CHIRAL SEPARATIONS BY ENZYME ENHANCED ULTRAFILTRATION: FRACTIONATION OF RACEMIC BENZOIN

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I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis and for the degree of Master of Science.

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ABSTRACT

CHIRAL SEPARATIONS BY ENZYME ENHANCED ULTRAFILTRATION: FRACTIONATION OF RACEMIC BENZOIN

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In this study, a methodology for separation of chiral molecules, by using enhanced ultrafiltration system was developed. Benzoin was the model chiral molecule studied.

In the scope of developing this methodology, some parameters were investigated in the preliminary ultrafiltration experiments in order to set the operation conditions for enhanced ultrafiltration experiments. Due to the slight solubility of benzoin in pure water, 15% (v/v) Polyethylene glycol (PEG 400) and 30 % (v/v) Dimethyl sulfoxide (DMSO) were selected as cosolvents. Because of the high retention capacity of RC-10000 Da membranes for benzoin, a membrane saturation strategy was developed.

In polymer enhanced ultrafiltration (PEUF) experiments bovine serum albumin (BSA) was used as ligand. Effects of ligand concentration and pH on total benzoin retention and on enantiomeric excess (ee %) were

investigated. Benzoin concentration was almost kept constant at ~10 ppm and ~50 ppm for 15% (v/v) PEG 400 and 30 % (v/v) DMSO cosolvents, respectively. It was observed that the increase either in pH or in BSA concentration yielded an increase in total benzoin retention. In 15% (v/v) PEG 400-water, with BSA concentration of 10000 ppm, at pH 10, total benzoin retention reached to 48.7%. For this cosolvent, at different pH values and at different BSA concentrations, all ee % values were about or less than 10%. When 50000 ppm BSA was dissolved in 30 % (v/v) DMSO-water, total benzoin retention increased to 41.3% at pH 10 and ee % reached 16.7 % at pH 11.

In enzyme enhanced ultrafiltration (EEUF) experiments, specific to benzoin, apo form of Benzaldehyde Lyase (BAL, E.C. 4.1.2.38) was used as ligand. These experiments were performed with constant ~ 10 ppm benzoin concentration in only 15% (v/v) PEG 400 –water solvent. Effect of BAL concentration on total benzoin retention and ee% was investigated. It was found that; for all the studied BAL concentrations in the range of 650- 1936 ppm total benzoin retention and ee % were kept almost constant at ~75% and ~60%, respectively.

Keywords: Chiral Separations, Enhanced Ultrafiltration, Benzoin, Bovine Serum Albumin, Benzaldehyde Lyase.

ÖZ

ENZİM DESTEKLİ ULTRAFİLTRASYON İLE KİRAL AYIRMALAR: RASEMİK BENZOİNİN AYRILMASI

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Bu çalışmada kiral moleküllerin ayrılması amacıyla destekli ultrafiltrasyon sistemi kullanılarak bir metodoloji geliştirilmiştir. Model kiral molekül olarak benzoin kullanılmıştır.

Bu metodolojiyi geliştirme kapsamında; destekli ultrafiltrasyon deneylerinin çalışma koşullarını belirleyebilmek amacıyla, ön ultrafiltrasyon deneylerinde bazı parametreler araştırılmıştır. Benzoinin saf suda cok az çözünmesinden dolayı % 15 (v/v) Polietilen glikol (PEG 400) ve % 30 (v/v) Dimetil sulfoksit (DMSO) eş cözücü olarak seçilmiştir. RC-10000 Da membranlarının yüksek benzoin tutma kapasitelerinden dolayı membran doyurma stratejisi geliştirilmiştir.

Polymer destekli ultrafiltrasyon deneylerinde bovin serum albumin (BSA) ligand olarak kullanılmıştır. Ligand derişiminin ve pH'ın toplam benzoin tutunması ve enantiyomerik fazlalık (% ee) üzerindeki etkisi incelenmiştir.

Benzoin derişimi % 15 (v/v) PEG 400 ve % 30 (v/v) DMSO eş çözücüleri için sırasıyla yaklaşık 10 ppm ve 50 ppm olarak sabit tutulmuştur. pH yada BSA derişimindeki artışın toplam benzoin tutunmasını arttırdığı gözlemlenmiştir. %15 (v/v) PEG 400 –su içerisinde , 10000 ppm BSA derişimi ile, pH 10'da, toplam benzoin tutunması %48.7' ye ulaşmıştır. Bu eş çözücü için farklı pH değerlerinde ve farklı BSA derişimlerinde elde edilen tüm % ee değerleri % 10 civarında ya da daha düşüktür. 50000 ppm BSA, % 30 (v/v) DMSO –su içinde çözündüğünde pH 10'da toplam benzoin tutunması %41.3'e ve pH 11'de, % ee, % 16.7'ye ulaşmıştır.

Enzim destekli ultrafiltrasyon deneylerinde, benzoine spesifik olarak Benzaldehit Liyaz (BAL, E.C. 4.1.2.38) enziminin apo hali ligand olarak kullanılmıştır. Bu deneyler sabit ~10 ppm benzoin derişiminde sadece % 15 (v/v) PEG 400- su çözücüsünün içinde gerçekleştirilmiştir. BAL derişiminin toplam benzoin tutunma ve % ee üzerindeki etkileri incelenmiştir. 650-1936 ppm aralığında çalışılan tüm BAL derişimleri için toplam benzoin tutunması ve % ee değerleri sırasıyla %75 ve % 60 civarında sabit olarak bulunmuştur.

Anahtar Kelimeler: Kiral Ayırmalar, Destekli Ultrafiltrasyon, Benzoin, Bovin Serum Albumin, Benzaldehit Liyaz.

To my mother, father and brother

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TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

LIST OF SYMBOLS

$C_{BAL,f}$	BAL concentration in the feed, ppm
$C_{BSA,f}$	BSA concentration in the feed, ppm
$C_{benzoin，f}$	Benzoin concentration in the feed, ppm
$C_{perm,\ predominant}$	Predominant enantiomer concentration in the permeate, ppm
$C_{perm,\ minor}$	Minor enantiomer concentration in the permeate, ppm
N	Stirring rate, rpm
P	Pressure, bars
V_{cell}	Volume of the feed content in the stirred cells, ml

Abbrevations

BAL	Benzaldehyde Lyase
BSA	Bovine Serum Albumin
CTA	Cellulose Triacetate
DMSO	Dimethyl sulfoxide
MWCO	Molecular weight cut off
PEG	Polyetylene Glycol
PWF	Pure water flux
PES	Polyether sulfone
RC	Regenerated Cellulose

CHAPTER 1

INTRODUCTION

Isomers are compounds with the same molecular formula but different arrangements of atoms. Due to the differences in atom arrangements, isomers have different properties. There are two major kinds of isomers:

Constitutional (Structural) Isomers are isomers that differ in how the atoms are joined together. That is, the order, in which the atoms are bonded to each other, differs.

Stereoisomers (Optical Isomers), on the other hand, are isomers in which the atoms are bonded to each other in the same order but differ in the precise arrangement of the atoms in space [1].

Stereoisomers are mainly divided into two categories: Enantiomers and diastereomers. **Enantiomers** are stereoisomers whose molecules are nonsuperimposable mirror images of each other. **Diastereomers** are stereoisomers whose molecules are not mirror images of each other [2].

Chiral molecule is the one that is not identical with its mirror image. Enantiomers occur only in chiral molecules. Namely, the chiral molecule and its mirror image are enantiomers.

Two enantiomers have almost the same chemical and physical properties with the only exception of their rotational behavior for the plane polarized light. A substance that rotates the plane polarized light in the

clockwise direction is called **dextrorotatory (right handed),** and the one that rotates the plane-polarized light in a counter clockwise direction is said to be **lavatory (left handed).** These substances have the names of **d- or r-enantiomer** and **l-or s- enantiomer,** respectively [2].

A mixture containing equal amounts of the two enantiomeric forms of a chiral molecule is called a **racemic mixture** or **racemate.**

Most of the molecules that make up plants, animals and human body are chiral, and usually only one form of the chiral molecule occurs in a given species [2]. For example, amino acids that make up naturally occurring proteins are chiral, and all of them are classified as being left handed. Since stereoselectivity is a characteristic feature, only one of the enantiomers reacts with the enzyme in enzymatic reactions.

Moreover, enantiomerically pure compounds are important in pharmaceuticals, agrochemicals, and flavors because only one of the two enantiomers perform the biological action. The other might be side product and it might have unwanted effects. For instance, one enantiomeric form of limenone is primarily responsible for the odor of oranges, and the other enantiomer, for the odor of lemons. When penicillamine is considered it is observed that one enantiomer is toxic whereas the other is antiarthritic. In Figure 1.1 different biological activities of different enantiomers are presented.

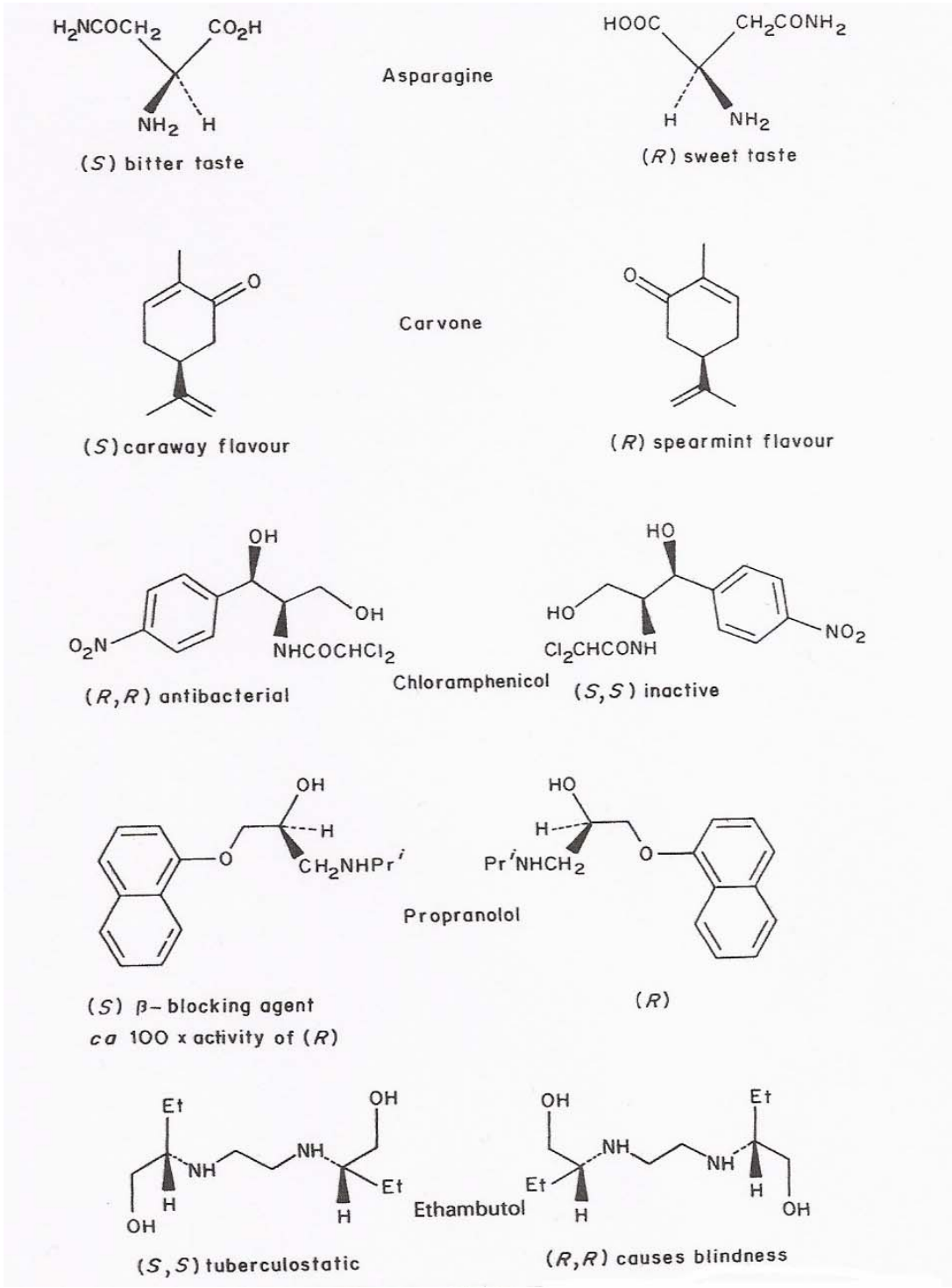


Figure 1.1 Differences in properties of enantiomers [3]

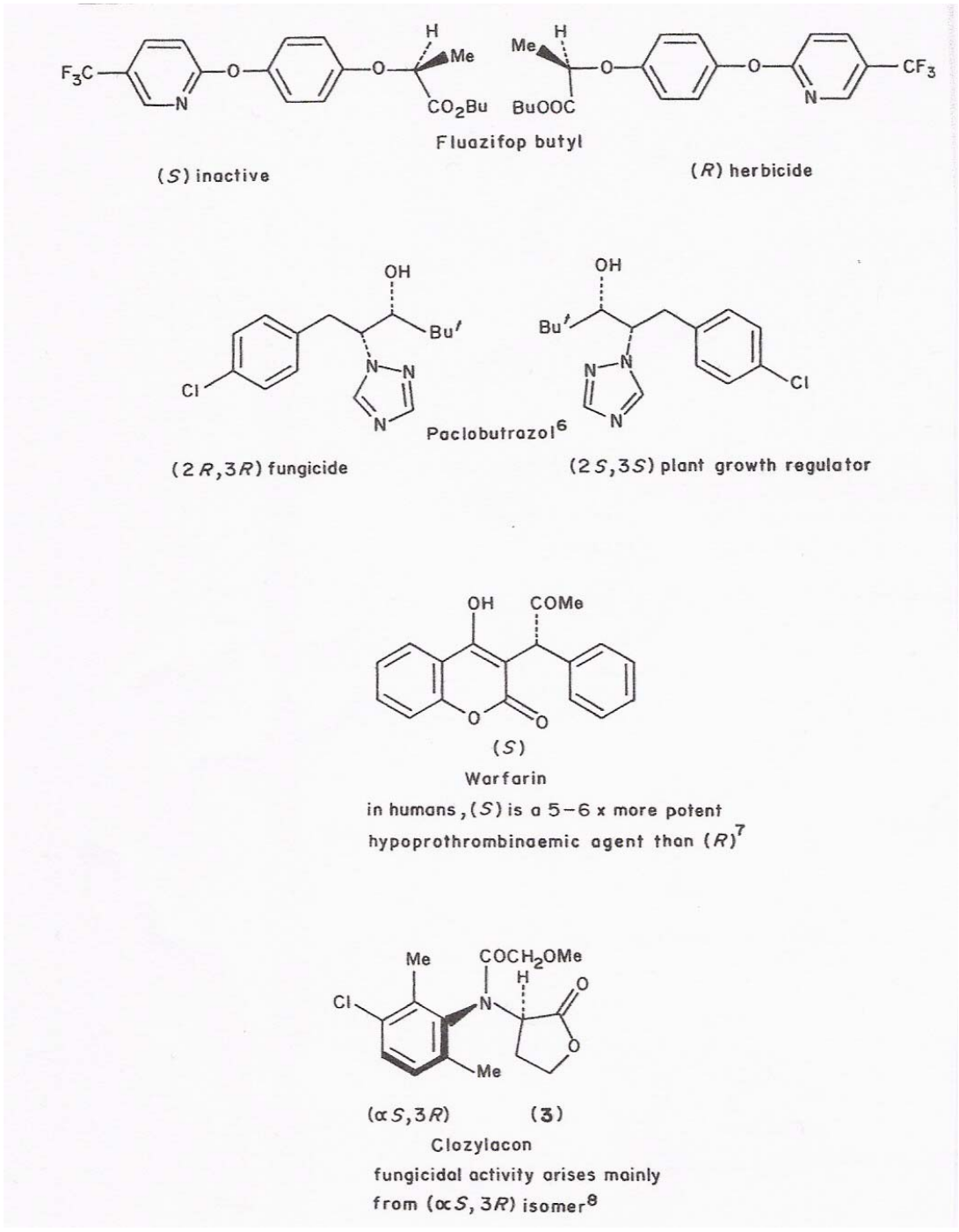


Figure 1.1 Differences in properties of enantiomers (cont'd) [3]

Due to one to one similarity in the physical and chemical properties of enantiomers, enantiomeric separation is not an easy task. On the other hand, their different biological activities make this kind of a separation very critical and important.

Several methods are being used for chiral separations. Capillary electrophoresis (CE) and liquid- liquid extraction are utilized at analytical level only. Capillary electrochromatography (CEC), gas chromatography (GC) thin layer chromatography (TLC), and some types of liquid chromatography (LC) applications are also observed for chiral separations. Although highly pure products are obtained with these chromatographic methods; low productivity, high capital costs and significant dilution of the product, requiring an additional unit operation for solvent removal are the main drawbacks of these methods [4]. Finally, crystallization is another method for chiral resolutions, however it is also limited due to its high cost [5]. In addition it is inflexible and requires the detailed investigation of system behaviour before use [4].

On the other hand, membrane based techniques in chiral separations are emerging. Membrane separations often provide cost efficient opportunities to the separations which are troublesome or even impossible, by using classical methods. In addition most membrane processes are performed at ambient temperature, therefore they can offer clear advantages compared to other conventional separation processes [6]. Besides, continuous separation, low energy consumption and easy scaling up can be considered as the other benefits of membrane separations [7].

Complexation enhanced ultrafiltration (CEUF) is a membrane process which has recently been developed with its three main types, namely; **Colloid enhanced, micellar enhanced and polymer enhanced ultrafiltration.** In these processes, colloids, surfactants and polymers are being used as binding agents (ligands), respectively.

Among these three methods, only polymer enhanced ultrafiltration (PEUF) is a one phase (homogeneous) operation, whereas the other two are heterogeneous phase operations. Therefore, when compared with the other

two; there is no difficulty related with heterogeneous reactions, interphase transfer and long contact time problems of multiphase separation processes in PEUF. This method is widely investigated for removal of heavy metals in waste waters [8-15] and chiral separation of racemic amino acids via PEUF were also studied [16-21]

Principally, there is a specific complex formation between macromolecule ligand polymer and only one enantiomer in the feed side of the membrane. Chiral resolution can be achieved by means of permeation of the free enantiomer to the permeate side, due to its small molecular weight which is smaller than molecular weight cut off (MWCO) of the membrane. As the polymer- enantiomer complex can not pass through the permeate, enantiomer separation is achieved. Namely, the free enantiomer is obtained on the permeate side, dominantly. After then, in order to decomplex the macromolecule- enantiomer complex on the feed side; a specific property, such as ionic strength or the pH of the feed solution should be manipulated.

In all CEUF systems, performance evaluation for chiral resolution is mainly based on enantiomeric excess and enantioselectivity.

Enantiomeric excess is defined as:

$$ee\,\% = \frac{C_{perm,predominant} - C_{perm,minor}}{C_{perm,predominant} + C_{perm,minor}} \times 100 \qquad (1.1)$$

where $C_{perm,\ predominant}$ and $C_{perm,\ minor}$ are the concentrations of the predominant and minor enantiomers in the permeate, respectively.

On the other hand, enantioselectivity (α) can be formulized as below:

$$\alpha = \frac{C_{perm,predominant} / C_{perm,minor}}{C_{feed,predominant} / C_{feed,minor}} \qquad (1.2)$$

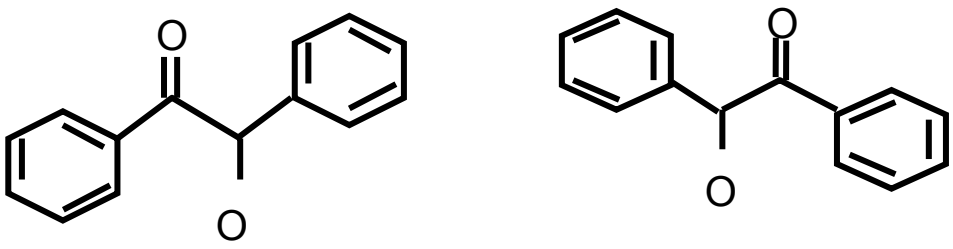
Since the feed is generally racemate, then equation (1.2) takes the form of:

$$\alpha = \frac{C_{perm,predominant}}{C_{perm,minor}} \quad (1.3)$$

Where C_{perm} and C_{feed} represent the concentration of predominant and minor enantiomers in the permeate stream and in the feed, respectively.

The aim of this study was to develop a new methodology for separation of chiral molecules by using enhanced ultrafiltration system. A systematic approach, focusing on effect of basic operating parameters, was followed. Since almost all of the existing chiral PEUF studies targeted amino acids, a non acidic chiral molecule (with a potential to be used as a drug intermediate) was searched as target. Therefore, benzoin molecule was selected as the model chiral molecule.

Benzoin ($C_{14}H_{12}O_2$) is a ketone alcohol (alpha hydroxyl ketone) with a molecular weight of 212.25 g/mol. As shown in Figure 1.1 it is a chiral molecule.



R- Benzoin S-Benzoin

Figure 1.2 Structure of chiral benzoin molecule

Both pure R- and S- enantiomers have applications in drug industry. However, they should be used in separate forms. They are used as a drug

intermediate for synthesis of antifungal azoles and deacetyltaxoles which are used in cancer treatment. It is a slightly soluble molecule in distilled water.

Two types of complexation ligands were utilized, for the application of polymer enhanced ultrafiltration process. Bovine serum albumin (BSA), which is a widely available protein with low price and a well established structure, is known to have a high capacity for binding only one form of amino acid enantiomers [8, 9]. However; here, it was aimed to investigate its binding capacity for a ketone alcohol. In addition, in order to develop a methodology, such an economical and widely available protein would be advantageous. So, in the first part of this study, in Polymer Enhanced Ultrafiltration (PEUF) experiments, BSA was selected as the ligand.

For the second part, one of the properties of apoenzymes was notable: They can not convert their substrate into product; however they can still bind them. So, it might be possible to utilize them as ligands. Moreover, in literature, there has been no study in which apoenzymes were used as ligands in an enhanced ultrafiltration system. Therefore, in the second part of experiments, this new and different approach was tried. This method which was a special sub group of PEUF was called as **"Enzyme Enhanced Ultrafiltration (EEUF)".** Specific to benzoin, apoenzyme form of Benzaldehyde Lyase (BAL, E.C. 4.1.2.38) was used.

At constant benzoin concentration; effect of ligand concentration on total benzoin retention and on enantiomeric excess (ee %) was investigated in both PEUF and EEUF experiments. In addition, in PEUF experiments, pH effect was also investigated.

CHAPTER 2

LITERATURE SURVEY

2.1 Chiral Separation Methods

For chiral separations many methods are used either at analytical level or at preparative level. At analytical level, high enantiomeric purity of the starting materials and products are the main criteria. However, at preparative level, apart from appropriate enantioselectivity, a high loading capacity, chemical inertness, thermal stability of chiral stationary phase and enantiomers to be resolved are important. For preparative applications chiral selectors should be readily available in terms of economic aspects. Moreover, for preparative level production; in terms of productivity, solubility of the chiral sample in the media where separation takes place should be considered. In contrast at the analytical level this would not be a major problem [22].

In recent years capillary electrophoresis (CE) application has undergone an enormous development, at analytical level. Its speed, high efficiency, low cost and small sample volume requirement make it more advantageous over other methods. Separation is based on differential complexation between enantiomers and a chiral selector added to the buffer. By the addition of these selectors, noncovalently bound diastereoisomers are formed. So, they can be easily separated based on their different physical properties [23]. Usually cyclodextrins (CDs) are used as chiral additives. If the interaction of enantiomers of the analyte with the cyclodextrin differs from each other, then a chiral separation is feasible. The hydroxyl groups on the cyclodextrin can be chemically substituted with groups such as methyl,

hydroxypropyl or sulphate [24]. In many studies, chiral drug separations were achieved by the use of CDs, in capillary electrophoresis [23, 25-28]. For instance, separation of norepinephrine, epinephrine and isoprinaline enantiomers was studied by capillary electrophoresis and recoveries obtained were greater than 90 % [27]. While studying the enantioselective determination of pheniramine in CE, charged cyclodextrins were used. Effects of type and concentration of the chiral selector, carrier cation and the counterion, and the pH of the buffer on separation were investigated [28].

Capillary electro chromatography (CEC) combines the efficiency of capillary zone electrophoresis and the selectivity of liquid chromatography (LC) with the use of a solid stationary phase [29]. It is used at analytical level. Mangelings et al. [30], studied the chiral separation of non-acidic pharmaceuticals by using capillary electrochromatography. They investigated the effect of temperature and buffer concentration, on separation of several non acidic test compounds and showed that enantioselectivity was obtained for most of these compounds by using CEC.

Liquid-liquid extraction is a mass transfer operation. A liquid solution (the feed) is contacted with an immiscible or nearly immiscible liquid (solvent) that exhibits preferential affinity or selectivity towards one or more of the components in the feed. The extract stream is the solvent rich solution containing the desired extracted solute. The raffinate stream is the residual feed solution containing little solute [31].

It is an analytical level technique utilized in chiral separations. Kellner et al. [32] studied the enantioseparation of racemic DNB-Leucine with liquid- liquid extraction by using quinine carbamate type chiral selector. For solvent extraction experiments, the racemic mixture of the target molecule to be separated was dissolved in the buffered aqueous donor phase (feed phase). The organic acceptor phase (extract phase) contained the lipophilized selector, which has very low solubility in the aqueous phase. During the liquid-liquid extraction, the two S- and R- enantiomers were stereoselectively extracted. Effect of selector /target molecule ratio, pH and ionic strength, on enantiomeric excess and yield was studied. In addition, it

was observed that organic solvent type was also effective for the enantiomeric excess and yield values.

On the other hand, crystallization is widely used for chiral separations at preparative level. It is an attractive method, in which auxiliaries and reagents other than solvent are not required [3]. In principle, it was based on obtaining crystals from a saturated solution. For the resolution of a racemate, addition of one chiral resolving agent to a racemate is followed by a suitable waiting period in order to observe crystallization of one diastereomeric salt [33]. Menahem et al. [34] proposed to use chiral polymers as additives in order to induce the enantioselective crystallization of racemic amino acids.

In addition, in order to separate the enantiomers in a mixture, chromatographic methods might be considered. These methods are based on differences in partitioning behavior between a flowing mobile phase and a stationary phase. Stationary phase is in the column and the mobile phase carries the sample through it [35].

For liquid chromatography (LC), mobile phase is the solvent and stationary phase is the liquid on a solid support, a solid, or an ion-exchange resin. High performance liquid chromatography (HPLC), simulated moving bed (SMB) chromatography and thin layer chromatography (TLC) are the main sub-groups of liquid chromatography (LC) utilized in enantiomeric separations. Except TLC, all other liquid chromatographic methods are at preparative level.

High performance liquid chromatography (HPLC), in which high-pressure pumps are used to increase the efficiency of the separation, is widely used for chiral separations. Guo et al. [36] studied the chiral separation of ketoprofen racemate in HPLC using acetonitrile–triethylamine acetate (TEAA) buffer containing antibiotics; norvancomycin or vancomycin as the mobile phase. Effect of antibiotic concentration, content of acetonitrile and TEAA buffer pH on the enantioseparation were investigated. Maximum enantioselectivity of 1.24 was found at 40% (v/v) acetonitrile concentration. For satisfactory enantioselectivity, pH of TEAA buffer was selected to be 5.2

at a fixed concentration of 20 mM. The two antibiotics had similar effects and for both, good enantioselectivity (α ~1.2) and resolution was obtained at 2 mM concentration.

A simulated moving bed (SMB) system consists of an array of columns connected in series and several pumps and valves. One recycling pump is used for delivering the mobile phase flow through all columns. Other pumps are required to inject the feed and fresh eluent and withdraw the raffinate and extract flows. The valve system controls opening and closing of the inlet and outlet stream of each column at definite intervals. A countercurrent movement of stationary and mobile phase is simulated by controlled switching of the recycle fluid stream and the external and internal fluid flow streams on different columns [37].

Chiral resolution of an ester of quinoline mevalonic acid [38], Tramadol [39] and two new drug candidates [40] was achieved by using simulated moving bed (SMB) chromatography in different studies. It was claimed that SMB has significant benefits with respect to batch chromatography in terms of solvent consumption and productivity. Furthermore it is possible to achieve high purities even when the resolution on a single column is poor.

