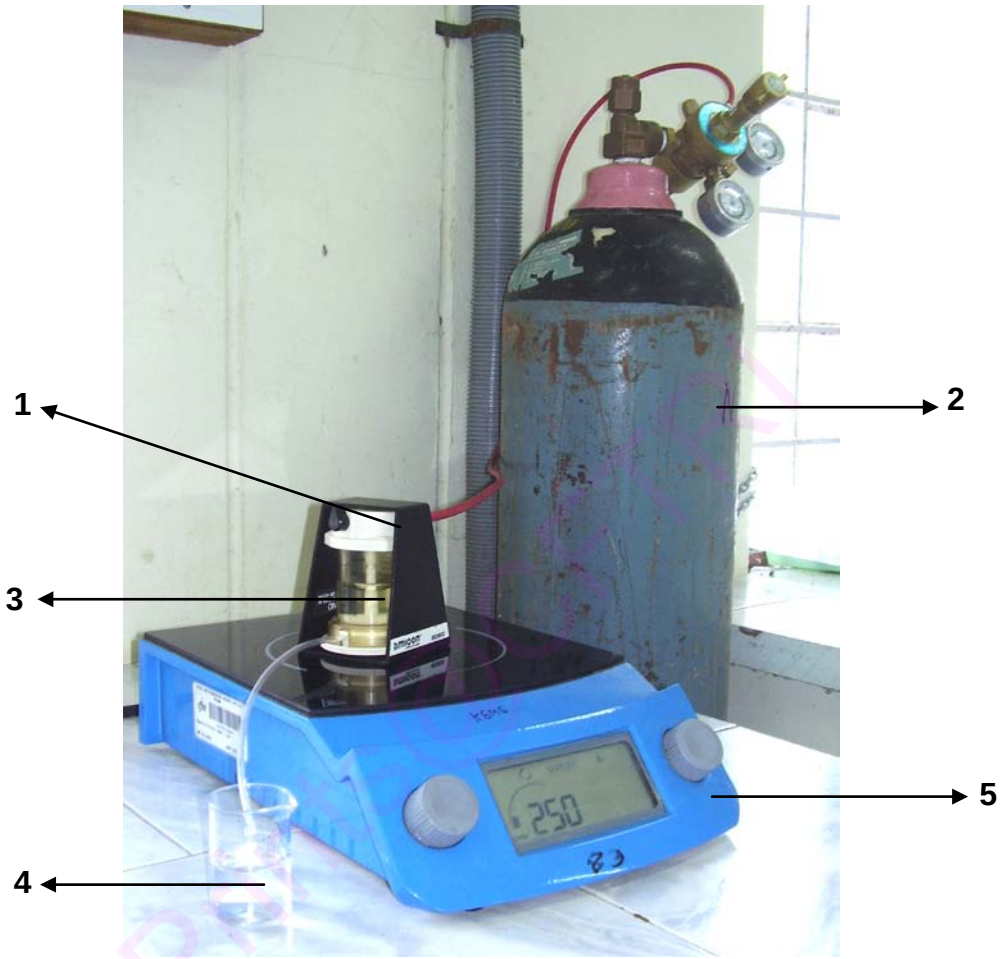


Figure 5A.1: Photograph of ultrafiltration stirred membrane cell. (1. membrane cell; 2. nitrogen cylinder; 3. retentate; 4. permeate; 5. magnetic stirrer)

