ENZYME ENHANCED ULTRAFILTRATION FOR THE RESOLUTION OF RACEMIC MANDELIC ACID

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ABSTRACT

ENZYME ENHANCED ULTRAFILTRATION FOR THE RESOLUTION OF RACEMIC MANDELIC ACID

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In this study, resolution of racemic mandelic acid by enyzme enhanced ultrafiltration (EEUF) was studied. In order to develop a methodology, bovine serum albumin (BSA) was used as a model protein for polymer enhanced ultrafiltration (PEUF) experiments and the enzyme *S*-mandelate dehydrogenase was used for EEUF experiments. To be used for enzyme enhanced ultrafiltration experiments, the gene which is responsible from the production of *S*-mandelate dehydrogenase was isolated from *Pseudomonas putida*, expressed in *Escherichia coli* and the recombinant enzyme was produced. For PEUF experiments, effects of pH and ligand ratio were investigated. Total retention of mandelic acid increased with decrease in pH and total retention of mandelic acid reached to a maximum value of 74.4% at pH 4.3. For EEUF experiments, pH and ligand ratio

effect on total retention, enantiomeric excess, enantioselectivity were investigated. Although apoenzyme was tried to be obtained by diafiltration and conversion was tried to be prevented, conversion occured especially at high pH values. To create the apoenzyme effect, three methods were studied. Enzyme conversion was prevented by sodium sulfite inhibition but enzyme did not retain mandelic acid. By oxygen saturation of enzyme, conversion was prevented, binding was achieved but enzyme showed no enantioselectivity. When the enzyme was diafiltrated at pH 10.0, total mandelic acid retention, enantiomeric excess and enantioselectivity reached to 77.2%, 38.9%, 2.27, respectively and the enzyme selectivity was reversed as *R*-selective.

Keywords: Chiral separation, Enzyme Enhanced Ultrafiltration, Mandelic acid, *S*-mandelate dehydrogenase, Recombinant Enzyme Production

ÖZ

RASEMİK MANDELİK ASİDİN ENZİM DESTEKLİ ULTRAFİLTRASYON İLE AYRILMASI

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Bu çalışmada, rasemik mandelik asidin enzim destekli ultrafiltrasyon ile ayrılması çalışılmıştır. Enzim destekli ultrafiltrasyon deneyleri için yöntem geliştirmek amacıyla polimer destekli ultrafiltrasyon deneyleri gerçekleştirilmiş ve bovin serum albumin model protein olarak kullanılmıştır. Enzim destekli ultrafiltrasyon deneyleri için (*S*)-mandelat dehidrogenaz enzimi rekombinant olarak üretilmiştir. Bu amaçla, enzimin üretiminden sorumlu gen *Pseudomonas putida'*dan izole edilmiş ve *Escherichia coli'*de ekspres edilmiştir. Polimer destekli ultrafiltrasyon deneylerinde pH ve ligand oranı etkisi araştırılmıştır. Bu deneylerde toplam tutma azalan pH ile artmış ve pH 4.3 değerinde toplam tutma %74.4 maksimum değerine ulaşmıştır. Enzim destekli ultrafiltrasyon deneylerinde toplam mandelik asit tutması, enantiyomerik fazlalık ve enantiyomerik seçicilik üzerinde pH ve

ligand oranı etkisi araştırılmıştır. Diafiltrasyon yardımı ile kofaktörün uzaklaştırılması ve bu sayede enzimin substratını ürüne dönüştürmesinin engellenmesi amaçlansa da özellikle yüksek pH değerlerinde enzim reaksiyonu gerçekleşmiştir. Apoenzim etkisini oluşturmak için üç farklı yöntem çalışılmıştır. Kofaktörün sodyum sülfit ile inhibisyonu ve enzimin oksijenle doyurulması yöntemlerinde enzim reaksiyonu engellenmiş, fakat sodyum sülfit inhibisyonunda tutma gerçekleşmemiş, oksijenle doyurmada bağlanma gerçekleşirken enantiyomerik seçicilik elde edilmemiştir. Enzimin diafiltrasyonunun pH 10 değerinde gerçekleştirilmesi deneyinde enzimin seçiciliği *R*-mandelik aside dönmüş ve toplam mandelik asit tutması, enantiyomerik fazlalık ve enantiyomerik seçicilik değerleri sırasıyla %77.2, %38.9, 2.27 olarak hesaplanmıştır.

Anahtar Kelimeler: Kiral Ayırma, Enzim Destekli Ultrafiltrasyon, Mandelik Asit, (*S*)-Mandelat Dehidrogenaz, Rekombinant Enzim Üretimi

To my mother and father

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NOMENCLATURE

A	Enzyme activity (U/L)
A_x	Specific enzyme activity (U/mg DCW)
$C_{feed,predo\min ant}$	Concentration of the predominant enantiomer in the feed, g/L
$C_{feed,\min or}$	Concentration of the minor enantiomer in the feed, g/L
$C_{permeate,predo\min ant}$	Concentration of the predominant enantiomer in the permeate, g/L
$C_{permeate,\min or}$	Concentration of the minor enantiomer in the permeate, g/L
C_x	Cell concentration, g dry cell/L
N	Stirring rate, rpm
P	Pressure, bar
PCR	Polymerase chain reaction
RE	Restriction enzyme
U	One unit of an enzyme
t	Bioreactor cultivation time, h
T	Bioreaction medium temperature, °C

Greek Letters

λ	Wavelength, nm

Abbreviations

ATCC	American Type Culture Collection
BSA	Bovine serum albumin
DCPIP	Dichloroindophenol
DCW	Dry cell weight
DSMZ	The German Resource Centre for Biological Material
ee	Enantiomeric excess
EEUF	Enzyme enhanced ultrafiltration
IPTG	Isopropyl *β-D*-thiogalactopyranoside
MA	Mandelic acid
MDH	*S*-mandelate dehydrogenase
mdIB	Gene of *S*-mandelate dehydrogenase
MWCO	Molecular weight cut-off
PCR	Polymerase Chain Reaction
PEUF	Polymer enhanced ultrafiltration
PMS	Phenazine methosulfate
RC	Regenerated cellulose
TEAA	Triethyl ammonium acetate

CHAPTER 1

INTRODUCTION

Isomers are different from each other with the same molecular formula, they can be classifed as: "constitutional (structural) isomers" and "stereoisomers".

Constitutional isomers have the same molecular formula with a different connectivity of atoms. Pentane and 2-methylbutane can be given as examples to this kind of isomers (Hart et al., 2003; Brown et al., 2009).

Pentane (C_5H_{12}) 2-methylbutane (C_5H_{12})

Figure 1.1 Constitutional isomers pentane and 2-methylbutane

Stereoisomers have the same molecular formula in the same order of attachment of atoms but different orientations in space. Stereoisomers are divided into two types: configurational isomers and

conformational isomers. If single bond rotation can interconvert two isomers, they are called conformational isomers. For configurational isomers (conformers), it is necessary to break and remake the bonds to interconvert. Eclipsed and staggered ethane can be given as example to conformational isomers (Hart et al., 2003; Brown et al., 2009).

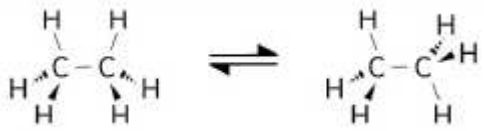


Figure 1.2 Conformational isomers: eclipsed and staggered ethane

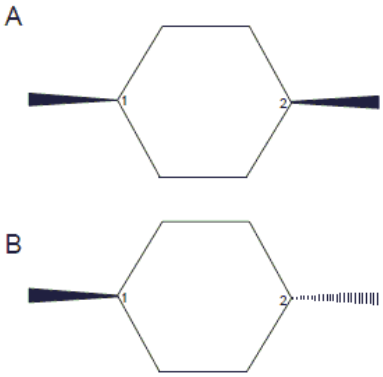


Figure 1.3 Configurational isomers A: *cis*-1,4-dimethylcyclohexane and B: *trans*-1,4-dimethylcyclohexane (Guenther et al.)

Cis-trans isomers of cycloalkanes can be given as examples to configurational isomers (Figure 1.3) (Brown et al., 2009).

Stereoisomers can be categorized in other ways. A molecule is either chiral or achiral. Molecules that are not superposable on their mirror images are called chiral. The mirror image of a chiral molecule is not superimposable on the molecule whereas the mirror image of an achiral molecule is identical with the molecule or superimposable on the molecule itself. Enantiomers are nonsuperimposible mirror images of each other whereas diastereomers are not mirror images of each other (Hart et al., 2003).

If four different groups are attached to a carbon atom, it is called a stereogenic carbon atom (stereogenic center). How these four groups arranged is said to be the configuration of that center. Enantiomers are another type of configurational isomers and they have opposite configurations. These four groups are placed in a priorty order and "the stereogenic center is observed from the opposite the lowest priority group". If a clockwise array is obtained for other three groups, the configuration is said to be *R* or *D*, if a counterclockwise array is seen, the configuration is said to be *S* or *L* (Hart et al., 2003).

"An ordinary light beam consists of waves that vibrate in all possible planes perpendicular to its path". The waves will vibrate in parallel planes if the light beam is passed through certain substances. This light beam is said to be plane polarized. Optically active substances rotate plane polarized light whereas optically inactive substances can not rotate (Hart et al., 2003).

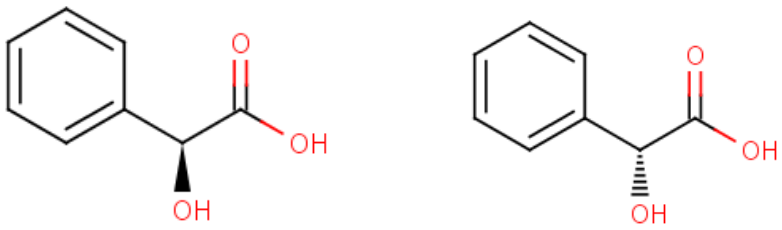
Enantiomers have identical achiral properties like melting point, boiling point and solubilities. However they have different chiral properties. The rotational behaviour of plane polarized light for enantiomers are different. "A substance that rotates the plane polarized light in the clockwise (right) direction is called

dextrorotatory(+), and the one that rotates the plane-polarized light in a counterclockwise (left) direction is said to be levorotatory(-)". There is no relationship between configuration *(R,S)* and sign of rotation *(+,-)*. Racemic mixture is defined as 50:50 mixture of enantiomers and it is optically inactive (Hart et al., 2003).

Many drugs, agrochemicals, food additives and fragrances are racemic mixtures. Life is based on biomolecules, like proteins, enzymes, DNA which are all of a "single handedness". Therefore, the left- and right-handed enantiomers show different effects in terms of pharmacology and metabolism for living organisms (Tang et al., 2007). As a result, enantiomers are desired to be obtained as pure materials. Only one of the enantiomers may show biological activity. One enantiomer may be useful as a pharmaceutical whereas other enantiomer is ineffective. For example, *R*-chloroamphenicol is antibacterial whereas *S*-chloramphenicol is inactive. Moreover, enantiomers may show different biological activities. One enantiomer may be useful whereas other one is not desired or both of them may be useful. For example, *S*-ethambutol is tuberculostatic whereas *R*-ethambutol causes blindness. Furthermore, an enantiomer may be more active than its racemate because of antagonism (Collins et al., 1992).

Mandelic acid is a chiral molecule, an aromatic α-hydroxy acid with the molecular formula of $C_8H_8O_3$ and molecular weight of 152.14 g/mol. It is soluble in water. Mandelic acid and its derivatives are important products for chemical and pharmaceutical industries. They are used in synthesis and investigations related to stereo-chemistry. Enantiomers of mandelic acid are used for the separation of racemic alcohols and amines (Yadav and Sivakumar, 2004). *(R)*-Mandelic acid is the key intermediate of semisynthetic penicillin, cephalosporin,

anti-tumor and anti-obesity drugs whereas (S)-mandelic acid is used in the synthesis of substituted cyclopentenones and commercial drugs, including the nonsteroidal anti-inflammatory drugs (Ju et al., 2010). Figure 1.4 shows the enantiomers of mandelic acid.



(S)-mandelic acid *(R)*-mandelic acid

Figure 1.4 Structure of mandelic acid enantiomers

Therefore, production of enantiomerically pure products is an important task. It can be achieved by the synthesis of one of the enantiomers or by resolution of racemic mixtures.

Since the resolution is simple, reliable and practical when compared to asymetric synthesis of enantiomers, it is the most widely applied method for the production of optically pure fine chemicals, pharmaceuticals and pharmaceutical intermediates. "Resolution and chirality are like twins until the day Louis Pasteur had separated crystals of salts of *D*-tartaric acid and *L*-tartaric acid under the microscope" (Ager, 1999).

For chiral resolution, chromatographic techniques like high pressure liquid chromatography, thin layer chromatography, gas

chromatography, supercritical fluid chromatography, capillary electrophoresis, liquid- liquid extraction and crystalization are used. Chromatographic methods have disadvantages like low productivity, high cost and necessity of an additional unit operation to remove the solvent. For crystalization high cost and detailed investigation of system is necessary (Pickering and Chaudhuri, 1997).

Membrane processes have emerged with the advantages of being simple, efficient, economical, easy to scale up, and suitable for continuous operations (Singh et al., 2010; Xie et al., 2008).

Pressure driven membrane processes are microfiltration, ultrafiltration, nanofiltration and reverse osmosis. Classification of membrane processes according to the size range of retentate components are given in Figure 1.5 (Wang et al., 2009). Ultrafiltration membranes have a separation range from about 0.005 μm to about 0.1 μm. Ultrafiltration is used for separation of virus particles and large organic molecules according to its molecular weight cut-off (MWCO) potential (Sutherland, 2008). Molecules with larger molecular weight than MWCO of the membrane are remained on the membrane whereas other smaller molecules pass through the membrane.

Complexation enhanced ultrafiltration (CEUF) is a new membrane process with three main types: colloid enhanced, micellar enhanced and polymer enhanced ultrafiltration. In these type of ultrafiltrations, colloids, surfactants and polymers are being used as binding agents, respectively. When compared to other types of CEUF, only polymer enhanced ultrafiltration (PEUF) is a one phase (homogeneous) operation, whereas others are heterogeneous phase operations. Therefore, in PEUF the problems like interphase transfer and long

contact time for multiphase separation processes are not observed. (Ölçeroğlu, 2006). PEUF is widely investigated for removal of heavy metals in waste waters (Müslehiddinoğlu et al., 1998).

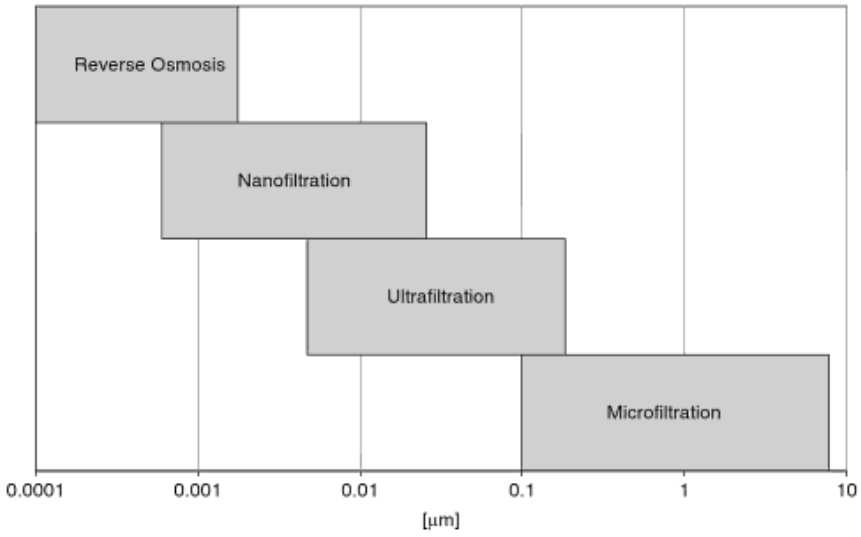


Figure 1.5 Classification of membrane processes according to the size range of retentate components

In CEUF for chiral separations, there is a specific complex formation between ligand polymer and one of enantiomers in the feed solution. This complex remains on the membrane whereas free enantiomer passes to the permeate side due to its smaller molecular weight than molecular weight cut off (MWCO) of the membrane. Therefore, enantioseparation is achieved. To be able to decomplex the ligand-enantiomer complex; a specific property, like pH or ionic strength should be changed (Ölçeroğlu, 2006).