Finally, compared to other liquid chromatographic techniques, thin layer chromatography (TLC) has been used less frequently for chiral separations and it is the only analytical level liquid chromatography. TLC might not be able to compete with HPLC or GC in terms of separation efficiency; however, it has several advantages. It is a very simple, inexpensive, rapid and flexible technique; many samples can be processed parallel on one plate and very selective detection can be carried out by using spray reagents [37].

Beretnitzki et al. [41] focused on the advantages of TLC in their review. It was stated that performing chiral separations by TLC has three major advantages: More flexible detection of the analytes can be obtained in TLC when compared to HPLC. Sample throughput in TLC is higher than in

HPLC. Finally, by using different types of interactions in TLC yields very good separations. It was also stated that there were many possibilities for chiral additives in TLC and the interactions used in TLC to achieve enantiomeric separation were dependent on additive or stationary phase.

Another preparative level chromatographic method is gas chromatography (GC), in which mobile phase is a gas and the stationary phase is usually a liquid on a solid support or sometimes a solid adsorbent. It is preferable due to its speed, simplicity, reproducibility and sensitivity. If an electronically and coordinatively unsaturated transition metal compound is added to the liquid stationary phase, then this is called complexation GC. Schurig V. [42] showed that enantiomeric separation with this method represented a high efficiency for determination of ee%. Solute volatility and thermal stability requirements were the main limitations in this method.

Among the methods utilized for resolution of chiral molecules, as mentioned, some are applicable only at analytical level; i.e, Capillary electrophoresis (CE) and liquid- liquid extraction.

Crystallization might be considered as the traditional method for chiral separations, but it is also limited due to its high cost. In addition, costly scale up and having a high energy requirement can be considered as the main drawbacks of this method. Moreover, it requires relatively inflexible multi-step processing, thus inducing low product yields [5].

Except thin layer chromatography (TLC), all other chromatographic applications are at preparative level and highly pure products are obtained with these chromatographic methods. However, low productivity, and significant dilution of the product, requiring an additional unit operation for solvent removal are the main drawbacks of these methods. High capital cost is the other important point for chromatographic methods. For liquid chromatography, high amount of solvent consumption and the high column costs are considerable affects in the economies of these methods. Especially in simulated moving bed chromatography, the need for several pumps, columns and valves; makes the process more expensive.

2.1.1 Membrane Based Chiral Separation Methods

Among the chiral separation techniques, membrane based separations are also emerging at preparative level. Mainly three membrane methods are used in this field. The first two methods are separations performed on **liquid membranes** and **ligand immobilized membranes.** The third method is a developing approach which is called as **complexation enhanced ultrafiltration.** In all three methods, binding agents (ligands) are used to achieve enantioseparation.

2.1.1.1 Liquid Membranes

Liquid membranes have great potential in the various separation areas such as selective metal extraction, removal of toxic components from waste water, and extraction of biochemicals from fermentation broths [4]. Working principle of these membranes is shown in Figure 2.2 [6]. In general, they contain an enantiospecific carrier which selectively forms a complex with one of the enantiomers of the racemic mixture at the feed side, transports it across the membrane, where it is released into the receptor phase by means of decomplexation occurring at the second interphase. Then the free carrier diffuses back [7].

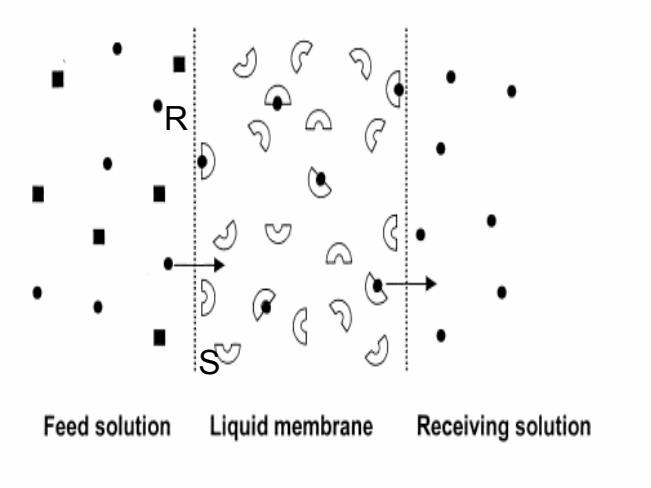


Figure 2.1 Representation of liquid membrane for chiral separation [6].

Liquid membranes are mainly investigated in three groups: Supported, Emulsion and Bulk liquid membranes.

In emulsion liquid membranes (ELM), droplets of the receiving phase are with in the organic membrane phase, in the form of a water-in-oil emulsion as shown in Figure 2.2. Then dispersion of this into the aqueous source phase takes place. The solute of interest is extracted from the external to the internal phase via the membrane phase. If the target molecule is initially in the internal phase, transport may occur in the opposite direction.

Main advantages of ELMs are relatively low capital costs and good stability. In addition they have the fastest mass transfer rates of all liquid membranes [4].

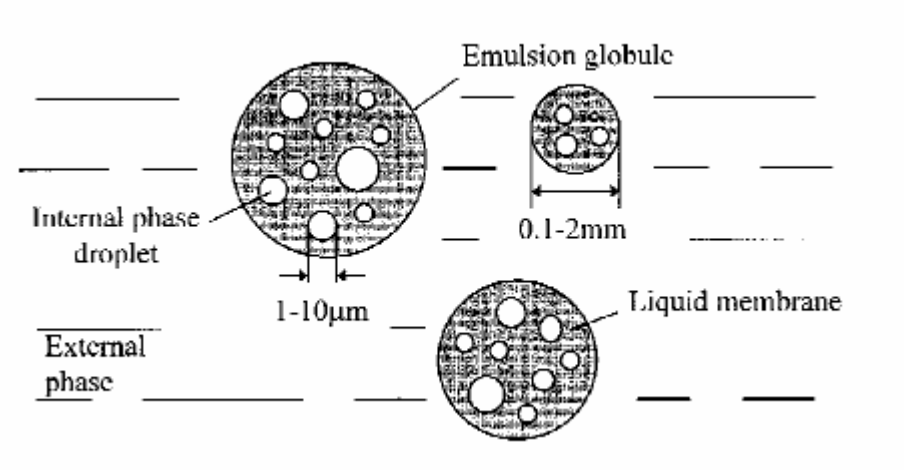


Figure 2.2 Emulsion liquid membrane configuration [4]

In supported liquid membrane (SLM) configuration, there are capillary and interfacial tension forces, by which a chiral liquid is immobilized in the pores of a membrane [4, 6]. Between the source phase and receiving phase transport takes place, passing over either side of the membrane surface.

Problem of membrane solubilization/ stabilization is the main drawback for these membranes [4].

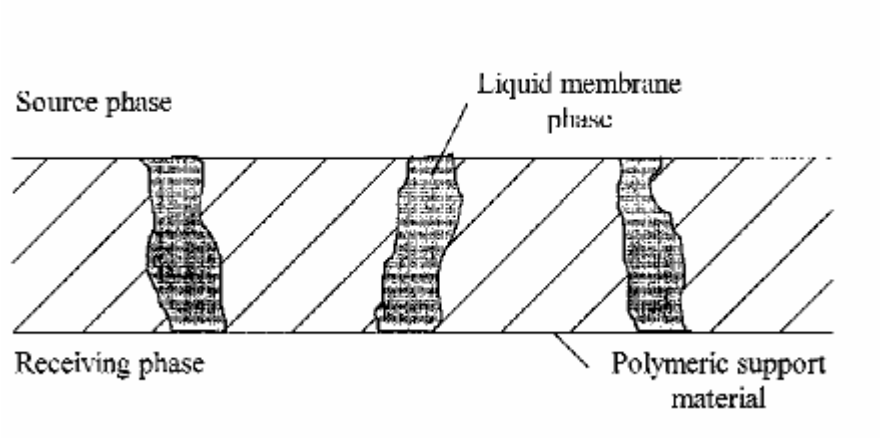


Figure 2.3 Supported liquid membrane configuration [4]

In bulk liquid membranes (BLM), which are less common, different than ELM, and SLM, membrane phase is a well-mixed bulk phase instead of an immobilized phase within a pore or film. Enantioselective extraction takes place from the source phase to the membrane phase and finally to receiving phase [6]. The main drawback of this method is the low interfacial surface areas which lead slow mass transfer rates [4].

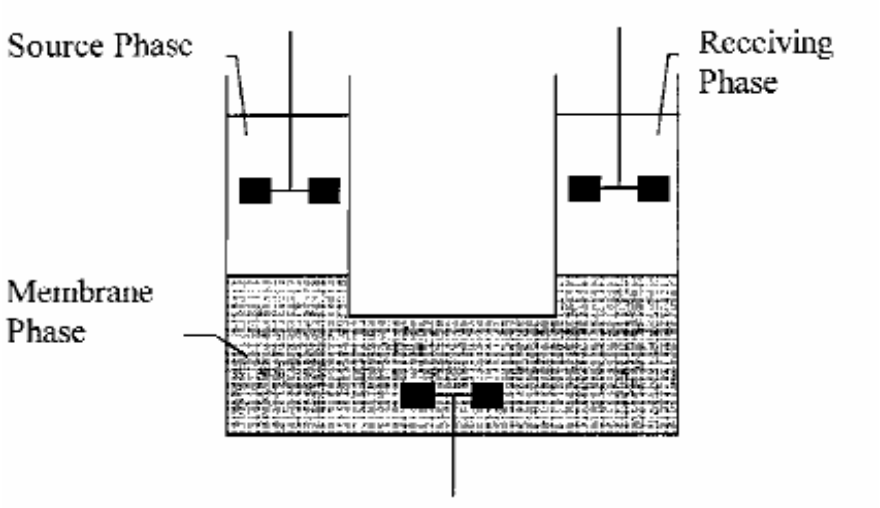


Figure 2.4 Classical bulk liquid membrane set-up [4]

Pickering et al. [4] studied the chiral extraction of phenylalanine by using emulsion liquid membranes. Copper (II) N-decyl-(L)- hydroxyproline was used as a chiral selector in an EML configuration. Investigation of the effect of pH gradient between the source (pH=5.0) and receiving phase (pH =1.0) yields that, this gradient provided increased rate of extraction, with respect to the case without any pH gradient. (pH=5.5 for both source and receiving phases). Enantioselectivity obtained was around 40 %.

Stella et al. [43] studied enantiomeric enrichment of mandalic acid and phenylglycine racemics by using a liquid membrane. Cinchonidine was used as chiral carrier. Effect of amount of modifier added, the initial carrier: analyte ratio in the feed, pH of the feed and receiving solutions were the parameters investigated. Selectivity of α =1.5 and α =0.9 were obtained for mandelic acid and phenylglycine, respectively.

In another study [44], D-L- Lactic acid racemic mixture was separated by using supported liquid membranes. N-3,5-Dinitrobenzoyl-L-alanine-octylester (L-SO) was the chiral selector. The average selectivity obtained was changing in the range of 1.2 and 2.

Lack of long term stability and slow mass transfer rates are the main drawbacks of SLMs and BLMs, respectively. Among these three liquid membranes, due to their relatively good stability, ELMs seem to be the most advantageous ones. However, they might sometimes have problems because of the reduced extraction efficiency and selectivity. These problems are observed because of swelling and leakage in processes by which water is transported across the membrane phase to balance the osmotic pressures which may exist across it [45]. Because of these problems, liquid membrane applications can not be considered as the best alternative among the membrane based chiral separation methods.

2.1.1.2 Ligand Immobilized Membranes

In some studies, the ligand is immobilized on the membrane support. During the ultrafiltration, ligand binds the target enantiomer specifically, forming a huge complex. Then the unbound enantiomer permeated through the membrane, due to it small size. Therefore, the separation is achieved. As a final step, the elution of the target enantiomer takes place by changing the ionic strength of the medium.

In literature, Bovine serum albumin (BSA) was widely used as ligand in immobilized form, due to its high-affinity binding sites especially for one of the amino acid enantiomers. It is a kind of plasma protein extracted from bovine. It is available in crystalline form therefore it is one of the most widely investigated proteins, with a molecular weight of 68 000 Da. It is easily dissolved in pure water and binds with various metal ions e.g. Zn^{2+}, Mn^{2+}, Co^{2+} etc. [46]

Higuchi et al. [47] studied the chiral separation of tryptophan by using immobilized BSA membranes, which were called as affinity membranes. The separation was strongly pH dependent, so that at pH 7, D-tryptophan preferentially existed in the permeate. However when the pH was reduced to 3, due to the ejection of L-tryptophan from the BSA binding site, L-tryptophan preferentially existed in the permeate. This meant that there were some conformational changes in the structure of BSA at low pHs. The

selectivity, α, was the concentration ratio of L-tryptophan to D- tryptophan in the permeate and it was found to be α = 8.70 for pH 3.

In their other article [48], optical resolution of phenylalanine was investigated again by using immobilized BSA membranes. For phenylalanine, similar to tryptophan, D-phenylalanine preferentially existed in the permeate at pH 7. Separation factor, α, was defined as concentration ratio of D- isomer to L-isomer in the permeate. The obtained separation factor is α =1.22. Schematic representation of ligand immobilized membranes is shown in Figure 2.

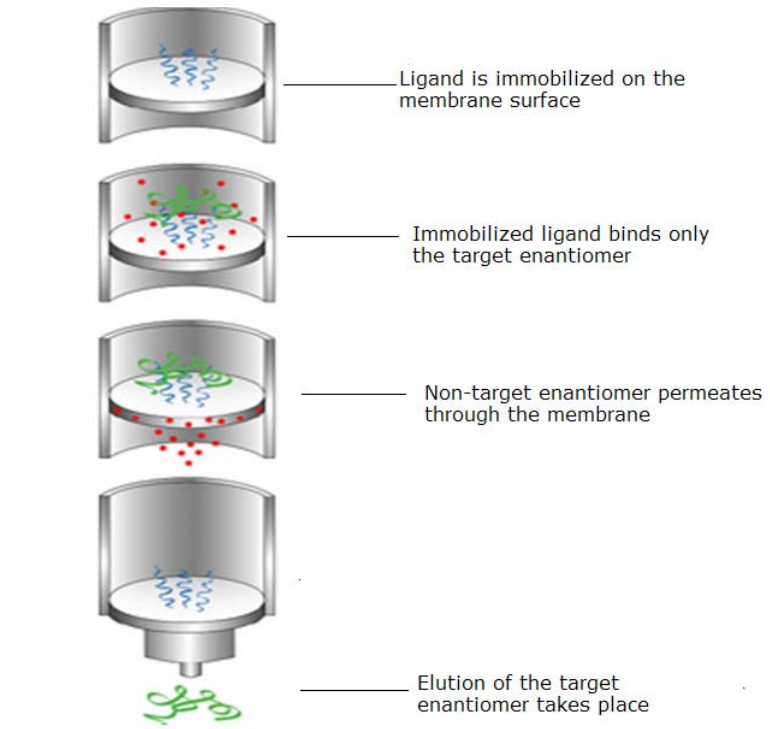


Figure 2.5 Schematic representation of ligand immobilized membranes [49]

In another study [50], immobilized DNA membranes were used in order to resolve phenylalanine. Different than previous studies [47, 48], these membranes were called as channel type membranes, not as affinity membranes. The reason could be explained as follows: Although DNA

preferentially bound to L-phenylalanine, in permeate and concentrate solutions L-phenylalanine and D- phenylalanine preferentially existed, respectively. This phenomenon showed that L- phenylalanine preferentially entered into the pores of the immobilized DNA membranes and permeated due to the interaction between DNA and L-phenylalanine. Separation factor was dependent on both time and feed concentration and it was always less than unity. ($\alpha < 1$) This means that for all racemic feed concentrations (C_{feed}= 0.002- 0.012 mmol/l) and for all periods of the study (0-8 hours), L-phenylalanine preferentially existed in the permeate, and with separation factor of the concentrated side, $\alpha_{c,}$ greater than unity ($\alpha_{c,} > 1$); D-phenylalanine existed in the concentrated feed side.

In their next study [51], it was stated that in case of ligand BSA immobilization, due to the too strong binding affinity of the L- enantiomer to the membrane, this enantiomer could not permeate through the membranes, but adsorbed on the membrane. Whereas, weaker binding affinity of DNA to L-amino acids compared to that of BSA, lets the permeation of L-form and makes the immobilized DNA membranes called to be channel type membranes. In this study, in addition, effect of membrane pore size on chiral resolution was investigated. It was found that pore size of the immobilized DNA membranes regulated preferential permeation of the enantiomer through the membranes. Different than experiments performed with MWCO>5000; if MWCO<5000; D-phenylalanine preferentially permeated through immobilized DNA membranes ($\alpha > 1$). Namely, in membranes with MWCO<5000, it was estimated that DNA was immobilized only to the surface of the membrane and not inside the pores, resulting in permeation of D-phenylalanine. Whereas, in membranes with MWCO>5000, DNA was expected to be immobilized inside the pores as well as on the surface, resulting in permeation of L-phenylalanine.

These systems might be thought to be similar to chromatographic systems, especially the affinity chromatography, in which a column with a chiral stationary phase was utilized, for chiral resolution. Therefore problems observed in ligand immobilized membranes are similar to chromatography; such as the limited amount of ligand bound on the membrane. These

problems might be reduced, by using ligands in free form in the feed solution.

2.1.1.3 Complexation Enhanced Ultrafiltration (CEUF)

Complexation enhanced ultrafiltration is an emerging method, which can be mainly investigated in three groups: **Colloid enhanced, micellar enhanced and polymer enhanced ultrafiltration** in which colloids, surfactants and polymers are being used as binding agents (ligands), respectively.

2.1.1.3.1 Colloid Enhanced Ultrafiltration (CoEUF)

This is a separation method that can remove both organic and inorganic species from aqueous solution. As a macromolecular species micelles or polyelectrolytes are used. Interaction between the metal and colloid is charge dependent. There is an electrostatic attraction between colloid with negatively charged surface and positively charged ion. The formed macromolecules are prevented from passing through the pores of the membrane into the permeate stream, whereas noninteracted metals can easily pass [52].

Roach et al. [52] stated that in conventional CEUF there is no selectivity in the process, except on the basis of the charge of the cation. Therefore they tried to achieve the selective removal of lead from aqueous solution by using ligand modified CEUF. Colloids used were cationic polyelectrolyte poly(diallyldimethylammonium) chloride (PDAD-MAC) and the cationic surfactant cetyllprydinum nitrate ($CPNO_3$). Four nitrilotriacetic acid (NTA) were investigated as ligands. Effect of pH, ionic strength, competing ions and colloid concentration were the parameters studied on the chiral separation.

2.1.1.3.2 Micellar Enhanced Ultrafiltration (MEUF)

In micellar enhanced ultrafiltration method, surfactants are added to the polluted water stream [53].

Baek et al. [53] studied MEUF system by using a cationic surfactant cetylpyridinium chloride (CPC) for the removal of nitrate and chromate from water streams. Effect of CPC molar ratio on the removal of equimolar nitrate and chromate was investigated. They showed that there is a competition between nitrate and chromate in case of coexistence, which resulted in significant inhibition in removal of nitrate. Removal of chromate with -2 as valance was higher than that of the nitrate with -1 as valance. For the molar ratio of 1:1:10 (nitrate: chromate: CPC), 91% and >99% removal were achieved for nitrate and chromate, respectively.

Problems such as, mass-transfer limitations, interphase reactions and membrane instability are observed in these two complexation methods, since they are two phase (heterogeneous) operations. On the other hand, polymer enhanced ultrafiltration (PEUF), is a one phase (homogenous) operation. Therefore, these problems are not observed in this method. In addition, in PEUF, possibility of binding several functional groups to the polymer provides an adjustment in polymers' molecular weight. So PEUF might be considered to be more advantageous [8].

2.1.1.3.3 Polymer Enhanced Ultrafiltration (PEUF)

Polymer enhanced ultrafiltration (PEUF) is widely applied for removing the heavy metals from aqueous solutions [8-15]. The process is the combination of two phenomena: binding of metal ions to water soluble polymer and ultrafiltration [9] Addition of water soluble polymers to the feed solution is followed by the ultrafiltration operation. The water soluble polymers are used to bind metals to form macromolecular complexes. This large molecule will be retained by the membrane in the retentate stream, while the non-complexed ions pass through the membrane to the permeate

stream [10]. In Figure 2.6 PEUF utilizing metal-polymer complexation is presented.

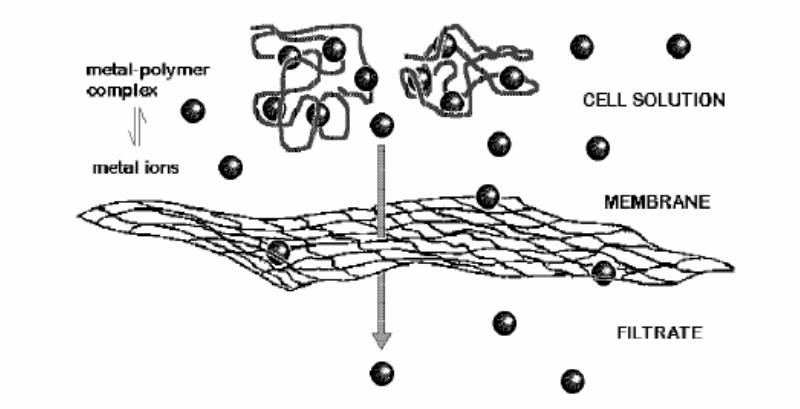


Figure 2.6 Schematic representation of PEUF which utilizes metal-polymer complexation [14].

There are many studies performed by using different water-soluble polymers as the macromolecule to form metal polymer complex. In their review, Rivas et al [14], mentioned the polymers used in several studies as the macromolecule. tPoly(N-hydroxyethyl)ethyleneimine (PHEI), Poly(N-acetyl)ethyleneimine (PAEI), Poly[(N-hydroxyethyl)ethyleneimine-co-N-acetyl) ethyleneimine] (PHEI-co-PAEI), Poly(ethyleneimine) (PEI), Poly(allylamine) (PALA), Poly(acrylamide) (PAm), Poly(acrylic acid) (PAA), Poly(methacrylic acid) (PMA), Poly(a-acetylamino acrylic acid) (PAAA), Poly(sodium 4-styrenesulfonate) (PSS) can be given as examples for these polymers. These polymers were used as ligands for the complexation of the several metal ions including; Cd^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, Cr^{3+}, Co^{2+}, Ag^{+}, Hg^{2+}, Ni^{2+}, Ca^{2+}. It was stated that, interactions of metal ions with water-soluble polymers are mainly due to electrostatic forces and the formation of coordinating bonds.

Geckeler et al., [15] stated that there are mainly two types of interactions for polymer binding: Ionic interactions and complex binding. For the removal of several ions including arsenate, iron, manganese and calcium, the polymer-ion interaction was ionic. Complex binding is more selective than ionic interactions. Binding of heavy metal ions into Polyethyleneimine (PEI) is an example. Selection of polymeric agent is critical for successful separation. High affinity towards target molecule, inactivity towards non target molecule, high molecular mass, chemical and mechanical stability, low toxicity and low cost are the main requirements that should be met by the polymeric agent. Then the binding conditions; including pH value, solution composition and binding degree are effective on the separation. Sometimes addition of low molecular weight synergetic agent provides stability. These triple compounds are more stable thermodynamically than just polymer-target compound complexes.

Rivas et al [14] studied the interactions of water-soluble polymers with metal ions under the ultrafiltration technique. The equilibrium distributions of divalent metal ions were experimentally obtained by ultrafiltration at a constant ionic strength. They showed that, the values adjust to Freundlich and Langmuir isotherms for the polyelectrolyte PSS interacting with Cd2+, and to a Freundlich isotherm for the chelating polymer PALA interacting with Ni2+.

Uludağ et al. [8] studied first the single metal containing solutions and focused on separation of mercury from aqueous solutions by PEUF. Polyethyleneimine (PEI) was used as the complexing agent. Effects of mercury to polymer ratio, pressure drop and feed solution circulation rate on retention of mercury and permeate flux were investigated. It was observed that, rather than individual concentrations, retention of mercury depends on mercury-to-PEI ratio. Performance depends on flux and retention. At pH=5, constant retention of R=0.98 was obtained until ratio of mercury to PEI is 1 kg/kg. After this value, there is a sharp decrease in retention. Then, mercury capacity of 1 kg of PEI can be taken as 1 kg of mercury at pH 5 for practical purposes.

In a continuing study, Muslehiddinoglu et al. [9], focused on the selective separation of heavy metals (cadmium and mercury) from binary mixtures using continuous PEUF by adjusting pH and loading (kg mercury / kg PEI). Increase in pH and decrease in loading resulted in higher retention of both metals. However, retention of cadmium was affected more than that of mercury as the pH decreased and loading increased. Besides, metal binding capacity of PEI for mercury is higher than its capacity for cadmium. Therefore selective separation of two metals was achieved at selected pHs. At pH=5.5, cadmium /PEI~0.35 and mercury / PEI~0.39, the highest separation factor was obtained as 49.

Similarly, in different PEUF studies, removal of cadmium in the presence of chitosan [11], Boron removal by using polyvinyl alcohol (PVA) [12], calcium removal by utilizing Poly(acrylic acid–maleic anhydride) sodium salt (PAM-Na) [13] were studied. Commonly effect of polymer/metal loading ratio and pH were investigated.

2.1.1.3.3.1 PEUF for Chiral Separations

In addition to metal removals from waste water streams, PEUF has some applications in chiral separations. However, these limited number of studies most of the time focused on chiral amino acid resolutions, without any parametric investigations. On the other hand, the potential of this method for separating molecules other than amino acids were studied to a more limited extent. Therefore, there is a need to develop a methodology for these kinds of separations.

In the existing model, chiral selectors are used as ligands and only one enantiomer has ability to bind with the macro molecule polymer and form a macromolecular complex, which can not pass through the membrane pores. The free enantiomer can easily pass. As a result separation is achieved. The schematic representation of this process is shown in Figure 2.6.

Similar to ligand immobilized systems, most widely used protein in PEUF for chiral separations was again BSA.

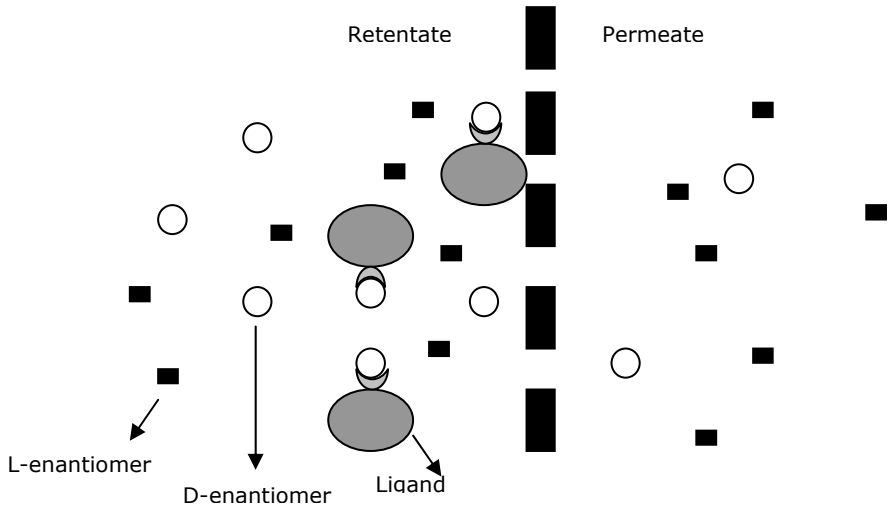


Figure 2.7 Schematic representation of PEUF for chiral separations

Garnier et al. [16] studied separation of racemic tryptophan by using PEUF system, with BSA as the binding agent. D-enantiomer does not have a specific binding site, whereas the L-enantiomer exhibits a high specific binding site on BSA. It was found that in the pH range from 7 to 11, the most probable complexation mechanism was a competitive binding of D- and L-enantiomers on a single site.

Similarly, Romero el al. [17] focused on pH and salt effects on chiral separations using BSA as the stereoselective microligand. It was shown that around pH 9.2 the highest purification factor and yield for L-tryptophan was achieved. But these conditions actually lead to a low D-tryptophan yield in the filtrate. In their continuing study [18], it was aimed to examine the performance of a multistage-diafiltration process for chiral separation of tryptophan, with BSA again. In a two-stage system purification factors of more than 20 at greater than 90% yield were obtained.