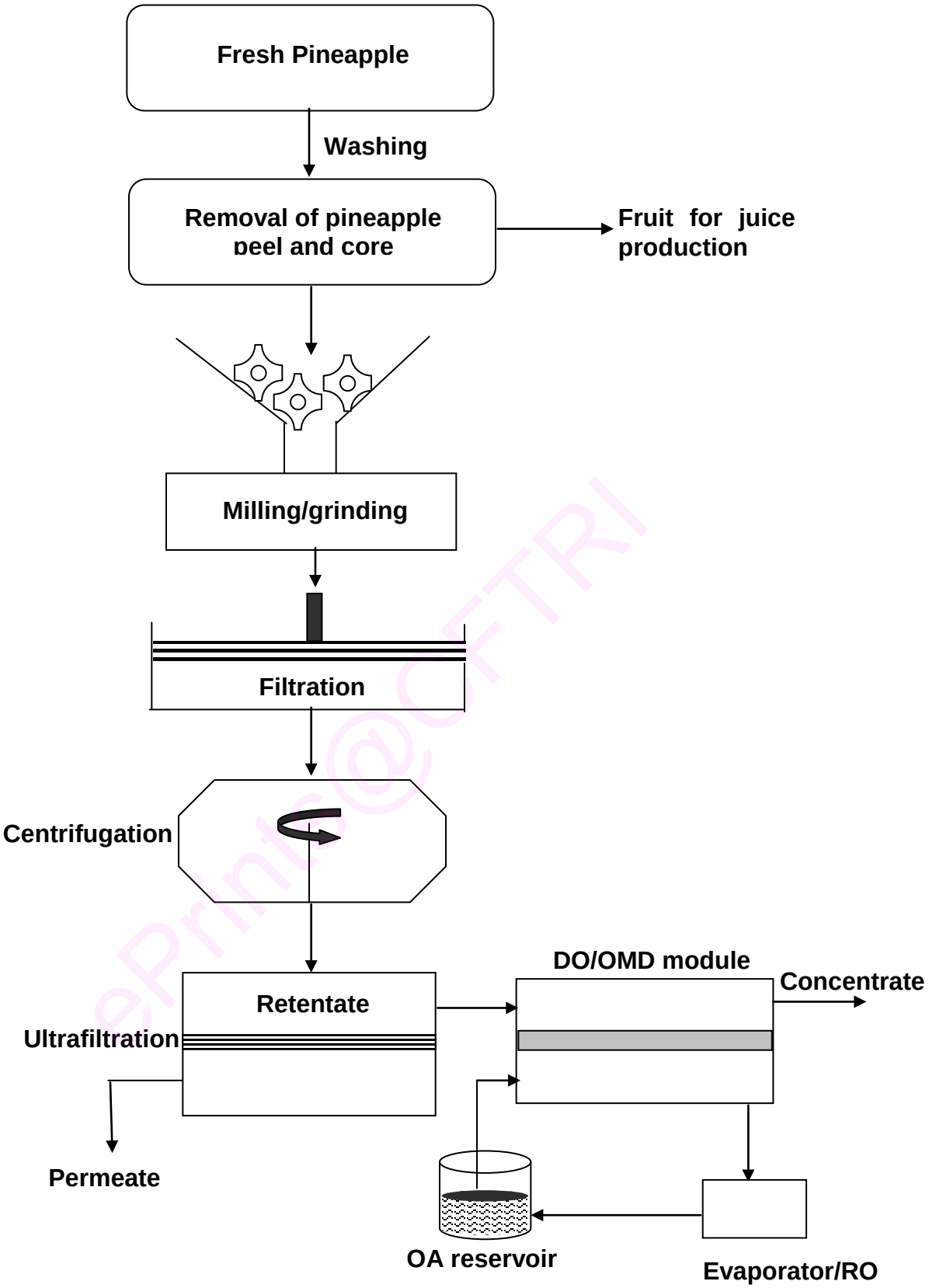


Figure 5A.2: Integrated membrane process for the purification and concentration of bromelain from pineapple waste