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

Enantiomeric excess and enantioselectivity are defined as:

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

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

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

Since the feed is a racemic mixture, the Equation 1.2 becomes:

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

Enzyme enhanced ultrafiltration method (EEUF) is a specific type of PEUF, which utilizes the apoenzymes as binding agent. Apoenzymes can bind to their substrates; however they can not convert them into product. Therefore, they can be used as ligands.

BSA is a single polypeptide chain with 583 amino acid residues and molecular weight of 66 kDa. Albumins are a group of acidic proteins and they are found in body fluids, tissues of mammals and in some

plant seeds. They are soluble in water and can be easily crystallized (Sigma Aldrich, Product Information).

In this study, the objective was the separation of racemic mandelic acid to its enantiomers by enzyme enhanced ultrafiltration method (EEUF). In order to develop a methodology, bovine serum albumin (BSA) was used as the separating agent for PEUF experiments in the first part of the study. To achieve enzyme enhanced ultrafiltration, the enzyme *S*-mandelate dehydrogenase was produced. For this production, the gene which is responsible from the production of *S*-mandelate dehydrogenase (MDH) was isolated from *Pseudomonas putida* and expressed in *Escherichia coli*. For the batch ultrafiltration, regenerated cellulose membranes (MWCO=10 kDa) were used. Operation parameters which are relative concentrations and pH were manipulated.

CHAPTER 2

LITERATURE SURVEY

2.1 Chirality and Chiral Separations

Stereochemistry is important when processes in biological metabolism are considered. Two enantiomers show different activities in these processes. For drugs, agrochemicals, food additives and flavours, chirality has scientific and economic importance.

The bronchodilator levalbuterol, the antidepressants (*S*)-citalopram and *(S)*-fluoxetine, CNS stimulant *d-threo*-methylphenidate, the gastrointestinal drug (1)-norcisapride, the antiulcerative perprazole are some of the recent examples of enantiomers (Maier et al., 2001).

2.2 Chiral Separation Methods

Enantiomers can be obtained by asymmetric synthesis or by enantioseparation of a racemic mixture. Asymmetric synthesis is more expensive than chiral resolution and provides low overall yields. Since enantiomers have similar physical and chemical properties, chiral resolution techniques have some difficulties. However they are effective on both analytical and industrial scales (Singh et al., 2010; Xie et al., 2008). Enantioselective separations have been achieved

with different separation techniques including chromatographic techniques, capillary electrophoresis, liquid–liquid extraction, crystalization, etc. These techniques for separation of enantiomers are summarized in Figure 2.1 (Maier et al., 2001; Singh et al., 2010).

Crystallization can be divided into direct (or preferential) and diastereomeric crystallization methods. Direct (or preferential) crystallization is simple and has low cost. However, since the product crystals are mixtures of two different enantiomorphic crystals, it is difficult to employ on an industrial scale. This method is available only when the racemate is a conglomerate. On the other hand, only 5% to 10% of all organic racemates form conglomerates. In diastereomeric crystallization method, racemic compound is resolved using an optically pure resolving agent. This method is expensive and finding an appropriate resolving agent is difficult (Xie et al., 2008). Mandelic acid was enantioseparated by "diastereomeric crystalization" using *L*-phenylalanine as the resolving agent and enrichment of *L*-mandelic acid achieved up to 85% in the final diastereomeric crystals (Pham et al., 2009).

Chromatographic techniques for chiral separation include gas chromatography (GC), supercritical fluid chromatography (SFC), capillary electrochromatography (CEC), and liquid chromatography which includes thin-layer chromatography (TLC), simulated moving bed chromatography (SMB) and countercurrent chromatography (CCC) (Maier et al., 2001; Xie et al., 2008). Supercritical fluid chromatography and simulated moving bed chromatography provides lower operating costs and continuous operation when compared with high performance liquid chromatography. Other chromatographic techniques are generally expensive, inefficient and must be performed as batch operations (Xie et al., 2008). By Sicoli et al.

racemic alkanes with seven and eight carbon atoms (3-methylhexane, 2,3-dimethylpentane, 3-methylheptane, 3,4-dimethylhexan, 2,4-diniethylhexane, 2,3-dimethylhexane and 2,2,3-trimethylpentane) was enantioseparated by gas chromatograhy on different modified cyclodextrins (Sicoli et al, 2009).

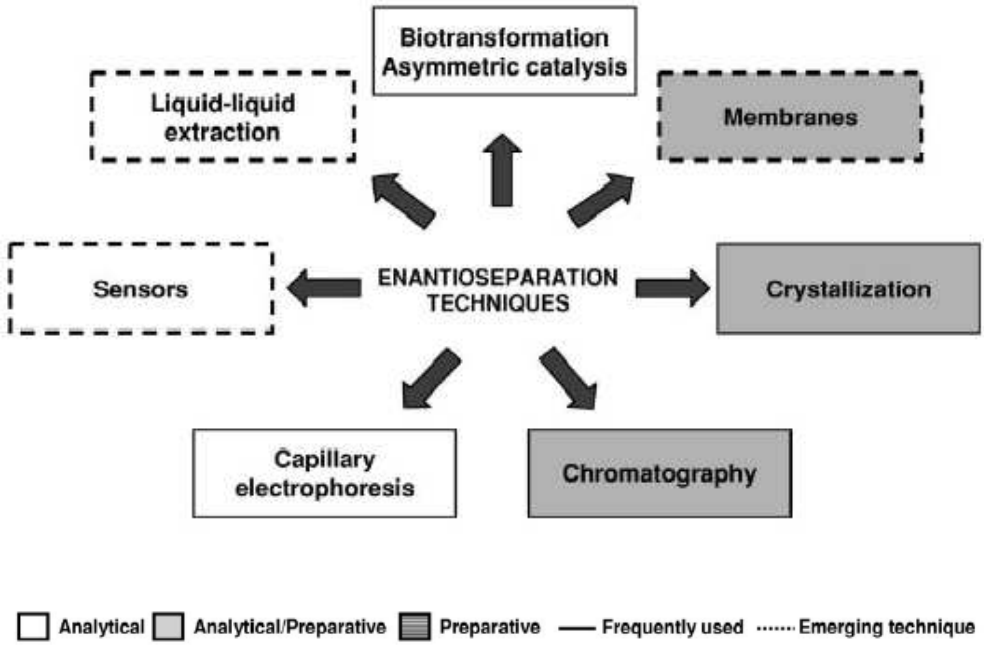


Figure 2.1 Techniques for separation of enantiomers (Maier et al., 2001)

In enantioselective liquid–liquid extraction (ELLE), an enantiopure host is used as an extractant to bind enantiospecifically and reversibly with a racemic substrate. Enantiomeric separation can occur in one step if the host is confined to one phase in a two-phase system. This

approach is preffered because of its potential versatility and ease of operation. For example, by Verkuijl et al. chiral palladium phosphine complexes were used for chiral separation of amino acids and phenylalanine analogues (Verkuijl et al., 2010).

For enantioseparation with capillary electrophoresis, "the enantiomers must migrate with different velocities along the longitudinal axis of a separation capillary". Since enantiomers have same effective charge-to-mass ratio in an achiral medium, chiral selectors should be added (Chankvetadze, 2009). By Prokhorova et al., enantioseparation of carboxylic acids bearing α-C*-asymmetric carbon atom (ibuprofen, indoprofen, ketoprofen, fenoprofen, flurbiprofen, mandelic acid, 3-phenylbutiric acid, 2-phenoxypropionic acid, and α-methoxyphenylacetic acid) were achieved (Prokhorova et al., 2010). Although these techniques have many advantages, they have disadvantages like high energy consumption, high cost, low efficiency, and discontinuous operation (Xie et al., 2008).

2.2.1 Membrane Based Chiral Separation Methods

Membrane processes are advantageous when compared to other separation processes due to being simple, efficient, economical and easy to scale up. Membrane technologies provide high efficiency, low energy usage, and they are suitable for continuous operations. By ultrafiltration processes high permeability can be obtained at low pressure. Hence, they are suitable for enantioseparations with chiral selective ligands like bovine serum albumin (BSA), deoxyribonucleic acid (DNA), etc. They can be immobilized on the membranes or added to the feed solution (Singh et al., 2010; Xie et al., 2008).

Membranes can be either enantioselective or non-enantioselective for chiral separation. The enantioselective membranes achieve the separation of enantiomers because of their chiral recognition sites such as chiral side chains, chiral backbones, or chiral selectors and they are usually liquid or solid. However, the non-enantioselective membranes provide separation of enantiomers by the help of a chiral ligand using the principle of size selectivity. Therefore, these non-enantioselective membrane-assisted processes are combined with other chiral recognition approaches such as enzymatic kinetic resolution, solution systems with micelles, and systems using chiral selectors as complexing agents (Xie et al., 2008).

2.2.1.1 Enantioselective Membranes

The enantioselective membranes are barriers which selectively transport one of two enantiomers due to the stereospecific interaction such as hydrogen bonding, hydrophobic, Coulombic, van der Waals interactions between the enantiomer and chiral recognition sites. Separation by enantioselective membranes can occur with two different mechanisms which are facilitated transport (liquid and solid membranes) and retarded transport (solid membranes). The factors for the classification of the enantioselective membranes are the magnitude of the binding affinity force, the kinds of driving force (i.e. concentration, pH, pressure or electrical potential) and the magnitudes of the driving force.

Facilitated transport is based on the different diffusion rates of two enantiomers and the membranes based on this mechanism are called diffusion-enantioselective membranes. Due to a higher binding affinity, one enantiomer preferentially adsorbs to the chiral recognition sites in the enantioselective membranes near the feed

phase, it continuously adsorbs and desorbs from one chiral site to the next and is transported toward the stripping phase, usually by concentration or electrical potential difference. The other enantiomer, which has no or less specific binding affinity for the chiral recognition sites, passes through the membrane by diffusion. This type of mechanism can be seen in most chiral liquid and solid membranes composed of a chiral polymer (polysaccharides like chitosan and sodium alginate), membranes coated with an enantioselective polymeric layer and chiral selector-immobilized membranes.

Membranes based on the retarded transport mechanism are called adsorption-enantioselective membranes which pressure difference is generally the driving force. In contrast to the facilitated transport mechanism, in retarded transport adsorbed enantiomer is retained in the membrane phase, while other enantiomer passes through the membrane more easily since it has no or lower affinity for the chiral recognition sites. In these membranes, the binding affinity between chiral environment and enantiomers is stronger than diffusion-enantioselective membranes, and this interaction occurs always between one enantiomer and one chiral site. Therefore, the adsorption-enantioselective membranes are expected to have relatively high flux and high enantioselectivity, and they have more potential than diffusion-enantioselective membranes to perform industrial-scale chiral separations (Xie et al., 2008).

In enantioselective liquid membrane processes, liquid-liquid extraction and membrane separation incorporates in one device. The transport mechanism is solution-diffusion in these membranes. The solute dissolves in the liquid membrane and diffuse across the membrane due to a concentration gradient. Different solutes have different solubilities and diffusion coefficients in the liquid membrane.

If a mobile complexation agent (carrier) is used, carrier in the membrane phase reacts rapidly and reversibly with the desired solute and makes a complex which increases the efficiency and selectivity and this process is called as facilitated or carrier-mediated liquid membrane separation (Kislik, 2010).

Liquid membranes may be in three types according to the module design configurations: "bulk liquid membranes (BLM), emulsion liquid membranes (ELM) and supported or immobilized liquid membranes (SLM)".

In bulk liquid membranes there is a bulk aqueous feed and receiving phases separated by a bulk organic, water-immiscible liquid phase. In emulsion liquid membranes, receiving phase is emulsified in an immiscible liquid membrane and the emulsion is dispersed in the feed solution. In supported or immobilized liquid membranes, liquid impregnated (or immobilized) in the pores of a thin microporous solid support is defined as a supported liquid membrane. Figure 2.2 shows the configurations of liquid membranes (Kislik, 2010).

By Ferreira et al, propranolol was separated to its enantiomers with heptakis (2,3,6-tri-O-acetyl)-β-cyclodextrin (TA-β-CD) that interacts with the *(S)-(*2)- propranolol by liquid membranes. In this study bulk liquid membrane (BLM) and supported liquid membrane (SLM) were tried and close values were obtained for recovery and enantiomeric excess with BLM and SLM. Obtained recovery was 30% and an enantiomeric excess was 12% with SLM (Ferreira, 2006).

In another study of bulk liquid membranes, by Jiao et al. racemic propranolol separation was studied with "a complex of *(S,S)*-di-n-dodecyltartrate and boric acid" as chiral carriers. Concentration ratio

of propranolol to chiral carrier and the pH of the buffer in the aqueous solution was found suitable as 1:20 and 5, respectively (Jiao, 2008).

Emulsion liquid membrane was used for chiral separations on low molecular weight species by Pickering et al. Racemic phenylalanine was separated using copper(II) *N*-decyl-(L)-hydroxyproline as chiral selector. Around 40% enantiomeric excess was achieved (Pickering, 1997).

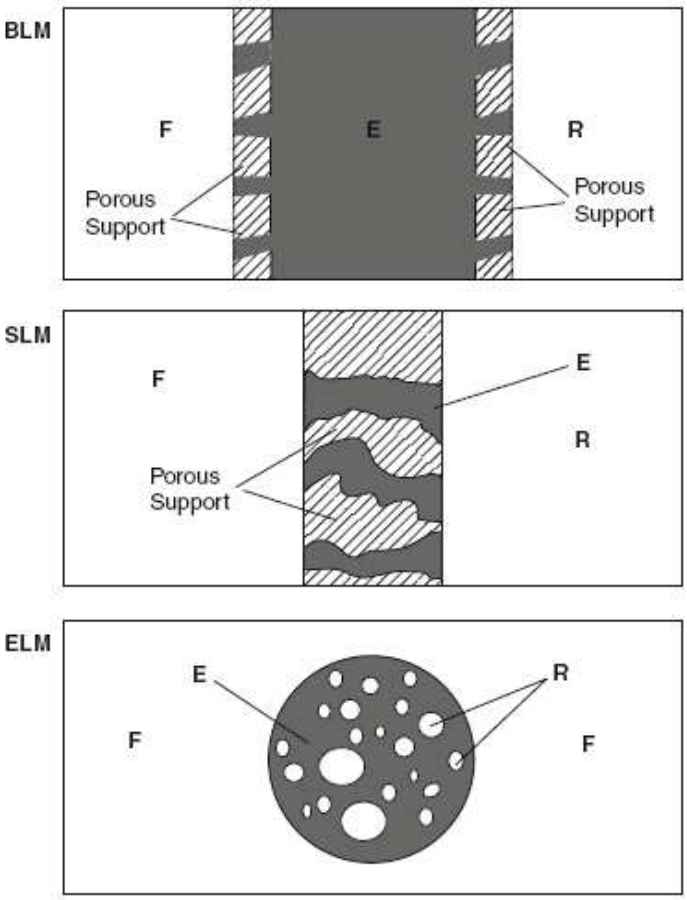


Figure 2.2 Configurations of liquid membranes (BLM, ELM, SLM) F: Feed phase R: Receiving phase E: Liquid membrane (Kislik, 2010)

Enantioselective solid membranes can be formed by chiral polymers or achiral polymers with immobilized chiral selectors (Xie et al., 2008). Chiral polysulfone polymer membrane obtained by bonding chiral carrier, N-dodecyl-4(R)-hydroxy-L-proline to the polymer was used for enantioseparation of propranolol by Gumi et al. 25% content of chiral polysulfone was found as necessary for the highest enantioselectivity which is 1.1 at t= 96 h. It was also found that facilitated transport mechanism took place and S- propranolol transport rate was higher than the rate of *R*-propranolol (Gumí, 2005).