Depending on the racemic amino acid to be separated, the conformation of BSA might change when it was used in immobilized form or freely in the solution. This was demonstrated in the study performed by Higuchi et al. [48]. Different than, phenylalanine and tryptophan; utilization of BSA in immobilized form or freely in the solution, changed the form of the leucine in the permeate; Namely, D-Leucine was found preferentially in the permeate with immobilized BSA membrane; whereas, L-leucine preferentially existed in the permeate of the ultrafiltration when BSA was free in the solution. This meant that recognition site of immobilized BSA membranes for D-leucine was abolished during immobilization.

Bowen et al. [54] again utilized BSA as chiral selector for ibuprofen separation in PEUF system. This study was important since it was the only study in which a chiral molecule different than an amino acid was investigated for resolution in a PEUF system. The effectiveness of optical resolution was characterized in terms of enantiomeric enrichment and solute recovery in the permeate. Effect of pH and loading ratio (BSA content in the feed) were examined. Enantiomeric excess (ee %) is strongly pH dependent and reaches a maximal value of 24 % at pH 9.0-9.2 when Ibuprofen /BSA (mol /mol) ratio is 4. At low pH values (below isoelectric point of BSA) total solute binding exceeds 90% for both enantiomers, showing a high nonspecific binding of ibuprofen. Increase in pH reduces nonspecific binding. At pH ~9, solute binding drops to 75% and ~ 60 % for R- and S-enantiomers, respectively. It was found that nonspecific binding was still significant at pH=9.1.These nonspecific interactions are suppressed in the presence of organic solvents (acetonitrile, methanol) in the feed solution. In addition, increase in BSA content of feed solution results in increase in both enantioselectivity and solute binding.

2.1.1.4 Enzyme Associated Membrane Systems

Enzymes catalyze or speed up chemical reactions. They play important roles on many biological reactions with high regioselectivity and stereospecificity under mild conditions. Some enzymes require non-protein molecules for activity. These molecules are called as **cofactor**. Cofactors can

be either inorganic or organic compounds. Enzymes that require a cofactor, but do not have one are called **apoenzymes** [55]. By means of the bound cofactors catalytically active enzyme complexes are formed as seen in Figure 2.8

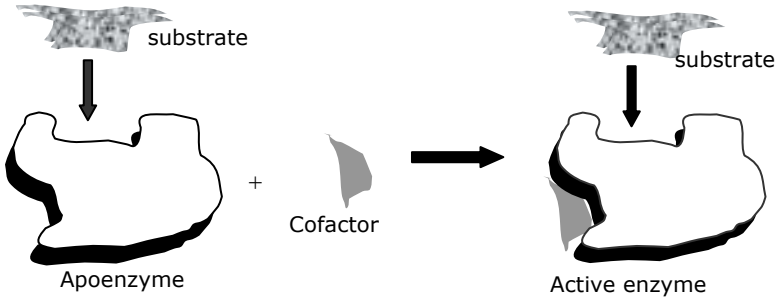


Figure 2.8 Schematic illustration of a mechanism for enzyme catalysis requiring a cofactor [55].

In Figure 2.8 it was also noted that, the binding sites for the substrate and cofactor are different from each other. This is the most crucial point of apoenzymes used in enzyme loaded membrane systems. Namely, without their cofactors they may still selectively recognize and bind their substrates, but they do not convert it to product.

Lakshmi et al. [56] studied enantioseparation of D- phenylalanine and L- phenylalanine by using apoenzymes immobilized in a porous polymeric membrane. The membrane consists of a microporous polycarbonate filter sandwiched between two thin films of polymer polypyrrole. Within the pores of the membrane, the apoenzyme is physically trapped by means of polypyrrole films. Apoenzyme is also entrapped within the polypyrrole films.

Before D- and L- phenylalanine enantioseparation, in order to verify that apoenzyme selectively recognizes its substrate molecule and transports it across the membrane, resulting in an increase in flux of the substrate; ethanol-phenol pair was studied. Membrane was entrapped with alcohol dehydrogenase apoenzyme (apo-ADH) Ethanol flux of this membrane was

compared with the membrane without any apo-ADH. The loaded membrane had a higher flux, which indicated that apo-enzyme facilitated the transport. Moreover, when the nonsubstrate molecule (phenol) flux was investigated, it was lower than the ethanol flux. Selectivity coefficient for ethanol vs. phenol transport is 9.2.

In order to confirm the transport mechanism apo aldehyde dehydrogenase (apo-AdDH) was used as the second apoenzyme. It was seen that apo-AdDH loaded membranes facilitated flux of aldehyde when the concentration dependence of aldehyde flux across apo-AdDH loaded and free membranes were compared.

After confirming the transport mechanism, they studied the enantioselectivity of D- L- phenylalanine this time, by using D-amino acid oxidase apoenzyme and obtain a selectivity coefficient of D- vs. L-phenylalanine as 4.9.

Giorno et al. [57] studied a multiphase system which was used for enantioselective conversion of racemic ester of naproxen into the corresponding S-naproxen acid. Transport properties of the system were measured in the absence of biochemical reaction. Therefore, deactivated enzyme loaded membranes were used for measuring overall mass transfer coefficients of reactant and product through the two phase membrane system. They showed that enzyme immobilization improved the transport. Moreover, use of enzyme in the presence of emulsion made the transport better.

2.2 Benzoin Separation Methods

When the chiral separation methods for benzoin was investigated; it was observed that in literature racemic benzoin was separated into its enantiomers by using several different methods, which are in consistency with the chiral separation methods explained in section 2.1.

Ding G.S. et al. [58] studied chiral separations of some racemates of drugs, including benzoin by High Performance Liquid Chromatography (HPLC). A novel Norvancomycin-bonded chiral stationary phase was utilized. After investigating effect of organic modifier concentration among ethanol, methanol and acetonitrile; 35:65(v/v) methanol: TEAA (1% Triethylammonium acetate buffer) was used as the mobile phase in reversed phase mode, due to the best results obtained in terms of separation time and resolution. Column temperature, mobile phase flow rate and pH effects were investigated. It was observed that for benzoin as the column operating temperature decreased, the retention factor and enantioselectivity increased similar to other drugs. When the effect of flow rate was investigated, it was seen that flow rate did not affect enantioselectivity however it did affect the separation efficiency. At lower flow rates the efficiency was high. On the other hand, benzoin chiral resolution was less affected by pH changes. At pH=4.0, 5.5 and 7.0 the obtained enantioselectivies were α=1.313, 1.317 and 1.322, respectively. This was due to its non-ionizable structure when compared to other ionizable drug compounds.

Ding W. et al. [23] studied chiral resolution in capillary electrophoresis (CE) with several surfactants, which are synthesized from an amino acid or alkyl chloroformate with a chain length of C_4 to C_{12}. Benzoin was one of the tested chiral drugs in this study. Variation of chain length, amino acid type and surfactant concentration were effective on resolution. When the effect of surfactant chain length was investigated, the surfactant obtained by the reaction of C10-leucine and alkyl chloroformate yielded the highest resolution of benzoin with a value of 1.75. At 50 mM C_8-Valine, C8-Leucine and C_8–isoleucine, the enantioselectivity of benzoin was almost kept constant at a value of α =1.02-1.03. In addition, two fold increase in surfactant concentration, results in enhancement of 200% for benzoin resolution.

Katayama et al. [59] also studied the separation of benzoin in capillary electrophoresis (CE) by using flavoprotein as chiral selector for benzoin.

As mentioned, capillary electrochromatography (CEC) is a promising analytical separation technique. Kato et al. [29] developed a protein encapsulation technique for the preparation of monolithic columns for CEC. Chiral selectivity was evaluated for separation of again some enantiomers including benzoin. Benzoin separation was achieved on a column which was encapsulated by a glycoprotein from chicken egg white, ovomucoid (OVM). Obtained enantioselectivity for benzoin was α=1.07.

Similarly, Chen et al. [60] focused on Cyclodextrin-modified monolithic columns for resolving benzoin enantiomers by CEC. Chemically β-CD modified monolithic chiral stationary phase columns has been used for the separation of the racemates of benzoin.

Haginaka et al. [61] studied the enantioselectivity of the BSA-bonded columns produced with isolated protein fragments. BSA fragment, BSA-FG75 was isolated by size exclusion chromatography following peptic digestion of BSA. BSA-FG 75 was mainly N-terminal half peptide with as average molecular weight of 35000. BSA and BSA-FG75 fragment protein were bound to aluminopropylsilica gels activated by N,N'-disuccinimidyl carbonate; at amounts of 2 and 5.5 μmol / g, respectively. When the chiral recognition of these proteins for benzoin was investigated; BSA-FG75 fragment was observed to show better performance in terms of enantioselectivity. It was stated that higher capacity of BSA-FG 75 for benzoin was due to the increase in the number of chiral recognition sites for benzoin on the BSA-FG75 column compared with BSA column. For the different amounts of benzoin injected into the columns, this behaviour was observed. For instance, when 0.5 nmol benzoin was injected; for BSA and BSA-FG75 loaded columns, the obtained enantioselectivities were α =1.49 and α =2.18, respectively.

2.3 Enzyme Enhanced Ultrafiltration (EEUF)

In this study, first, PEUF with ligand BSA was studied for benzoin resolution, in order to investigate binding capacity of BSA against a ketone alcohol. Furthermore, investigation of the effect of ligand concentration on

total benzoin retention and on ee% with such an economical and widely available protein would be reasonable in terms of developing and strengthening the methodology.

Then, in the light of conventional PEUF studies and principles used in enzyme loaded membrane systems [56, 57]; apo-enzymes were proposed to be used as ligands in an ultrafiltration system. This method was called as **"Enzyme Enhanced Ultrafiltration (EEUF)".** As an apo-enzyme, apo form of Benzaldehyde Lyase (BAL) (E.C.4.1.2.38) was selected.

2.3.1 Benzaldehyde Lyase (BAL, E.C. 4.1.2.38)

Benzaldehyde lyase (BAL, EC 4.1.2.38) catalyses cleavage of the carbon-carbon bond of benzoin to form two benzaldehydes. It also catalyses the reverse acyloin condensation of benzaldehydes resulting in the synthesis of R-benzoins. The corresponding reactions are shown in Figure 2.9

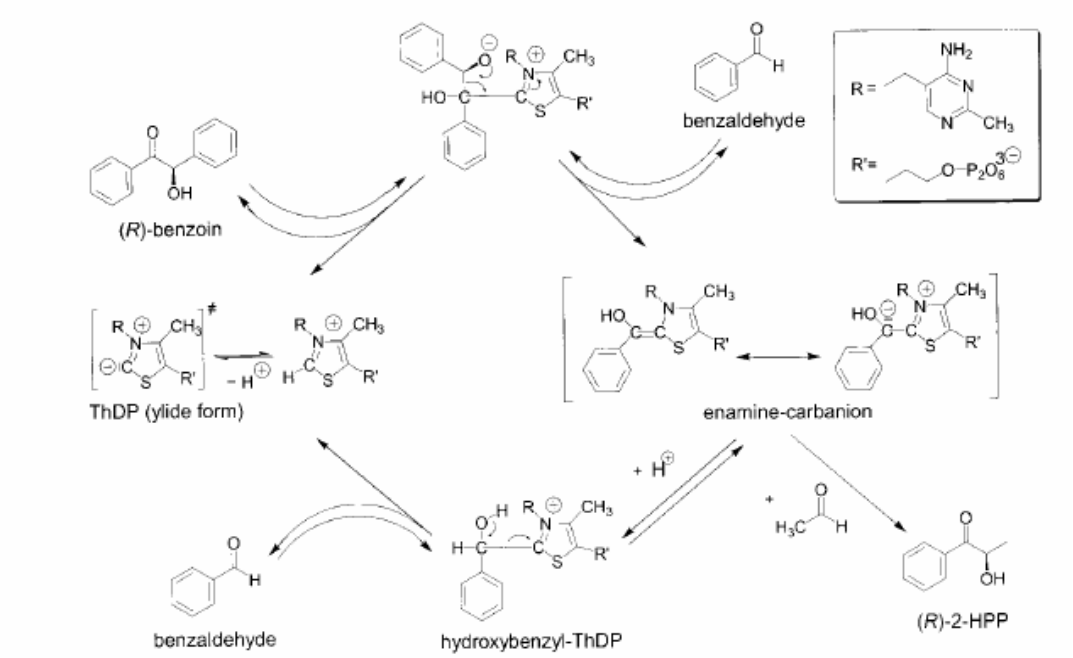


Figure 2.9 Benzaldehyde lyase catalyzed cleavage and synthesis of benzoin [62]

BAL is a thiamin diphosphate (ThDP) and Mg^{+2} dependent enzyme as cofactors, and it was first reported by Gonzáles et al. [63]. It is naturally produced by wild-type *Pseudomonas fluorescens* Biovar I. In their study, purification and characterization of the enzyme were described and the ability of BAL to catalyse the cleavage of acyloin linkage of benzoin to form benzaldehyde irreversibly was described. They also showed that maximal activity is in the pH range of 7.5-8.5 and it is inactive below pH 6 [63].

Recently, in a study in which enzyme structure has been modeled [62], it was affirmed that BAL is a homotetramer, where each subunit binds to one THDP molecule using one Mg^{+2} ion. The enzyme is composed of 4 x 563 amino acid residues and has a molecular weight of 4 x 58919 Da.

Afterwards, Maria et al. [65] studied enzymatic activity and stability of BAL. Influence of co solvent DMSO, cofactors and pH on the stability and activity were examined in a test reaction of BAL catalyzed synthesis of benzoins by carboligation of benzaldehyde derivatives. It was stated that 30% DMSO content in the presence of potassium phosphate buffer with 0.5 mM Mg^{+2}, 0.5 mM ThDP and 1 mM DTT, a well-known stabilizer of hydrolases, was optimum, for enzyme activity and stability. Enzyme prepared in potassium phosphate buffer supplemented with ThDP, Mg^{+2}, DTT as cofactor and 30% DMSO as co solvent lost its activity after 30 h, while in water it lost almost all of its activity in 3 hours. It was also shown that when the pH of the reaction medium was increased from 8.0 to 9.5, the best enzymatic activity was obtained.

In 2004, Yılgör [66] investigated recombinant BAL production. Host *Escherichia Coli* strain having the highest BAL production capacity was investigated and they concluded that *E.coli K12* (ATCC10798) was the best producer among four types of *E.coli* strains.

In the continuing study, in 2006, Kaya [67] showed that the activity of BAL obtained in microorganism E.coli BL21 carrying PRsetA:bal gene in the optimized production medium was 1.2 times better than that in the *E.coli* K12.

BAL is used for the synthesis of enantiopure α-hydroxy ketones, an important class of compounds in natural product and drug synthesis with its chital enzyme property. Namely, only R- enantiomer of benzoin is converted into benzaldehyde through BAL catalysis, while S-benzoin gives no reaction at all, as seen in Figure 2.10

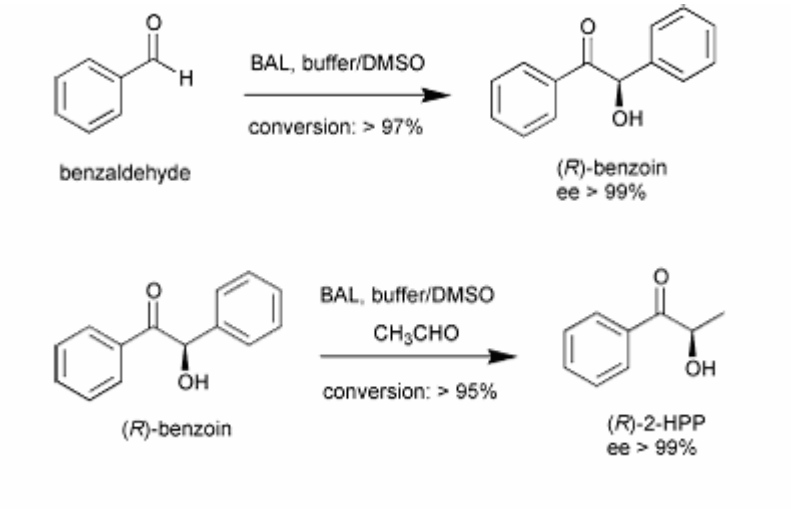


Figure 2.10 Benzaldehyde lyase catalyzed reactions [62]

In our study also, conversion of only R-enantiomer into benzaldehyde, was the most critical point. In the developed EEUF method, in order to resolve benzoin molecule; cofactor of BAL, thiamine diphosphate (ThDP) should be removed from the enzyme production medium, hence from the separation medium. So that, by means of the obtained apo-BAL the catalytic reaction would not take place, instead; without the cofactor, apo-BAL would just selectively recognize and bind its substrate R-benzoin.

It was expected that, similar to the principle of PEUF studies mentioned above, while the macromolecule BAL- R-benzoin complex could not permeate through the membrane pores, free S- benzoin could easily pass. As a result, enantiomeric benzoin separation would be achieved.

In this study; it was aimed to contribute to the literature; by developing an enhanced ultrafiltration system; first with model ligand BSA. Systematic optimization of the operating parameters with this ligand for the separation of chiral molecules would make this study one of the leading ones in this field. During this systematic optimization; first stirring rate effect on flux was searched. Then effect of cosolvent type and ratio were the parameters investigated for better dissolution of benzoin and for better flux values. Noticing the retention capacity of membranes for benzoin necessitated development of membrane saturation strategy. Accordingly, it was decided to use regenerated cellulose (RC) membranes.

Secondly, on the basis of these parameters set in PEUF experiments, a completely new ligand; an apoenzyme, namely apo-BAL was used as ligand for benzoin resolution. There has been no study in which apoenzymes were used as ligands in such an enzyme enhanced ultrafiltration (EEUF) system, yet.

Finally, it should also be noted that, specific for benzoin chiral resolution, not only the EEUF with ligand apo-BAL but also PEUF with ligand BSA would be first in the literature.

CHAPTER 3

EXPERIMENTAL PROCEDURE

3.1 Materials

Racemic Benzoin (catalog no: 12510) purchased from Fluka Chemika, Albumin from bovine Serum minimum 98% (BSA) obtained by electrophoresis (catalog no: A7906) was purchased from Sigma Ltd. Polyethylene Glycol 400 (PEG 400) (catalog no: 8.07485), Dimethyl Sulfoxide (DMSO) (catalog no: 1.02952), and HPLC grade solvents; n-Hexane (catalog no: 1.04391) and 2- propanol (catalog no: 1.01040) were obtained from Merck Ltd. In addition, all chemicals used in Benzaldehyde lyase (BAL) production are analytical grade and obtained from, Sigma Ltd., Difco Laboratories, and Merck Ltd.

All membranes utilized have MWCO of 10000 Da. Regenerated cellulose (RC) (catalog number: PLGC 02510 and PLGC04710) and polyether sulfone (PES) (catalog number: PBGC 02510) membranes were purchased from Amicon,Millipore. Cellulose triacetate (CTA) (catalog number: 14539-047-D) membrane was from Sartorius.

3.2 Ultrafiltration Experiments

3.2.1 Operating Conditions for Ultrafiltration Experiments

Due to the low solubility of benzoin in pure water, Polyethylene glycol 400 (PEG 400) and Dimethyl sulfoxide (DMSO) were used as co solvents. Two types of stirred cells were utilized in this study: Amicon 8010 dead-end stirred membrane cell with a total volume of 10 ml, was used with co-solvent PEG 400 and for the compatibility of cosolvent DMSO, Millipore- XFUF 04701 -Solvent Resistant stirred cell with a total volume of 75 ml was preferred. According to the cosolvent type, utilized membrane materials were also changing for these cells.

Cosolvent DMSO could only be used with regenerated cellulose membrane due to its incompatible structure with other membrane materials studied. It was only compatible with regenerated cellulose material. On the other hand, cosolvent PEG 400 was used with Cellulose triacetate and polyethersulfone membranes in addition to regenerated cellulose membranes.

For Amicon 8010 dead-end stirred membrane cell, the pressure should not exceed 75 psi (5.2 bar), and for the Millipore- XFUF 04701-solvent resistant stirred cell pressure should be less than 90 psi (6.2 bar). Therefore, in both cells, to be on the safe side, the system was pressurized to 3 bars.

For Amicon 8010 dead-end stirred membrane cell, total solution volume and effective membrane area were 10 ml and 4.1 cm^2, respectively. On the other hand, for Millipore-XFUF 04701-solvent resistant stirred cell the same quantities were 50 ml and 15 cm^2, respectively.

During the experiments, retentate at a minimum volume must be left in the cells. These volumes were 1 ml and 10 ml for the Amicon 8010-stirred cell and Millipore-XFUF 04701-solvent resistant stirred cell, respectively.

PEUF experiments with ligand BSA were performed in both cells, with co-solvents PEG 400 and DMSO. However, EEUF experiments were performed only in Amicon 8010 dead-end stirred cell, by using PEG 400. The reason of using only this cell for EEUF experiments was mainly the limited amount of enzyme produced from one erlenmeyer of production medium content. The obtained enzyme volume was almost less than 10 ml. Namely, in order to use Millipore-XFUF 04701- solvent resistant stirred cell the volume of the produced enzyme should be greater. Moreover, when it was necessary to concentrate the enzyme, the produced volume decreased further and hence the obtained volume would be very close to stirred minimum volume of the Millipore- XFUF 04701 solvent resistant cell as indicated in its catalogue, which was 2.5 ml. To prevent this, many erlenmeyers of enzymes should be used at once, which would not be practical at the development stage of the EEUF method. Therefore, Amicon 8010 dead-end stirred cell with 10 ml volume was used with 15% (v/v) PEG 400 cosolvent, in EEUF experiments.

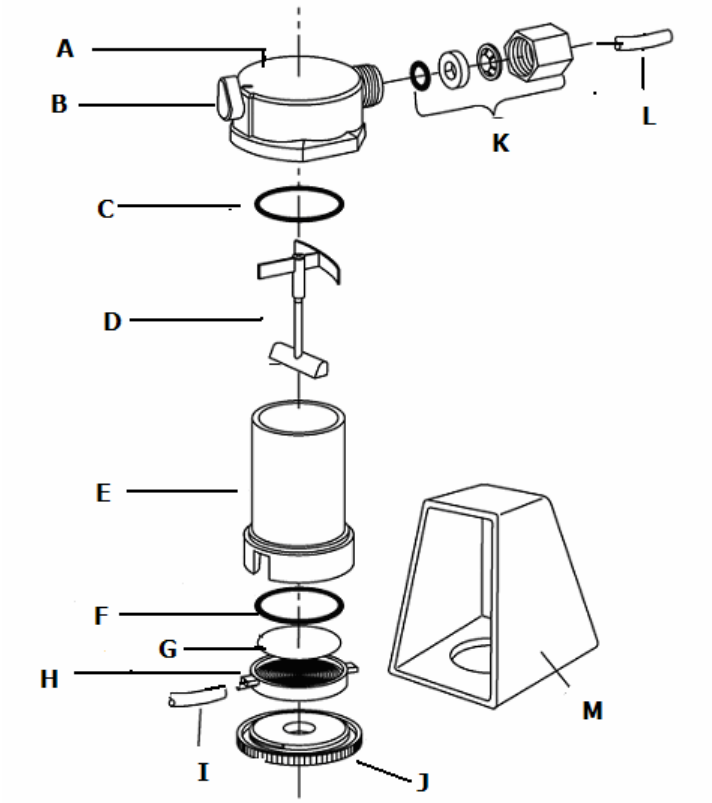
Benzoin concentration was kept constant at 10 ppm and 50 ppm for Amicon 8010 dead-end stirred cell and Millipore-XFUF 04701-solvent resistant stirred cell, respectively. In Table 3.1 operation conditions of the two cells are summarized.

Table 3.1 Operation conditions for the two cells.

	Amicon 8010 Stirred Cell	Millipore-XFUF 04701-Solvent Resistant Stirred Cell
Pressure (bars)	3	3
Total solution volume (ml)	10	50
Effective membrane area (cm2)	4.1	15
Membrane diameter (mm)	25	47
Co-solvent type	PEG 400	DMSO
Membrane type	RC,PES, CTA	RC
Retentate volume left in the cell (ml)	1	10
Ligand	BSA and BAL	BSA

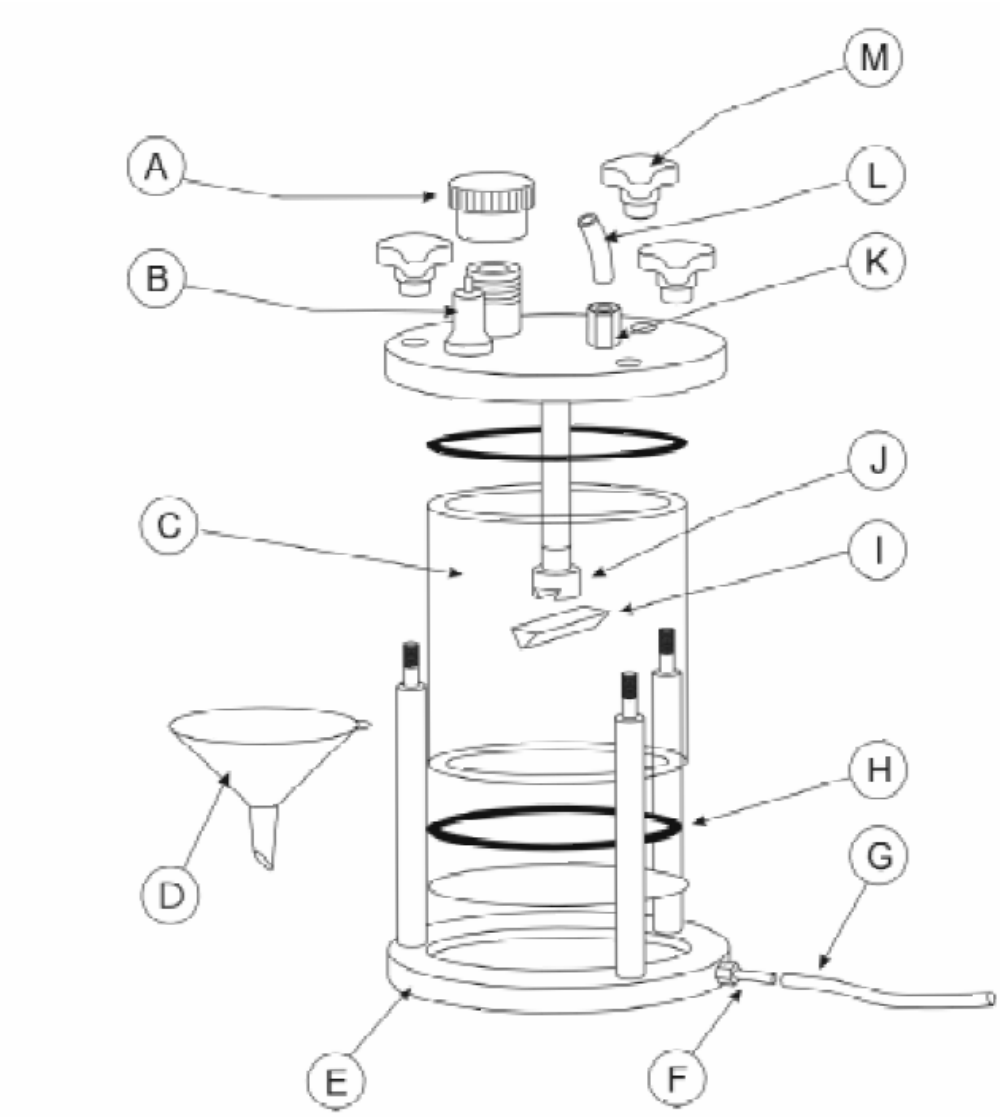
The cell was operating on a stirrer and was pressurized by means of a nitrogen cylinder.

In Figure 3.1 and Figure 3.2 exploded views of the two cells are shown. In Figure 3.3 flow diagram for the use of the Amicon 8010 stirred cells is given.