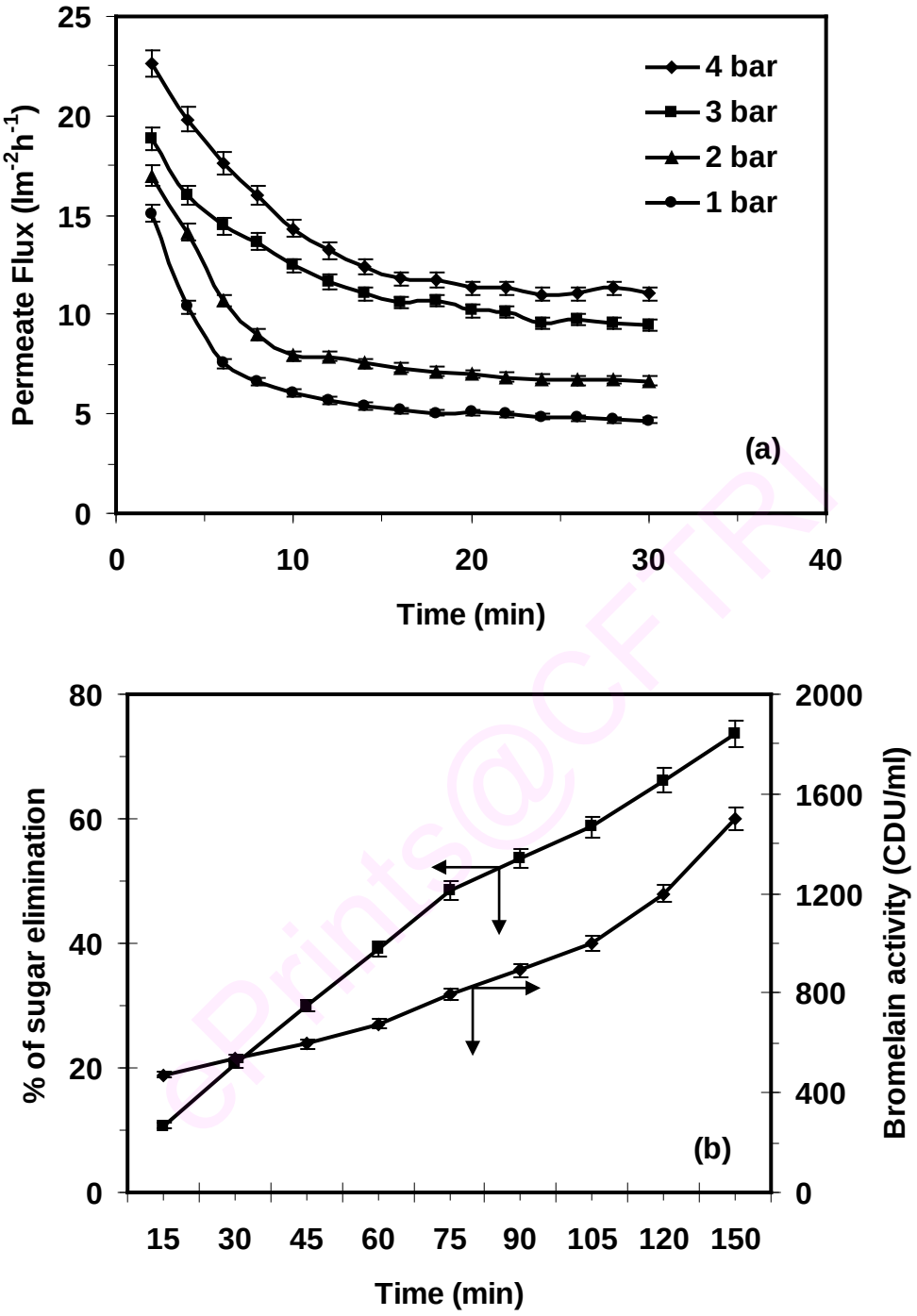


Figure 5A.3: (a). Effect of transmembrane pressure on permeate flux; (b). Variation in sugar eliminated and bromelain activity at 4 bar transmembrane pressure during ultrafiltration