2.2.1.2 Non-enantioselective Membranes

Non-enantioselective membranes are used as supports to capture chiral selectors or to separate particles by size. They do not recognize one of the enantiomers in a racemic feed solution, they are utilized to retain larger molecules. Therefore, enantioseparation by non-enantioselective ultrafiltration membranes was achieved by size diffference. For example, after formation of a complex by binding of one enantiomer with a large chiral recognition molecule like bovine serum albumin (BSA) or selective hydrolysis one of the enantiomers by an enzyme, ultrafiltration by a porous non-enantioselective membrane with a suitable molecular weight cut-off (MWCO) can be used to achieve the enantioseparation (Xie et al., 2008).

2.2.1.3 Complexation Enhanced Ultrafiltration (CEUF)

In complexation enhanced ultrafiltration, colloids, micelles and polymers are used as binding agents (ligands).

Enantioseparation by micellar enhanced ultrafiltration (MEUF) was firstly performed by Creagh et al. Separation of phenylalanine was achieved by *L*-5-cholesterol glutamate, mixed micelles that prefer D-phenylalanine to bind. These micelles were formed with a "chiral ligand-exchange cosurfactant", and a "nonionic surfactant". Batch system pressure was 3 bars and speed was 400 rpm and Amicon300-mL stirred cell with regenerated cellulose membrane with MWCO of 5 kDa was used. The system for MEUF is shown in Figure 2.3 (Creagh et al., 1994).

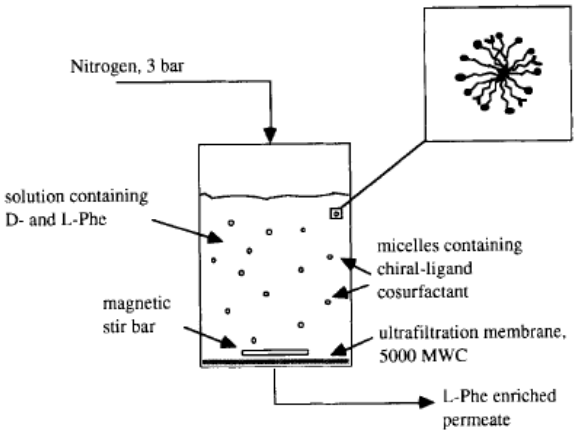


Figure 2.3 System for MEUF for phenylalanine enantioseparation (Creagh et al., 1994)

Most widely used ligand in PEUF for chiral separations is bovine serum albumin (BSA). In the literature, BSA was used widely as a ligand for enantioseparations of chiral molecules like tryptophan, phenylalanine, ibuprofen by adding to the feed solution and also by immobilization on the membrane. By Poncet et al., racemic tryptophan was enantioseparated by ultrafiltration using BSA and 91% purity with a

89% recovery of *D*-tryptophan was achieved (Poncet et al., 1997). One of the latest studies about chiral separation of tryptophan with BSA in the feed solution was performed by Singh et al. and ee% of 33 was obtained at pH 9.2 and with 0.07% BSA (w/v) (Singh and Bajaj, 2007).

By Bowen et al. racemic ibuprofen was separated by ultrafiltration with BSA added to the feed solution. In this study, an Amicon dead-end stirred filtration cell, cellulose membrane with diameter of 44.5 mm and MWCO of 5 kDa were used. The system was operated at 100 kPa with a speed between 200 and 400 rpm. A 0.1M sodium hydrogen phosphate solution was used as solvent whereas organic modifiers methanol and acetonitrile were used as cosolvents with feed solutions of 80 mg/L ibuprofen. *R*-ibuprofen was preferentially bound by BSA. Enantiomeric excess was about 23% in the permeate for 15% of acetonitrile at pH around 9.1 and ibuprofen/BSA ratio about 3.4. At pH 9.0-9.2 maximum enantioselectivity was obtained, enantioselectivity increased with BSA feed concentration. By a multistage separation with six steps the permeate contained about 95% of *(S)*-ibuprofen (Bowen, 2002).

By Ölçeroğlu et al., chiral molecule benzoin was separated by enzyme enhanced ultrafiltration and to optimize the process parameters for EEUF experiments, for PEUF, BSA was used. In PEUF experiments, while BSA concentration and pH increased, total benzoin retention increased. Total benzoin retention was 48.7% at pH 10 for BSA concentration of 10000 ppm. With DMSO as the cosolvent total benzoin retention was 41.3% at pH 10 and ee % increased to 16.7 % at pH 11. Therefore, enantiomeric separation of benzoin by PEUF using BSA was not achieved (Ölçeroğlu, 2006).

BSA was also immobilized to be used as a ligand. Tryptophan was separated by ultrafiltration using "BSA immobilized polysulfone membrane" and "polysulfone membrane having BSA semi-IPN network" which was formed of cross-linked network of BSA chains in polysulfone matrix by Singh et al. It was found that enantiomeric excess (ee%) increased until 8 hours and then decreased for both types of membranes. Separation factor of 1.89 and enantiomeric excess of 30.8% was achieved with membrane that BSA was immobilized whereas separation factor of 1.62 and enantiomeric excess of 23.8% was obtained with BSA-IPN membrane at 8 hours (Singh et al., 2010).

2.2.1.4 Enzyme Enhanced Ultrafiltration

Enzyme enhanced ultrafiltration method (EEUF) is a specific type of CEUF, which utilizes the apoenzymes as binding agent. In the study of Ölçeroğlu et al. chiral molecule benzoin was separated by EEUF using apoenyzme benzaldeyhde lyase (BAL, E.C. 4.1.2.38). Amicon dead-end stirred membrane cell and Millipore solvent resistant stirred cell were used with regenerated cellulose membranes of 10 kDa molecular weigth cut-off. The system pressure was kept constant at 3 bars. Since benzoin is only slightly soluble in water, polyethylene glycol (PEG 400) and dimethyl sulfoxide (DMSO) were used as cosolvents. Also, there was a membrane saturation step because benzoin was retained by the membrane. To develop a methodology for EEUF experiments, in PEUF experiments with BSA, concentration and pH effects were investigated. According to this study, while BSA concentration and pH increased, total benzoin retention increased. For 15% (v/v) PEG 400-water and BSA concentration of 10000 ppm total benzoin retention was 48.7% at pH 10. However for PEUF experiments with PEG 400 as the cosolvent ee % values were about

or less than 10% whereas For PEUF experiments with DMSO as the cosolvent total benzoin retention was 41.3% at pH 10 and ee % increased to 16.7 % at pH 11. For EEUF experiments, to bind Mg^{2+} ions which were necessary for the enzyme to be active, EDTA was added to the solution and the conversion of *R*-benzoin was prevented. In EEUF experiments effect of BAL concentration was studied and total benzoin retention and ee % were at around 75% and 60%, respectively for 15% (v/v) PEG 400 as the cosolvent (Ölçeroğlu, 2006; Ölçeroglu et al., 2008).

2.3 Mandelic Acid Separation Methods

Different methods for enantioseparation of mandelic acid was studied in the literature. Resolution of mandelic acid investigated with chromatographic methods like gas chromatograpghy, thin-layer chromatography, liquid chromatography. By Kezic et al. enantioseparation of mandelic acid in urine was achieved by gas chromatography using chiral CP Chirasil-Dex-CB column. Mandelic acid is a major metabolite of styrene and accepted as a biological indicator of occupational exposure to styrene which has adverse effects on the central nervous system in case of long-term exposure and accepted as a possible human carcinogen. Since *R*- and *S*-enantiomers which are metabolised from styrene epoxide show different mutagenicity and toxicity, precise determination of enantiomer compositions of mandelic acid has toxicological importance. In that study, ECD method was found as less sensitive when compared to FID method (Kezic et al., 2000). Furthermore, mandelic acid enantiomers in urine were determined by a reversed-phase HPLC method with A ZORBAX SB-C18 column by Wang et al. in 2006 (Wang et al., 2006).

Mandelic acid and its derivatives were enantioseparated by thin-layer chromatography and "molecularly imprinted polymers" were employed as chiral stationary phases by Rong et al. In that study, obtained chiral separation factor was 1.45 for *L*-mandelic acid (Rong et al., 2006).

In the study of Franco et al., as the stationary phase, a monoclonal anti-α-hydroxy acid antibody was immobilized onto a synthetic chromatographic support material for enantioseparation of model mandelic acid in high-performance immunoaffinity chromatography. Temperature, ionic strength, flow rate and pH of mobile phase were investigated. L-enantiomer eluted with the void volume whereas the D-enantiomer was retained and eluted secondly. For 6 ml/min flow rate, the resolution was 2.31 ± 0.01 (Franco et al., 2008).

High-performance liquid chromatography (HPLC) with chiral stationary phases is a widely used approach for enantiomeric separation. Immobilized 1,2,3-triazololinked quinine tert-butyl carbamate was prepared as the chiral stationary phase for the enantioseparation of mandelic acid in HPLC and its derivatives and α values between 1.08 and 1.68 were obtained. (Kacprzak et al.,2010). Moreover, a chiral stationary phase derived from 4-(3,5-dinitrobenzamido) tetrahydrophenanthrene was used by Aneja et al. and relations between structures separation factors were discussed (Aneja et al., 2010).

By Jandera et al. the effects of the mobile phase composition on the retention, selectivity, adsorption isotherm profiles of enantiomers of mandelic acid were investigated and it was found that methanol or ethanol concentration and pH of mobile phase affected retention, selectivity, the saturation capacity, isotherm profile and solubility for

liquid chromatography with Chirobiotic T column. According to this study, retention of the *L*-mandelic acid did not changed significantly with pH, whereas the retention of the *D*-mandelic acid and the selectivity increased with increasing pH up to 6.5. Also, for the mobile phases which contain 60–70% methanol or ethanol in water, methanol or ethanol concentration did not significantly change the retention, but the retention and enantioselectivity increased for mobile phases with 80–100% methanol or ethanol. It was stated in the paper that to prevent changing acid–base equilibrium during separation for high concentrations of mandelic acid, it is necessary to add a buffer to the feed and to the mobile phase. Moreover, triethyl ammonium acetate (TEAA) buffer was proposed because of its volatility and removability from the product. (Jandera et al., 2001)

Capillary electrophoresis was studied for the chiral atenolol, isoprenaline, verapamiland, mandelic acid and effects of concentration of the chiral selector, pH and organic modifier percentage were investigated and optimized by Deeb et al. with the aim of quantifying enantiomeric impurities for chiral drugs. A resolution between 3 and 4 for the racemic mixture was provided during the screening and optimization steps and limit of quantification which is 0.1% (enantiomeric impurity relative to the major constituent) was reached for these four drugs (El Deeb et al., 2008).

Antibiotic eremomycin was combined with coupled chitosan coating and used as a chiral selector for enantioseparation of carboxylic acids which have α-C* asymmetric carbon atom (including mandelic acid as well as ibuprofen, indoprofen, ketoprofen, fenoprofen, flurbiprofen, 3-phenylbutiric, 2 phenoxypropionic, and α-methoxyphenylacetic acids). They have been separated to their

enantiomers and more than 1.04 selectivity was obtained by capillary electrophoresis (Prokhorova, 2010).

Mandelic acid was separated to its enantiomers by forming diastereomeric molecular complexes with *(1R,3S)*-camphoramic acid and randomly distributing in a disordered crystal structure with an approximate molar ratio of 3:1. Therefore, this study resulted as 3:1 separation of enantiomers (Hu et al., 2002).

Solubility, nucleation, and solid-phase transformation in aqueous solutions for enantioseparation of mandelic acid by direct crystallization were studied. It was found that a metastable conglomerate which has reasonable stability that makes possible to separate by direct crystallization could be formed (Profir et al., 2002).

Considering five different chromatographic systems, the influence of different adsorption isotherms were compared based on the achievable productivities using SMB chromatography (Kaspereit et al., 2002). In 2005, By Kaspereit et al. a shortcut method which could be used a tool for design and estimation of hybrid processes was discussed; simulated moving bed (SMB) chromatography and selective crystallisation were combined and demonstrated for resolution of mandelic acid (Kaspereit et al., 2005).

Simulated moving-bed chromotography was also used by Lee et al. using Kromasil TBB (O,O'-bis(4-tert-buylbenzoyl) -N,N'- diallyl-L-tartar diamid) column and purity between 82% to 94% was obtained (Lee et al., 2010).

Cooling crystallization of a non-racemic solution of mandelic acid were discussed by Lorenz et al. in 2006. Moreover, preferential

crystallization was limited to conglomerate forming systems which is possible for only a minor part of chiral substances until a hybrid process with preferential crystallization which could be applied to more common racemic systems was proposed by Lorenz at al. This study contained a preliminary step like chromatographic enantiomeric enrichment and then preferential crystallization which produced the pure enantiomers and racemic mandeic acid was used for a model. The obtained product purities were more than 96% (Lorenz, 2006). By Polenske et al, two different techniques of preferential crystallization which were "seeded isothermal preferential crystallization" and "auto-seeded polythermal preferential crystallization" were developed and for mandelic acid a cyclic auto seeded polythermal preferential crystallization was found as significantly more efficient than the seeded isothermal preferential crystallization (Polenske, 2009). By Elsner et al. Two batch crystallizers which two enantiomers were seeded and crystallized separately were coupled by an exchange of their liquid phases. In that study, higher concentrations of the preferred enantiomers than single batch crystallization were obtained and it was resulted with an increase of the driving forces for the crystallization which enhanced the productivity (Elsner et al., 2009).

Mandelic acid resolution using chiral ligands and chiral selectors was also investigated in the literature. By Tang et al. enantioseparation of mandelic acid by chiral ligand, the distribution behavior of enantiomers in the two-phase system containing copper iron(II) and N-n-dedecane-L-hydroxyproline was investigated. The effect of pH, concentrations of copper iron(II) and chiral ligand, and solvents on partition coefficients and separation factor were studied and found that pH and solvents affect significantly partition coefficients and separation factor. Also, it was stated that Li forms more stable

ternary complex with *D*-mandelic acid than with *L*-mandelic acid (Tang et al.,2004).

A binary chiral selector system with *L*-dipentyl tartrate and beta-cyclodextrin as binary chiral selectors was proposed by Jiao et al. for separation of mandelic acid to its enantiomers and the enantioselectivity was found as 2.1 at optimum concentration of beta-cyclodextrin (Jiao et al., 2007).

Enantiomers of mandelic acid were separated by reaction of the enantiomers and precipitation of the diastereomeric salts simultaneously in a supercritical carbon dioxide environment with *R(+)*-α-methylbenzylamine as the chiral agent. When a partial diastereomeric salt formation and the precipitation was conducted simultaneously the highest resolution efficiency which was e.e = 63% was obtained (Martin et al.,2007).

In the study of Guo et al. L-phenylalanine was used as chiral selector and immobilized on a quartz crystal microbalance (QCM) sensor surface to predict the chiral recognizability of a chiral selector for a racemic mixture. Mandelic acid was also separated by diastereomeric crystallization using *L*-phenylalanine as the resolving agent and effects of molar ratio of mandelic acid to resolving agent, pH, agitation speed, cooling rate and crystallization temperature were discussed. According to the results of this study, the results of the diastereomeric crystallization and quartz crystal microbalance matched. Furthermore, QCM provided a simple solution for screening resolving agents (Guo et al.,2011).