A - Cap assembly
B - Pressure relief valve
C - O-ring
D - Stirrer Assembly
E – Body
F - O-ring
G - Membrane
H – Membrane Holder
I – Elastomeric Tubing
J - Base
K – Tube fitting Assembly
L- Tubing plastic
M – Stand Assembly

Figure 3.1 Exploded View- Amicon 8010 Stirred Ultrafiltration Cell



A	Filler/pressure–relief cap and O–ring	H	O-ring seal
B	Safety valve and O–ring	I	Stirring bar
C	Glass cylinder	J	Stirring bar grip, grip screw and washer
D	Funnel		
E	Bottom plate	K	Pressure inlet port
F	Filtrate port	L	Pressure tubing
G	Filtrate tube	M	Cross knob

Figure 3.2 Exploded view- XFUF 04701- Solvent Resistant Stirred Cell

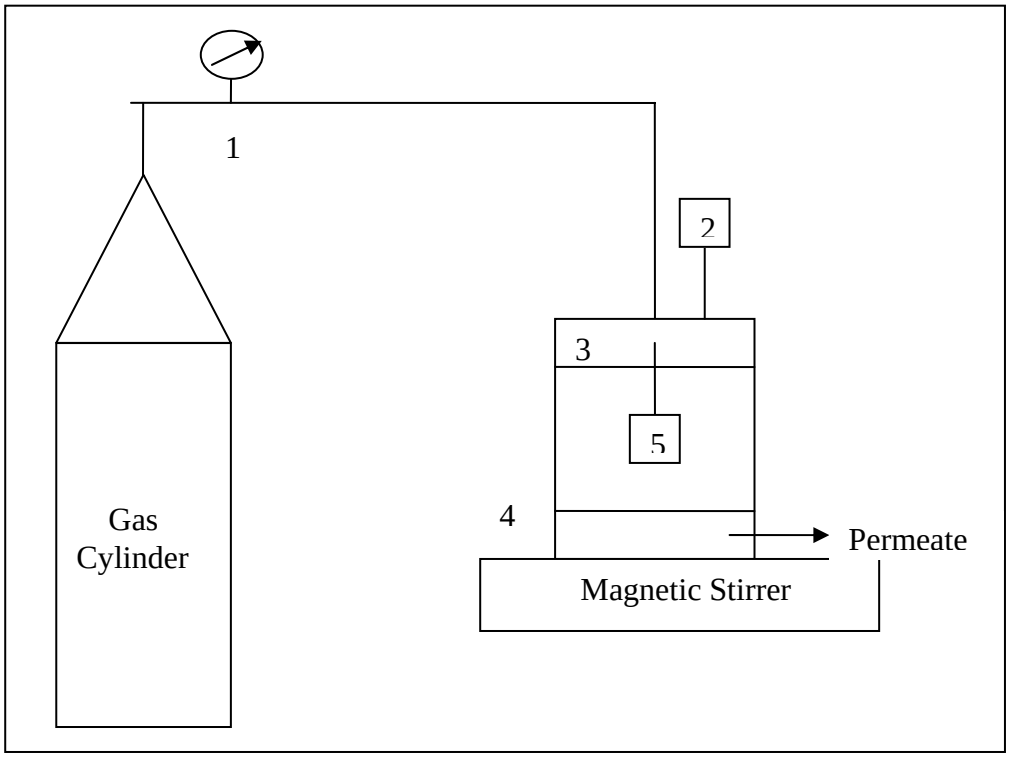


Figure 3.3 Flow diagram for the use of the stirred cell (1-Pressure Gauge, 2-Pressure release valve, 3-Stirred cell, 4-Membrane, 5-Stirrer bar)

3.3 Experimental Procedure

3.3.1 Polymer Enhanced Ultrafiltration (PEUF) Experiments

In PEUF experiments, as indicated in previous section two types of co solvents were used. Accordingly, in the first group of PEUF experiments 10 ppm benzoin- 15% (v/v) PEG 400-water and in the second group 50 ppm benzoin- 30 % (v/v) DMSO-water solutions were prepared, 24 hours before the experiment by stirring at a rate of 700-1000 rpm. In Figure 3.4, experimental procedure for PEUF experiments is shown.

In these experiments BSA was used as ligand. Prior to ultrafiltration experiments, the feed solution should be prepared. According to the predetermined concentration value, BSA was dissolved in benzoin-solvent

solution. This concentration was in the range of 1000 -10000 ppm for 15% (v/v) PEG 400-water and 5000-50000 ppm for 30 % (v/v) DMSO-water solvents, respectively.

After adjusting the pH of the feed by using HCl or NaOH, It was stirred at 200 rpm for at least 2 hours to make sure that complexation equilibrium between enantiomer and BSA was achieved. Effect of pH was examined in the range of pH 4-10 for cosolvent 15% (v/v) PEG 400 and pH 4-11 for cosolvent 30 % (v/v) DMSO. Although pH was not adjusted for every experiment, it was monitored continuously, in each experiment.

On the other hand membrane saturation process should take place after having pure water and then pure solvent ultrafiltered through the cell the day before the experiment. In order to keep the membrane saturated, it was stored in concentrated benzoin-solvent solution for the night. The details and the necessity of membrane saturation process are given in Chapter 4.

Just before the experiment, benzoin solution was filtered in order to see whether the retention was less than 10 % or not. Almost in all experiments the effectiveness of the membrane saturation process, which yielded less than 10% benzoin retention in this control stage, was observed.

Then the prepared BSA-benzoin-solvent solution was filtered from the membrane in order to perform the PEUF.

As mentioned, initial feed volume was 10 ml and 50 ml for Amicon 8010 stirred cell and Millipore- XFUF 04701- solvent resistant stirred cell, respectively, with 1 ml and 10 ml retentate volumes. Therefore, the permeate stream for experiments with 15% (v/v) PEG 400 was 9 ml, which was divided into three consecutive portions of 3 ml volumes. Similarly, for experiments with 30 % (v/v) DMSO, 40 ml permeate stream was divided into equal four consecutive portions of 10 ml volumes.

After PEUF, in order to clean the membrane; full cell of solvent-water solution, 0.1 M NaOH and pure water were filtered respectively. After washing, membrane was stored in 10% (v/v) ethanol-water solution at $+4^{o}C$.

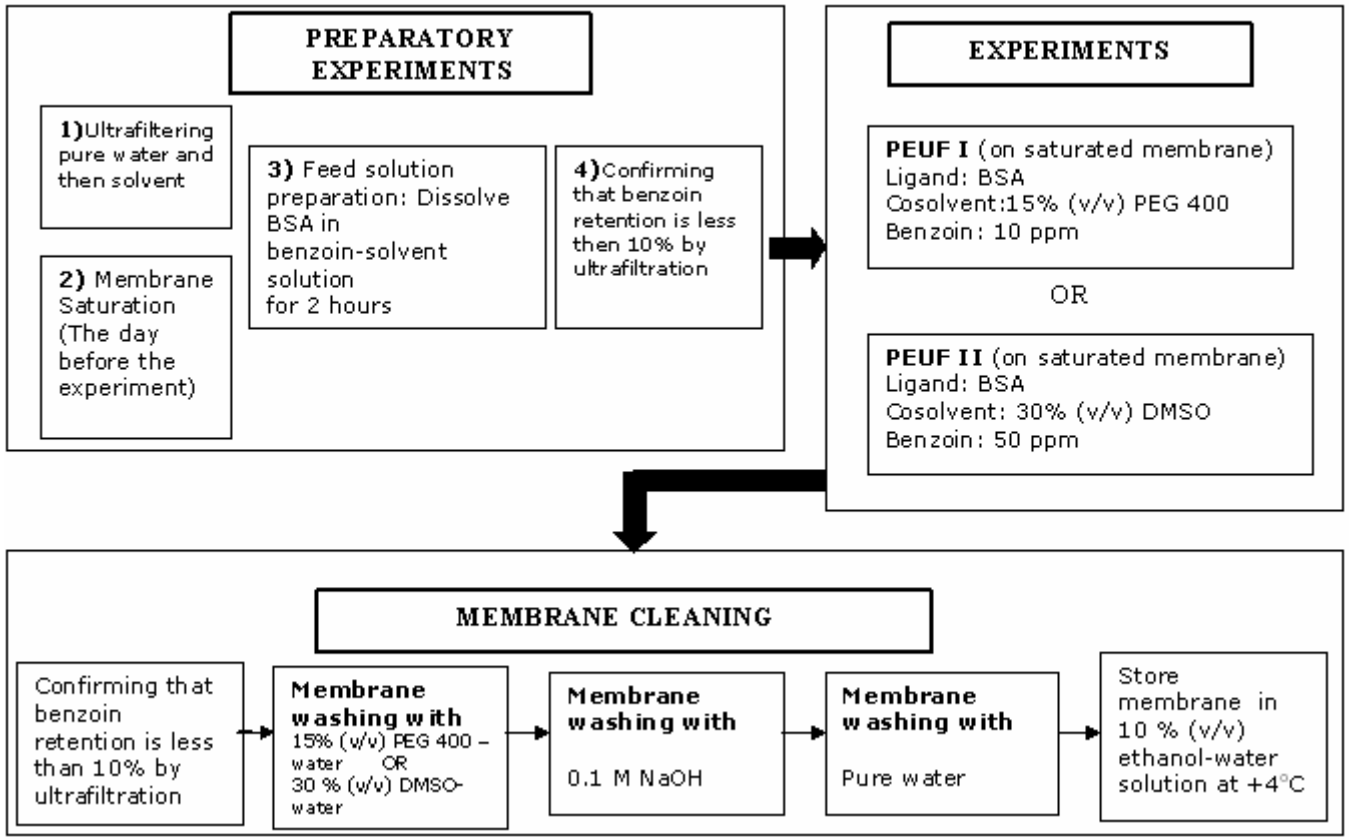


Figure 3.4 Experimental Procedure for PEUF experiments

3.3.2 Enzyme Enhanced Ultrafiltration (EEUF) Experiments

For these experiments, 20 ppm benzoin- 30% (v/v) PEG 400 solution was prepared 24 hours before the experiment by stirring at a rate of 700-1000 rpm.

Apo form of Benzaldehyde Lyase (BAL, E.C. 4.1.2.38) was used as ligand. Although enzyme production was not the main purpose of this study, some details are given, in the following section. Explained procedure was completely the same with the one applied in Kaya, 2006. [67].

3.3.2.1 Procedure for Benzaldehyde Lyase Production

Benzaldehyde Lyase (BAL, EC 4.1.2.38) was produced from *Eschericha Coli (E.Coli)* BL21 carrying *pRSETA::bal* gene. It was first inoculated into solid medium, then transferred into precultivation medium and finally inoculated into the production medium. The details were given in the following sections:

3.3.2.1.1 Solid Medium

Luria-Bertani (LB) Agar was used as the solid medium. Microorganisms were received as very few drops from the microbank stored at -55°C and inoculated onto the LB agar slants in sterile conditions, and incubated at 37 ^{0}C for 10 hours. According to the antibiotic resistance ability of the microorganism, ampicillin and chloramphenicol were added in to solid medium. In Table 3.2 the composition of the solid medium is given.

Table 3.2 Composition of the reference solid medium (pH =7.5) [67]

Compound	Concentration, kg m^{-3}
Soytryptone	10.0
Yeast Extract	5.0
NaCl	10.0
Agar	15.0
Ampicilin	0.100
Chloramphenicol	0.035

3.3.2.1.2 Precultivation Medium

Microorganisms grown in the solid medium were inoculated into precultivation medium and incubated at 37ºC and N=200 min^{-1} for 14 h. Experiments were conducted in agitation and heating rate controlled orbital shakers, using air-filtered Erlenmeyer flasks 150 ml in size that had working volume capacities of 33 ml. LB medium was used as the precultivation medium for biomass production, and its constituents is given in Table 3.3.

Table 3.3 Composition of the reference precultivation medium [67]

Compound	Concentration, kg m-3
Soytryptone	10.0
Yeast Extract	5.0
NaCl	10.0
Ampicilin	0.100
Chloramphenicol	0.035

3.3.2.1.3 Production Medium

After incubation in the precultivation medium for 12 hours, microorganisms were inoculated into the production medium, contained in 150 ml volume bioreactor, with 1/10 inoculation ratio. In agitation and heating rate controlled orbital shakers, incubation took place at T=37 ^{0}C and N= 200 min^{-1} for 12 hours. At the 4th hour of the production period, isopropyl β-D-thiogalactopyranoside (IPTG) to obtain a final concentration of 1 mM was added. The contents of the production medium are shown in Table 3.4.

Table 3.4 Composition of the reference BAL production medium [67]

Compound	Concentration, kg m^{-3}
Glucose	20.0
Na_2HPO_4	6.7
KH_2PO_4	3.1
NaCl	0.5
$MgSO_4.7H_2O$	0.5
$(NH_4)_2HPO_4$	11.8
Ampicilin	0.100
Chloramphenicol	0.035

3.3.2.1.4 Storage of the BAL produced

After 12th hour, production media were centrifuged at 13500 rpm (24000 rcf) and +4 ^{o}C for 10 minutes. Then the supernatant was removed; and the intracellularly produced BAL within the *E.coli* cells, was stored at -50 ^{o}C, in centrifuge tubes.

Before each EEUF experiment, lysis of the cell wall was conducted at f=10 s^{-1} for 10 minutes in agitator bead mill using 30% suspension glass beads in PBS buffer (pH = 7.2, did not contain any Mg^{+2} ion). At the end of

the final centrifugation at 13500 rpm (24000 rcf) and +4 ^{o}C for 10 minutes, the apo form of the enzyme (apo-BAL) was collected in the supernatant.

3.3.2.2 Procedure for EEUF Experiments

After enzyme production, diafiltration step was applied for the enzyme solution on an RC-10000 Da membrane (other than the saturated one). 10000 Da MWCO was suitable for removing small MW components in the medium. Enzyme with 4 X 59 kDa molecular weight was expected to be fully retained on the permeate side. The procedure applied for 10 ml volume Amicon 8010 stirred cell can be summarized as follows (If the produced enzyme volume is larger, 50 ml Amicon 8050 stirred cell might be used):

Amicon 8010 stirred cell with 10 ml volume capacity was filled with the enzyme solution. This solution was filtered until 1 ml solution is left inside the cell. Then, the cell was filled up to its initial volume with pure water. This procedure continues until the absorbance of the permeate stream at 250 nm, in UV- spectrophotometer was very close to 0.0, which means that almost all of the small MW molecules (< 10000 Da) was removed. In the final diafiltration step, the enzyme could be concentrated or diluted by the desired ratio.

Prior to EEUF experiments, again preparation of the feed solution took place. However, enzyme concentration was measured after the EEUF experiment by Bradford Method. Therefore, after the diafiltration step (before feed solution preparation), 100-500 µL enzyme was taken into an eppendorf tube and stored at -20 ^{0}C until the application of Bradford Method.

For feed solution preparation, 5 ml apoenzyme solution was mixed with 5 ml 20 ppm benzoin-30% (v/v) PEG 400 solution at room temperature, yielding enzyme at a certain concentration and 10 ppm benzoin in 15% (v/v) PEG 400. 7.5 µL, 0.5 M (139.5 ppm) ethylenediaminetetraacetic acid (EDTA), as chelating agent, was added in order to bind the ions such as Mg^{+2} in the medium. These ions might be effective as catalyst in benzoin to

benzaldehyde conversion. Therefore they are not desired. After the pH measurement, the feed solution was stirred at 300 rpm for 2 hours [8-10, 21] to make sure that complexation equilibrium between enantiomer and apo-BAL was achieved.

Similar to PEUF experiments, EEUF experiments were also performed on the membrane, which was saturated, the day before.

The stages after the EEUF experiment were similar to those stages after PEUF experiments. Membrane was cleaned with pure solvent, 0.1 M NaOH and pure water, respectively. Finally it was stored in 10% (v/v) ethanol /water solution at +4^{o}C. In Figure 3.5 experimental procedure for BAL production; and in Figure 3.6 experimental procedure for EEUF are shown.

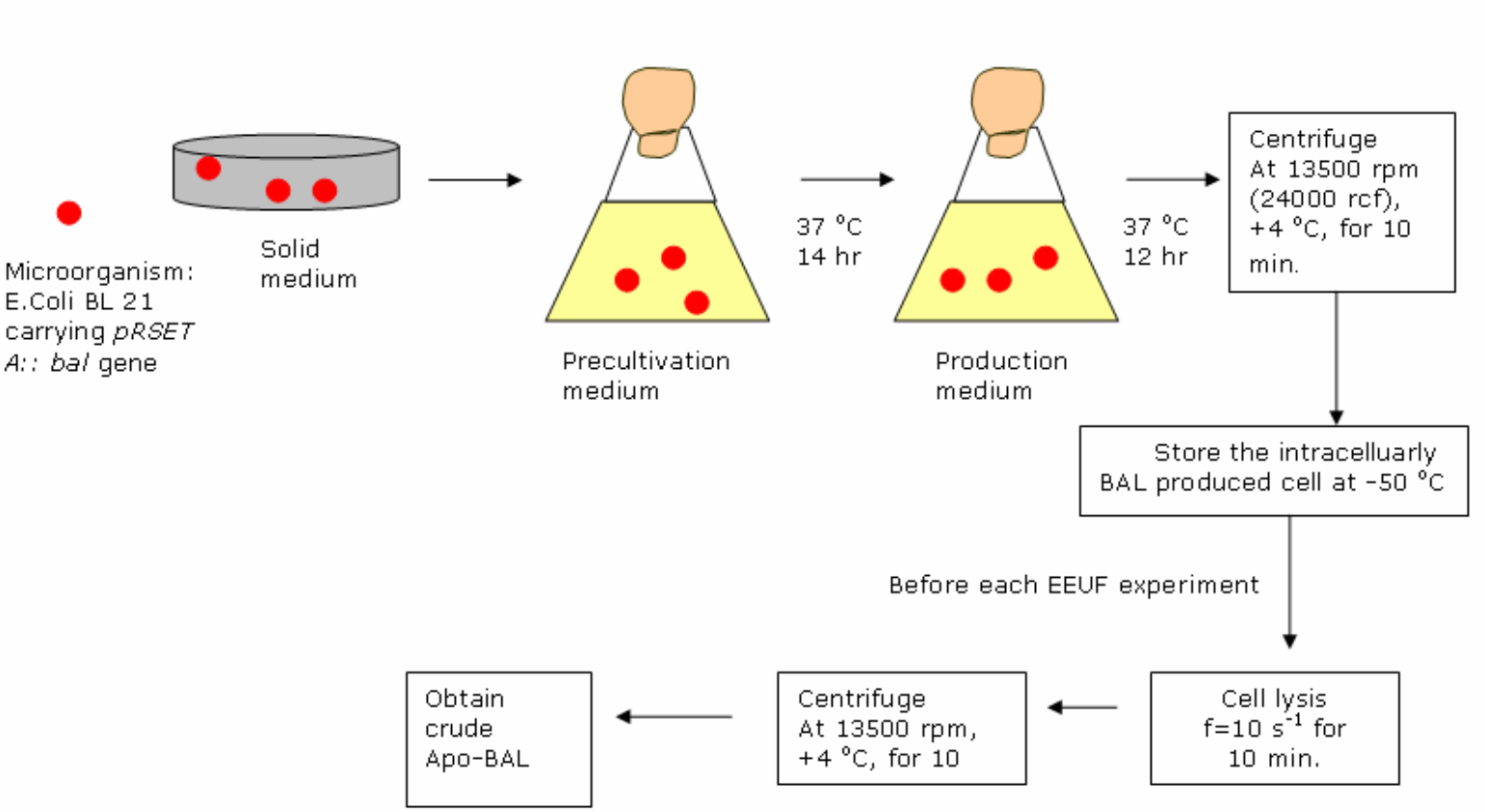


Figure 3.5 Benzaldehyde Lyase (BAL, E.C. 4.1.2.38) production

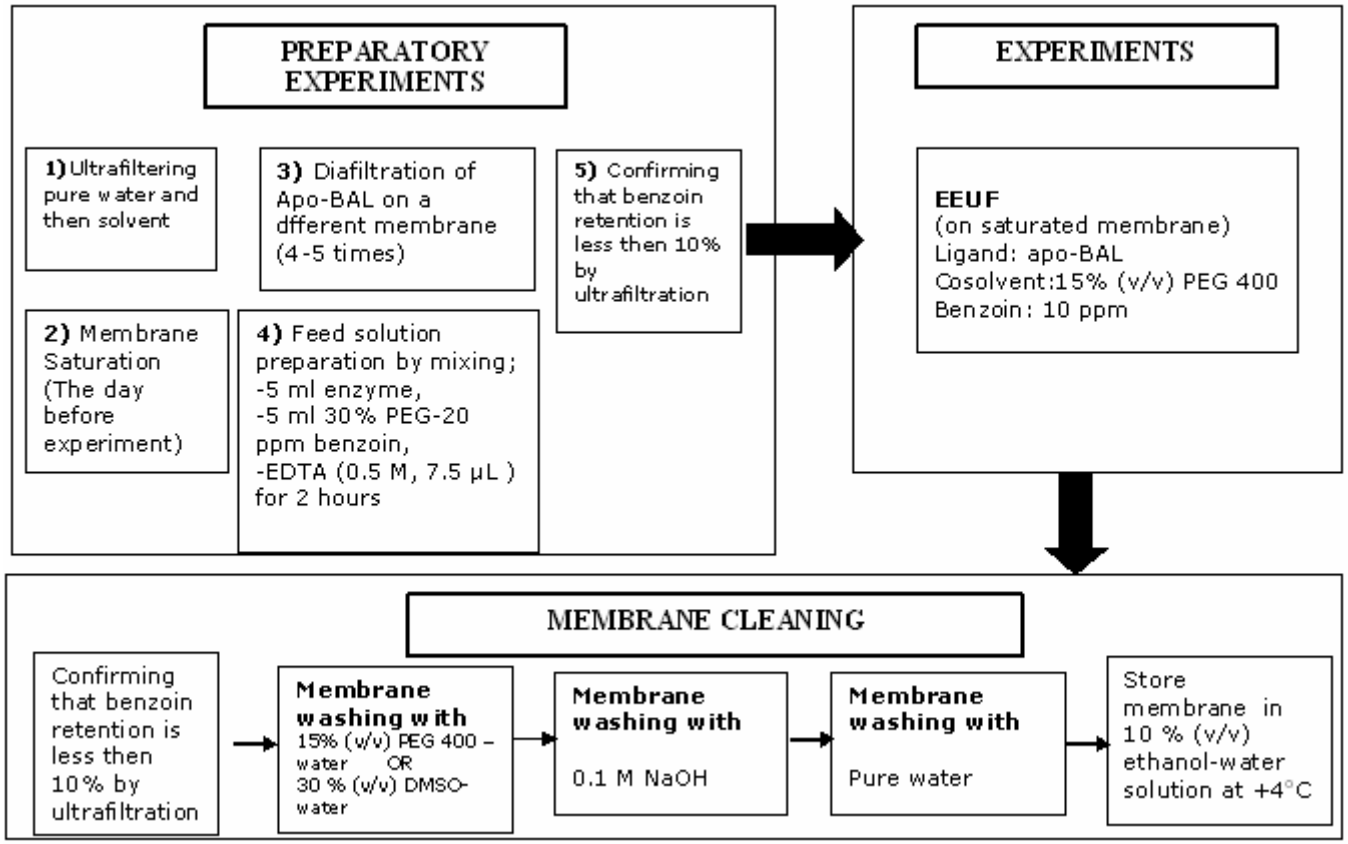


Figure 3.6 Experimental procedure for EEUF experiments

3.4 Analyses

3.4.1 Flux Measurements

By measuring the flow rate flux was calculated during experiments.

3.4.2 Total Benzoin Retention Analysis in PEUF

Total benzoin retention in PEUF experiments was determined by UV-Spectrophotometer (Shimadzu UV-1601 visible spectrophotometer) at 250 nm. Calibration curves of benzoin-15% (v/v) PEG 400 and benzoin- 30 % (v/v) DMSO were used in finding out the permeate concentration. Calibration curves are shown in Appendix A.

3.4.3 Determination of Enantiomeric Excess (ee %)

Enantiomeric excess was defined as; $ee\,\% = \frac{C_{predominant} - C_{minor}}{C_{predominant} + C_{minor}}$.

BAL has ability to catalyze the reaction with R- benzoin, therefore apo-BAL was expected to form a complex with only R- benzoin. In addition, in literature for PEUF studies with BSA, it was observed that BSA was able to bind R- enantiomers. Therefore, in this study S- and R- benzoin were expected to be predominant and minor components of permeate, respectively.

For ee% analysis, HPLC (Agilent 1200 series) was used. The analyses were performed under the conditions specified below:

- Column: Daicel OD-H Chiral Column
- Column dimensions: 4.6 mm x 250 mm
- Guard Cartridge dimensions: 4 mm x 10 mm
- System: Normal phase chromatography

- Mobile Phase: n-Hexane (90 % (v/v)) - 2-propanol (10% (v/v))
- Mobile phase flow rate: 1 ml/min
- Column temperature: 25 °C
- Detector and wavelength: UV/VIS, 254 nm
- Injection volume: 20 µl
- Analysis period: 20 min

It should be pointed out that in the literature [68], for the utilized OD Chiral column in racemic benzoin separation, the first peak observed in chromatogram was reported to correspond to S- benzoin. Therefore, in our study in the HPLC analyses performed with OD-H column, due to completely the same packing material with the OD column (Cellulose tris (3,5-dimethylphenylcarbamate) coated on a silica support) except only the particle size difference, the first peak in the HPLC chromatogram was also accepted to be the S- benzoin.

3.4.4 Total Benzoin Retention Analysis in EEUF

Due to the complex medium of enzyme solution, total benzoin retention could not be determined spectrophotometrically in EEUF experiments. Therefore retention was calculated by using the calibration curves obtained for R- and S- benzoin in HPLC which are shown in Appendix C. The calculation details are also shown in Appendix D.

3.4.5. Determination of Enzyme Concentration

Assuming that in the complex production medium, enzyme itself constitutes the biggest portion; protein concentration was determined by using Bradford Method [69]. Bovine serum albumin (BSA) was used as the standard protein. Composition of reagents, procedure and standard curve are given in Appendix F.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Preliminary Ultrafiltration Experiments

Prior to complexation enhanced ultrafiltration experiments, some preliminary experiments were performed in order to decide on operation conditions i.e, stirring rate and cosolvent ratio. Then retention and adsorption of ligand BSA was checked on regenerated cellulose RC-10000 Da membranes.

4.1.1 Effect of BSA Concentration on Flux

In these preliminary experiments, BSA was dissolved in pure water. No benzoin was used in these experiments. As seen in Figure 4.1, as the BSA concentration increased the flux decreased. Fluxes remained constant with respect to time at each BSA concentration. This indicated that probably there was no concentration polarization or gel formation. Namely, there was probably no accumulation of BSA on the RC-10000 Da membrane surface which would result in flux decrease with time. However, in order to be sure about the existence of concentration polarization effect of stirring rate or the pressure must also be investigated.

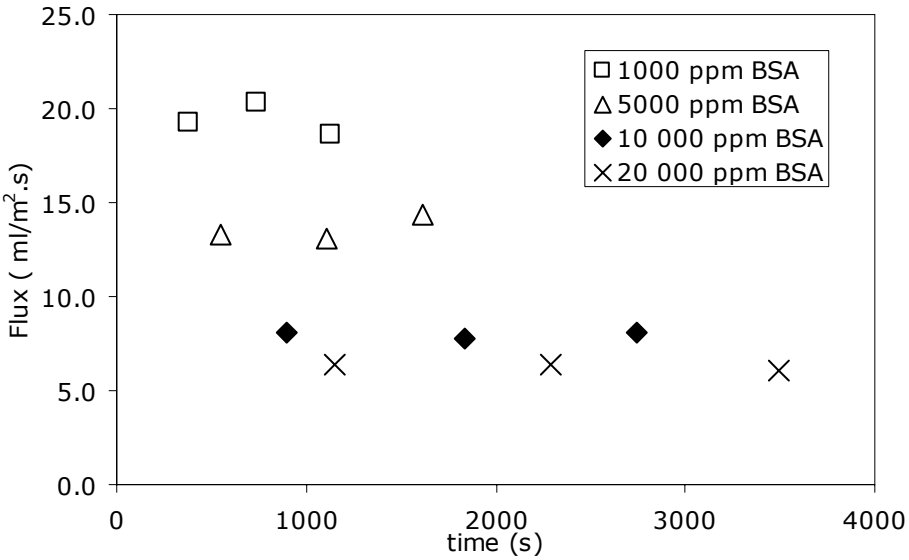


Figure 4.1 Effect of BSA concentration on flux (Membrane= RC- 10000 Da, P= 3 bars, pH ~ 7, N=200 rpm, V_{cell} = 10 ml, solvent= pure water)

4.1.2 Stirring Rate Effect on Flux

In the experiments performed to determine the stirring rate effect, 5000 ppm BSA was again dissolved in pure water. As can be seen in Figure 4.2 the increase in stirring rate did not affect the flux significantly. This case again confirmed that there were no concentration polarization and no gel formation on the membrane surface, since no flux increase was observed with increasing stirring rate.