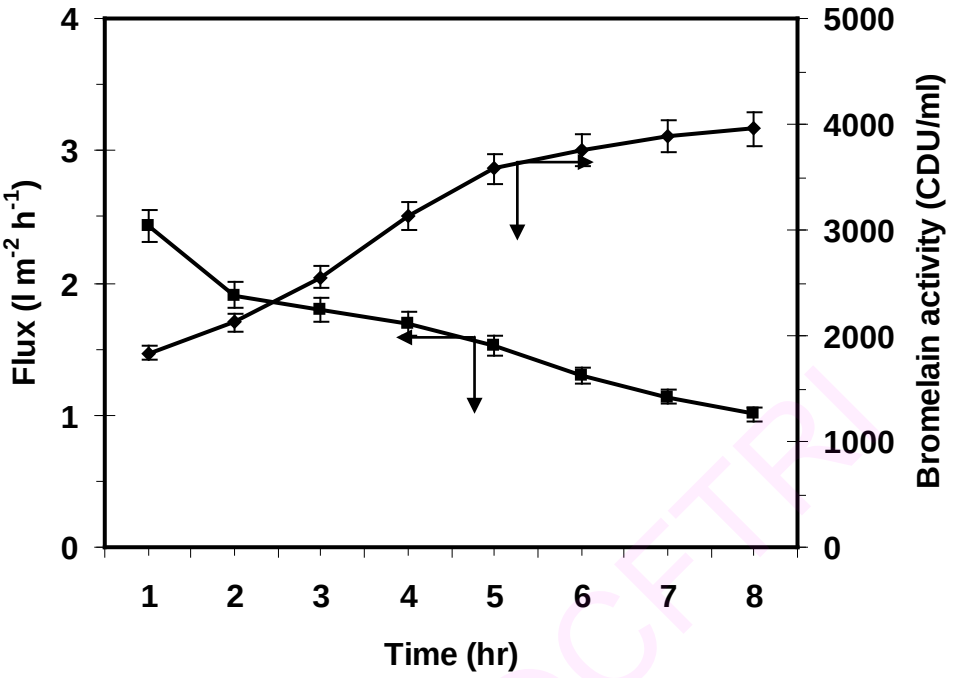


Figure 5A.4: Variation in transmembrane flux and bromelain activity during concentration of purified bromelain extract (ultrafiltration retentate) by direct osmosis process

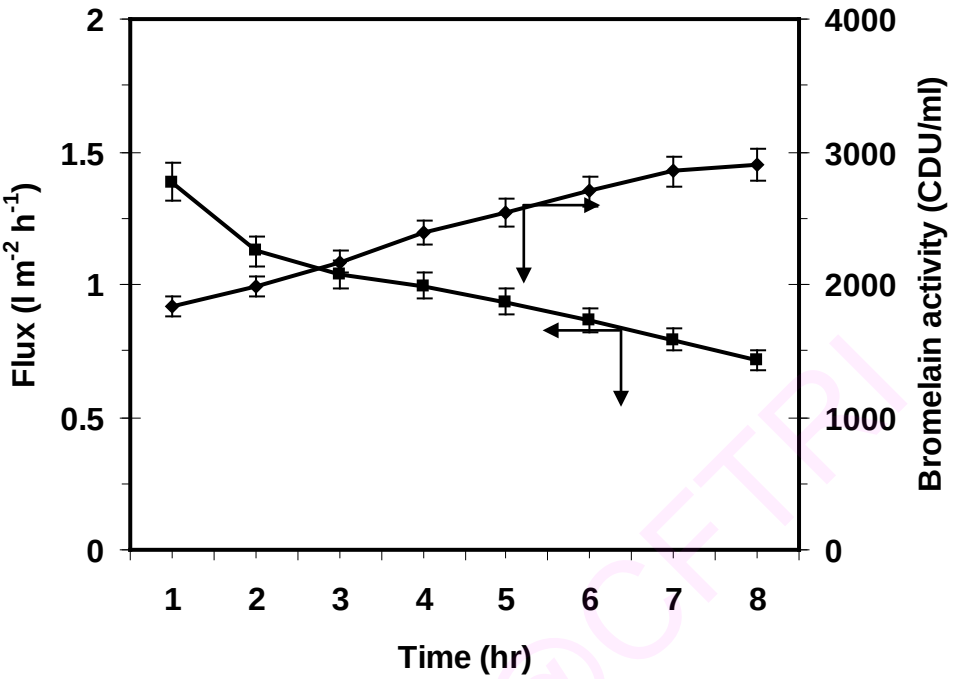


Figure 5A.5: Variation in transmembrane flux and bromelain activity during concentration of purified bromelain extract (ultrafiltration retentate) by osmotic membrane distillation process

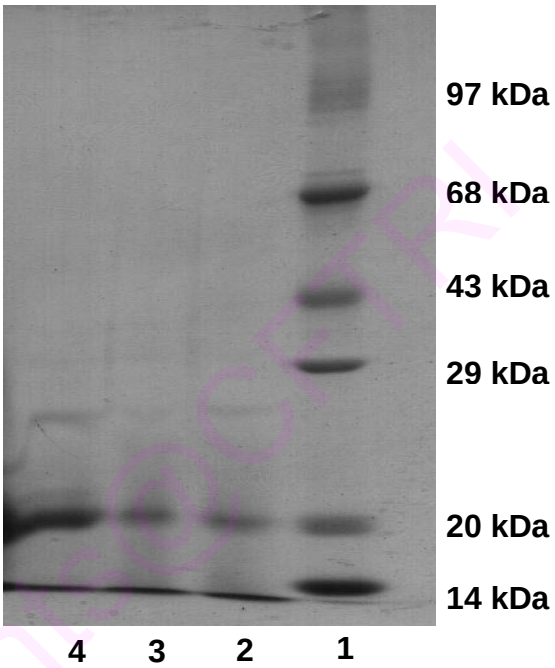


Figure 5A.6: SDS-PAGE profile of bromelain: lane 1: molecular marker; lane 2: crude bromelain extract; lane 3: dialyzed bromelain extract lane 4: concentrated bromelain extract.