Chiral calix[4]arenes with aminonaphthol were synthesized and used for recognition of chiral carboxylic acids like mandelic acid, *o*-chloro

mandelic acid, dibenzoyl tartaric acid, hydroxyisovaleric acid and 2-chloropropionic acid by Durmaz et al. Two of them, calix[4]arenes 9 and 16 were found suitable to be used as chiral NMR solvating agents to determine the enantiomeric purity of mandelic acid (Aneja et al., 2010).

Bulk liquid membrane (BLM) method consisting a chiral carrier which was complex of di(2-ethylhexyl) phosphoric acid and *O,O′*-dibenzoyl-(2*R*, 3*R*)-tartaric acid (*L*-(-)-DBTA) was developed for the separation of racemic mandelic acid. The effects of resolution time, concentration of racemic mandelic acid, stirring speed and temperature were studied. According to the paper, the greatest separation factor (α) was 2.74 and the enantiomeric excess (ee %) was 46.47%. These results were obtained when the resolution time was about 4 h, the molar concentration ratio of racemic mandelic acid, di(2-ethylhexyl) phosphoric acid and *L*-(-)-DBTA 0.05:0.2:0.3 for stirring speed of 400 rpm and the temperature 35°C (Yang et al.,2009).

Racemic mandelic acid was enantioseparated by native cellulose membrane prepared with cellulose and LiCl in the DMA solution. For 8.1 wt % cellulose and 8.1 wt % LiCl in the DMA solution, over 90% of enantiomeric excess was achieved at the operating pressure as 0.0125 MPa and feed concentration of racemic mandelic acid as and 0.5 mg/ml (Ma et al.,2011).

Pertraction in which a supported liquid membrane applied and preferential crystallization were combined as a hybrid process for chiral separation. The liquid membrane and the selective carrier types were investigated. Usage of tetrahydronaphthalene as liquid membrane and hydroquinine-4-methyl-2-quinolylether (HMQ) as chiral carrier resulted with enantiomeric excesses of 15% in average.

With subsequent preferential crystallization a purity above 96% for *(+)-(S)*-mandelic acid was obtained (Gou et al.,2011).

Enzymes were also used for the resolution of racemic mandelic acid. "Biocatalytic enantioconvergent separation" of racemic mandelic acid was achieved. *(R)*-mandelic acid ester was obtained from racemic mandelic acid by an aqueous/organic two-phase system with two enzymes. Firtsly, a commercial "lipase-catalyzed enantioselective esterification" of racemic mandelic acid in organic solvent and secondly "in situ racemization" of *(S)*-mandelic acid in aqueous phase by recombinant mandelate racemase which was cloned from *Pseudomonas putida* in *E. Coli* were performed. The dynamic kinetic resolution was achieved in a hollow fiber membrane bioreactor. In this study, *(R)*-mandelic ethyl ester was obtained from racemic mandelic acid in 65% yield and with enantiomeric excess of 98% (Choi et al.,2007).

2.4 Enzymes

Enzymes are "life catalysts". Enzymes are protein molecules by which each biochemical reaction in cell is catalyzed specifically. They can be classified as: "oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases" according to the International Union of Biochemistry (IUB). Enzymes are substrate specific catalysts which work efficiently and rapidly at low concentrations and mild pH values and temperatures. Their toxicity is low which is an important advantage. Moreover, inactivating the enzyme is easy to be able to terminate the reaction. The enzymes are also highly biodegradable (Illanes, 2008; Sanchez and Demain, 2011).

"Enzyme activity is a measure of rate at which an enzyme converts substrate to products in a biochemical reaction". Temperature, pH, substrate and enzyme concentration or even vigourous shaking may effect enzyme activity (Stoker, 2010).

Enzymes can be "simple enzymes" and "conjugated enzymes". Simple enzymes are only composed of aminoacid chains whereas conjugated enzymes also contain non-protein parts. An apoenzyme is the protein part and cofactor is the non-protein part of a conjugated enzyme. Holoenzyme is biochemically active conjugated enzyme which includes both apoenzyme and cofactor. Cofactors may be small organic molecules (i.e. coenzyme) or inorganic ions. Substrate is reactant in an enzyme catalyzed reaction (Stoker, 2010).

2.5 *S*-Mandelate Dehydrogenase

(S)-mandelate dehydrogenase (MDH) (EC 1.1.99.31) is an enzyme in the mandelate pathway of Pseudomonads and *Pseudomonas putida* is a microorganism that naturally produces this enzyme. Also *Rhodotorula graminis* and *Acinetobacter calcoaceticus* are source organisms for MDH (Fewson et al., 1988; Fewson et al., 1988). It is a membrane-associated enzyme (Mitra et al., 1993) which converts *(S)*-mandelic acid to benzoylformic acid (phenylglyoxylate) as shown in Figure 2.5. Flavin mononucleotide (FMN) with molecular weight of 456 g/mol is the cofactor of this enzyme (Mitra et al., 1993). Mandelate pathway for *Pseudomonas putida (ATCC 12633)* was shown in Figure 2.4 (Tsou et al.,1990).

The responsible gene for the production of MDH had been cloned and sequenced by Tsou et al. In that study, *(S)*-Mandelate dehydrogenase

was predicted to be 393 amino acids in length and it had a molecular weight of 43352 (Tsou et al.,1990).

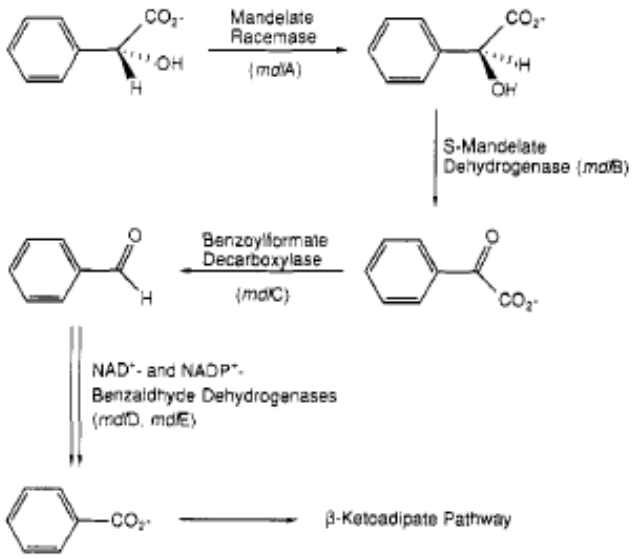


Figure 2.4 Mandelate pathway of *Pseudomonas putida* (ATCC 12633) (Tsou et al.,1990)

R-mandelic acid was reported as the competitive inhibitor of the enzyme (Xu et al.,2002; Lehoux and Mitra, 1999a; Sukumar et al., 2001).

It was found that MDH reaction was reversible and reoxidation of reduced MDH by the benzoylformic acid was catalyzed by MDH (Dewanti et al., 2003). Also, it was stated that MDH reaction had two rate-limiting steps with similar activation energies. These are the formation and breakdown of a distinct intermediate which is formed during FMN reduction reaction and breakdown is slightly more rate limiting (Dewanti et al., 2003).

The seven active site residues in the structure of the enzyme, Tyr^{26}, Tyr^{131}, Asp^{158}, Arg^{165}, Lys^{231}, His^{255}, and Arg^{258} are believed to be important for catalyzing the reductive half-reaction (Sukumar et al., 2004). The surroundings of cofactor FMN were shown in Figure 2.6. FMN structure was given in purple. As shown in Figure 2.6, the ribityl phosphate chain of the FMN makes 13 hydrogen bonds with three main chain atoms, six side chain atoms, and four water molecules whereas flavin ring forms seven hydrogen bonds with one main chain atom, four side chain atoms, and one water molecule. In the figure, hydrogen bond distances are in Å, carbon atoms are black, oxygen atoms are red and nitrogen atoms are cyan. Residues making hydrophobic contact to FMN are indicated as shown in the lower right of figure. One of the hydrogen bonds is between FMN and the side chain of active site residue Lys^{231}. Also, the flavin ring is in close contact (below 4 Å) with four other conserved active site residues, Arg^{258}, Tyr^{26}, Tyr^{131} and His^{255} (Sukumar et al., 2004). Therefore, FMN interacts selectively and non-covalently with the enzyme.

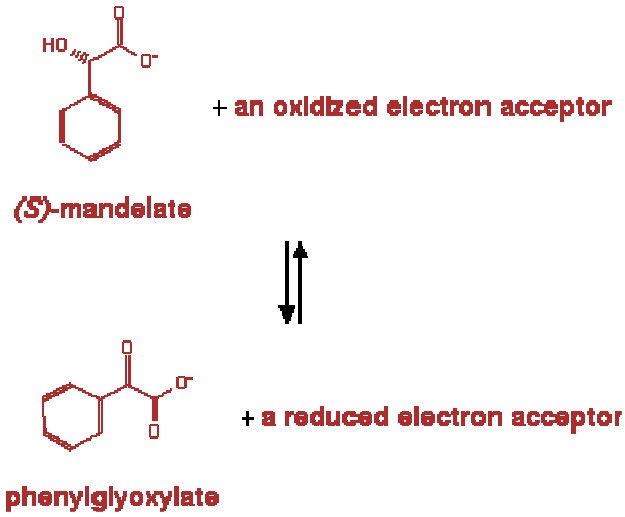


Figure 2.5 MDH reaction

2.6 Enzyme Production and *Escherichia coli*

Enzymes play an important role in the industry. For enzyme production, microbial sources are prefered since enyzmes can be obtained rapidly and cheaply by fermentation. Production levels of enzymes increased with the improvements in recombinant DNA technology (Sanchez and Demain, 2011).

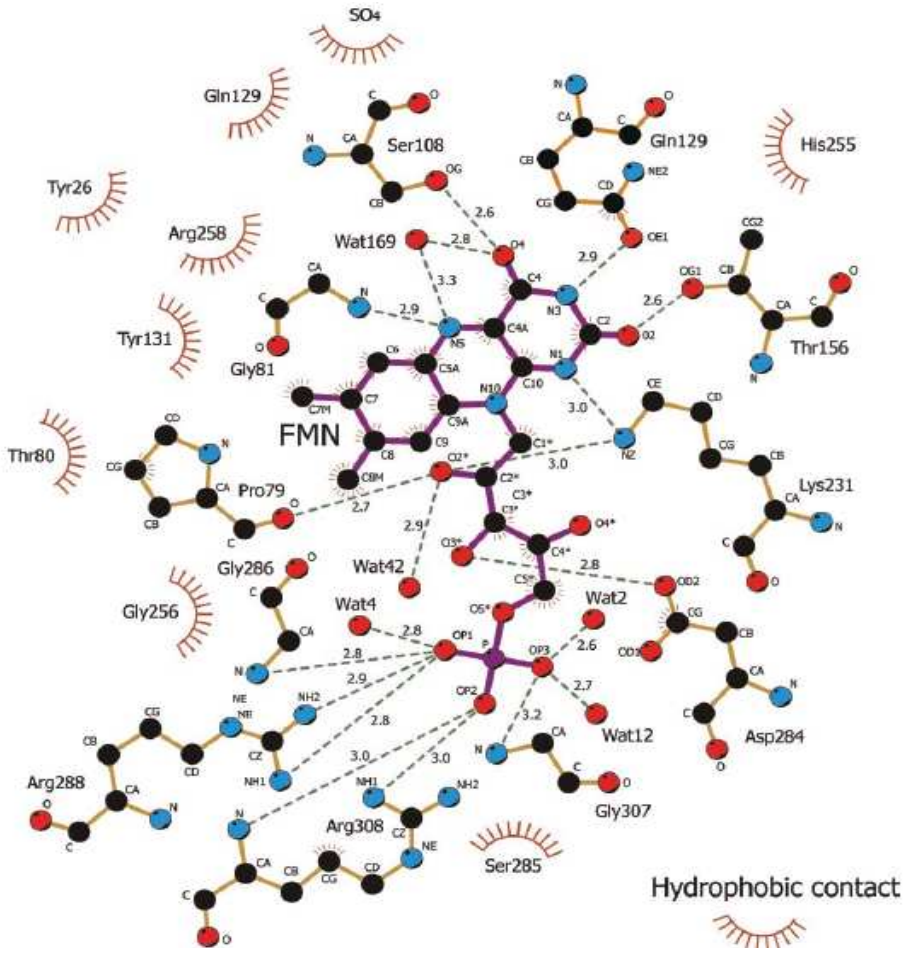


Figure 2.6 Protein environment of cofactor FMN in the oxidized form of MDH (Sukumar et al., 2004).

To increase the yields of products, recombinant technologies are important alternatives to traditional methods. *Escherichia coli*, which is a gram-negative bacterium is preferred with the advantages of genetic feasiblity, being sensitive to antiobiotics to select genetically modified strains, availability of genetic, metabolic information, growing quickly at high density in simple media and the availability of many vectors and mutant strains (Baneyx, 1999; Yu et al., 2010).

Escherichia coli is a widely used microorganism for recombinant enzyme production in the literature for many years and it is still used for this purpose. For example, genes from different microorganisms like *Bacillus licheniformis* for the production of enzyme *β*-mannanase (Songsiriritthigul et al., 2010), *Bacillus sp. MD2* for the production of phytase (Tran et al., 2010), *Geobacillus pallidus* for the production of amidase (Olaofe et al., 2010), *Rhodococcus sp.* for the production of cytochrome P450 monooxygenase (Zhang et al., 2010), *Enterobacter sp.* for the production of ferulic acid decarboxylase (Gu et al., 2011) were cloned and expressed in *Escherichia coli.*

For production of penicillin G acylase by *Escherichia coli* strain DH5α, effect of carbon sources were investigated. Batch cultivations were performed in Erlenmayer flasks of 250 ml at 30°C and 200 rpm in shaker whereas in 3.7 L bioreactor at 30°C and keeping the dissolved oxygen concentration above 30%. Optimum enyzme production with activities of 43385 U/L for shaker and 79880 U/L for biorector was achieved with 45 g/L of dextrin (Cheng et al., 2007).

Effects of amino acids, organic solvents and surfactants were investigated on phenylalanine ammonia lyase activity using recombinant *Escherichia coli* JM109. For the fermentation, two subsequent cultivations were performed in LB medium of 50 ml

working volume with ampicillin at 100 µg/ml concentration at 30°C and 200 rpm for 12 h followed by cultivation in 90 ml medium with the same parameters. Enzymes were induced by cultivating at 42°C for 4 h. The highest enzyme activity was achieved by induction with *L*-tyrosine as a single-inducer and *L*-phenylalanine and *L*-tyrosine as a combination. It was found that by treating the cells with acetone or cetyl trimethyl ammonium bromide, enzyme activity could be enhanced more (Cui et al., 2008).

Although the microorganism *Escherichia coli* has many advantages, it has disadvantage of incorrect folding of synthesized polypeptides and formation of inclusion bodies during high level expression of heterologous recombinant proteins. To recover the products from aggregated state, unfolding with chaotropic agents and refolding were conducted. In the study of Gupta et al., it was found that low temperature and co-expression of bacterial chaperonin GroEL and its co-chaperonin GroES together could enhance the production of active aconitase and prevent aggregation of aconitase (Gupta et al., 2009).

It was found that glycine could enhance the extracellular secretion of recombinant *R*-cyclodextrin glycosyltransferase in *Escherichia coli* BL21(DE3) and the problem of cell growth inhibition induced by glycine can be solved with Ca^{2+}. At 150 mM concentration of glycine, 23.5 U/mL activity at 40 h of culture was achieved (Li et al., 2009).

Effect of inducer IPTG concentration on activity of novel thermostable amidase enzyme was investigated in batch cultivations with glucose based medium using microorganism *Escherichia coli* BL21 (DE3). Amidase activity increased with increasing IPTG concentration up to 400 µM. Induction with 400 µM IPTG at early exponential phase of growth (5th hour) increased the volumetric amidase activity

compared to induction at mid exponential phase (8th hour). However, induction at late lag phase of growth (3rd hour) inhibited cell growth (Olaofe et al., 2010).