On the other hand, at high stirring rates BSA solutions tend to foam. Therefore, since there was no concentration polarization and no gel formation of BSA, it was decided to use relatively low stirring rate of 200 rpm in future PEUF experiments.

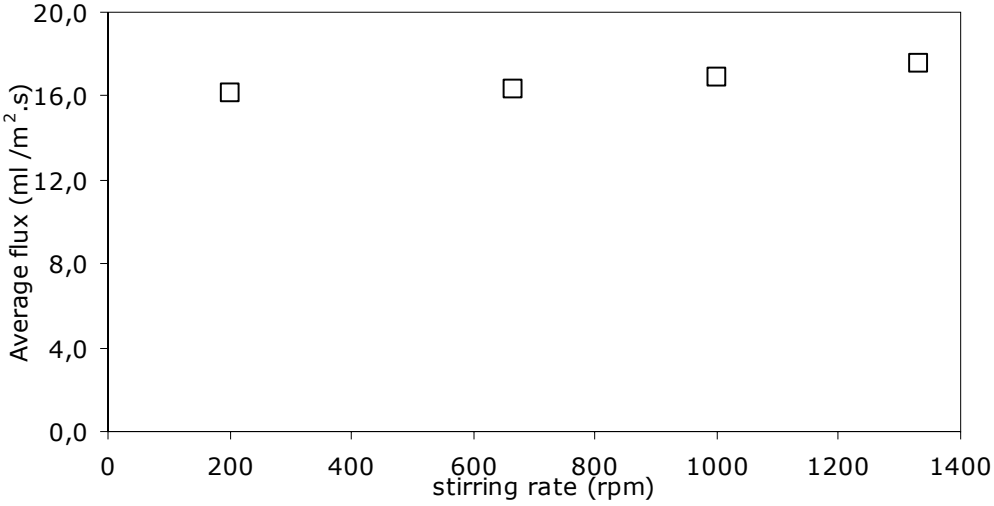


Figure 4.2 Effect of stirring rate on average flux values
(Membrane= RC- 10000 Da, $C_{BSA, f}$ = 5000 ppm, P= 3 bars, solvent= distilled water, V_{cell} = 10 ml)

4.1.3 Controlling the Retention and Adsorption of Ligand BSA

In the preliminary experiments BSA retention on RC- 10000 Da membrane was also investigated. As seen in Table 4.1 retention values of the BSA were greater than 99% for all BSA concentrations studied by dissolving it in pure water. This was expected since BSA has a molecular weight of 67 000 Da, which was about seven times greater than the MWCO of 10 000 Da regenerated cellulose membrane. These very high retention values also showed that there were no smaller contaminating proteins or no BSA fragments in feed, since BSA could be retained greater than 99% on RC-10000 Da membrane.

Table 4.1 BSA retention values at different concentrations (membrane= RC- 10000 Da, P = 3 bars, N = 200 rpm, solvent = pure water, V_{cell} = 10 ml)

$C_{BSA,f}$ (ppm)	BSA retention %
1000	99.9
5000	99.7
10000	99.6
20000	99.6

Then the possibility of adsorption of BSA on the RC-10000 Da membrane surface was checked. After filtering the 5 ml of the 10 ml dissolved BSA, the feed, retentate and permeate were analyzed spectrophotometrically at 278 nm. Calibration curves used for BSA adsorption control are given in Appendix G. In Table 4.2 the calculated BSA concentrations for feed, retentate and permeate are shown. About two fold increase in retentate concentration with respect to permeate was due to the two fold volume decrease. This means that there was almost no BSA adsorption on the membrane surface. It should be noted that, the observed ~10% deviation for the reported BSA concentrations in feed and retentate was probably be due to the experimental errors and uncertainities in spectrophotometric analysis, since it was too difficult to obtain the same absorbance value even in two consecutive readings in spectrophotometer.

Therefore, BSA was suitable to be utilized as ligand, due to its high retention values and no adsorption on RC-10000 Da membranes.

Table 4.2 BSA concentrations in feed, retentate and permeate during controlling the adsorption on membrane surface (membrane= RC-10000 Da, P = 3 bars, N = 200 rpm, V_{cell} = 10 ml, solvent= pure water)

Portion	Volume (ml)	BSA Concentration (ppm)
Feed	10	5075
Retentate	5	11180
Permeate	5	0.0

4.1.4 Selection of Cosolvent Ratio

Solubility of benzoin in pure water is very low. Therefore, cosolvents should be used for dissolution. Polyethylene glycol 400 (PEG 400) was tried as the cosolvent. In literature, it was observed that utilizing 15% (v/v) PEG 400 not only increased the low solubility of benzoin in aqueous buffer but also enhanced benzaldehyde formation [62]. On the basis of this study, two ratios were initially used in Amicon 8010 dead-end stirred membrane cell, one of which was 15% (v/v) PEG 400-water and the other was -to get a better solubility- 30% (v/v) PEG 400 –water solvents. 20 ppm benzoin was dissolved in PEG 400 cosolvent by stirring on a magnetic stirrer for 24 hours with a stirring rate of 700-1000 rpm. Benzoin solutions at concentrations greater than 20 ppm could not be obtained in either of these two solvents even after 24 hours. Heating might be taught to be an alternative for better solubility. However, due to the possibility of spontaneous conversion of benzoin into benzaldehyde occurring at 37^0C, the solution could not be heated during stirring.

Effect of PEG 400 content on the flux of preliminary experiments with BSA and on the solubility of benzoin is shown in Figure 4.3. As seen, with the

increase in PEG 400-water ratio; the flux in experiments with BSA decreased. At 30 % (v/v) PEG-water; a significant decrease in flux was observed.

In terms of solubility of benzoin, although slightly better benzoin solubility values were obtained with cosolvent 30 % (v/v) PEG 400; because of the considerable decrease in flux, and difficulties in spectrophotometric analysis due to the oscillations occurred, cosolvent 15% (v/v) PEG 400 was decided to be used.

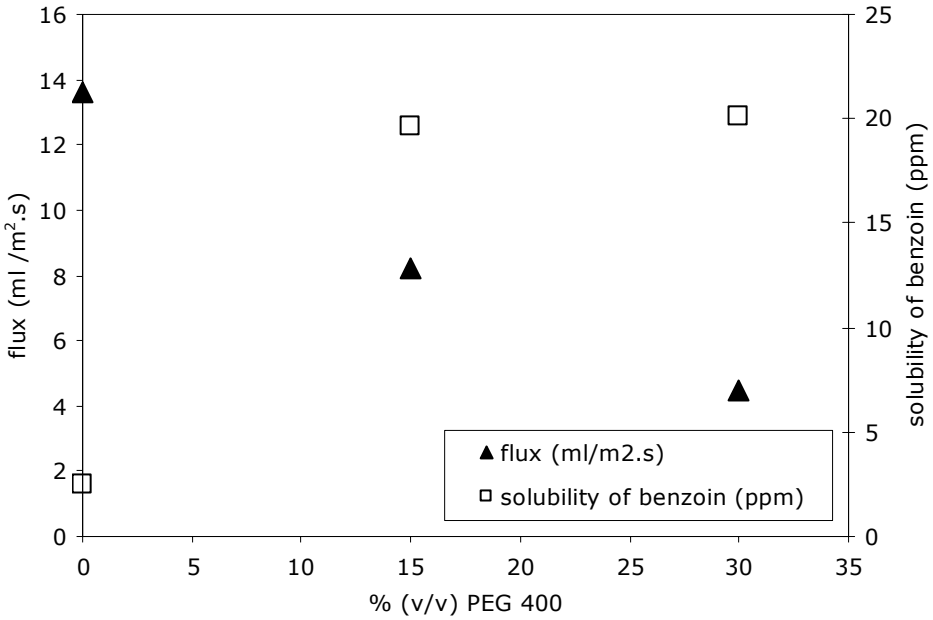


Figure 4.3 Effect of % (v/v) PEG 400 on flux in experiments with BSA (Membrane = RC 10000 Da, $C_{BSA,\ f}$ =5000 ppm, P=3 bars, N= 200 rpm, V_{cell} = 10 ml) and on solubility of benzoin (after 24 hours stirring at 700-1000 rpm)

In Table 4.3 in addition to flux values it was also observed that addition of cosolvent had no affect on BSA retention value which was still greater than 99 % similar to the case of solvent pure water. Then, in terms

of the utilization of the BSA as ligand selection of cosolvent 15 % (v/v) PEG 400 was also suitable.

Table 4.3 Fluxes and retention values of BSA dissolved in different cosolvent ratios (Membrane = RC-10000 Da, P=3 bars, N =200 rpm, V_{cell} =10 ml)

C_{BSA} (ppm)	Solvent	Flux ($ml/m^2.s$)	BSA retention %
5000	Pure water	13.6	99.7
5000	15 % (v/v) PEG –water	8.2	99.4
1000	30% (v/v) PEG –water	4.5	99.4

On the other hand, dimethyl sulfoxide (DMSO) was tried as the second cosolvent in order to increase the solubility. However for this cosolvent, not only the solubility of benzoin, but also membrane compatibility should also be considered. Since DMSO is an aprotic solvent, it is incompatible with a very wide range of materials. So, it is not suitable for every membrane material. It could only be used with regenerated cellulose (RC) membranes. Having Millipore-XFUF04701-Solvent Resistant stirred cell, provided the trial of 30% (v/v) DMSO as cosolvent different than 20 %(v/v) DMSO used in literature [62]. It was observed that, at this selected high DMSO content, in contrast to 30 % (v/v) PEG 400, there were no flux decreasing and oscillation problem. Therefore it was decided to use 30 % (v/v) DMSO as the second cosolvent; with which benzoin solubility was about five times higher than that with 15 % (v/v) PEG 400 cosolvent. Namely 100 ppm benzoin could be dissolved in 30% (v/v) DMSO - water solvent, by again stirring on a magnetic stirrer at a rate of 700-1000 rpm for 24 hours without heating, at room temperature.

4.1.5 Retention of Target Molecule Benzoin by Membrane Materials

Benzoin is a relatively small molecule with a molecular weight of 212.25 Da. Theoretically; it was expected to permeate through RC-10000 Da membranes. However, very high total benzoin retention values, even at very low ligand concentrations were obtained in initial PEUF experiments as seen in Table 4.4.

Table 4.4 Total benzoin retention values obtained in preliminary PEUF experiments (membrane= RC-10000 Da, P = 3 bars, N = 200 rpm, V_{cell} = 10 ml, cosolvent = 15% (v/v) PEG 400)

$C_{BSA,f}$ (ppm)	$C_{benzoin,\ f}$ (ppm)	pH_{feed}	Total benzoin retention %
1000	8.5	6.7	30.31
5000	9.0	7.0	34.77
10000	9.0	7.2	41.87
20000	8.5	7.4	48.05

These results leaded to suspicion about retention of benzoin by RC-10000 Da membranes. Although in PEUF studies, no adsorption of target molecule on membrane material has been reported in the literature, it was decided to check retention of benzoin on RC-10000 Da membranes.

For this aim 10 ppm benzoin- 15 % (v/v) PEG 400 solution was prepared as described. Then this feed solution without containing ligand BSA was ultrafiltered on RC-10000 Da membrane, in Amicon 8010 stirred cell. Total benzoin retention capacity of this RC-10000 Da membrane was found to be 33.5 % in the first trial. After some other reproducibility experiments with different RC-10000 Da membranes, obtained total benzoin retention values were all in the same order of magnitude (~30 %) as shown in Table 4.5. This significant amount of retention was close to the retention value

expected in the presence of the ligand. This result showed that without the ligand membrane material could retain the benzoin. In order to understand whether this retention provided chiral resolution or not, two samples were detected by HPLC to determine ee %. Low ee % values of 4.6% and 2.3 % for these two samples indicated that retention on the RC-10000 Da membranes did not provide a chiral resolution.

Table 4.5 Trials for determining total benzoin retention (membrane= RC-10000 Da, $C_{benzoin,f}$ = 10 ppm, $C_{BSA,\ f}$ = 0, P = 3 bars, N = 700 rpm, V_{cell} = 10 ml, cosolvent = 15% (v/v) PEG 400)

Trial #	Total Benzoin retention %	ee%
1	33.5	Not measured
2	34.5	Not measured
3	29.3	4.6
4	29.6	2.3

In order to overcome this problem, as an alternative, membranes made from different materials, cellulose triacetate (CTA)-10000 Da and polyethersulfone (PES)-10000 Da, were tried. Initially, some flux analyses were performed. PES membranes were known to have high flux values [70] and this trend was observed in this study also as seen in Figure 4.4 and in Figure 4.5. The flux of PES membranes was better than the other two; in terms of both pure water and 15% (v/v) PEG 400-water solvent fluxes.

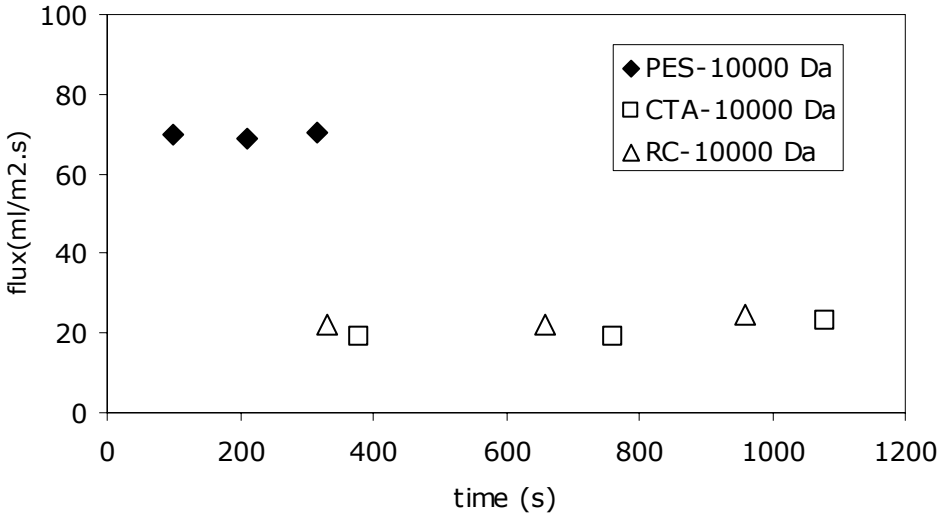


Figure 4.4 Pure water fluxes during ultrafiltration in three different membrane materials. (P= 3 bar, N= 700 rpm, V_{cell} = 10 ml)

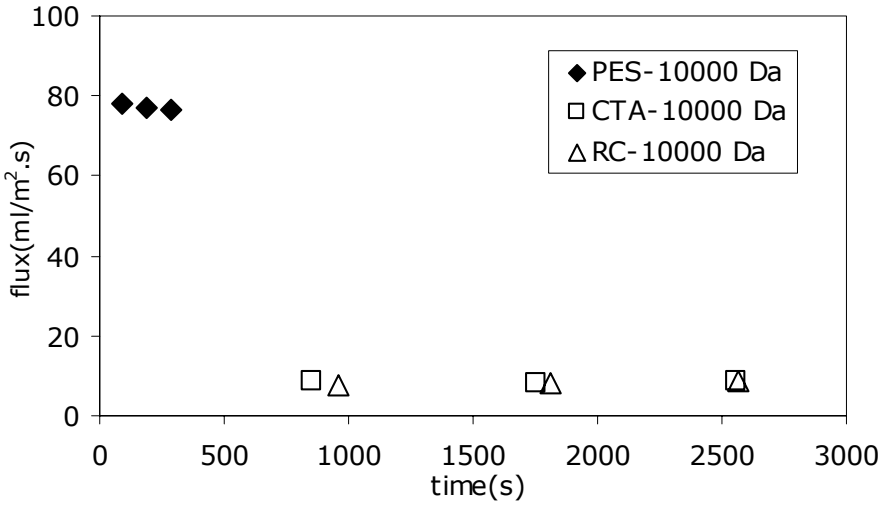


Figure 4.5 15% (v/v) PEG 400-water fluxes in three different membranes. (P= 3 bar, N = 700 rpm, V_{cell} = 10 ml)

These results showed that membrane material selection was important in terms of effective results. Although the MWCO of all of the membranes were reported by manufacturers as 10000 Da nominally, the differences in fluxes pointed out that, pore size distributions of these membranes might be different from each other. For PES membranes, pore size distribution seemed to be wider than the other two, yielding relatively high flux values in terms of both pure water and 15% (v/v) PEG 400 –water fluxes. In addition lower and closer flux values observed in CTA and RC membranes showed that narrower pore size distributions of these membranes were close to each other.

After then, pure water fluxes (PWFs) were compared before and after the ultrafiltration experiments performed with feed solution of 10 ppm benzoin dissolved in 15% (v/v) PEG 400 containing no ligand BSA. As seen in Table 4.6, decrease in PWF during membrane cleaning with respect to PWF in preparatory experiments was recorded to be around 67% and 39 % for CTA and PES membranes, respectively. Similarly, when decrease in 15% (v/v) PEG 400- water flux during membrane cleaning with respect to that in preparatory experiments were considered, 18 % and 32 % decreases were observed for the CTA and PES membranes, respectively. However, when RC membranes were considered, observed flux decrease was less than 5%.

Table 4.6 Comparison of flux decreases in three membrane materials. (P = 3 bars, N = 700 rpm, V_{cell} = 10 ml)

Membrane material	Decrease in PWF during membrane cleaning wrt PWF in preparatory exp. (%)	Decrease in 15% (v/v) PEG 400-water flux during membrane cleaning wrt. that in preparatory experiments
CTA-10000 Da	67	18
PES-10000 Da	39	32
RC-10000 Da	<5	<5

These high flux decreases in CTA-10000 Da and PES-10000 Da membranes might indicate the possibility of the existence of irreversible pore blocking or membrane compaction.

More importantly, when the total benzoin retentions were considered for these three membrane materials, again in ultrafiltration experiments performed with feed solution of 10 ppm benzoin dissolved in 15% (v/v) PEG 400-water containing no ligand BSA; it was observed that the retention capacity of RC membrane was lower than that of PES and CTA membranes as shown in Table 4.7. Similar to RC membranes, HPLC analysis again showed that the chiral separation could not be achieved either with PES or CTA membranes. Obtained ee% values were very close to 0% for these membranes. For these ee% values, deviations were so small that ee% values could still be accepted to be 0% in the margin of error.

Table 4.7 Comparison of three membrane materials in terms of their total benzoin retention and enantiomeric excess values ($C_{benzoin,f}$ = 10 ppm, $C_{BSA,f}$ = 0 ppm , P = 3 bars, N = 700 rpm, V_{cell} = 10 ml, cosolvent = 15% (v/v) PEG 400)

Membrane Material	Total benzoin retention %	ee%
CTA-10000 Da	61.2	0.15
PES-10000 Da	81.2	0.01
RC-10000 Da	33.5	4.6

It was concluded that, although molecular weight of benzoin was small, it was retained at a significant amount on all the three membrane types. Then, it was suspected if the membranes retain the benzoin molecules sorptively. To be sure about benzoin adsorption on membrane surface,

retentate portions of the experiments performed with 10 ppm benzoin dissolved in 15 % (v/v) PEG 400-water solvent without any ligand were also analyzed in spectrophotometer. The obtained benzoin concentrations in retentates are given in Table4.8 together with the expected benzoin concentrations if there had been no adsorption.

Table 4.8 Benzoin concentrations in the retentates for the three membrane materials (P = 3 bars, N = 700 rpm, Vcell = 10 ml) ($C_{,retentate}$ (expected) : Concentration of benzoin if there were no adsorption, $C_{,retentate}$ (actual) : Concentration of benzoin with adsorption)

Membrane Material	$C_{,feed}$ (ppm)	$V_{retentate}$ (ml)	$C_{,retentate}$ (ppm) (expected)	$C_{,retentate}$ (ppm) (actual)
RC-10000 Da	11.38	7	16.25	11.17
PES-10000 Da	11.47	7	16.39	11.29
CTA-10000Da	11.38	6	18.97	11.08

Actual benzoin concentrations were detected to be less than expected theoretical values in case of no adsorption. This case was an indication for the adsorption of benzoin on the membrane surfaces, yielding low benzoin concentrations in the retentate.

In addition, benzoin concentrations in 15% (v/v) PEG 400 – water washing solvent after the experiments were detected. The obtained values are shown in Table 4.9.

Table 4.9 Comparison of three membranes in terms of benzoin concentrations in 15% (v/v) PEG 400-water solvent during membrane cleaning (P = 3 bars, N = 700 rpm, V_{cell} = 10 ml)

Membrane	Benzoin concentration in 15% (v/v) PEG 400-water solvent after experiment(ppm)
RC-10000 Da	0.99
PES-10000 Da	3.54
CTA-10000Da	4.44

As seen in Table 4.9, benzoin at a certain concentration greater than zero in 15% (v/v) PEG 400-water washing solvent after experiment was detected spectrophotometrically, in all three membrane types. If there had been no adsorption, it would have been expected to observe no benzoin in 15% (v/v) PEG 400-water washing solvent streams. Therefore, it was concluded that the benzoin was sorptively retained on all three membrane materials. Moreover, observing benzoin in the washing solvent stream of 15% (v/v) PEG 400-water not only confirmed the adsorption but also showed that adsorption was reversible.

According to Table 4.8 and Table 4.9 it can be concluded that the highest adsorption was observed in CTA membrane, whereas the lowest was in RC membrane.

In addition, when Table 4.6 is revisited, the low flux decreases either in pure water fluxes or in 15 %(v/v) PEF 400-water solvents after experiments with respect to the fluxes before experiments on RC-10000 Da membranes showed that flux recovery in these membranes were sufficient. This might be considered as another indication that the adsorption on RC-10000 Da membranes was reversible. Namely, since the adsorption was reversible; by means of washing the membrane, adsorbed benzoin molecules

on the membrane surface could be removed and hence initial pure water fluxes could almost be recovered.

The adsorption phenomena observed in these membranes would probably be due to the inherent sorption capacity of the membrane materials. However, as seen in Table 4.7, according to ee % values for three membranes, it was pointed out that the adsorptive retention was nonspecific in all three membrane material types. This kind of retention was not favoured in this study since it was desired to have stereospecific selectivity. However this selectivity would be undesirably prevented by such a non specific adsorption and this case would counter with principles of PEUF systems.

Another reason for benzoin retention by the membranes might be slight dissolution of benzoin even in the existence of cosolvents. The solubility was so bad that, after at most three days the prepared benzoin solutions started to precipitate especially in 15% (v/v) PEG 400-water solvent. Moreover, although cosolvent was used, the solubility could reach just 20 ppm with 15% (v/v) PEG 400. Since it was a very small solubility value, during the ultrafiltration there might be a benzoin precipitation, which caused benzoin to be retained on the feed side as a result of precipitation on the membrane surface.

According to these results, due to the lowest retention capacity for benzoin, moderately less adsorption observed, and reversibility of this adsorption, RC-membrane was decided to be used for further experiments, by developing a membrane saturation strategy.

4.1.5.1 Development of Membrane Saturation Strategy

In this study it was desired to have stereospecific complex formation between the ligand and the target molecule. However, nonspecific sorptive retention of benzoin on RC-10000 Da membrane would completely prevent or would decrease this formation. Therefore, minimizing the retention capacity of RC-10000 Da membrane for benzoin could be an alternative for

better and reliable results. Accordingly, in order to be able to use the RC-membrane material a membrane saturation strategy was developed. The developed method can be summarized as follows:

Initially pure water and then 15% (v/v) PEG –water solution were filtered in the Amicon 8010 stirred cell. After these preliminary stages, the developed strategy was applied in order to saturate the RC-10000 Da membrane. This strategy consisted of several consecutive saturation sets in each of which 10 ppm benzoin dissolved in 15% (v/v) PEG –water solution was filtered through the RC- 10000 Da membrane.

For all saturation sets, the initial feed volume was 10 ml and at the end of the operation 1 ml retentate was left in the cell. 9 ml permeate stream was equally portioned into three tubes, during ultrafiltration process. Between any two consecutive experiment, 1 ml retentate was thrown away and the cell was filled up with fresh 10 ml feed solution. Total benzoin retention was calculated for each set by considering the absorbance value of the first 3 ml portion. The detailed calculations are shown in Appendix B.

These saturation experiments continued, until the total benzoin retention reduced to around 10 % and kept constant for at least two sets of saturation sets.

After obtaining about 10 % retention, the membrane was saturated with a one more set in which 20 ppm benzoin dissolved in 15% (v/v) PEG –water solution was filtered. This time instead of throwing away the 1 ml retentate with a benzoin concentration of about 200 ppm, it was left in the cell, covering the membrane at room temperature overnight, in order to have the membrane saturated as much as possible.

After applying this membrane saturation strategy; before performing all enhanced ultrafiltration experiments, solution of 10 ppm benzoin dissolved in 15% (v/v) PEG –water was filtered through the saturated membrane once more in order to control the final retention capacity of RC-10000 Da membrane. Almost in all trials this retention capacity was obtained to be less then 10% before the enhanced ultrafiltration experiment. This

10 % retention capacity can be tolerable since it was consistent with the study performed by Bowen et al [54]; in which the target molecule retention was reported to be not more than 10 %.

This membrane saturation procedure was applied both for PEUF experiments with ligand BSA and for EEUF experiments with ligand apo-BAL and cosolvent 15% (v/v) PEG 400.

Similarly, in PEUF experiments with cosolvent 30% (v/v) DMSO which were performed later on because of the poor ee% obtained with cosolvent 15% (v/v) PEG 400 the same saturation procedure was applied. According to data shown in Table 4.10, it is seen that RC-10000 Da membrane nonspecific sorptive retention was again observed with cosolvent 30% (v/v) DMSO.

Table 4.10 Total benzoin retention values in experiments with 30 % (v/v) DMSO. (Membrane = RC-10000 Da, $C_{benzoin,f}$ = 50 ppm, $C_{BSA,\ f}$ = 0 ppm, P = 3 bars, N = 700 rpm, V_{cell} = 50 ml, cosolvent = 30% (v/v) DMSO)

Trial #	Total Benzoin retention %
1	21.37
2	23.89
3	21.61
4	20.03

It should be noted that obtained retention values were this time about 20% which was less than the retention values with cosolvent 15 % (v/v) PEG 400. The reason for this trend could be due to the better dissolution of benzoin with cosolvent 30 % (v/v) DMSO. By the better dissolution of

benzoin with cosolvent 30 % (v/v) DMSO, membrane might allow more benzoin to permeate instead of retaining it adsorptively. From an other point of view it could be thought that, if there was a benzoin precipitation on the membrane material, it would be prevented by the better dissolution; yielding low retention values.

For these experiments, after filtration of first pure water and then 30% (v/v) DMSO –water solution in the solvent resistant stirred cell, 50 ppm benzoin-30% (v/v) DMSO solution was filtered. For all saturation sets with this cosolvent, initial feed volume was 50 ml. 40 ml permeate was filtered in consecutive four portions. Total benzoin retention calculation was based on the first 10 ml permeate sample. After some sets of saturation experiments the retention was reduced to around 10 %. In the final set 100 ppm benzoin-30% DMSO solution was filtered. In this last step, 5 ml retentate with an approximate concentration of 1000 ppm was left in the cell covering the RC-10000 Da membrane overnight at room temperature.

Similar to experiments with cosolvent 15% (v/v) PEG 400; total benzoin retention values were less than 10 % in almost all control trials in which solution of 50 ppm benzoin dissolved in 30% (v/v) DMSO –water was filtered through the saturated membrane once more in order to control the final retention capacity before the PEUF experiments. For experiments with cosolvent 30% (v/v) DMSO; the absorbance values were determined by dilution in order to fit the benzoin-30 % (v/v) DMSO calibration curve shown in Appendix A.

In Figure 4.6 the decrease in total benzoin retention during saturation stages with both cosolvents were shown as an example. It was notable that with cosolvent 30 % (v/v) DMSO the saturation was quicker in terms of the number of necessary sets to reach 10% retention. Namely, due to the initial total benzoin retention of about 23% with cosolvent 30 % (v/v) DMSO, number of necessary saturation sets to achieve 10 % retention decreased. It was noted that for the two cosolvents the same trend was observed for the reduction of total benzoin retention. There was just a decreasing shift in the trend observed for cosolvent 30 % (v/v) DMSO, reaching 10% total benzoin retention in only three sets. Therefore due to not only better solubility of

benzoin but also easier membrane saturation experiments, cosolvent 30 % (v/v) DMSO seemed to be more advantageous.