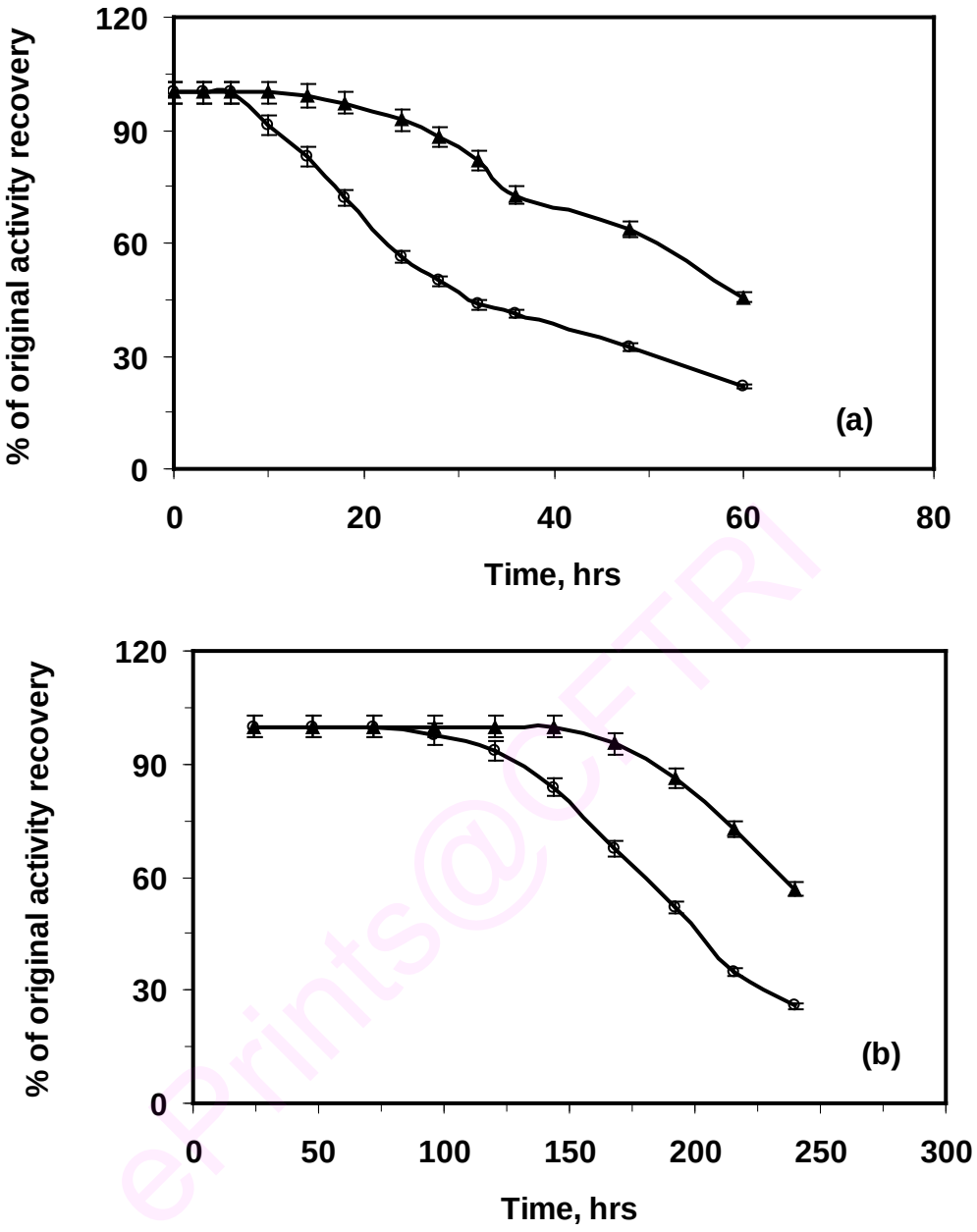


Figure 5A.7: Stability of bromelain enzyme (a). ambient (25.0 ± 2°C) temperature; (b). refrigeration (5.0 ± 2°C) temperature (-▲- concentrated bromelain extract; -○- crude bromelain extract).