A fed-batch exponential feeding strategy for cultures of *Escherichia coli* BL21 (DE3) was provided for production of enzyme 2-Deoxyribose-5-phosphate aldolase. Most efficient growth rate for enzyme production was found as 0.15 h^{-1}. At this specific growth rate, the glucose concentration was kept below 1.0 g/L and acetate concentration which inhibits the cell growth and target protein expression was kept below 0.4 g/L which prevented the inhibition. After 4 hours of IPTG induction, the target protein expression was stable at about 40%. Obtained enzyme concentration with this fed-batch strategy which was 5.12 g/L was 10 time higher than batch cultivation. Also total volumetric productivity (0.256 gL^{-1} h^{-1}) was 5 time higher than batch cultivation. Moreoever, highest enzyme concentration was obtained at highest biomass concentration (Pei et al., 2010).

A flavoprotein human D-amino acid oxidase was over-expressed in *Escherichia coli* BL21(DE3) cells. Volumetric productivity and specific activity of the enzyme were improved by investigating the effect of medium components, time and amount of inducer, pH and production was optimized for about 770 U/L enzyme with a specific activity of 0.4 U/mg protein and a specific productivity of 24.9 U/g biomass (Romano et al., 2009).

The enzyme glutaryl- 7-aminocephalosporanic acid acylase which was naturally produced in *Pseudomonas* N176 was over-expressed in *Escherichia coli* BL21 (DE3) pLysS. The induction was optimized with 0.6 mM IPTG and 25 g/L NaCl and maximum enzyme expression was

obtained using 44 g/L tryptone, 30 g/L yeast extract and 10 g/L NaCl. 80-and 120-fold increase in specific and volumetric enzyme productivity were obtained and 1380 U/g cell and 16,100 U/L of enzyme were produced (Volontè et al., 2008).

S-mandelate dehydrogenase (MDH) is a naturally produced enzyme by *Pseudomonas putida, Acinetobacter calcoaceticus* and *Rhodotorula graminis*. Also, in the literature, MDH gene from *Pseudomonas putida* and *Rhodotorula graminis* was cloned and expressed in *Escherichia coli.* The MDH gene which was cloned from *Pseudomonas putida* (ATCC 12633) was expressed in *Escherichia coli* JM105 using vector pKK223-3. Cells including the recombinant plasmid were grown in LB medium with 50-100 µg/mL ampicillin (Mitra et al., 1993). Moreover, *Rhodotorula graminis* was used as the source microorganism and the MDH gene was expressed to *Escherichia coli* strains TG1, JM109 and NF1 with the plasmids pTZ19r and pTZ18r for cloning. Recombinant microorganisms were grown in Terrific Broth with 150 µg/mL ampicillin (Illias et al., 1998).

CHAPTER 3

MATERIALS AND METHODS

3.1 Metabolic Engineering Experiments

3.1.1 Chemicals

For metabolic engineering experiments, *Taq* DNA polymerase, *Pfu* DNA polymerase, Ribonuclease A (DNase and protease free), T4 DNA ligase, dNTP mixture, ATP, restriction enzymes, *Nde*I, *EcoR*I and their buffers were purchased from Fermentas. Also, GeneJET™ PCR Purification Kit, GeneJET™ Plasmid Miniprep Kit, λ DNA/HindIII Marker and 6X Loading Dye were purchased from Fermentas. Gel elution kit was purchased from GeneMark.

The buffers and the solutions used in metabolic engineering experiments and their preparations were given in Appendix A.

3.1.2 The Microorganisms and Plasmid

The main microorganism that naturally produces the enyzme whose chromosomal DNA was isolated and the gene of interest was amplified was *Pseudomonas Putida* DSM 291 (DSMZ). The host microorganism

for recombination was *E.coli* XL1Blue whereas the plasmid used was pRsetA (Invitrogen Life Technologies).

3.1.3 Chromosomal DNA Isolation from *Pseudomonas Putida*

Chromosomal DNA of *Pseudomonas Putida* DSM 291 was isolated according to the method given below (Kaya, 2006):

1. Pick a single colony from agar plate and inoculate in 30 ml LB medium. Grow for 12h (overnight) at 30°C and 200 rpm,
2. Centrifuge at 6000 rpm and 4°C for 10 min after removing the supernatant, resuspend the bacterial pellet in 5 ml SET buffer,
3. Add lysozyme (1mg/ml) and incubate at 37°C for 90 min to lysate the bacterial cell wall,
4. Add 1/10 volume of 10% SDS, mix and immediately add proteinase-K (0.5mg/ml) over the mixture. Incubate the mixture at 4°C for 2 h,
5. Add 1/3 volume of 5M NaCl and equal volume of chloroform and incubate at room temperature for 30 min with gently shaking,
6. Centrifuge the two-phase mixture at 7100 rpm for 15 min. After phases are separated, take the water phase carefully with micropipette,
7. Precipitate chromosomal DNA by adding equal volume of room temperature isopropanol. Mix and centrifuge immediately at 7100 rpm for 15 min. Decant the supernatant without disturbing the pellet,
8. Redissolve the DNA in a suitable volume of water.

3.1.4 Primer Design

Primers were designed in accordance with the sequence of *mdlB* (MDH) gene (Appendix B). Nucleotide sequence of *mdlB* gene

(Accession no: AY143338.1) was obtained from EMBL-EBI European Bioinformatics Institute (http://www.ebi.ac.uk/). Sequence including the restriction sites for plasmid pRsetA was found using "Invitrogen User Manual for pRSET A, B, and C For high-level expression of recombinant proteins in *E. Coli* (Cat. no. V351-20, 2008)". Restriction enzymes and their recognition sites were determined by the help of Restriction Mapper web-page of ABD Molecular Biology Resources (http://www.restrictionmapper.org). In primer design, following rules were considered:

1. The nucleotide sequence of the primers should agree with the template region of the DNA that will be amplified.
2. Primers should be 18-45 bases in length.
3. The melting temperatures of two primers should have close values.
4. Primer self-complementarities should be avoided.
5. 3' of primers should not be complementary, as otherwise primer dimers can be formed.
6. 3' ends of the primer should end with one or two G or C nucleotides in order to increase correct annealing at the site of addition of bases.
7. The G+C base composition of primer should be at least 43%.

Designed primers were checked using Oligo Analyzer 1.0.2. and synthesized in Alpha DNA.

Designed primers are shown in Table 3.1.

3.1.5 PCR Amplification

PCR amplification was performed using thermal cycling program (Techgene, Flexigene). Optimized PCR process parameters and

components of the reaction mixture are shown in Table 3.2 and Table 3.3, respectively.

Table 3.1 Primers designed for pcr amplification of desired gene fragment

Name	Sequence
Forward primer (MDH)	CGAATTCCTCATGCGTGTGTTCCTTTACCAA
Reverse primer (MDH)	CGCATATGAGCCAGAATCTCTTTAACGTTGAGGACTA

3.1.6 Purification of PCR Products

PCR products were purified using GeneJET™ PCR Purification Kit according to manufacturer's instructions. The products were eluted with proper amount of water.

3.1.7 Restriction Digestion Reactions

Restriction digestion for DNA fragment to be cloned and plasmid vector were performed by incubating DNA fragments with restriction enzymes *Eco*RI and *Nde*I and specified buffers in 20µl final volume at 37ºC for approximately 4h for each restriction enzyme. The composition of the restriction digestion reaction mixtures were

demonstrated in Table 3.4. After restriction digestion was completed, the enzymes were inactivated at 65°C for 20 minutes.

3.1.8 Purification of Restriction Digestion Products

Restriction digestion products were purified using Gel Elution Kit according to manufacturer's instructions. The products were eluted with proper amount of water.

Table 3.2 PCR process parameters

1 cycle	94°C	4 min
1 cycle	94°C	1 min
	50°C	1 min
	72°C	1.5 min
35 cycle	94°C	1 min
	55°C	1 min
	72°C	1.5 min
1 cycle	72°C	10 min
	4°C	∞

3.1.9 Agarose Gel Electrophoresis

PCR products, digested DNA fragments were analyzed by agarose gel electrophoresis with 0.8% (w/v) agarose gel (Sigma) which was supplemented by ethidiumbromide (Sigma) and 1XTBE buffer. DNA samples were mixed with 1/5 volume of 6X loading dye and were applied to the gel. At the end of the electrophoresis, bands were

visualized with a UV transilluminator and photographs were taken using gel imaging and documentation system (UVP Biolmaging Systems, Hamamatsu Digital CCD Camera).

The concentrations of the DNA fragments were determined using UVP Biolmaging Systems Labworks Image Acquisition and Analysis Software.

3.1.10 Ligation Reaction

PCR amplified genes were cloned into suitable expression vectors after restriction digestion with the gene/vector molar ratio 5.

$$amount\ of\ \ vector(ng)\times\frac{size\ of\ \ insert(bp)}{size\ of\ \ \mathrm{vector}(bp)}\times ratio = amount\ of\ \ insert(ng)$$

Table 3.3 Components of PCR reaction mixture

Taq Buffer	5 µL
Pfu Buffer	1 µL
$MgCl_2$	4 µL
dNTP	10 µL
Forward primer (MDH)	1 µL
Reverse primer (MDH)	1 µL
Template DNA	1 µL
Pfu DNA polymerase	0.2 µL
Taq DNA polymerase	1 µL
Sterile dH_2O	To 50 µL

Table 3.4 Restriction digestion reaction mixture

Component	Amount
DNA fragment	5 µl
*Nde*I RE (10U/µl)	1 µl
*Eco*RI RE (10U/µl)	1 µl
Buffer O	2 µl
dH_2O	Up to 20 µl

The ligation reactions were performed by incubating the reaction mixture of 20µl final volume at 16°C for 16h. The composition of the ligation reaction mixture was shown in Table 3.5.

Table 3.5 Ligation reaction mixture

Insert DNA	109 ng
Vector DNA	51 ng
T4 DNA Ligase Buffer	1 µl
ATP	1 µl
T4 DNA ligase	1 µl
dH_2O	To 20 µL

3.1.11 Transformation by $CaCl_2$ Method to *E.coli*

1. Incubate *Escherichia coli* for 24 h in LB-solid medium at 37°C,
2. Pick a single colony and inoculate in 5 ml LB medium. Grow for 12h (overnight) at 37°C and at 200 rpm,
3.Take 1ml sample from the precultivation medium and transfer it into 100 ml- LB medium, incubate at 37C and 200 rpm for 2.5 hours,
4. Transfer 50 ml from medium into sterile polypropylene tubes; and place on ice for 10 minutes,
5. Centrifuge at 4000 rpm at 4°C for 10 minutes,
6. Remove the supernatant; place the tubes on a paper tissue for 1 minute to dry the cells,
7. Add 30 ml of $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$-20mM $CaCl_2$) onto the cells and make a complete solution, set on ice for 10 minutes,
8. Centrifuge at 4000 rpm, 4°C for 10 minutes,
9. Remove the supernatant; place the tubes on a paper tissue for 1 minute to dry the cells,
10. Add 2 ml of 0.1M $CaCl_2$ solution onto the cells and make a complete solution by pipetting up and down, set on ice for 10 minutes,
11. Transfer 200 µl from the solution to eppendorf tubes and add 7 µl from ligation mixture to this solution. Then, place the tubes on ice for 30 minutes,
12. Apply heat-shock to the solution at 42°C for 90 seconds and immediately place the tube on ice for 10 minutes,
13. Transfer the cell suspension to sterile tubes which contains 800 µl of LB medium without antibiotics and incubate at 37°C for 45 minutes with shaking at 200 rpm.
14. Transfer 250 µl from the cultured cells onto LB agar plate with the desired antibiotic. With a sterile glass rod, spread the cells over the

LB agar plate immediately and incubate at 37°C overnight (Kaya, 2006).

3.1.12 Isolation of Plasmid DNA

1. Pick a single colony from agar plate and inoculate in 30 ml LB medium. Grow for overnight at 37°C at 200 rpm,
2. Take 1ml of culture into microfuge tube and centrifuge at 12000 rpm, 4°C, for 30 s,
3. Remove the supernatant and repeat the second step two times,
4. Remove the supernatant and take off all fluid by micropipette; place the tube on ice,
5. Resuspend the bacterial pellet in 100 µl of ice-cold alkaline lysis solution I one by vigorous vortexing. The bacterial pellet should be completely dispersed in alkaline lysis solution I,
6. Add 200 µl of freshly prepared alkaline lysis solution II. Close the tube tightly, and mix the content by inverting the tube rapidly for 5 minutes,
7. Add 150 µl of ice-cold alkaline lysis solution III. Close the tube and disperse alkaline lysis solution III. Place the tube on ice for 5 minutes,
8. Centrifuge the bacterial lysate at 12000 rpm, 4°C, for 5 minutes. Transfer the supernatant to a fresh tube,
9. Add 1/10 volumes of NaAc and 2 volumes of EtOH. Mix the solution by vortexing and place the tube at -20°C for at least 10 minutes,
10. Centrifuge at 12000 rpm at 4°C for 5 minutes and collect the precipitated plasmid DNA,
11. Remove the supernatant gently and place the tube on a paper towel drain away the fluid,
12. Dissolve the plasmid DNA in suitable amount of dH_2O and store the solution at -20ºC (Kaya, 2006).

3.1.13 Purification of Plasmid DNA

Plasmid purification was carried out by using GeneJET™ Plasmid Miniprep Kit according to manufacturer's instructions. The products were eluted with proper amount of water.

3.1.14 DNA Sequencing

The DNA sequencing was performed in Refgen Biotechnology and METU Central Laboratory Molecular Biology-Biotechnology R&D Center using the primers designed.

3.2 Enzyme Production

3.2.1 Chemicals

All chemicals used in enzyme production were purchased from Sigma-Aldrich, Fluka and Merck.

3.2.2 The Microorganism

The recombinant *E.coli* BL21 (DE3) pLySs p*RSET A:: mdIB* was used for enzyme production experiments.

3.2.3 The Solid Medium

The recombinant *E.coli* strains stored in the microbanks (PRO-LAB) were inoculated onto the freshly prepared LB agar plates and were incubated at 37ºC for 24h. Ampicillin (100 mg/ml) and chloramphenicol (35 mg/ml) were used as antibiotics in the agar slants.

3.2.4 The Precultivation Medium

The recombinant *E. coli* strains grown in the solid medium were inoculated into LB Broth medium containing ampicillin (100 mg/ml) and chloramphenicol (35 mg/ml) as the precultivation medium. They were incubated at 37ºC and N=200 rpm in agitation and heating rate controlled orbital shakers (B.Braun, Certomat BS-T) using air-filtered Erlenmeyer flasks 150 ml with working volume capacities of 33 ml. Precultivation was carried out overnight for laboratory-scale bioreactor experiments.

3.2.5 The Production Medium

The microorganisms inoculated in precultivation medium were transfered to the production medium with an inoculation ratio of 1:10. The production experiments were performed in laboratory-scale bioreactor of erlenmeyer flasks of 150 ml with working volume of 30 ml.

For laboratory-scale bioreactor experiments, microorganisms were inoculated at 37ºC with an agitation rate of 200 rpm in agitation and heating rate controlled orbital shakers (B.Braun, Certomat BS-T). At the 4th hour of the production period, isopropyl *β-D*-thiogalactopyranoside (IPTG) to obtain a final concentration of 1 mM was added. Composition of the production medium for laboratory-scale bioreactor is given in Table 3.6.