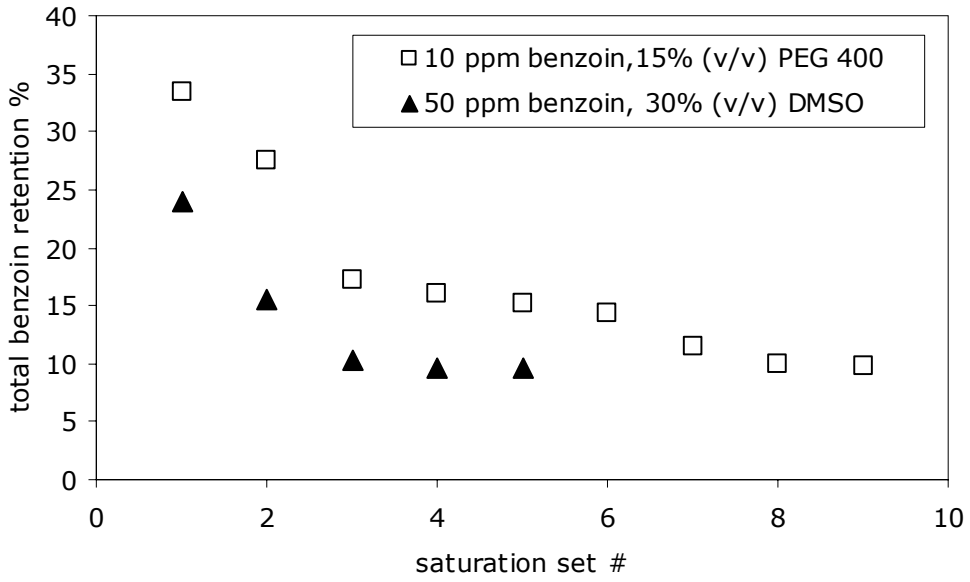


Figure 4.6 Decrease in total benzoin retention (Membrane = RC-10000 Da, P= 3 bars, N = 700 rpm, V_{cell}= 10 ml)

After developing membrane saturation strategy for RC-10000 Da membranes, PEUF experiments were performed first with 15% (v/v) PEG 400. Then, because of the low ee% values obtained with this cosolvent, 30% (v/v) DMSO was tried. Finally, EEUF experiments with ligand apo-BAL were performed with 15% (v/v) PEG 400.

In all enhanced ultrafiltration experiments, it was aimed not only to obtain high ee%, but also to obtain high total benzoin retention %. Because high ee % values would be meaningful and valuable from the economical point of view, if only they were together with high total benzoin retentions.

4.2 Polymer Enhanced Ultrafiltration (PEUF) Experiments with Ligand BSA

For PEUF experiments BSA was used as ligand. Benzoin concentration was kept constant at 10 ppm and 50 ppm for experiments with 15 % (v/v) PEG 400 and 30% (v/v) DMSO, respectively. The cells were stirred at a rate of 200 rpm and at a pressure of 3 bars.

4.2.1 Flux Analyses in PEUF Experiments

Effects of change in BSA concentration and pH on flux were examined during PEUF experiments.

4.2.1.1 Effect of BSA Concentration on Flux

In these experiments pH was not controlled but measured to be in the range of 7-7.5. Volumetric flow rate was measured continuously during the flow; then the flux was calculated. In Figure 4.7 and in Figure 4.8 the effect of BSA concentration on flux was shown. As expected when the BSA concentration increased in the feed solution, the flux decreased for experiments with both 15% (v/v) PEG 400 and 30 % (v/v) DMSO cosolvents at constant benzoin concentration of 10 ppm and 50 ppm, respectively. On the other hand, flux could be kept constant during the experiments which showed that there were no concentration polarization and fouling on the membrane.

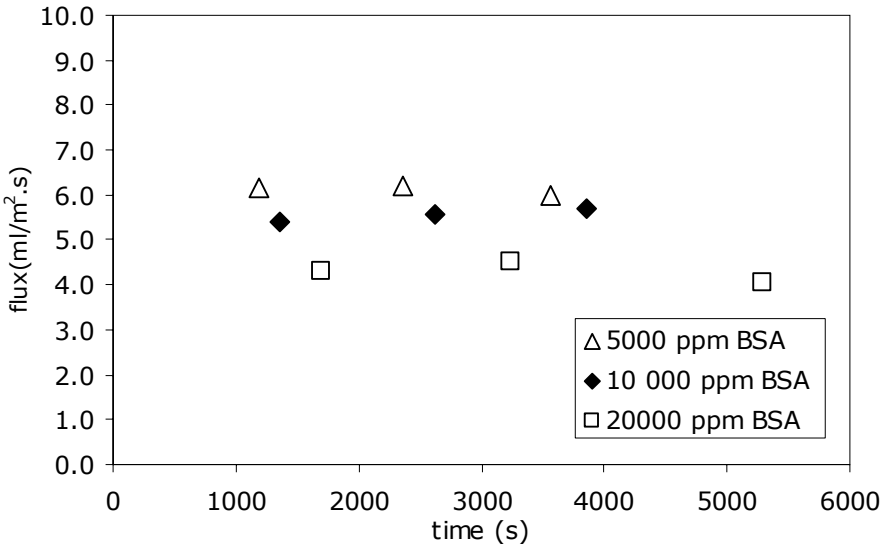


Figure 4.7 Effect of BSA concentration on flux in experiments with cosolvent 15% (v/v) PEG 400 (Membrane =RC- 10000 Da, $C_{benzoin,\ f}$ ~10 ppm, P = 3 bars, pH ~ 7, N= 200 rpm, V_{cell} = 10 ml)

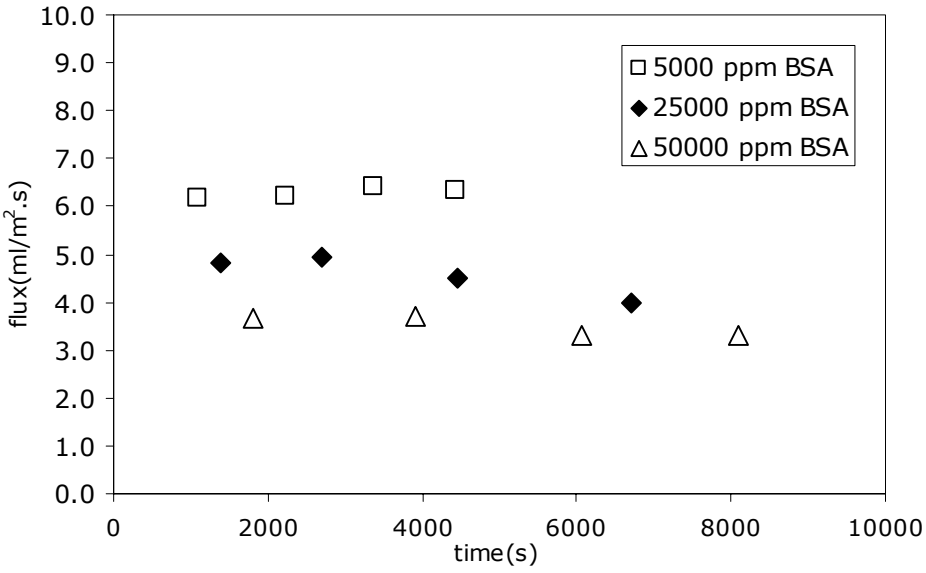


Figure 4.8 Effect of BSA concentration on flux in experiments with cosolvent 30% (v/v) DMSO (Membrane = RC-10000 Da, $C_{benzoin,\ f}$ ~50 ppm, P = 3 bars, pH ~ 7, N= 200 rpm, V_{cell} = 50 ml)

In addition in Figure 4.9 effect of cosolvent type on the average flux values can be investigated as follows: Benzoin was dissolved at a concentration of 10 ppm and 50 ppm in these solvents, respectively. These concentrations could be negligible when compared with the ligand BSA concentration. Then it was noted from Figure 4.9 that when the BSA concentrations were the same (5000 ppm) for the two cosolvents; due to the polymeric structure of PEG 400, although the utilized ratios were different, the flux was slightly better with 30 % (v/v) DMSO than in 15 % (v/v) PEG 400. On the other hand, at different BSA concentrations, in addition to BSA concentration, effect of cosolvent type on flux values could be observed more clearly. For example the flux with 25000 ppm BSA in 30 % (v/v) DMSO was greater than the flux with 20000 ppm BSA in 15 % (v/v) PEG 400. As seen although BSA was dissolved in 30 % (v/v) DMSO-water solvent at high concentrations, better fluxes could be obtained. Therefore, it can be concluded that both ligand concentration and cosolvent type influence permeate flux.

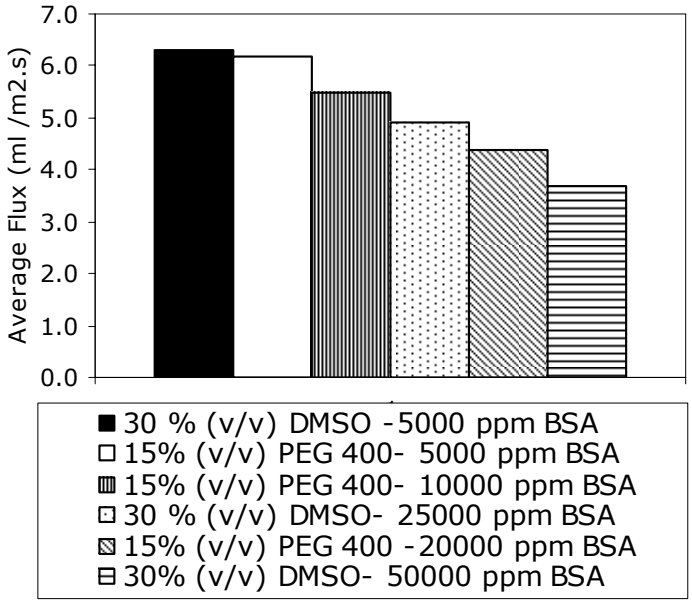


Figure 4.9 Comparison of average flux values with different BSA concentrations dissolved in two cosolvents. Membrane = RC-10000 Da, P = 3 bars, pH ~ 7, N= 200 rpm. For cosolvent 15% (v/v) PEG 400: $C_{benzoin,\ f}$ =10 ppm, V_{cell} = 10 ml; For cosolvent 30% (v/v) DMSO: $C_{benzoin,\ f}$ =50 ppm, V_{cell} = 50 ml)

4.2.1.2 Effect of pH on Flux

Effect of pH on flux was examined for the experiments in which BSA concentration was kept constant at 10000 ppm and 50000 ppm for 15% (v/v) PEG 400 and 30 % (v/v) DMSO cosolvents, respectively. When the pH effect on flux was examined, it was seen that for 15% (v/v) PEG 400 cosolvent there was a slight increase in flux as the pH increased, whereas for 30 % (v/v) DMSO cosolvent the flux almost remained constant as the pH changed. The obtained results are shown in Figure 4.10. The observed slight increase in flux with increasing pH might be due to the changes occurring in the molecular structure of BSA at high pH values.

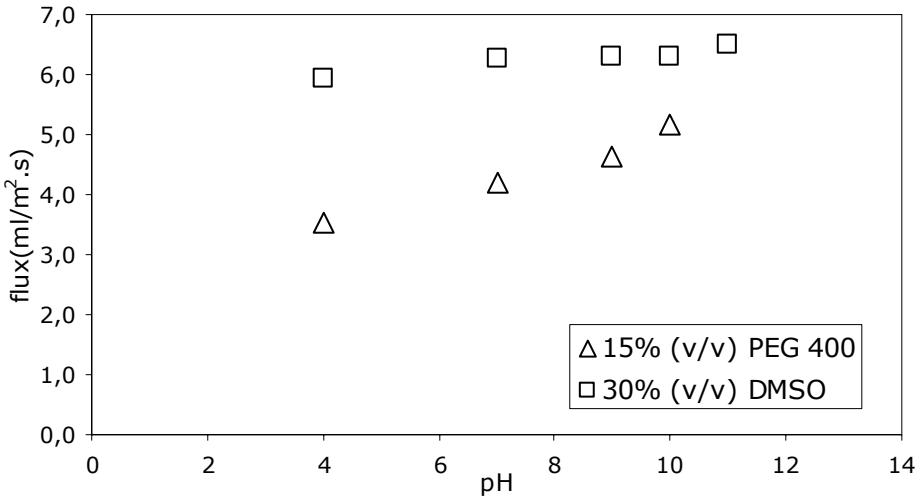


Figure 4.10 Effect of pH on flux (Membrane = RC-10000 Da, P = 3 bars, N= 200 rpm) (For cosolvent PEG 400; $C_{benzoin,f}$ =10 ppm, $C_{BSA,\ f}$ = 10000 ppm; For cosolvent DMSO ; $C_{benzoin,f}$ =50 ppm $C_{BSA,\ f}$ = 50000 ppm)

As seen in Figure 4.10, fluxes in 15 % (v/v) PEG 400-water solvent were again lower than that in 30 % (v/v) DMSO-water, due to the polymeric structure of solutions containing PEG 400, although their BSA contents were lower. This result is consistent with the ones discussed in Figure 4.9.

4.2.2 Determination of Total Benzoin Retention in PEUF Experiments

Prior to HPLC analysis, in order to determine the total benzoin retention percentage, spectophotometric measurements were performed at 250 nm. Effect of pH and ligand BSA concentration were the parameters investigated, on total benzoin retention.

4.2.2.1 Effect of pH on Total Benzoin Retention

Effect of pH was investigated again on total benzoin retention for the experiments with BSA concentration of 10000 ppm and 50000 ppm with 15% (v/v) PEG 400 and 30% (v/v) DMSO cosolvents, respectively. It was noted that the retention values were very close to each other in two types of the solvents used, as shown in Figure 4.11.

Effect of pH was not found to be very important on total benzoin retention up to pH 9, with constant retention value of about 20 %. However starting from pH 9 there was an increase. At pH 10 obtained total benzoin retention values were 48.7 % and 41.3 % for 15% (v/v) PEG 400 and 30% (v/v) DMSO cosolvents, respectively, which might be considered to be good retention values when compared to literature [54].

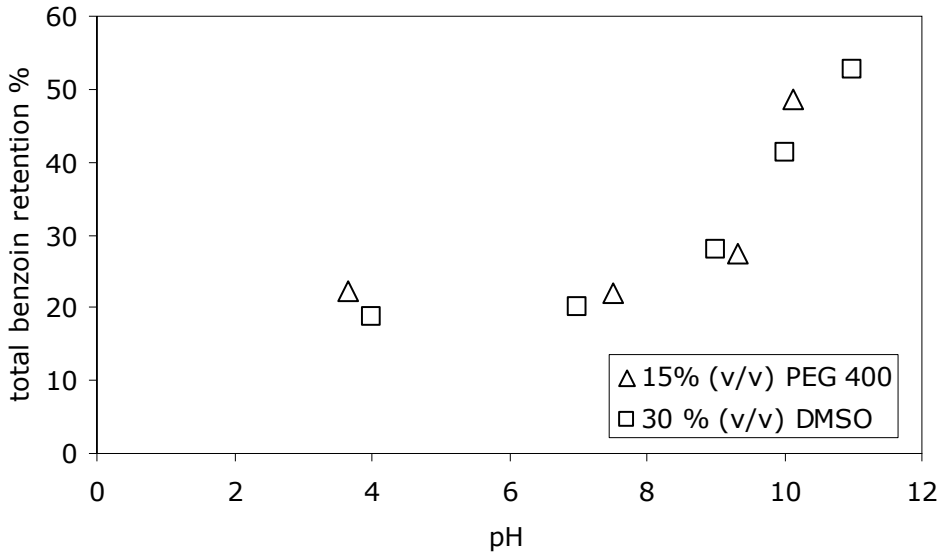


Figure 4.11 Effect of pH on total benzoin retention Membrane = RC-10000 Da, P = 3 bars, N= 200 rpm, for 15% (v/v) PEG 400; $C_{benzoin,f}$ =10 ppm, $C_{BSA,\ f}$ = 10000 ppm; for 30% (v/v) DMSO ; $C_{benzoin,f}$ =50 ppm, $C_{BSA,\ f}$ = 50000 ppm)

The increase in total benzoin retention with pH might be due to the some conformational changes which were not destroying the structure but having some effects on the binding sites of BSA at high pH values. At very high pH values, the hydrophobic tail structure in the center of binding sites of BSA molecule might move to outside, displacing with the hydrophilic head structures; which resulted in better benzoin binding to BSA at high pH values.

For 30 % (v/v) DMSO experiments pH 11 was also tried and it was observed that the increasing trend in total benzoin retention still continued. However to be on the safe side in terms of membrane life and benzoin structure pH 10 was selected in the experiments followed.

4.2.2.2 Effect of BSA Concentration on Total Benzoin Retention at pH 10

Effect of BSA concentration on total benzoin retention was studied at pH 10 due to better retention values obtained at this pH. As seen in Figure 4.12 and in Figure 4.13, as the BSA concentration increased, total benzoin retention also increased independent of the cosolvent type. In Figure 4.12 for the cosolvent 15% (v/v) PEG 400 retention values at different BSA concentrations are available for both pH ~7 and pH ~10. The results are consistent with the results shown in Figure 4.11. At each BSA concentration the total benzoin retention values at high pH (pH 10) were higher than that at low pH (pH 7). For cosolvent 30 % (v/v) DMSO effect of BSA concentration on total benzoin retention was investigated only at pH~10; yielding again high benzoin retention % values at high BSA concentrations.

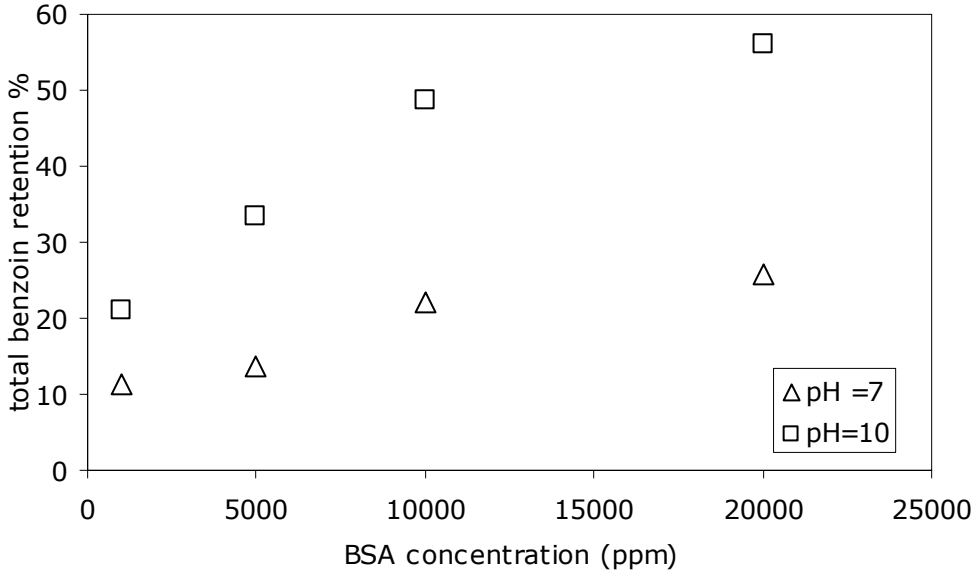


Figure 4.12 Effect of BSA concentration on total benzoin retention with cosolvent 15 % (v/v) PEG 400 (Membrane= RC-10000 Da, $C_{benzoin,f}$ =10 ppm, P= 3 bars, N = 200 rpm, V_{cell} = 10 ml)

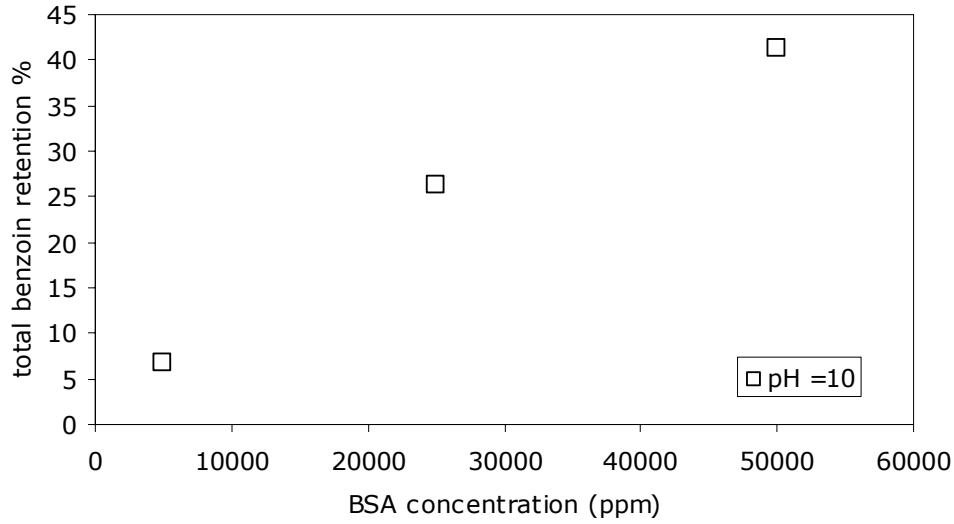


Figure 4.13 Effect of BSA concentration on total benzoin retention with cosolvent 30 % (v/v) DMSO (Membrane= RC-10000 Da, $C_{benzoin,f}$ =50 ppm, P= 3 bars, pH =10, N = 200 rpm, V_{cell} = 50 ml,)

From Figure 4.12 and Figure 4.13 it can be also noted that the increase in total benzoin retention with the increase in BSA concentration was linear up to a point, then the retention leveled off at high BSA concentrations either at pH ~7 or at pH ~10 with cosolvent 15 % (v/v) PEG 400.

On the other hand, for cosolvent 30 % (v/v) DMSO, nonlinearity in the increase of total benzoin retention was observed for all concentrations studied, due to the higher BSA concentrations.

So it might be concluded that, at high BSA concentrations due to crowded medium, the interactions between the BSA and benzoin might be prevented. Namely, in such a crowded medium it might not be easy for benzoin to reach BSA and bind it exactly. Hence, starting from 10000 ppm BSA concentration, the increase in total benzoin retention continued with a slight deviation from the linearity. Since the concentrations with 30 % (v/v) DMSO cosolvent were higher than that with 15 % (v/v) PEG 400 cosolvent,

the linear trend in the increase was not observed starting from the beginning.

4.2.3 Chiral Resolution of Benzoin in PEUF Experiments

Chiral resolution was based on enantiomeric excess %, which was defined as:

$$ee\,\% = \frac{C_{perm,\,predominant} - C_{perm,\,minor}}{C_{perm,\,predominant} + C_{perm,\,minor}} \times 100 \qquad (4.1)$$

In addition enantioselectivity was also evaluated.

$$\alpha = \frac{C_{perm,predominant}}{C_{perm,minor}} \qquad (4.2)$$

The analyses for determining the enantiomeric excess or enantioselectivity were performed by using HPLC.

In PEUF experiment with cosolvent 15 % (v/v) PEG 400 neither changing the concentration of BSA nor changing the feed solution pH was effective on chiral resolution of benzoin. Unfortunately, the chromatogram areas for both R- and S- benzoin were obtained to be very close to each other, yielding too low enantiomeric excess values. The results are shown in Table 4.11.

Table 4.11 Chiral resolution of benzoin in PEUF experiments at different BSA concentrations and pH values (membrane= RC-10000 Da, P = 3 bars, N = 200 rpm, V_{cell} = 10 ml, cosolvent = 15% (v/v) PEG 400)

$C_{BSA, f}$ (ppm)	$C_{Benzoin.f}$ (ppm)	Feed pH	Total benzoin retention %	ee %	α
5000	10.4	**~7**	13.8	7.5	1.2
10000	10.0	**~7**	22.1	7.8	1.2
20000	10.8	**~7**	25.7	0.7	1.0
5000	10.3	**10.2**	33.4	2.1	1.0
10000	10.3	**10.1**	48.7	6.7	1.1
20000	10.0	**10.2**	56.1	2.7	1.1
10000	10.0	3.7	22.3	2.2	1.0
10000	10.3	9.3	27.5	2.5	1.1
10000	10.3	10.1	48.7	6.7	1.1

As seen in Table 4.11 either keeping pH constant at pH 7 and pH 10 while changing BSA concentration or keeping the BSA concentration constant at 10000 ppm while changing the pH could not result in an increase of ee%. The obtained ee % values were all less than 10 %. Although there were some differences between ee % values, they could all be considered to be the same and very low. In addition, enantioselectivities (α's) with almost constant value at α ~1 confirmed that the enantiomeric separation in all trials were too low.

After obtaining these results, the stirring period parameter was investigated, whether it was effective on chiral resolution of benzoin. Namely, the feed which had been being stirred for 2 hours to obtain fully complexation was left for stirring overnight, for better complexation in these

trails. However, the result did not change. As seen from Table 4.12, obtained enantiomeric excess was still less than 10%.

Table 4.12 Enantiomeric excess obtained when the feed was long stirred (membrane= RC-10000 Da, P = 3 bars, N = 200 rpm, V_{cell} = 10 ml, cosolvent = 15% (v/v) PEG 400)

$C_{BSA, f}$ (ppm)	$C_{Benzoin.f}$ (ppm)	Feed pH	Total benzoin retention %	ee%
10000	10.3	7.2	19.35	2.42

From another point of view, it was thought that, due to the very low benzoin concentration of 10 ppm, it was really difficult to obtain distinct peaks in chromatograms. This revealed the need for dissolving benzoin at higher concentrations which could be achieved by utilizing 30% (v/v) DMSO as cosolvent.

As seen from Table 4.13 in experiments with 30% (v/v) DMSO; the concentration effect on enantiomeric excess was still not too much effective. Again, ee% values were less than 10 % (α values were around 1.0)

Table 4.13 Effect of BSA concentration on enantiomeric excess in PEUF experiments (membrane= RC-10000 Da, P = 3 bars, N = 200 rpm, V_{cell} = 50 ml, cosolvent = 30% (v/v) DMSO)

$C_{BSA, f}$ (ppm)	$C_{Benzoin.f}$ (ppm)	Feed pH	Total benzoin retention %	ee%	α
25000	48.7	7	13.60	7.01	1.2
50000	50.4	7	20.07	6.36	1.1

However when the results in Table 4.14 and Figure 4.14 were examined, it was noted that there was a small increase in ee% as the pH increased from 4 to 11.

Table 4.14 Effect of pH on enantiomeric excess in PEUF experiments (membrane= RC-10000 Da, P = 3 bars, N = 200 rpm, V_{cell} = 10 ml, cosolvent = 30% (v/v) DMSO)

$C_{BSA, f}$ (ppm)	$C_{Benzoin.f}$ (ppm)	Feed pH	Total benzoin retention %	ee%
50000	49.8	4	18.62	4.74
50000	50.4	7	20.07	6.36
50000	46.7	10	41.26	12.32
50000	45.1	11	52.61	16.74

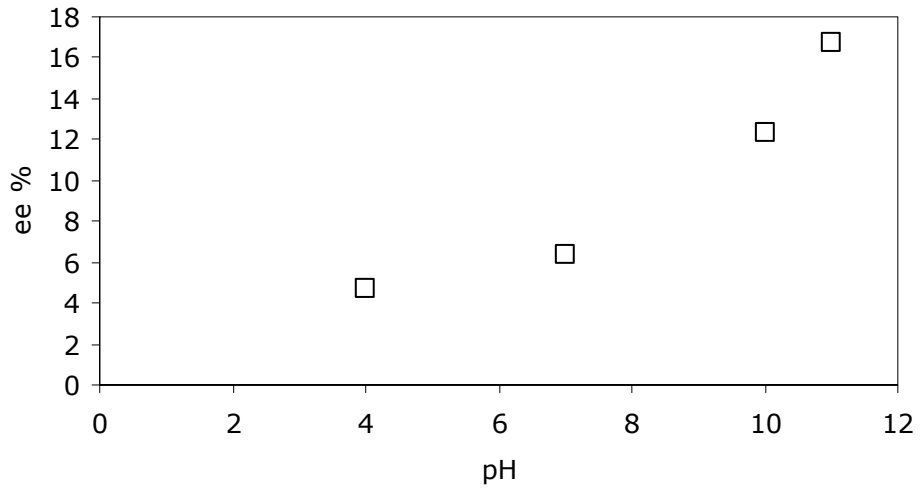


Figure 4.14 Effect of pH on ee %. (Membrane = RC- 10000 Da, $C_{benzoin, f}$ =50 ppm, $C_{BSA,f}$ = 50000 ppm, P= 3 bars, pH =10, N = 200 rpm, V_{cell} = 50 ml, cosolvent =30% (v/v) DMSO)

The obtained results for ee% at pH 10 and pH 11, were 12.3% and 16.7 %, respectively. They have the same order of magnitude when compared to literature [54]. In that study maximum enantioselectivity of 24 % for ibuprofen was obtained at pH 9.0-9.2 in a similar PEUF system with ligand BSA.