CHAPTER – 5B

Integration of aqueous two phase extraction with membrane processes

5B.1 Introduction

In recent years downstream processing (that is the recovery and purification of the product) is faced with a strong demand for intensification and integration of process steps to increase yield, to reduce process time and to cut down in running costs and capital expenditure (Schugerl and Hubbuch, 2005). Process integration, wherein two or more unit operations are combined into one in order to achieve specific goals not effectively met by discrete processes, offers considerable potential benefit for the recovery and purification of biological products (Rito-Palomares, 2004). Integration of aqueous two phase extraction with other processes (such as fermentation, cell disruption and membrane processes) is one approach which is gaining considerable attention, in recent years. Application of ATPS for extractive fermentation is a meaningful approach to overcome low product yield in a conventional fermentation process and by proper design of the two-phase systems, it is feasible to obtain the product in a cell-free stream. Recently, extractive fermentation using ATPS have been developed for the recovery of different protein products which has resulted in an increase in the productivity (Guan *et al*., 1996; Li *et al*., 2000; Sinha *et al*., 2000). Rito-Palomares and Lyddiatt (2002) have reported the integration of cell disruption and aqueous two phase systems for the recovery and purification of intracellular proteins. There are only a few research articles available on integration of ATPE with membrane processes for the purification and concentration of various biological products (Tanuja *et al*., 2000; Srinivas *et al*., 2002; Rito-Palomares, 2004; Ganapthi and Raghavarao, 2007a). In this chapter integration of aqueous two-phase extraction (ATPE) with membrane processes such as

ultrafiltration and direct osmosis has been discussed for the purification and concentration of bromelain and polyphenol oxidase (PPO) from pineapple.

5B.2 Materials and Methods

5B.2.1 Materials

5B.2.1.1 Membranes

Hydrophilic direct osmosis (Osmotek, Inc., Corvallis, OR), and polysulphone membranes (MWCO 10,000 Da, M/S, Permionics, Baroda, India) were used in the present study.

5B.2.1.2 Chemicals

Polyethylene glycol (PEG) 1500 was purchased from Merck Chemicals, India. Potassium hydrogen phosphate (mono-basic and di-basic), trichloracetic acid, sodium acetate, sodium chloride were procured from Ranbaxy Limited, India. Casein (Hammarsten grade), catechol were procured from Loba Chemicals, India. All other chemicals were of analytical grade.

5B.2.2 Methods

5B.2.2.1 Enzyme Extraction

The enzyme extract from pineapple fruit was prepared as per procedure given in Chapter 2B. The bromelain as well as PPO activity and protein concentration in the enzyme extract were determined as per detailed procedures given in Chapter 2B.

5B.2.2.2 Osmotic agent solution

Osmotic agent solution was prepared by dissolving 350 gm of sodium chloride in one kg of distilled water.

5B.3 Results and Discussion

5B.3.1 Aqueous two phase extraction

The phase system comprising of PEG 1500 (18 %) and phosphate (14 %) was selected for the fractionation and purification of bromelain and PPO from pineapple based on the results obtained in Chapter 2B. In a typical experiment, 20% of enzyme extract was mixed with phase forming solutes. The mixture was mixed thoroughly using a magnetic stirrer for equilibration and was allowed to separate for about five hours in a separating funnel. After clear separation of two phases, the volumes of top and bottom phases of the system were noted, and analyzed for bromelain as well as PPO enzyme activities and protein concentration. The bromelain as well as PPO activity and protein concentration in top and bottom phases are presented in Table 5B.1. From the table, it can be observed that bromelain preferentially partitioned to the top phase and PPO to the bottom phase.

5B.3.2 Integration of aqueous two phase extraction with ultrafiltration and direct osmosis

The schematic representation of integrated approach employed for the purification and concentration of bromleain and PPO is presented in Fig.5B.1.

The top and bottom phases rich in bromelain and polyphenol oxidase were separated and further purified/concentrated by ultrafiltration process in a stirred cell (Amicon cell, having a working volume of 50 ml) using 10 kD MWCO cellulose acetate membrane. The integration of aqueous two-phase extraction with ultrafiltration resulted in 4.20 ± 0.2 and 2.80 ± 0.2 fold increase in purity of bromelain and polyphenol oxidase, respectively (Table 5B.2). Further, the purified enzyme extracts were concentrated by using DO process. During direct osmosis process the sodium chloride (6 molality) was used as an osmotic agent. The final activity of concentrated enzyme extracts is presented in Table 5B.2. Direct osmosis process resulted in 1.2 ± 0.1 and 1.8 ± 0.1 fold increase in activity (concentration) of bromelain and PPO, respectively.