Table 3.6 Composition of the production medium for laboratory-scale bioreactor (Taşpınar, 2010)

Component	Concentration, kg m^{-3}
Glucose	8.0
Na_2HPO_4	6.7
KH_2HPO_4	3.1
$(NH_4)_2HPO4$	5.0
NaCl	0.5
$MgSO_4.7H_2O$	0.5
$ZnSO_4.7H_2O$	0.2×10^{-5}
$MnSO_4.7H_2O$	0.1
Ampicillin	0.1
Chloramphenicol	0.007

3.2.6 Analysis

During laboratory-scale fermentations, samples were taken with certain time intervals to measure cell concentrations and *S*-mandelate dehydrogenase activities. The medium was centrifuged at 13200 rpm for 10 minutes at 4°C. Precipitated cells were used for the determination of the enzyme activity, and supernatant was stored at -55°C for further analysis.

3.2.7 Storage of the Produced Enzyme

Production medium of 30 ml was centrifuged at 13200 rpm at 4°C for 10 minutes. After removing its supernatant, the precipitated cells

were stored in -55°C. Before each ultrafiltration experiment, these precipitated cells were dissolved in 36 ml distilled water and separated to eppendorf tubes, each of them contained 1.2 ml from solution which contains the cells. The cells in each eppendorf tube were lysed at f=10 s^{-1} for 10 minutes in agitator bead mill with 30% suspension glass beads. Then, all supernatants which contain the produced enzyme was collected and stored. Before using for the ultrafiltrations, this enzyme solution was diafiltrated until a constant permeate absorbance was obtained. The enzyme production steps for EEUF experiments were shown in Figure 3.1.

3.2.8 Determination of Enzyme Activity

Activity assays were performed in 3 ml assay mixture with 0.1 M potassium phosphate buffer, at pH 7.5, with 1 mg/ mL BSA, 1 mM phenazine methosulfate (PMS), 100 μM dichloroindophenol (DCPIP) and 10 mM *S*-mandelate at 20°C and wavelength of 600 nm (Lehoux and Mitra, 2000). One unit of enzymatic activity is defined as one micromole of DCPIP reduced by MDH catalysis at 20°C and pH 7.5 in one second. Sample calculations for enzyme activity was given in Appendix K.

3.3 Ultrafiltration Experiments

3.3.1 Materials

DL-Mandelic acid was purchased from Merck, *S*-mandelic acid and *R*-mandelic acid were purchased from Sigma. Albumin from bovine serum minimum 98% (BSA), benzoylformic acid were also purchased from Sigma.

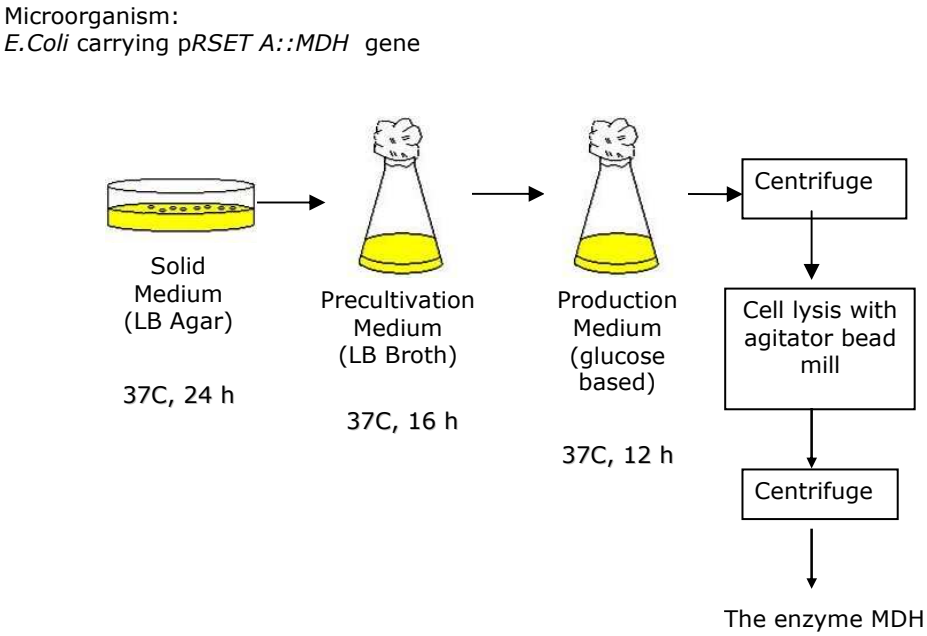


Figure 3.1 Enzyme (MDH) production

For HPLC, triethyl ammonium acetate (TEAA) buffer was purchased from Fluka, methanol was purchased from Merck.

Ultrafiltration membranes utilized were regenerated cellulose (RC) membranes with 10 kDa MWCO (Catalog number: PLGC 02510 and PLGC04710) which were purchased from Amicon, Millipore.

3.3.2 Experimental Setup

Amicon 8010 dead-end stirred membrane cell with a total volume of 10 ml was used for the experiments. In Figure 3.2, exploded view of the stirred ultrafiltration cell was shown.

This cell was operating on a stirrer and was pressurized by means of a nitrogen cylinder. In Figure 3.3 experimental set-up was given.

3.3.3 Operating Conditions

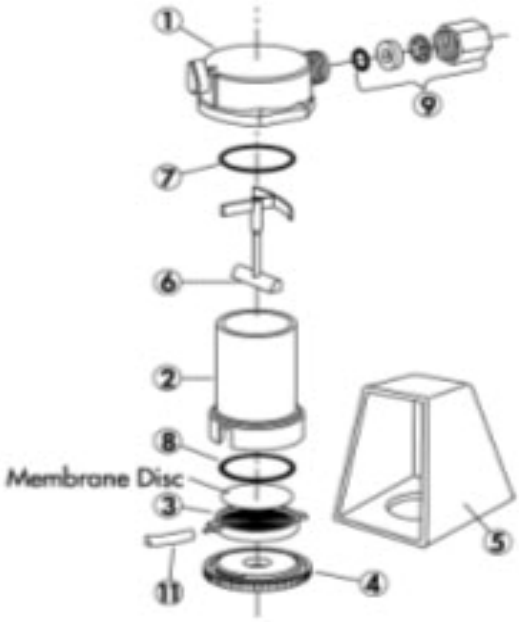
For the membrane cell, the pressure should be below 75 psi (5.2 bar). The system pressure was kept constant at 3 bars for all the experiments. Total solution volume was 10 ml. In Table 3.7 operation conditions were given.

3.3.4 Experimental Procedure for PEUF Experiments

BSA was used as ligand in PEUF experiments. Since mandelic acid and BSA are both soluble in water it was not necessary to add a cosolvent to the feed solutions.

Firstly, BSA solution which would be used in the feed solution was prepared and diafiltrated. Even existence of very small amounts of BSA in permeate interfered with analysis of mandelic acid via UV-spectrophotometer. Therefore a diafiltration procedure with several ultrafiltration steps was applied for BSA solutions. For each ultrafiltration experiment with mandelic acid and BSA mixture, diafiltrated BSA solution was used.

For mandelic acid-BSA mixture experiments, feed solutions of ultrafiltration which contain only racemic mandelic acid and BSA were prepared. BSA concentration was kept constant as 10000 ppm during experiments.



1-Cap assembly

2-Body

3- Membrane Holder

4-Base

5 Retaining Stand Assembly

6- Stirrer Assembly

7- O-ring for cap assembly

8- O-ring

9- Tube fitting Assembly

11- Tubing

Figure 3.2 Exploded view of Amicon 8010 stirred ultrafiltration cell (Blossom Biotechnologies)

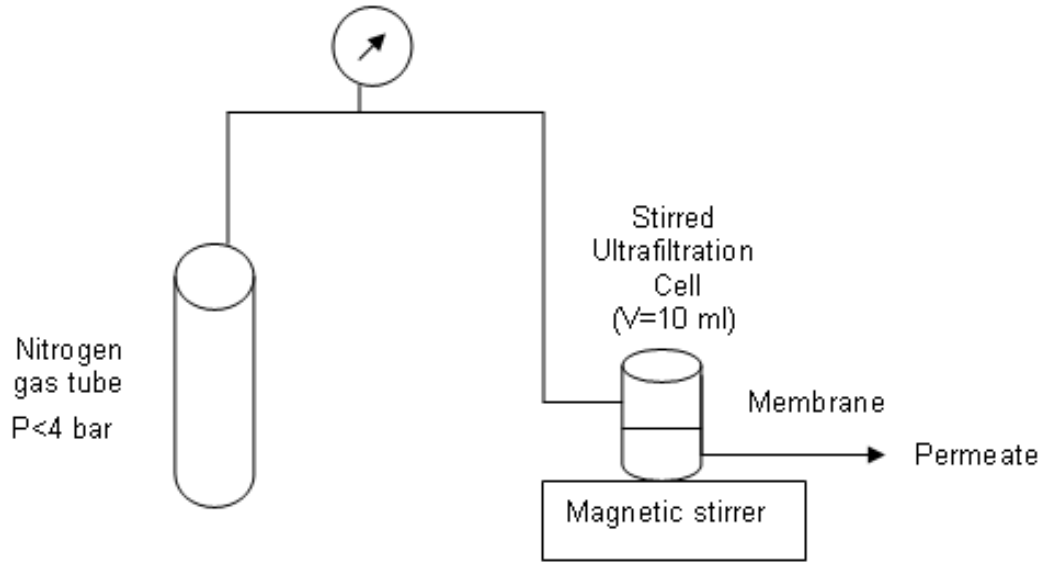


Figure 3.3 Experimental set-up

Table 3.7 Operating conditions

Pressure (bar)	3
Membrane material	Regenerated cellulose
Membrane diameter (mm)	25
Effective membrane area (cm^2)	4.1
Total solution volume (ml)	10
Stirrer rate	300 rpm

After preparing the feed solution, pH was checked for each experiment and adjusted to the necessary pH value for that experiment by using HCl or NaOH solution.

Then the prepared BSA-mandelic acid solution was used for the PEUF experiment. Volume of feed solution was kept at 10 ml and permeate was collected at every 3 ml, three times for each experiment. After ultrafiltration, the membrane was cleaned by filtering 0.1 M NaOH and distilled water respectively. Membrane was stored in distilled water.

3.3.5 Experimental Procedure for EEUF Experiments

Details of enzyme production were given in Section 3.2. The collected and stored enzyme solutions were used for EEUF experiments.

Before EEUF, diafiltration step was applied for the enzyme solution on an regenerated cellulose membrane with 10 kDa MWCO and 44.5 mm diameter which was suitable for removing the cofactor and small MW components in the solution and could retain the enzyme with 43 kDa molecular weight on the retentate side. Pressure and stirrer rate was kept constant at 3 bars and 300 rpm for diafiltration, respectively whereas feed solution volume was increased to 50 ml using Amicon stirred ultrafiltration cell with 50 ml.

The feed solution containing the enzyme was filtered until 15 ml solution remained in the cell. Then, the stirred cell was filled up to its initial volume with distilled water and this procedure continued until the absorbance of the permeate stream at 260 nm in UV-spectrophotometer became constant and very close to 0.0. In the final diafiltration step, necessary amount of distilled water was added to achieve the desired concentration.

For feed solution preparation, enzyme solution was mixed with mandelic acid solution at room temperature and the pH was checked and adjusted if necessary. After preparing the feed solution, EEUF was performed. Volume of feed solution was kept at 10 ml and permeate was collected at every 3 ml, three times for each experiment. To store the membrane, it was cleaned with 0.1 M NaOH and distilled water, respectively and stored in distilled water.

3.3.6 Analyses

3.3.6.1 Flux Measurements

Flow rate was measured for each permeate solution of 3 ml for PEUF experiments, 2.5 ml for EEUF experiments controlling the time period (min) with a chronometer and flux (ml/min) was calculated to control the membrane during ultrafiltration experiments.

3.3.6.2 Determination of Total Mandelic Acid Retention

Total mandelic acid retention in PEUF and EEUF experiments was determined by HPLC (Waters 2695 Separations Module, Waters 2487 Dual λ Absorbance Detector). When a mobile phase with same compositions was used for the analysis of two enantiomers in HPLC for EEUF experiments, peaks for *S*-mandelic acid and *R*-mandelic acid were overlapping. Therefore, for EEUF experiments, permeate samples were analyzed two times, firstly with mobile phase of 30/70; methanol / 1% TEAA for the analysis of *S*-mandelic acid and benzoylformic acid secondly with 10/90; methanol / 1% TEAA for the analysis of *R*-mandelic acid. The HPLC method was given in Table 3.8. Calibration curves were shown in (Appendix E,F,G).

3.3.6.3 Determination of Enantiomeric Excess (ee %) and Enantioselectivity

Enantiomeric excess and enatioselectivity were calculated using Equation 1.1 and Equation 1.3, respectively using results obtained by HPLC as explained in Section 3.3.6.2. For EEUF experiments, MDH catalyzes the reaction with *S*-mandelic acid and apo-MDH was expected to show more affinity to form a complex with *S*-mandelic acid. Therefore, for EEUF experiments *R*-mandelic acid and *S*-mandelic acid were expected to be predominant and minor components in the permeate, respectively.

Table 3.8 HPLC method

Column	Chirobiotic T HPLC Column- 5 µm particle size
Column dimensions	250 x 4.6 mm
System	Reversed Phase Chromatography
Mobile Phase	30/70; Methanol / 1% TEAA (pH=4.0) 10/90; Methanol / 1% TEAA (pH=4.0)
Flow rate	0.5 ml/min
Column temperature	20 ºC
Detector and wavelength	UV, 254 nm
Injection volume	5 µl

CHAPTER 4

RESULTS AND DISCUSSION

In this study, enantioseparation of mandelic acid by enyzme enhanced ultrafiltration (EEUF) was studied. The enzyme *S*-mandelate dehydrogenase was selected to be used for enzyme enhanced ultrafiltration experiments. The enzyme *S*-mandelate dehydrogenase which shows more affinity to bind with *S*-mandelic acid was produced to achieve the enantioseparation of racemic mandelic acid. For this production, the gene which is responsible from the production of *S*-mandelate dehydrogenase was isolated from microorganism *Pseudomonas putida* and expressed in microorganism *Escherichia coli.* Therefore, this study was carried out in three parts: cloning of the gene encoding *S*-mandelate dehydrogenase and expression in *E. coli* BL21 (DE3) pLySs, intracellular production of the enzyme *S*-mandelate dehydrogenase and lastly enantioseparation studies of racemic mandelic acid by EEUF using the produced enzyme.

4.1 Metabolic Engineering Experiments

Recombinant microorganism *E.coli* BL21 (DE3) pLySs p*RSET A:: mdIB* for *S*-mandelate dehydrogenase production was achieved by metabolic engineering techniques.

In order to combine the gene encoding *S*-mandelate dehydrogenase (*mdIB)* in pRSETA and obtain recombinant plasmid, forward primer (MDH) and reverse primer (MDH) were designed. Recombinant plasmid was transformed into *E. coli* XL1-Blue and transformed colonies were selected. Then recombinant plasmid was transformed into *E. coli* BL21 (DE3) pLySs and recombinant microorganism *E.coli* BL21 (DE3) pLySs p*RSET A:: mdIB* which was used for the production of the enzyme was obtained.

4.1.1 Primer Design

Chromosomal DNA of *Pseudomonas Putida* DSM 291 (DSMZ) was isolated and used as template for amplification of the gene encoding *S*-mandelate dehydrogenase. (Appendix B) Forward and reverse primers were designed. While designing the primers restriction enzyme recognition sequences for *Nde*I and *EcoR*I were added. The restriction enzymes that were added to the 5' ends were selected from which were not cutting the *mdIB* gene. Designed primers are shown in Table 3.1. Thermodynamic properties of designed primers and dimer and self-complimentary formation affinities were given in Appendix C.