Even the resolution capacity was low for the studied molecule benzoin; increase in ee% was observed at high pH values. Although this increase was slight, the main reason for such an increase in enantiomeric excess values at high pH values might probably be the change in binding affinity of BSA with pH. This was in consistency with many studies in which pH effect was mentioned to be important for the chiral recognition ability of BSA [17, 18]. Although the affinity of BSA was different for each target molecule, the binding capacity against any molecule was changing depending on the pH of the system.

In addition, as mentioned before, probable conformational changes occurring at high pH values might not have destructive effect on BSA structure. Contrarily, these changes might give better results not only in the enhancement of total benzoin retention but also in the improvement of enantiomeric excess.

On the other hand, the different solvent effect was found to be important at this point. It was observed that solubility of benzoin in 15 % (v/v) PEG 400-water was less stable. Namely, after at most three days time some lumpy structures were observed, indicating most probably the ineffective dissolution. However, better dissolution of benzoin in 30 % (v/v) DMSO-water was observed. This might provide better stereoselective binding interactions between BSA and benzoin. As a result of this better stereoselectivity the increase in pH was effective in experiments with 30 % (v/v) DMSO cosolvent as seen in Figure 4.14. On the other hand, for 15 % (v/v) PEG 400 experiments insufficient dissolution of benzoin might prevent the effect of pH change on stereoselective binding interactions, resulting in no increase in ee%.

When the effect of BSA concentration on ee % at pH 10, with cosolvent 30% (v/v) DMSO experiments was examined, it was observed that, although ee% values were all less than or about 10%, there was almost a linear relationship between ee% and BSA concentration. As the ligand concentration increased, ee% values increased. The obtained results are shown in Figure 4.15.

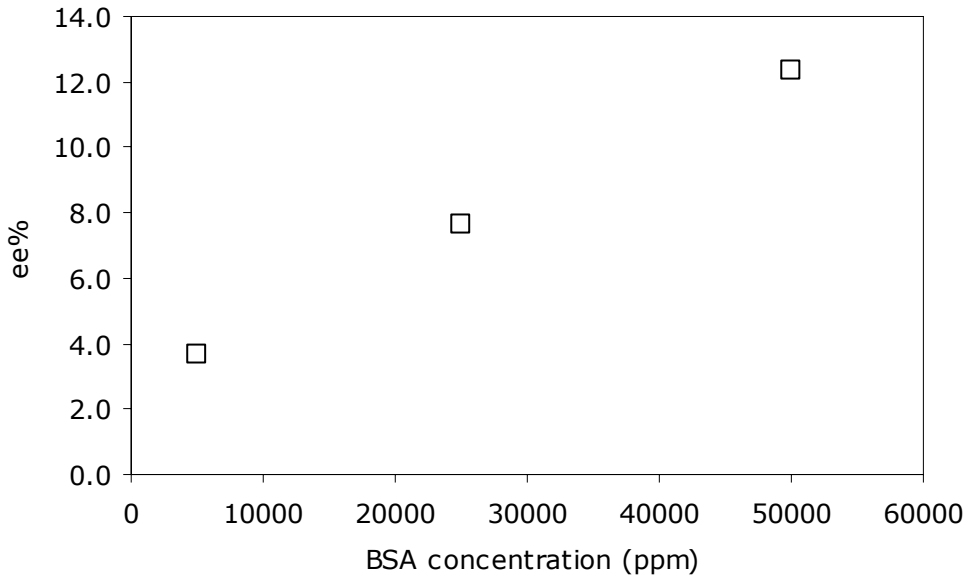


Figure 4.15 Effect of BSA concentration on ee% (Membrane = RC-10000 Da, $C_{benzoin,\ f}$ =50 ppm, P= 3 bars, pH =10, N = 200 rpm, Vcell = 50 ml, cosolvent =30% (v/v) DMSO)

For PEUF experiments, it can be concluded that, all the ee% values were about or less than 10% with either 15 % (v/v) PEG 400 or with 30 % (v/v) DMSO. In literature, Haginaka et al [61], studied chromatographic separation of benzoin in BSA bonded columns. BSA-FG75 was the BSA fragment which was isolated by size exclusion chromatography followed by peptic digestion of BSA. The selectivities were found as 1.51 and 2.19 for BSA and BSA-FG75 bonded columns, respectively. Namely, BSA FG-75

column provided about ~1.5 fold greater separation. It was concluded that higher capacity of BSA FG-75 column for benzoin was due to the increase in number of recognition sites for benzoin on the BSA-FG75 column compared with BSA column. Similarly, Erlandsson et al.[71] also showed BSA fragments were effective on benzoin resolution rather than BSA.

Then, in the light of this study, the results obtained in this work should not be so surprising. In addition, it should be noted that, BSA is an effective protein in binding especially the fatty acids, amino acids and some other pharmaceutical drugs due to its structure. Namely, hydrophobic residues, such as alkyl and aromatic groups, ionic groups, such as $-NH_3^+$ and $-COO^-$, hydrophilic polar groups such as –OH and $-NH_2$ etc. that BSA has, constitute the binding sites of the protein for the molecules with similar structures [72].

On the other hand, benzoin is an organic compound, an alpha hydroxyl ketone, consisting of an ethylene bridge flanked by phenyl groups and with a hydroxyl and a ketone functional group [73], which do not present high similarity with BSA . Therefore, the poor affinity of BSA for this molecule might be expected.

In the light of this consistency between the results of PEUF experiments with ligand BSA and the literature; it can be concluded that these results had many contributions in this study in terms of developing the methodology and setting the parameters before EEUF experiments. First of all since BSA is a widely available protein with low price and a well established structure, such an economical and widely available protein was advantageous while developing the methodology. In determining the cosolvent type and ratio the data obtained from the experiments with BSA were effective. Then, by means of PEUF experiments with ligand BSA, membrane saturation procedure was found to be very critical which had never been mentioned in any study yet. After setting these parameters fixed, enzyme enhanced ultrafiltration experiments with ligand BAL were performed.

4.3 Enzyme Enhanced Ultrafiltration (EEUF) Experiments with Ligand BAL

These experiments were performed with only cosolvent 15% (v/v) PEG 400 on RC-10000 Da membranes. Benzoin concentration was around 10 ppm. Since foaming was not a critical problem for BAL, stirring rate of the 10 ml volume stirred cell was 700 rpm at a pressure of 3 bars.

4.3.1 Determination of Total Benzoin Retention in EEUF Experiments

Different than PEUF experiments, due to the complex enzyme medium the total benzoin retention could not be determined by UV-spectrophotometer in EEUF experiments. Therefore, it was determined by "benzoin concentration –chromotogram area" calibration curves which were plotted by using the standard benzoin samples at different concentrations as shown in Appendix C. The related calculations were also available in Appendix D.

4.3.1.1 Effect of BAL concentration on Total Benzoin Retention

In EEUF experiments, the effect of ligand apo-BAL concentration was investigated. Obtained results in terms of total benzoin retention are shown in Table 4.15 and in Figure 4.16

Table 4.15 Total benzoin retention values in EEUF experiments (membrane= RC-10000 Da, $C_{benzoin,f}$ = 10 ppm, P = 3 bars, N = 700 rpm, V_{cell} = 10 ml, cosolvent = 15% (v/v) PEG 400)

$C_{BAL,f}$ (ppm)	$C_{Benzoin.f}$ (ppm)	Feed pH	Total benzoin retention %
649.5	11.49	~7.3	73.25
1129.2	10.96	~7.3	75.33
1935.8	11.63	~7.2	74.13

It was observed that for the studied BAL concentrations, the total benzoin retention was almost kept constant around 75%. Then, it was concluded that these concentrations were all at the highest level to enantiomerically bind ~10 ppm benzoin. Namely, even at the lowest BAL concentration studied (649.5 ppm), the total benzoin retention was about 75%, and it would not increase further with the increase in BAL concentration. This showed that for ~10 ppm benzoin, 649.5 ppm BAL was enough to achieve the highest retention capacity.

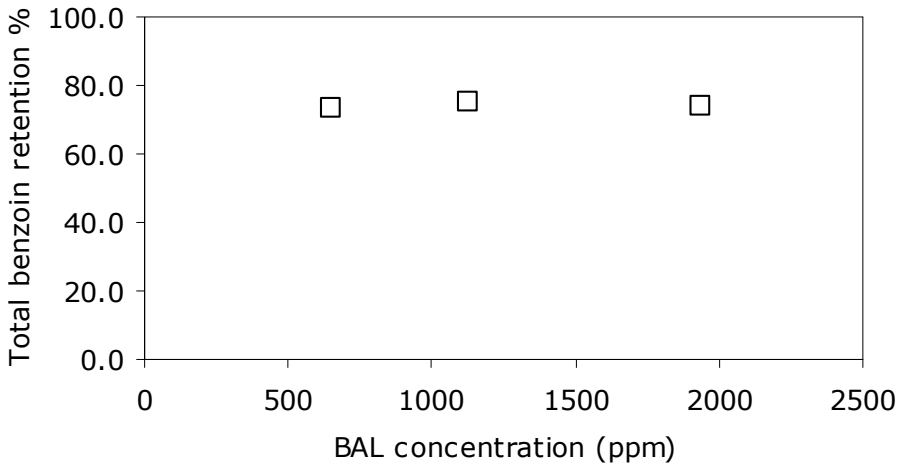


Figure 4.16 Effect of BAL concentration on total benzoin retention (Membrane = RC- 10000 Da, $C_{benzoin,\ f}$ =10 ppm, P= 3 bars, pH ~7.3, N = 700 rpm, Vcell =10 ml, cosolvent =15 % (v/v) PEG 400)

4.3.2 Chiral Resolution of Benzoin in EEUF Experiments

First, EEUF experiments, in which the effect of EDTA concentration was examined, were performed. The reason for using chelating agent EDTA was to bind Mg^{+2} ions, which are effective in activity of BAL in addition to Thiamin Diphosphate (ThDP), if some left from the production periods. It was

aimed to investigate whether the utilized amount was effective on chiral resolution or not . It was found that, as seen in Table 4.16 not only the enzyme concentration but also the EDTA concentration was effective on enantiomeric excess (ee %) values. The utilized EDTA concentrations were 1842.7 ppm and 139.5 ppm for two sets of experiments. Accordingly, the ee% values obtained were 28.2 % and 65.2 % for 535 ppm and 790 ppm apo- BAL, respectively. It might be thought that at the excess EDTA concentration, which was much greater than the enzyme concentration, EDTA was so concentrated for the system that although up to a certain concentration level it might be effective in complexing with the Mg^{+2} ions; it might affect and fill in the binding sites of the apo-BAL or it might have some inherent effects disturbing the enzyme structure at these very high concentrations. Then, in the light of these two experiments, EDTA at a concentration of 139.5 ppm was decided to be used, for future experiments.

Table 4.16 Effect of BAL and EDTA concentrations on ee% (membrane= RC-10000 Da, $C_{benzoin,f}$ = 10 ppm, P = 3 bars, N = 700 rpm, V_{cell} = 10 ml, cosolvent = 15% (v/v) PEG 400)

$C_{BAL,f}$ (ppm)	$C_{Benzoin,f}$ (ppm)	C_{EDTA} (ppm)	Feed pH	ee%
535	10.5	1842.7	7.1	28.2
790	10.9	139.5	7.2	65.2

After performing some more sets of EEUF experiments, it was concluded that similar to total benzoin retention, for enantiomeric excess values also, the same behavior continued as seen in Table 4.17 and in Figure 4.17. Namely, for the studied BAL concentrations; the ee% and

enantioselectivities were almost constant at around 60% and 4, respectively. This same behavior confirmed the idea that the studied concentrations are at the highest level for binding ~10 ppm benzoin enantiomerically. Hence, increase in BAL concentration even three fold did not increase the ee% further.

Table 4.17 Effect of BAL concentration on ee% (membrane= RC-10000 Da, $C_{benzoin,f}$ = 10 ppm, P = 3 bars, N = 700 rpm, V_{cell} = 10 ml, cosolvent = 15% (v/v) PEG 400)

$C_{BAL,f}$ (ppm)	$C_{Benzoin,f}$ (ppm)	Feed pH	Total benzoin retention %	ee%	α
649.5	11.49	7.3	73.25	60.05	4.0
1129.2	10.96	7.3	75.33	56.53	3.6
1935.8	11.63	7.2	74.13	64.01	4.6

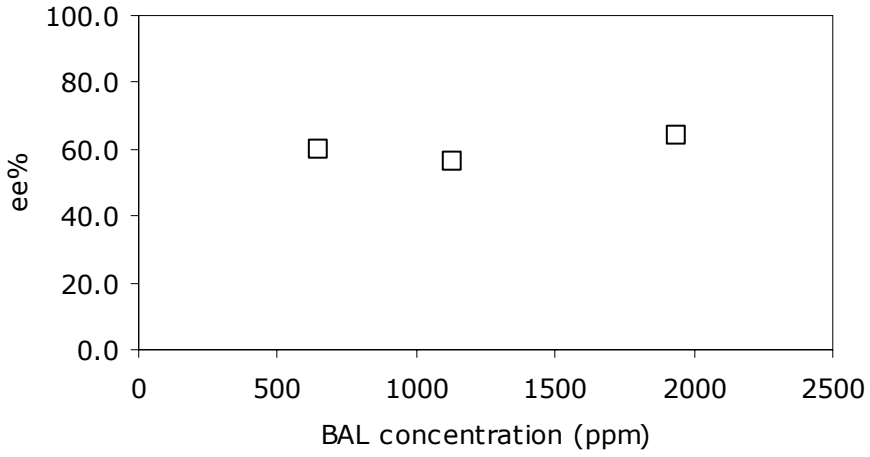


Figure 4.17 Effect of BAL concentration on ee % (Membrane = RC- 10000 Da, $C_{benzoin,\ f}$ =10 ppm, P= 3 bars, pH =7.3, N = 700 rpm, V_{cell} =10 ml, cosolvent =15 % (v/v) PEG 400)

Another possibility for almost constant total benzoin retention and ee % at the tried BAL concentrations might be explained as follows: Due to the very high enzyme concentrations, the complexation medium was so crowded that the binding sites of the enzyme were not easily available by the benzoin molecules. Since benzoin could not reach these sites easily, the complexation had to be limited even at the smallest BAL concentration studied.

On the other hand, it can be thought that, if the BAL concentration had increased starting from very small (~0 ppm) BAL concentration values, there would probably be an increase in either total benzoin retention or in ee% until a certain BAL concentration, after which they kept constant. In this study, even the studied minimum BAL concentration (649.5 ppm), was one of those highest level concentrations for ~10 ppm benzoin, therefore increase in BAL concentration did not increase either total benzoin retention % or ee%.

CHAPTER 5

CONCLUSION

In this study, by applying enhanced ultrafiltration systems, a methodology was developed for the separation of chiral molecules. Benzoin was selected as the model chiral molecule. First, BSA was used as ligand in polymer enhanced ultrafiltration (PEUF) experiments. Secondly, by combining the principles of classical PEUF and enzyme immobilized membrane systems, a new method was applied and it was named as enzyme enhanced ultrafiltration (EEUF). Apo form of Benzaldehyde Lyase (BAL, E.C. 4.1.2.38) was utilized as the ligand in these experiments. In this context, the following conclusions were drawn:

1. In the scope of developing a methodology, preliminary experiments were performed dissolving BSA in pure water. From these experiments, stirring rate to be utilized and behavior in flux values on RC- 10000 Da membrane were obtained.

2. For an enhanced ultrafiltration system, the solubility of the target molecule was a very important factor. Due to the low solubility of benzoin molecule in pure water, cosolvents had to be employed in order to obtain sufficient solubility. Polyethylene glycol 400 (PEG 400) was the first cosolvent tried in the preliminary experiments. After some trials 15 % (v/v) PEG 400 was selected to be the suitable ratio to be utilized in the experiments at which maximum 20 ppm benzoin could be dissolved.

Secondly, 30% (v/v) DMSO was utilized as the alternative cosolvent With this cosolvent, the solubility was relatively better, with a value of 100 ppm benzoin.

3. Regenerated Cellulose (RC) membrane material has the capacity to retain the benzoin about 30 %. In addition, it was also found out that the retention of benzoin on RC-membrane was sorptive type. After trying Polyether sulfone (PES) and Cellulose triacetate (CTA) membranes, the sorptive retention was again observed. After comparing the retention capacities of these membranes, it was decided to continue with RC -10000 Da membranes by developing a membrane saturation strategy, in which the retention capacity of the membrane for benzoin molecule was reduced to about 10 %.

   It was pointed out that sorption of the target molecule on the membranes was also too critical in deciding the membrane material. The one which had the lowest sorption capacity against the target molecule should be selected.

4. BSA retention was greater than 99% in RC membranes with MWCO of 10000 Da. Then, these membranes were assumed to have complete retention for BSA.

5. After completing the preliminary experiments, by having the many parameters set, PEUF experiments were performed. In these experiments, by keeping the benzoin concentration constant at ~10 ppm and ~50 ppm for experiments with 15 % (v/v) PEG 400 and 30% (v/v) DMSO cosolvents, respectively; effect of ligand concentration and pH on the total benzoin retention and on enantiomeric excess (ee%) were examined.

6. As the ligand BSA concentration increased, the total benzoin retention also increased.

7. With cosolvent 15% (v/v) PEG 400, all ee% values were less than 10 %, independent of change in pH and ligand BSA concentration.

8. When the cosolvent was turned to be 30 % (v/v) DMSO; still the change in BSA concentration was not effective on ee%. However, when the medium pH was changed, slightly better ee % results were obtained.

9. For EEUF experiments ~10 ppm benzoin was dissolved in only 15% (v/v) PEG 400-water solvent. Effect of BAL concentration on total benzoin retention and on ee% was examined. It was concluded that the studied BAL concentrations were all at the highest level to enantiomerically bind the ~10 ppm benzoin. Even the studied lowest BAL concentration was enough to reach the maximum binding capacity for ~10 ppm benzoin.

10. At the end of this study, due to the high total benzoin retention and ee % values obtained in EEUF experiments, it can be concluded that for the separation of racemic molecules a suitable and acceptable method has been developed.

CHAPTER 6

RECOMMENDATIONS

For the application of enhanced ultrafiltration system efficiently in chiral separations, the following suggestions should be considered:

1. First of all, the interactions between the target molecule and the membrane material should be checked. If possible, the model chiral molecule or membrane material should be selected so that, there is no or very few interaction between the membrane material and the target chiral molecule.

2. The utilized target molecule to be enantiomerically separated should be easily soluble. In this case, it would be possible to increase chiral molecule concentration.

3. For the ligand BSA, it would be better if the target molecule had acidic structure; since BSA is capable of binding molecules with acidic structure, enantiomerically. In addition, using such a molecule would make easier to observe the effect of pH better on both total retention and ee %, depending on the changes in binding capacity of BSA.

4. In terms of EEUF experiments, the target molecule should be selected so that the apo enzyme specific for this molecule would be more stable. If it were, it would be better in terms of being able to use it for complexation with benzoin even several hours after the diafiltration process. A good stability might also provide

the chance to store and reuse the enzyme when needed. Only in case of a good stability the application of EEUF could be cosidered at industrial level.

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APPENDIX A

CALIBRATION CURVES FOR SPECTROPHOTOMETRIC TOTAL BENZOIN RETENTION ANALYSIS

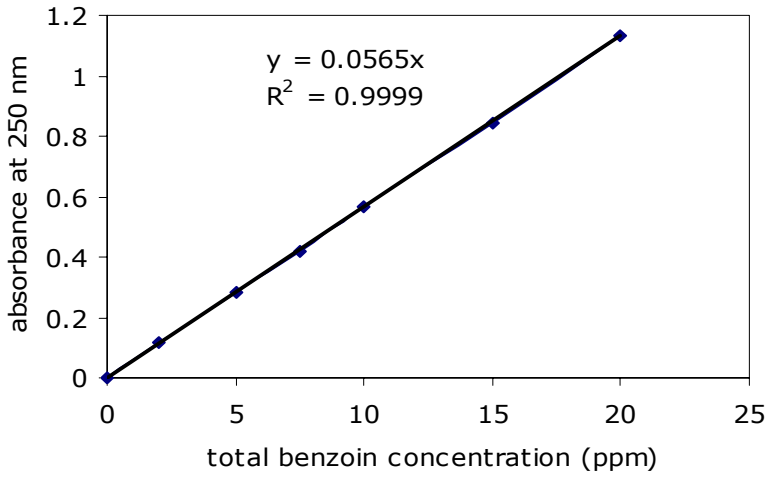


Figure A-1 Calibration curve for benzoin-15% (v/v) PEG 400-water solvent.

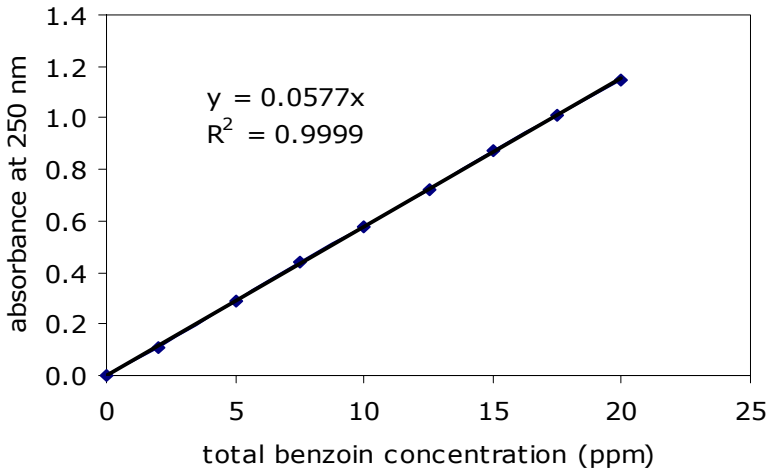


Figure A-2 Calibration curve for benzoin-30 % (v/v) DMSO-water solvent

APPENDIX B

TOTAL BENZOIN RETENTION PERCENTAGE CALCULATION FOR SPECTROPHOTOMETRIC ANALYSIS

This calculation was performed by considering the first 3 ml of the each 9 ml permeate stream (0-3 ml portion) for each experiment. The sample calculation is shown below, for the experiment performed at pH~10, with 10000 ppm BSA, 10 ppm benzoin in 15% (v/v) PEG 400-water solvent.

Table B-1 Raw experimental data. (Membrane= RC- 10000 Da, P = 3bars, N= 200 rpm, pH=10, V_{cell} =10 ml $C_{BSA,f}$ = 10000 ppm, $C_{benzoin,f}$ =10 ppm, cosolvent: 15% (v/v) PEG 400.

		A	B	C	D	E	F	G
1	initial benzoin	0.582	10.31*	0.103*	0.103*	10.30	-	**-**
2	0-3 ml	0.327	5.79	0.017	0.086	12.29	11.29	**48.73**
3	3-6 ml	0.342	6.05	0.018	0.068	17.00	14.64	**58.68**
4	6-9 ml	0.370	6.55	0.020	0.048	48.00	32.50	**79.85**

A- Absorbance at 250 nm

B- Benzoin concentration in the permeate (ppm)

C- Benzoin amount in the permeate (mg)

D- Benzoin amount in the cell (mg)

E- Benzoin concentration in the cell (ppm)

F- Average benzoin concentration (during filtering the corresponding portion) in the cell (ppm)

G- Total benzoin retention (%)

*- These data are for the feed

The sample calculation is shown for 0-3 ml portion:

After determining the absorbance value (A) in the spectrophotometer at 250 nm, it was converted to benzoin concentration in the permeate (B) by using the slope of the calibration curve given in Figure A-1.

$$\frac{A}{0.0565}=B \quad \Rightarrow \quad \frac{0.327}{0.0565}=5.79\,\text{ppm}$$

Then benzoin amount in the permeate (C) was calculated by multiplying B with the corresponding permeate volume (3 ml)

$$B\frac{mg}{L}\times\frac{1L}{1000\,ml}\times 3ml = C\ mg \Rightarrow$$

$$5.79\frac{mg}{L}\times\frac{1L}{1000\,ml}\times 3ml = 0.017\ mg$$

By subtracting the benzoin amount in the permeate from the previous portion's benzoin amount in the cell, current benzoin amount in the cell was found.

D1-C2=D2

0.103-0.017=0.086 mg

By dividing this value (D) with the corresponding solution volume in the cell benzoin concentration in the cell (E) was obtained. After filtering the first portion, the solution in the cell was 7 ml. Then,

$$E2=\frac{D2\ mg}{7\,ml}\times\frac{1000\,ml}{1\,L}=12.29\ ppm$$

Finally, the concentration of each portion was calculated by the average of the two consecutive portions:

$$F2 = \frac{E1 + E2}{2} \text{ppm} \Rightarrow \quad F2 = \frac{10.30 + 12.29}{2} = 11.29 \text{ ppm}$$

Total benzoin retention was calculated by taking the difference of average benzoin concentration in the cell and permeate and dividing this difference by the average benzoin concentration in the cell.

$$G2\ (\%) = \frac{(F2 - B2)}{F2} \times 100$$

$$\text{total benzoin retention} \quad = G2\,\% = \frac{11.29 - 5.79}{11.29} \times 100 \ = 48.7\,\%$$

APPENDIX C

CALIBRATION CURVES FOR TOTAL BENZOIN RETENTION ANALYSIS IN HPLC

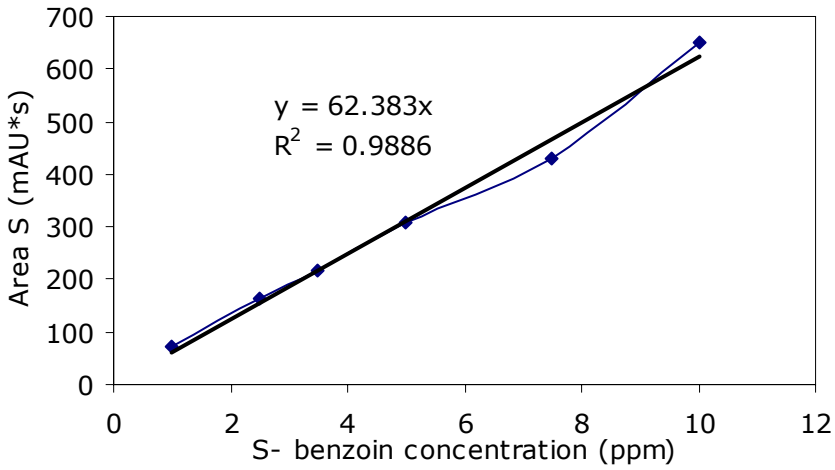


Figure C-1. HPLC Calibration curve for S- benzoin

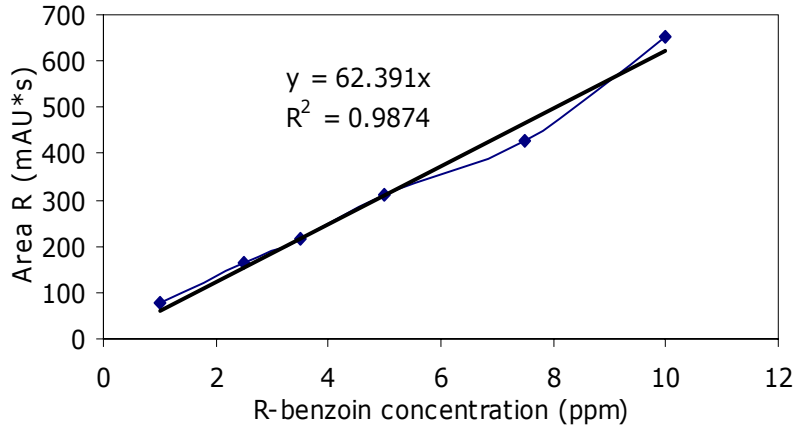


Figure C-2. HPLC Calibration curve for R- benzoin

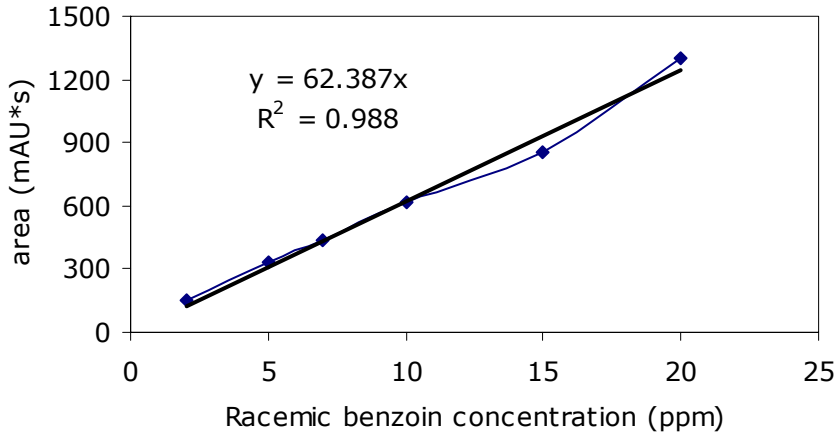


Figure C-3 HPLC Calibration curve for racemic benzoin

APPENDIX D

CALCULATION OF TOTAL BENZOIN RETENTION IN HPLC FOR EEUF EXPERIMENTS

The following sample calculation is given for BAL concentration of 649.5 ppm, and benzoin concentration of 10 ppm. For this experiment when the permeate stream was analyzed in HPLC, the following values for areas for R- and S- benzoin were obtained:

Area of S-benzoin: 675.8 mAU*s
Area of R- benzoin: 168.7 mAU*s

From Figure C-1:

$$\text{Area} = \text{Slope} \times C_{\text{S-benzoin}} \qquad \text{(D-1)}$$

$$675.8 = 62.383 \times C_{\text{S-benzoin}}$$

$$C'_{\text{S-benzoin}} = \mathbf{10.833\ ppm}$$

From Figure C-2:

$$\text{Area} = \text{Slope} \times C_{\text{R-benzoin}} \qquad \text{(D-2)}$$

$$168.7 = 62.391 \times C_{\text{S-benzoin}}$$

$$C'_{\text{R-benzoin}} = \mathbf{2.704\ ppm}$$

At this stage the concentration ratio utilized in extraction while preparing the samples for HPLC analysis, should be considered. (This procedure is explained in Appendix E) The obtained concentrations were

multiplied by this concentration ratio to obtain the actual concentrations as shown in equation D-3.

$$C_{benzoin} = \text{ratio X } C'_{benzoin} \qquad \text{(D-3)}$$

For this experiment;

Concentration ratio: 0.227

Then;

$C_{S\text{-benzoin}} = 0.227 \times 10.833$

$C_{s\text{-benzoin}}$ **= 2.459 ppm**

and

$C_{R\text{-benzoin}} = 0.227 \times 2.704$

$C_{R\text{-benzoin}}$ **= 0.614 ppm**

Hence, in the permeate total benzoin concentration was:

$C_{benzoin} = C_{R\text{-benzoin}} + C_{S\text{-benzoin}}$

$C_{benzoin} = 2.459 + 0.614$

$C_{benzoin}$ **= 3.073 ppm**

The feed benzoin concentration was the only one parameter which can be detected by spectrophotometer in EEUF experiments. The samples were analyzed in UV-spectrophotometer at 250 nm, by 1:1 dilution of 30% (v/v) PEG 400- 20 ppm benzoin solution with pure water. For this experiment, the obtained absorbance value was 0.649.