5B.3 Conclusions

The feasibility of integration of ATPE with ultrafiltration and direct osmosis process was demonstrated for the purification and concentration of bromelian and PPO from pineapple. The integrated approach resulted in about 4.2 ± 0.2 and 2.8 ± 0.1 fold increase in purity of bromelain and PPO, respectively.

Table 5B.1:

Purification and concentration of bromelain and PPO during aqueous two phase extraction integrated with membrane process

Fraction	Enzyme activity		Protein concentration (mg/ml)	Purification factor	
	Bromelain (CDU/ml)	PPO (U/ml)		Bromelain	PPO
Crude	490 ± 2.0	430 ± 2.0	0.39 ± 0.005	-	-
Aqueous two phase extraction					
Top Phase	612 ± 4.0	20 ± 1.0	0.13 ± 0.002	3.74 ± 0.04	0.14 ± 0.03
Bottom Phase	74 ± 1.0	180 ± 4.0	0.06 ± 0.001	0.98 ± 0.05	2.72 ± 0.02
Ultrafiltration (retentate)					
Top phase (Bromelain rich)	890 ± 6.0	-	0.17 ± 0.005	4.20 ± 0.2	-
Bottom phase (PPO rich)	-	340 ± 6.0	0.11 ± 0.005	-	2.80 ± 0.1
Direct osmosis – concentration of ultrafiltration retentate					
Concentrate (Bromelain rich)	1084 ± 10	-	0.216 ± 0.004	3.99 ± 0.07	-
Bottom phase (PPO rich)	-	630 ± 10	0.210 ± 0.005	-	2.72 ± 0.05

Fig. 5B.1: Integrated approach for the purification and concentration of bromelain and polyphenol oxidase.

CHAPTER - 6

Overall conclusions

6. Overall conclusions and suggestions

Physical properties of aqueous two phase systems (ATPSs), such as density, viscosity and interfacial tension determines the phase demixing rate. The density difference between top and bottom phases increased with an increase in phase forming polymer and salt concentrations. The interfacial tension of the PEG/potassium phosphate system increased with an increase in concentration of phase forming components and molecular weight of polymer (**Chapter 2A**). Physical properties of ATPSs for different polymer/salt systems and polymer/polymer systems at different temperatures need to be studied. Differential partitioning of bromelain to the top phase and polyphenol oxidase to the bottom phase could be achieved by ATPE (**Chapter 2B**). Polymer molecular weight and concentration were found to be prominent effect on partitioning coefficient. The polyethylene glycol/potassium phosphate system (comprising of 18% PEG 1500 and 14% phosphate) resulted in about 228.4 ± 4.20% activity recovery and 4.0 ± 0.10 fold increase in purity in case of bromelain and about 90 ± 1.0% activity recovery and 2.7 ± 0.10 fold increase in purity in case of polyphenol oxidase. All these experiments were carried out at lab scale, however more detailed study on large scale need to be performed. Attention needs to be paid on the design and development of equipments to increase the rate of mass transfer and rate of phase demixing during aqueous two phase extraction.

Interest in newer non-thermal membrane processes for concentrating biomolecules/fruit juices has increased significantly in the last few years and direct osmosis is one such process. The feasibility of direct osmosis as an

alternate method for the concentration of biomolecules (such as bromelain from pineapple waste) was demonstrated (**Chapter 3A**). The osmotic agent (sucrose and sodium chloride) concentration and flow rate are found to be prominent effect on transmembrane flux. In order to overcome the drawbacks associated with sucrose solution (low flux) and sodium chloride solution (saltiness in juice) as osmotic agents, an attempt was made to explore the use of mixed osmotic agent (sodium chloride and sucrose) solutions during the concentration of pineapple juice by direct osmosis process. The pineapple juice was concentrated up to 60°Brix at ambient temperature and atmospheric pressure (**Chapter 3B**). All these experiments were carried out at lab scale, however more detailed study on large scale need to be planned and performed in order to reach the industrial requirements.