4.1.2 Polymerase Chain Reaction (PCR) Amplification of *mdIB* gene

By polymerase chain reaction (PCR) with the designed forward and reverse primers, *mdIB* gene was amplified (1195 bp with restriction sites). Both *Taq* DNA polymerase which provides a higher yield and *Pfu* DNA polymerase which provides a higher specificity were used as polymerase enzymes. The critical parameters for PCR, annealing temperature and time which depend on the sequence to be amplified were optimized in terms of yield and specificity. Annealing

temperature was optimized as 50°C for 1 cycle and as 55°C for 35 cycles. Since for 1000 bp, 1 min amplification for each cycle was considered, it was determined to adjust as 1.5 min at 72°C. PCR process parameters and components of PCR mixture were given in Table 3.2 and Table 3.3, respectively.

4.1.3 Ligation of *mdIB* Gene into pRSETA Vector and Transformation in *E. coli XL1BLue*

Reaction parameters are critical for ligation reaction. Concentrations of insert DNA and vector DNA should be optimized. Also, purity of insert DNA and vector DNA are very important. Therefore, both insert DNA and vector DNA were extracted from the agarose gel with Gel Elution Kit to avoid contaminations and impurities. The ligation reaction was performed at two gene/vector ratio, 3 and 5, and ligation reaction was achieved correctly at gene/vector ratio of 5. Ligation mixture components and their amounts were given in Table 3.5.

Ligation samples were transformed into *E. coli* XL1-Blue strain by $CaCl_2$ method described in section 3.1.11. Plasmid DNA isolation was performed as explained in Section 3.1.12 for the selected recombinant colonies. The recombinant plasmids which have higher bands than pRSETA vector on the gel were double digested with restriction enzymes *Nde*I and *EcoR*I and PCR was performed using these plasmids as a template to check whether they were recombined correctly or not. Two of the selected colonies gave correct result according to gel electrophoresis. Figure 4.1 showed the result of this gel electrophoresis image for the better colony from two correct colonies in terms of yield.

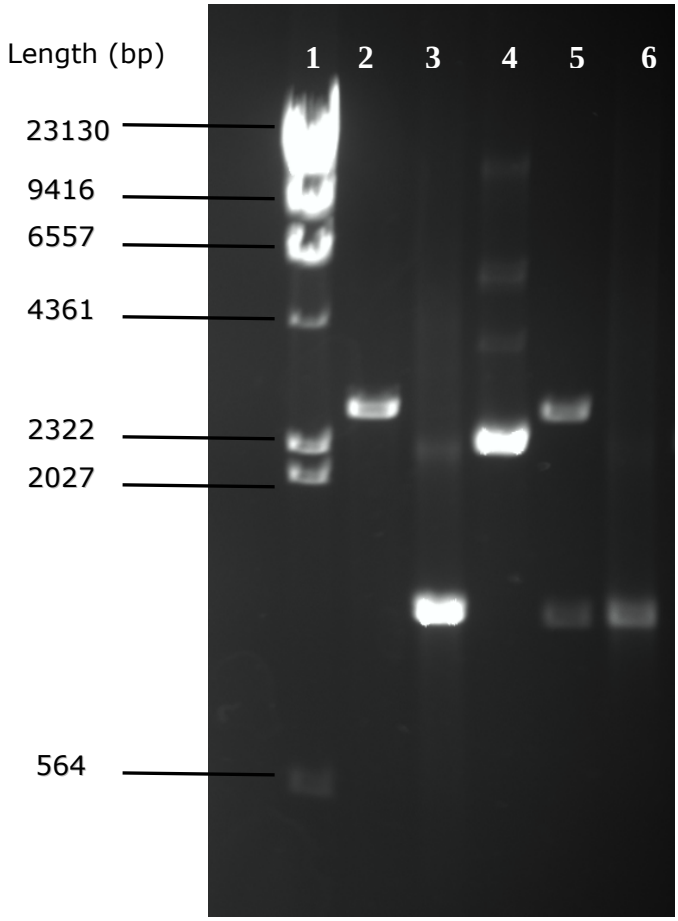


Figure 4. 1 Gel electrophoresis image of the recombinant plasmid before and after restriction digestion and PCR product of r-pRSETA plasmid. 1. λDNA/HindIII Marker; 2. pRSETA vector; 3. *mdIB* gene; 4. r-pRSETA plasmid; 5. r-pRSETA cuttted with *Nde*I and *Eco*RI; 6. PCR product *mdIB* gene amplified from r-pRSETA.

Double digested recombinant plasmid showed that both *mdIB* gene and pRSETA vector were present in the recombinant plasmid since the two bands were seen at the expected lengths. Checking with PCR showed that the correct expected *mdIB* gene was involved in the recombinant plasmid.

4.1.4 Analysis of DNA Sequence

Analysis of gene sequence was further controlled by DNA sequencers by Refgen Biotechnology and METU Central Laboratory Molecular Biology-Biotechnology R&D Center. The results were compared with the part of the DNA sequence of *Pseudomonas putida* which is responsible from the production of S-mandelate dehydrogenase (Appendix B) and proved that the cloning was successful.

Finally, the recombinant plasmid was transformed into *E. coli* BL21 (DE3) pLySs strain as explained in Section 3.1.11 to be used for enyzme production.

4.2 The Enzyme *S*-mandelate Dehydrogenase Production

Laboratory-scale bioreactor experiments were performed with recombinant microorganism *E.coli* BL21 (DE3) pLySs p*RSET A::mdIB* in agitation and heating controlled orbital shaker at 37°C and 200 rpm using air filtered Erlenmeyer flasks of 150 ml with a working volume of 33 ml. The components and compositions of the production medium were chosen as the optimum conditions in the study of Taşpınar (2010), only instead of molasses 8.0 kg m^{-3} glucose was used. Production medium compositions were given in Table 3.6.

4.2.1 Cell Growth Profile

Cell concentrations were measured with UV-Vis Spectrophotometer at 600 nm. Samples taken from the fermentation medium were diluted with distilled water. Cell concentrations, Cx, were calculated using the equation given in Appendix H which converts the absorbance to cell concentration on dry cell weight basis. Cell growth profile of

laboratory-scale bioreactor experiments were given in Figure 4.2 According to the figure, cell concentration increased until t=8h and the highest cell concentration was achieved as 2.4 g dry cell /L at t=8 h where the cell growth was reached to the stationary phase.

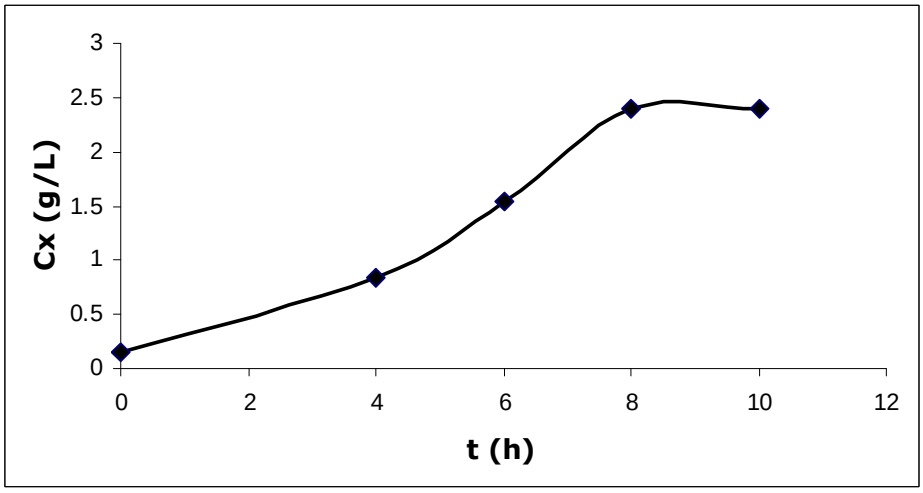


Figure 4.2 The variation in the cell concentration with the cultivation time

4.2.2 *S*-mandelate Dehydrogenase Activity Profile

The variation *S*-mandelate dehydrogenase activity with the cultivation time for laboratory-scale bioreactor experiments was given in Figure 4.3. The maximum *S*-mandelate dehydrogenase activity was obtained at t=8h as 0.0762 U/L and then enzyme activity decreased significantly.

Consequently, when the cell concentration reached its maximum value, maximum enzyme activity was obtained. Also, when cell

growth reached to the stationary phase, enzyme activity started to decrease significantly.

Since cell growth reached to the stationary phase and maximum activity was observed at t=8h, the production media were taken and centrifuged at t=8h to be stored and used for ultrafiltration experiments.

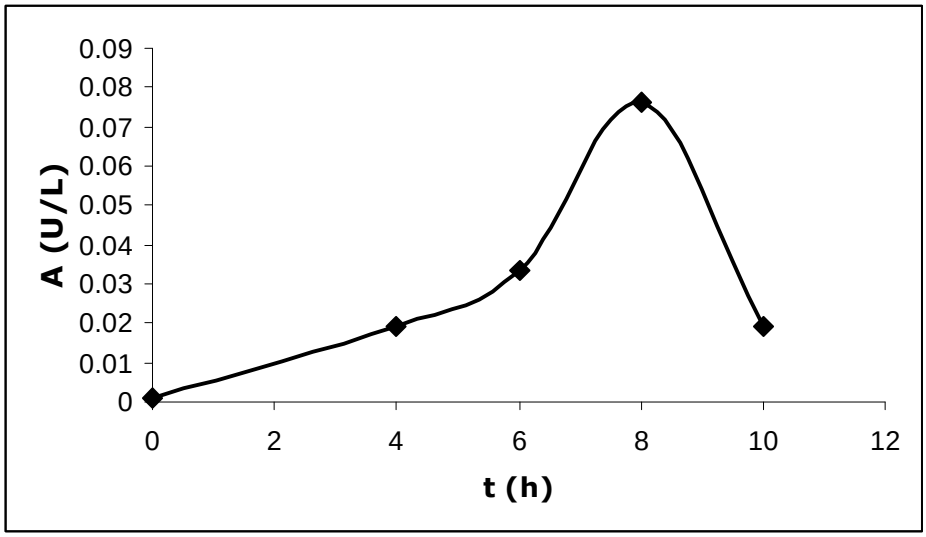


Figure 4. 3 The variation *S*-mandelate dehydrogenase activity with the cultivation time

Also, specific enzyme activity profile was given in Figure 4.4. The maximum specific enzyme activity was calculated for t=8h as $3.2\times10^{-5}\ U/mg\ DCW$.

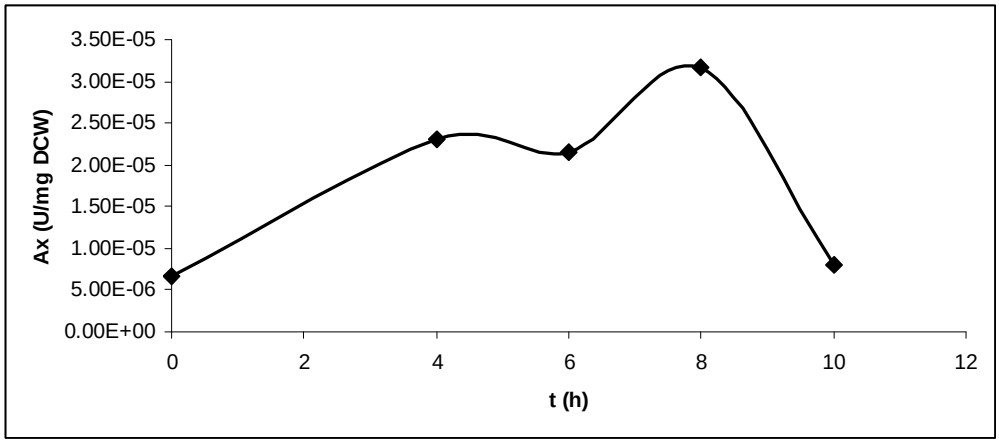


Figure 4.4 Variation in specific benzaldehyde lyase activity with the cultivation time

4.3 Enantioseparation Experiments for Mandelic Acid

4.3.1 Polymer Enhanced Ultrafiltration (PEUF) Experiments

4.3.1.1 Controlling of the Retention of Target Molecule Mandelic Acid by Membrane

BSA is large protein with molecular weight of 66 kDa. Therefore, it should be retained during ultrafiltration which employs a 10 kDa membrane. Nevertheless, BSA retention was checked on regenerated cellulose RC-10 kDa membranes by Ölçeroğlu et al.(2006) and found that BSA retention which was dissolved in water was more than 99.6% for different BSA concentrations at 3 bars and 300 rpm. Also, in the same study, possible adsorption of BSA by the membrane was controlled and no significant adsorption was detected.

In this study, for PEUF experiments BSA was used as ligand whereas mandelic acid was the target molecule. Mandelic acid is a small molecule with molecular weight of 152 g/mol and it is expected to pass from the 10 kDa membrane. However, any possible unwanted retention of mandelic acid via adsorption by regenerated cellulose membrane with MWCO of 10 kDa should be investigated. Therefore, as the first step before PEUF experiments, ultrafiltration experiments were performed with only mandelic acid to see if mandelic acid is retained significantly by membrane or not. Feed, permeate and retentate solutions were analyzed by HPLC. In that ultrafiltration, mandelic acid was dissolved in water, feed concentration was 0.493 g/L, solution volume was 10 ml and the unadjusted pH of feed solution was 3.0. Mandelic acid amounts for permeate and retentate were shown in Table 4.1. According to the results, around 3% of mandelic acid was retained by the membrane which is within experimental error limits of analysis techniques. Therefore it can be concluded that, unlike benzoin (Ölçeroğlu et al.,2006), employed membrane did not adsorb mandelic acid significantly.

Table 4.1 Mandelic acid amounts in feed, retentate and permeate during controlling retention by membrane surface (Regenerated cellulose membrane of 10 kDa, P = 3 bars, N = 300 rpm)

	Mandelic acid mass (mg)
Feed	4.930
Permeate	4.524
Retentate	0.273

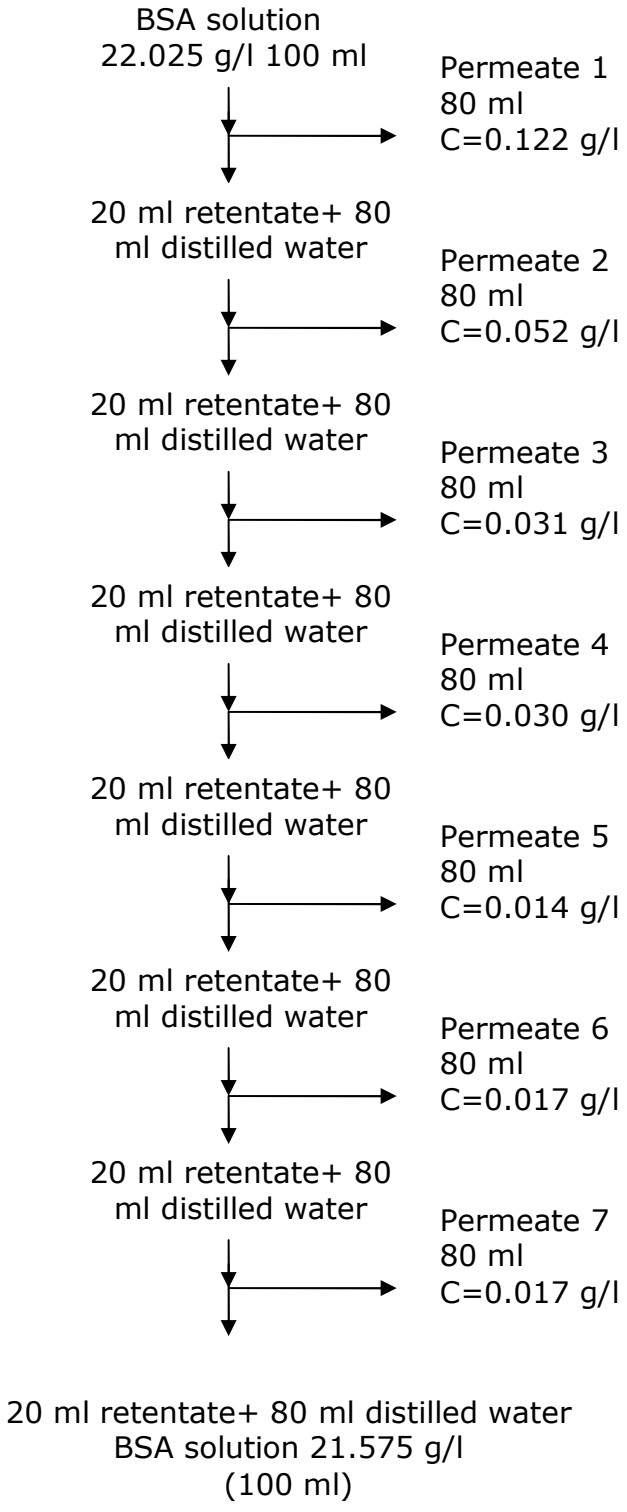


Figure 4.5 BSA Diafiltration steps (Regenerated cellulose membrane with MWCO of 10 kDa, P = 3 bars, N = 300 rpm)

4.3.1.2 Diafiltration of BSA Solutions

When BSA solutions were prepared and ultrafiltrated without mandelic acid, it was seen that permeate absorbances were high at the wavelength (260 nm) which is also detection wavelength of mandelic acid. Therefore a diafiltration procedure with several ultrafiltration steps was applied for BSA solutions until BSA concentration, absorbance at this wavelength, reached to a very low constant value. For each ultrafiltration experiment with mandelic acid and BSA, diafiltrated BSA solution by regenerated cellulose membrane of 10 kDa at 3 bars and with stirrer rate of 300 rpm was used. Figure 4.5 shows a sample diafiltration procedure.