Then by using calibration curve given in Figure A-1, the feed benzoin concentration was obtained:

$\text{Absorbance} = 0.0565 \times C_{benzoin}$

$0.649 = 0.0565 \times C_{benzoin, feed}$

$C_{benzoin, feed}$ **=11.487 ppm**

Finally, total benzoin retention in EEUF experiments was determined as follows:

$$\text{Total benzoin retention } \% = \frac{C_{\text{benzoin, feed}} - C_{\text{benzoin, permeate}}}{C_{\text{benzoin, feed}}} \times 100 \quad \text{(D-4)}$$

$$\text{Total benzoin retention } \% = \frac{11.487 - 3.073}{11.487} \times 100$$

Total benzoin retention **=** 73.2 %

APPENDIX E

SAMPLE PREPARATION FOR HPLC ANALYSIS: EXTRACTION

Each experiment sample was extracted before the HPLC analysis. Extraction procedure is explained below:

About ~1.25 ml chloroform was added to the permeate sample. Volumes of water phases utilized are given in Table E-1. After vortexing; the samples were centrifuged at 10000 rpm (18000 rcf) for 15 minutes. The phase separation was clear that, upper and lower phases were water phase (15% (v/v) PEG 400-water or 30 % (v/v) DMSO-water) and organic phase (chloroform), respectively. Then the organic phase was aimed to be transferred to the vial by means of a dropper, in which there was a piece of cotton near the front end of the dropper as shown in Figure E-1. In order to prevent the passage of water phase, which might be mixed with organic phase during transfer, into the vial; the dropper was filled with $MgSO_4$ which had been dried from $MgSO_4.6H_2O$, over the cotton piece. $MgSO_4$ captured the water phase and the organic phase dripped on to the vial over the cotton.

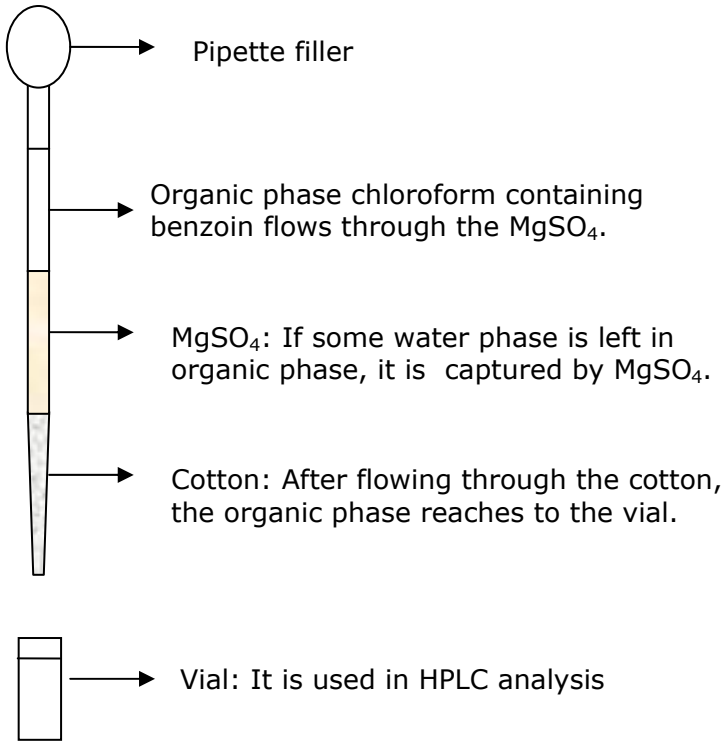


Figure E.1 Schematic view of transferring the organic phase chloroform into the vial.

In Table E-1, in addition to volumes of organic and water phases, the concentration ratios utilized are also given.

Table E-1. Concentration ratios of water and organic phases

BAL concentration (ppm)	Initial volume of water phase: V1 (ml)	Volume of added Chloroform:V2 (ml)	Concentration ratio (V2 /V1)
649.5	5.50	1.25	0.227
1129.2	6.50	1.25	0.192
1935.8	7.60	1.25	0.164

APPENDIX F

BRADFORD REAGENT METHOD

F-1 PREPARATION OF BRADFORD REAGENT

To prepare concentrated stock reagent solution (5x stock) chemicals given in Table F-1.1 were used at the indicated amounts.

Table F-1.1. Bradford reagent preparation procedure

Chemicals	Amount used
85% Ortho-phosphoric acid	500 ml
95% Ethanol	250 ml
Brillant Blue G-250 dye	500 mg

These chemicals were mixed and diluted to 1 L with pure water to prepare (5x) concentrated stock reagent solution. The stock solution was stored at 4°C.

To prepare diluted (1x) reagent solution 1 volume concentrate stock solution was mixed with 4 volumes of pure water. This solution was well mixed and filtered.

Bradford reagent should wait at least 24 hours at room temperature, before use.

F-2.PREPARATION OF PROTEIN STANDARD FOR BRADFORD METHOD

The Bradford assay is a very fast and fairly accurate method utilized for general use especially in determining the protein content of cell fractions and assessing protein concentrations for gel electrophoresis.

The principle of this assay was based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. A visible color change was observed due to the hydrophobic and ionic interactions stabilizing the anionic form of the dye. It was a useful assay since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. In addition, samples that are out of range can be retested within minutes.

As the protein standard Bovine serum albumin (BSA) was used. To prepare 1 mg/ml stock BSA solution, 25 mg BSA was dissolved in 25 ml of pure water. This stock solution was diluted at different ratios given in Table F-2.1

Table F-2.1 BSA dilution ratios for Bradford Method

Protein (mg/ml)	0	0.01	0.02	0.03	0.04	0.05
BSA stock (ml)	0	0.1	0.2	0.3	0.4	0.5
Distilled water (ml)	10	9.9	9.8	9.7	9.6	9.5

After preparation of diluted BSA samples, 0.5 ml BSA sample and 5 ml of Bradford reagent were mixed in a glass test tube and vortexed. 10 minutes later absorbance at 595 nm was measured in a spectrophotometer.

F-3. BSA STANDARD CURVE FOR BRADFORD METHOD

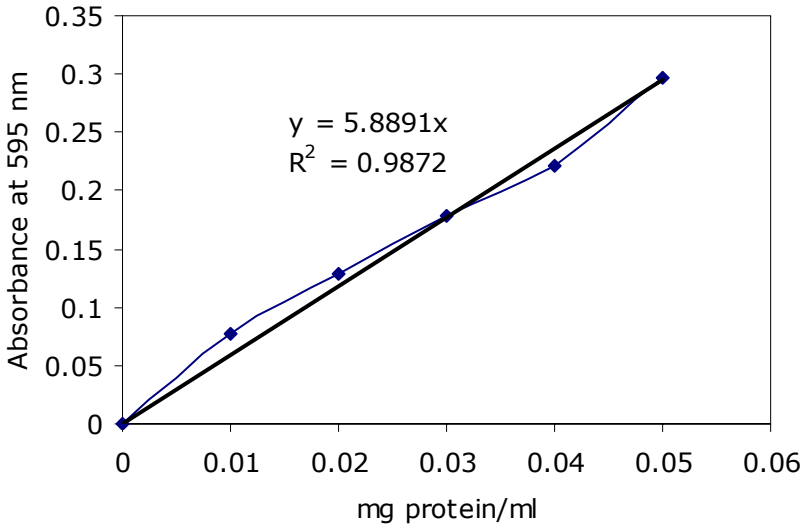


Figure F-3.1. BSA standard curve for Bradford Method

APPENDIX G

CALIBRATION CURVES FOR SPECTROPHOTOMETRIC BSA RETENTION ANALYSIS

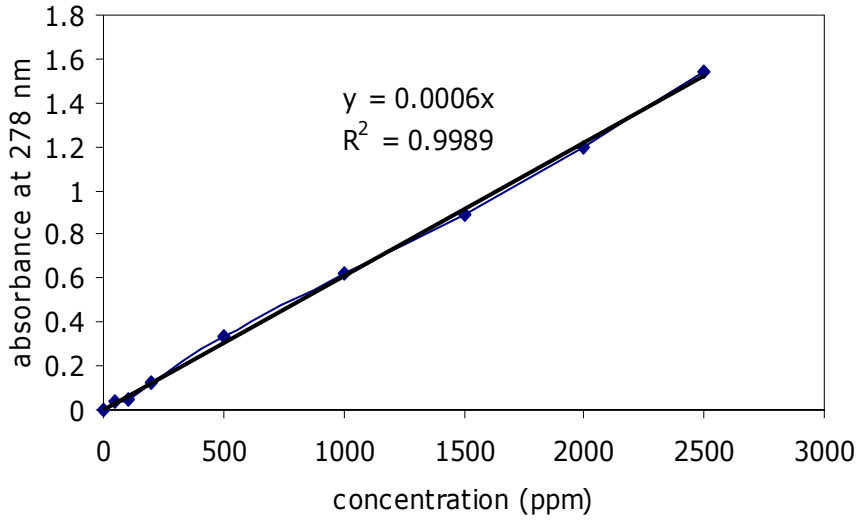


Figure G-1 Calibration curve for BSA -water solvent

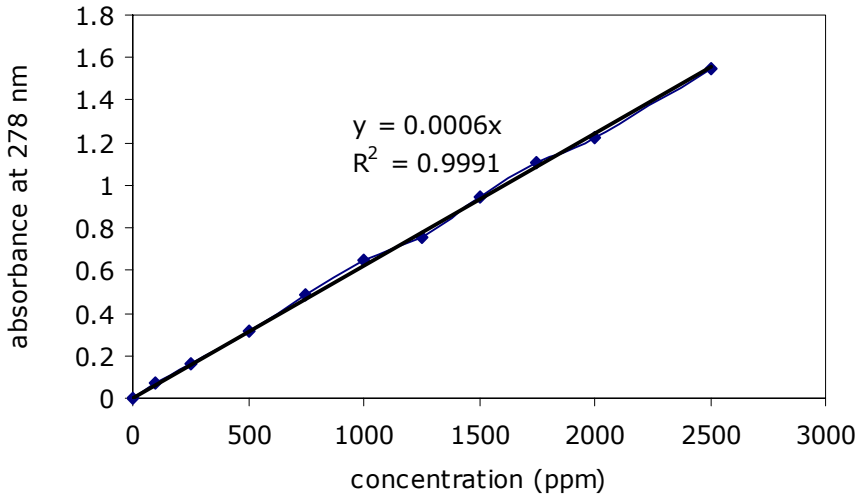


Figure G-2 Calibration curve for BSA –"15% (v/v) PEG 400-water" solvent

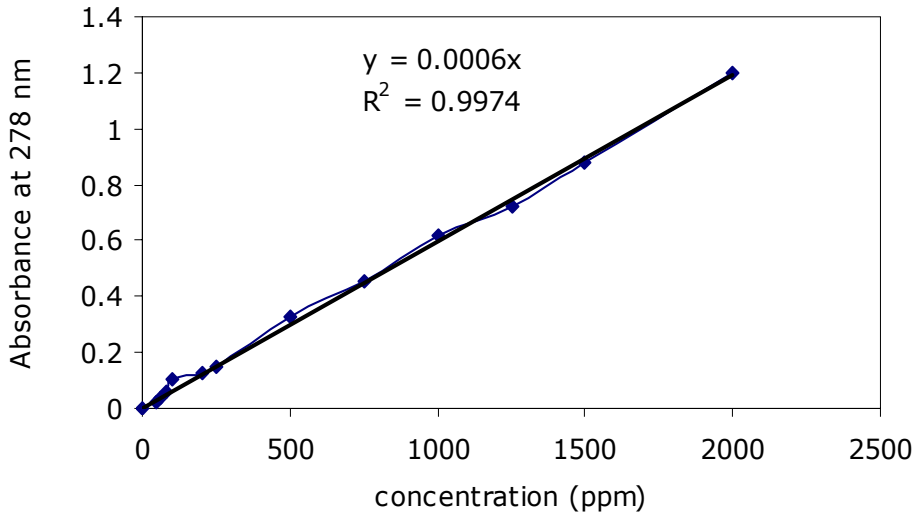
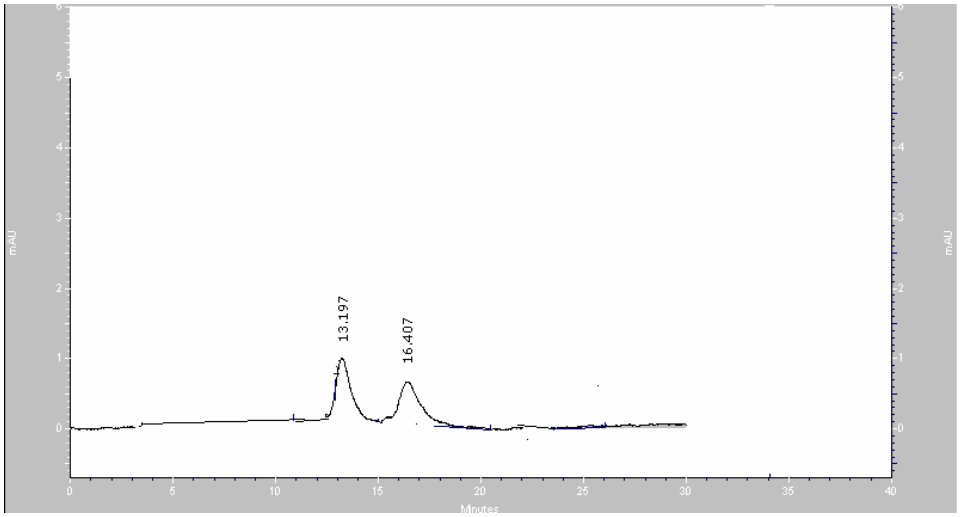


Figure G.3 Calibration curve for BSA –"30% (v/v) PEG 400-water" solvent

APPENDIX H

HPLC CHROMATOGRAMS

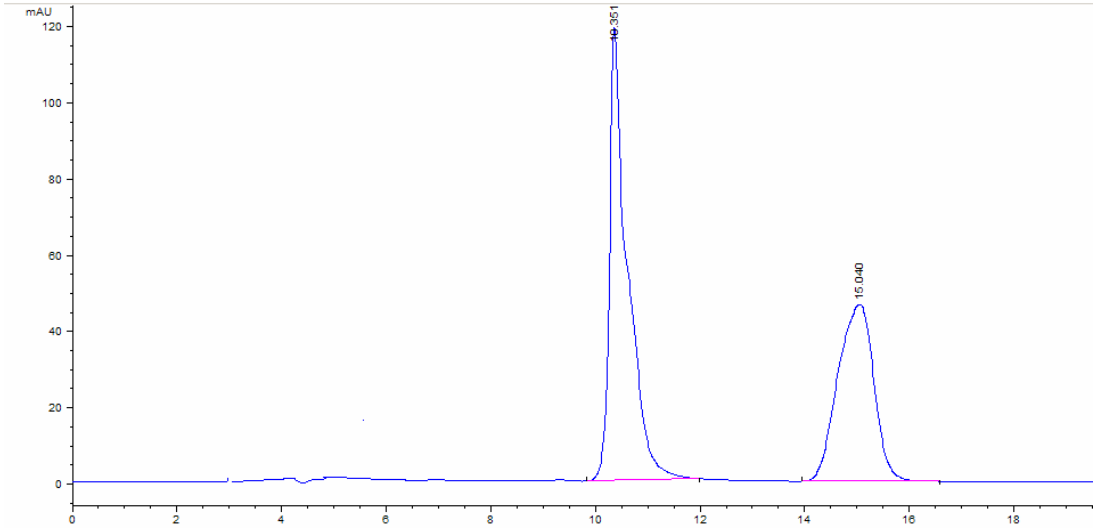
H-1 PEUF EXPERIMENTS



Area Percent Report

Name	Retention Time	Area	Area Percent	Integration Codes
	13.197	41702	53.342	vv
	16.407	36476	46.658	vv
Totals		78178	100.000	

Figure H-1.1 HPLC Chromatogram and Area Percent Report of PEUF experiment with cosolvent 15 % (v/v) PEG 400 (Membrane = RC- 10000 Da, $C_{benzoin,\ f}$ =10 ppm, $C_{BSA,f}$ = 10000 ppm, P= 3 bars, pH =10 , N = 200 rpm, V_{cell} =10 ml)



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Area Percent Report

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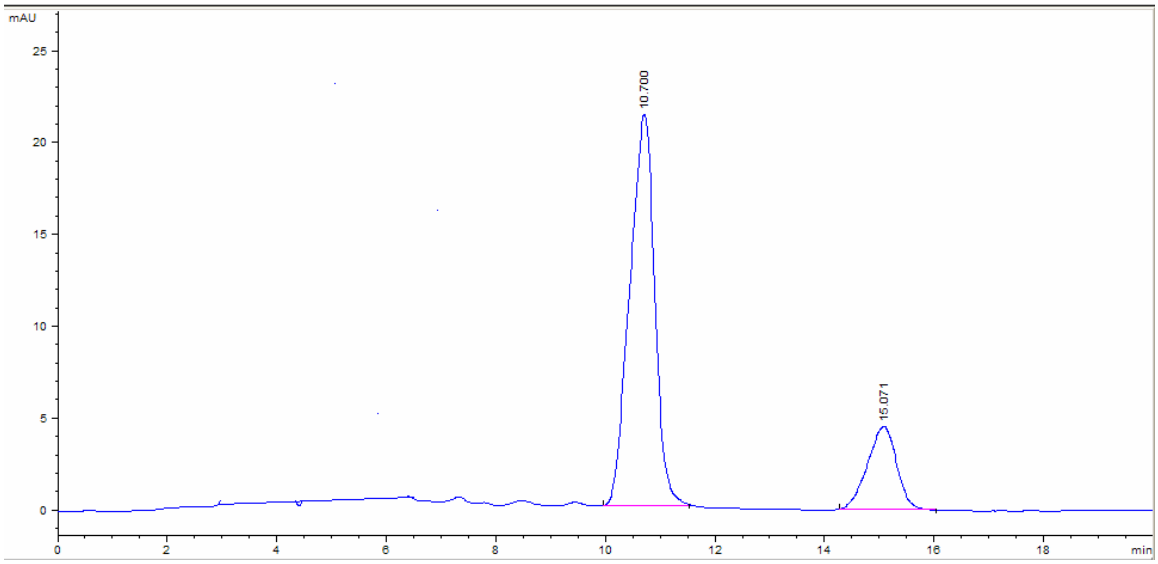
Signal 1: VWD1 A, Wavelength=254 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	10.351	BB	0.3198	2841.90991	118.83094	56.1601
2	15.040	BB	0.7879	2218.46338	46.39854	43.8399
Totals :				5060.37329	165.22948	

Figure H-1.2 HPLC Chromatogram and Area Percent Report of PEUF Experiment with cosolvent 30 % (v/v) DMSO (Membrane = RC- 10000 Da, $C_{benzoin,\ f}$ =50 ppm, $C_{BSA,f}$ = 50000 ppm, P= 3 bars, pH =10, N = 200 rpm, Vcell =50 ml)

H-2 EEUF EXPERIMENTS

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=====================================================================
                        Area Percent Report
=====================================================================

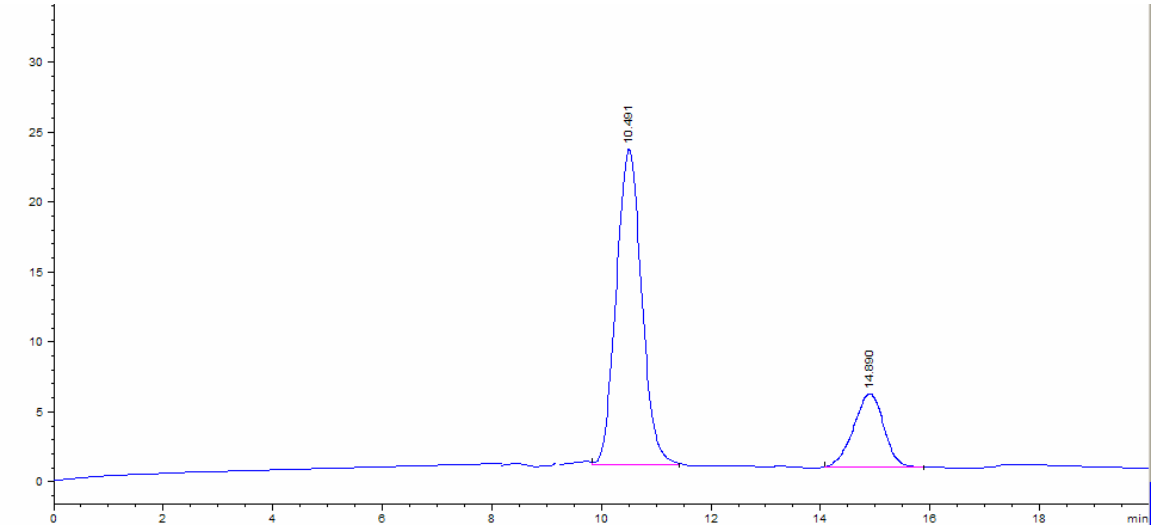
Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]  mAU   *s   [mAU   ]      %
----|-------|----|-------|----------|----------|--------|
   1  10.700 BB    0.4647  675.80151   21.30430  80.0284
   2  15.071 BP    0.5591  168.65080    4.53502  19.9716

Totals :                  844.45232   25.83932
```

Figure H-2.1 HPLC Chromatogram and Area Percent Report of EEUF experiment in which C_{BAL} = 649.5 ppm (Membrane = RC-10000 Da, $C_{benzoin,\ f}$ =10 ppm, P= 3 bars, pH ~7.3, N = 700 rpm, Vcell =10 ml, cosolvent =15 % (v/v) PEG 400)

.



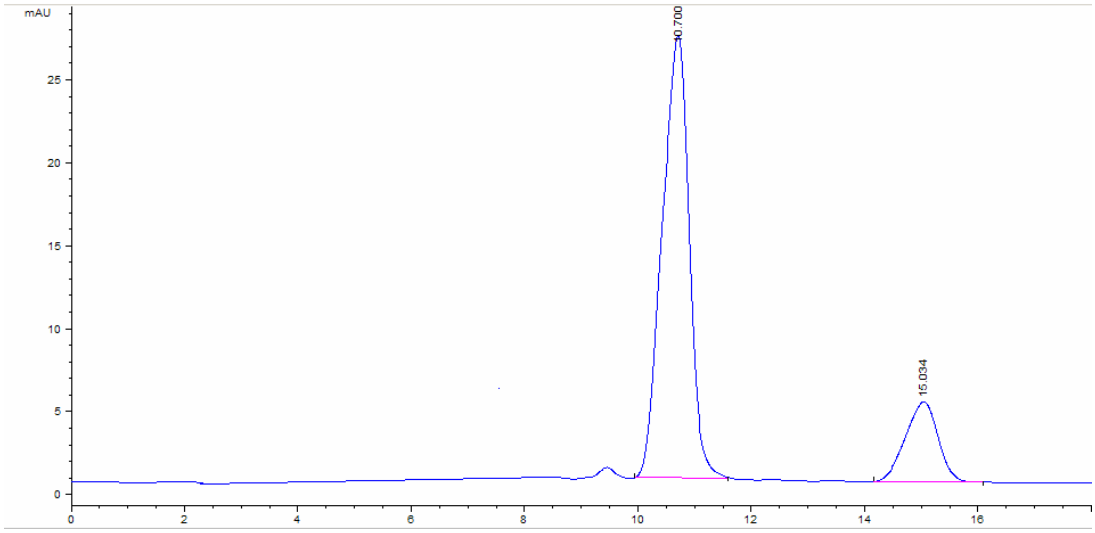

```
================================================================================
                              Area Percent Report
================================================================================

Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]  mAU   *s   [mAU   ]      %
----|-------|----|-------|----------|----------|--------|
   1  10.491 VB    0.5061  729.50488   22.60562  78.2509
   2  14.890 BB    0.5899  202.75861    5.27176  21.7491

Totals :                  932.26349   27.87738
```

Figure H-2.2 HPLC Chromatogram and Area Percent Report of EEUF experiment in which C_{BAL} = 1129.2 ppm (Membrane = RC-10000 Da, $C_{benzoin,\ f}$ =10 ppm, P= 3 bars, pH ~7.3, N = 700 rpm, Vcell =10 ml, cosolvent =15 % (v/v) PEG 400)




```
================================================================================
                          Area Percent Report
================================================================================
```

Signal 1: VWD1 A, Wavelength=254 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	10.700	BB	0.4786	884.08960	26.66365	82.0035
2	15.034	BB	0.6215	194.02292	4.84557	17.9965
Totals :				1078.11252	31.50922	

Figure H-2.3 HPLC Chromatogram and Area Percent Report of EEUF experiment in which C_{BAL} = 1935.8 ppm (Membrane = RC-10000 Da, $C_{benzoin,\ f}$ =10 ppm, P= 3 bars, pH ~7.2, N = 700 rpm, Vcell =10 ml, cosolvent =15 % (v/v) PEG 400)