Osmotic membrane distillation process could be effectively used for the concentration of real systems (phycocyanin colorant and bromelain enzyme extract) at ambient temperature and atmospheric pressure. Out of the process parameters examined during osmotic membrane distillation, osmotic agent concentration and flow rate were found to have significant influence on transmembrane flux. Phycocyanin solution was concentrated from 0.52 ± 0.02 to 1.14 ± 0.10 mg/ml and bromelain enzyme extract was concentrated from 462 ± 2.0 to 912 ± 4.0 CDU/ml at 25.0 ± 2.0°C (**Chapter 4A**). The bromelain present in pineapple juice was completely removed at bentonite concentration of 4000 ppm at pineapple juice pH 3.0. The osmotic agent concentration and flow rate were found to have significant effect on transmembrane flux during the concentration of clarified pineapple juice. Boundary layer mass and heat

transfer coefficients were estimated during osmotic membrane distillation process. It was observed that the contribution of concentration polarization on driving force (in turn flux) reduction was more prominent when compared to that of temperature polarization. A resistance-in-series model was employed for analyzing the mass transfer during the osmotic membrane distillation process. The experimental values are found to correlate well with the predicted ones. Under best experimental conditions pretreated pineapple juice was concentrated up to 62 ± 0.20°Brix while preserving the ascorbic acid content of the pineapple juice (**Chapter 4B**). Efforts are required in the development of suitable membranes with improved diffusional characteristics and with longer life cycles in order to make osmotic membrane distillation viable option. Improvements of process engineering in terms of module design as well as process design and optimization are required in order to overcome the drawbacks like low flux. A serious problem with commercial application of both direct osmosis and osmotic membrane distillation process is the management of diluted osmotic agent solution. It is essential to concentrate and re-use the osmotic agent solution several times before it is removed from the process. Possible integration of reverse osmosis (as a pre-concentration step) and evaporation (final concentration step) for the concentration of diluted osmotic agents needs to be studied.

The potential of newer membrane processes (such as direct osmosis/osmotic membrane distillation) for concentrating biomolecules, liquid foods and natural colours have been proved beyond doubt. However, newer membrane processes suffer from low flux, which limits their full commercial

application. In order to overcome such limitations and to improve the product quality, purification and concentration of biomolecules by membrane based hybrid (integrated) processes appears to be very attractive. An attempt has been made during this study (**Chapter 5A**) to increase the process efficiency by integrating ultrafiltration with direct osmosis/osmotic membrane distillation for the concentration of bromelain from pineapple waste. Also, the feasibility of integration of ATPE with ultrafiltration and direct osmosis has been demonstrated for the purification and concentration of bromelain and PPO from pineapple (**Chapter 5B**). The majority of the commercially available microfiltration/ultrafiltration membranes are inherently inhomogeneous (non-uniform in mass and thickness). This affects the operational performance. Nanofibrous microfiltration/ ultrafiltration membranes offer unique properties for filtration and adsorptions-based separations including high specific surface area, good interconnectivity of pores and the potential to incorporate active chemistry on a nano scale. The potential of nanofibrous microfiltration/ultrafiltration membrane in food/biotechnology applications needs to be studied in detail. Integration of aqueous two phase extraction with nanofibrous microfiltration/ultrafiltration and direct osmosis/osmotic membrane distillation needs to be explored.

In this chapter, after elucidating the overall conclusions a few suggestions for further research are indicated. In the continuous process of research, it observed that the present thesis work has opened up more areas/aspects for future investigation. Even if some of these aspects are addressed by future researchers the objective of this thesis can be considered fulfilled.

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Out come of the work

Research papers

1. *B. Ravindra Babu*, N.K. Rastogi, K.S.M.S. Raghavarao, Mass transfer in osmotic membrane distillation of phycocyanin colorant and sweet-lime juice, Journal of Membrane Science 272 (2006) 58–69.

2. *B. Ravindra Babu*, N.K. Rastogi, K.S.M.S. Raghavarao, Effect of process parameters on transmembrane flux during direct osmosis, Journal of Membrane Science 280 (2006) 185–194.

3. *B. Ravindra Babu*, N.K. Rastogi, K.S.M.S. Raghavarao, Liquid-liquid extraction of bromelain and polyphenol oxidase using aqueous two-phase system, Chemical Engineering and Processing 47 (2008) 83–89.

4. *B. Ravindra Babu*, N.K. Rastogi, K.S.M.S. Raghavarao, Study of polarization effects during osmotic membrane distillation, Journal of Membrane Science (revised and re-submitted to the journal, 2008).

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