4.3.1.3 Ultrafiltration Experiments with BSA

For mandelic acid-BSA mixture experiments, feed solutions of ultrafiltration contain only racemic mandelic acid and BSA. Mandelic acid is soluble in water, a cosolvent is not necesary while preparing solutions. Therefore mandelic acid and BSA was dissolved only in distilled water and prepared as stock solutions at high concentrations. After diafiltration of BSA solution, they were mixed in proper amounts to obtain the wanted final concentration.

During PEUF experiments, BSA concentration was kept constant at 10 g/L and mandelic acid concentration was changed. Permeates were collected at every 3 ml. First 3 ml of permeate solution collected was named as “Permeate 1”, the second 3 ml of permeate solution was named as “Permeate 2” and the third 3 ml of permeate solution collected was named as “Permeate 3”.

To have a general idea about total mandelic acid retention ultrafiltration samples were first analyzed with UV-spectrophotometer and then HPLC for PEUF experiments. However for the results presented in this study, to determine total mandelic acid retention, enantiomeric excess and enantioselectivity, calibration curves for *S*-mandelic acid, *R*-mandelic acid and BSA obtained by HPLC for PEUF Experiments (Appendix F) were used and ultrafiltration samples were analyzed by HPLC.

4.3.1.3.1 Effect of pH on Total Mandelic Acid Retention

To investigate the effect of pH on total mandelic acid retention, racemic mandelic acid (0.5 g/L) and BSA (10 g/L) mixtures were prepared and their concentrations kept constant whereas pH of solutions have been changed. Results of ultrafiltration experiments obtained with HPLC for feed solutions with same concentrations but different pH values were shown in Figure 4.6.

According to the results, mandelic acid was retained significantly by BSA. At the unadjusted pH of 4.3, total retention was 74.4%. It was found that pH was an important parameter for the total mandelic acid retention by ligand BSA. Total retention of mandelic acid decreased while pH increased, total mandelic acid retention decreased significantly to 38.7% at pH 7.1.

Since BSA is a protein, when the solution pH is moved from its isoelectric point, the conformation of the protein changes. BSA has an isoelectric point at pH=4.7. Because of the electrostatic repulsion of same charges on the surface of the protein, conformation change is favored. At pH values below its isoelectric point, BSA carries a net positive charge whereas at pH values above its isoelectric point, BSA

carries a net negative charge. Also, mandelic acid has a pK value of 3.41 and it has high affinity for protons. Since it was studied at pH values above pK value of mandelic acid, dissociated molecules dominated at these pH values. Therefore, at pH=4.3, which is a pH value below the pI value of BSA, BSA was carrying a net positive charge whereas dissociated molecules dominated for mandelic acid. This may be the reason that the highest total retention of mandelic acid was achieved at that pH. However, other pH values were above the pI value of BSA, the net charge on BSA was negative whereas dissociated molecules dominated for mandelic acid. Low retention values obtained at higher pH values can explained with these interactions.

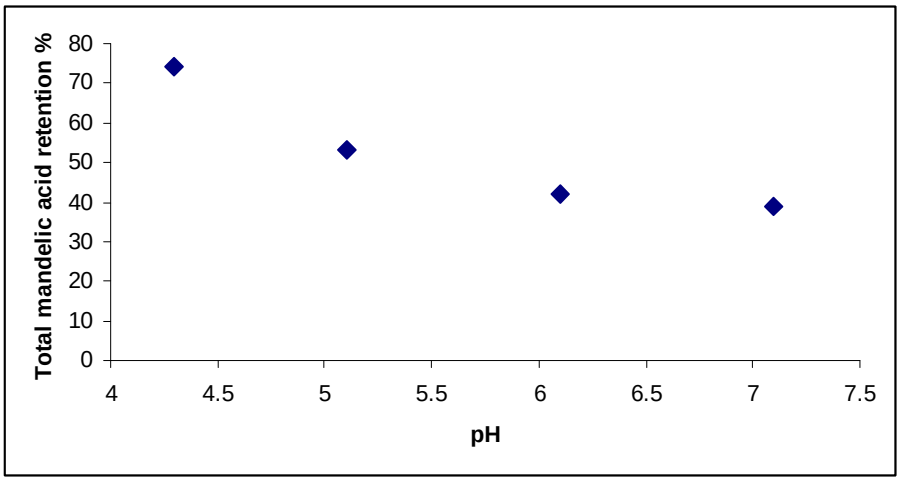


Figure 4.6 Effect of pH on total mandelic acid retention (0.5 g/L MA-10 g/L BSA, Regenerated cellulose membrane of 10 kDa, P = 3 bars, N= 300 rpm)

4.3.1.3.2 Effect of pH on Enantiomeric Excess (ee%) and Enantioselectivity

As explained in Section 1, enantioseparation of racemic mandelic acid is determined by enantiomeric excess and enantioselectivity. Enantiometric excess was calculated using Equation 1.1 and enantioselectivity was calculated using Equation 1.3. Racemic mandelic acid (0.5 g/L) and BSA (10 g/L) mixtures were prepared and pH of solutions have been changed to determine the effect of pH on enantiomeric excess and enantioselectivity. Results of ultrafiltration experiments obtained with HPLC were given in Table 4.2.

According to the results, for 0.5 g/L MA-10 g/L BSA mixture and for all pH values between 4.3 and 7.1, enantiomeric excess values were less than 10%. Enantioselectivity values were around 1.0 which means that BSA did not bind enantioselectively with enantiomers of mandelic acid. Therefore it can be concluded that, in PEUF experiments with BSA, pH showed no effect on enantioseparation of mandelic acid.

4.3.1.3.3 Effect of Mandelic Acid Ligand Ratio on Total Retention, Enantiomeric Excess and Enantioselectivity

To investigate the effect of mandelic acid ligand ratio on total retention, enantiomeric excess and enantioselectivity, different mandelic acid concentrations were tried. To eliminate the effect of pH, results of ultrafiltration experiments with feed solution of 0.25 g/L racemic mandelic acid and 10 g/l BSA with adjusted pH 5.1 and experiment with 0.5 g/L mandelic acid and 10 g/L BSA with pH=5.1 were given in Table 4.3.

According to the results, mandelic acid ligand ratio showed no significant effect on total mandelic acid retention. Also, ligand ratio of mandelic acid did not have an effect on enantiomeric excess and enantioselectivity, i.e, enantiomeric excess values were still less than 10% and enantioselectivities were around 1.0.

Table 4.2 Effect of pH on enantiomeric excess and enantioselectivity (0.5 g/L MA- 10 g/L BSA, Regenerated cellulose membrane of 10 kDa, P = 3 bars, N= 300 rpm)

0.5 g/L MA-10 g/L BSA	**Enantiomeric excess % (ee%)**	**Enantioselectivity**
pH=4.3 (unadjusted)	3.8	1.1
pH= 5.1	1.7	1.0
pH= 6.1	1.1	1.2
pH= 7.1	0.83	1.0

Table 4.3 Effect of mandelic acid ligand ratio on total retention, enantiomeric excess and enantioselectivity (0.5 g/L MA - 10 g/L BSA, Regenerated cellulose membrane with MWCO of 10 kDa, P = 3 bars, N= 300 rpm)

	Total retention %	**Enantiomeric Excess %**	**Enantioselectivity**
0.5 g/L MA-10 g/L BSA	53.3	1.7	1.0
0.25 g/L MA-10 g/L BSA	57.8	6.5	1.1

4.3.2 Enzyme Enhanced Ultrafiltration (EEUF) Experiments

In these experiments, the produced enzyme *S*-mandelate dehydrogenase (MDH) was studied as ligand. All the experiments were conducted with regenerated cellulose membrane with MWCO of 10 kDa at 3 bars and strirring rate of 300 rpm. For each enzyme preparation set, precipitated cells of 30 ml production medium was lysed and the enzyme was prepared in the same way as explained in Appendix K. However, activity was changed in the range of 0.08 U/L and 0.54 U/L. Therefore, lysed cell amount was considered for the enzyme. Mandelic acid concentration was changed if necessary.

To determine the total mandelic acid retention, enantiomeric excess and enantioselectivity, calibration curves of *S*-mandelic acid, *R*-

mandelic acid and benzoylformic acid for EEUF obtained by HPLC were used and ultrafiltration samples were analyzed by HPLC (Appendix G).

4.3.2.1 EEUF Experiments with *S*-Mandelate Dehydrogenase

As explained in Section 1, in EEUF, apoenzymes are used as ligands to enantioseparate the chiral molecules. Therefore, it was necessary to remove the cofactor from the enzyme medium to obtain the enyzme in apo-form. Accordingly, as in the case of Ölçeroğlu et al.(2006) the cofactor was tried to be removed from the medium by diafiltrating at unadjusted pH. In this part of the study, after diafiltrating the enzyme solution at unadjusted pH, racemic mandelic acid was mixed with diafiltrated enzyme and ultrafiltrated. Permeates were collected at every 2.5 ml. First 2.5 ml of permeate solution collected was named as "Permeate 1", the second 2.5 ml of permeate solution which was the subsequent during the ultrafiltration, was named as "Permeate 2" and the third subsequent 2.5 ml of permeate solution collected was named as "Permeate 3".

4.3.2.1.1 pH Effect on Enzyme-Substrate and Enzyme-Inhibitor Binding and Total Retention, Enantiomeric Excess, Enantioselectivity

In the first three ultrafiltration experiments, mandelic acid concentration was kept constant at 0.125 g/L (0.0625 g/L *S*-mandelic acid, 0.0625 g/L *R*-mandelic acid) and measured enzyme activity was in the range of 0.18 U/L and 0.54 U/L. For unadjusted pH (pH=3.5) permeate compositions were given in Table 4.4.

As can be seen from Table 4.4, product of the enzyme which is benzoylformic acid was observed in the permeate which means enzyme was not in its apoenzyme form and could convert *S*-mandelic acid to benzoylformic acid. Moreover, the inhibitor *R*-mandelic acid was also bound to the enzyme since its concentration also decreased.

While determining the total retention, enantiomeric excess and enantioselectivity, produced benzoylformic acid concentration which was seen in the permeate was added to the *S*-mandelic acid concentration since it was produced due to the enzyme reaction by conversion of *S*-mandelic acid. Applying this idea, total mandelic acid retention, enantiomeric excess and enantioselectivity values were calculated as 29.5%, 11.3%, 1.26, respectively.

Table 4.4 Ultrafiltration results with 0.125 g/L mandelic acid and diafiltrated MDH (Feed solution at unadjusted pH- pH=3.5, Regenerated cellulose membrane of 10 kDa, P = 3 bars, N= 300 rpm)

		Concentration* (g/L)
Permeate 1	Benzoylformic acid	0.0168
	S-mandelic acid	0.0259
	R-mandelic acid	0.0537
Permeate 2	Benzoylformic acid	0.0249
	S-mandelic acid	0.0174
	R-mandelic acid	0.0567
Permeate 3	Benzoylformic acid	0.0398
	S-mandelic acid	0.0078
	R-mandelic acid	0.0598

* 10% error due to HPLC analysis

For the same feed solution, feed pH was adjusted to pH=5.0 and ultrafiltration was performed. Permeate compositions for this ultrafiltration were given in Table 4.5.

Table 4.5 Ultrafiltration results with 0.125 g/L mandelic acid and diafiltrated MDH (Feed solution at pH 5.0, Regenerated cellulose membrane of 10 kDa, P = 3 bars, N= 300 rpm)

		Concentration* (g/L) pH=5.0	**Concentration* (g/L) pH=6.7**
Permeate 1	Benzoylformic acid	0.0476	0.0460
	S-mandelic acid	-	-
	R-mandelic acid	0.0517	0.0390
Permeate 2	Benzoylformic acid	0.0529	0.0487
	S-mandelic acid	-	-
	R-mandelic acid	0.0518	0.0447
Permeate 3	Benzoylformic acid	0.0569	0.0611
	S-mandelic acid	-	-
	R-mandelic acid	0.0543	0.0581

* 10% error due to HPLC analysis

As can be seen from Table 4.5, *S*-mandelic acid was totally consumed and converted to the product, therefore benzoylformic acid was produced with increasing concentrations.
For the same feed solution, feed pH was adjusted to pH=6.7 and ultrafiltration was performed. Permeate compositions for this ultrafiltration were also given in Table 4.5. According to the results, benzoylformic acid was still produced and *S*-mandelic acid was totally consumed whereas bound *R*-mandelic acid amount did not change significantly.

Consequently, although cofactor was attempted to be removed by diafiltration to obtain the apoenzyme and conversion was tried to be prevented, enzyme still converted its substrate (*S*)-MA to its product benzoylformic acid especially at high pH values. Reaction efficiency increased when pH increased. At adjusted pH values of 5.0 and 6.7, more benzoylformic acid was produced rather than at unadjusted pH of 3.5. Also at adjusted pH values of 5.0 and 6.7, all the *S*-mandelic acid in the medium was bound to the enzyme and consumed although at pH 3.5 *S*-mandelic acid binding was less. At higher pH values of 5.0 and 6.7 the increase in produced amount of benzoylformic acid and bound and consumed amount of *S*-mandelic acid was expected since the enzyme MDH was more active between pH 5.5 and 9.5 (Dewanti and Mitra, 2003). On the other hand, less R-mandelic acid inhibitor binding occured at pH 6.7 rather than pH 3.5 and pH 5.0.

Moreover, in Table 4.6, summary of pH effect on total retention, enantiomeric excess and enantioselectivity were given. It can be understood from this table that pH did not have an important effect on total retention. For pH values of 5.0 and 6.7, enantiomeric excess and enantioselectivity decreased and enzyme selectivity was reversed

as slightly *R*-selective. This may be because of the change in the structure of the enyzme at higher pH values.

Table 4.6 pH effect on total retention, enantiomeric excess and enantioselectivity (0.125 g/L mandelic acid, Regenerated cellulose membrane of 10 kDa, P = 3 bars, N= 300 rpm)

	Total retention %	**Enantiomeric Excess %**	**Enantioselectivity**
pH=3.5 (unadjusted)	29.5	11.3	1.26
pH=5.0	24.9	2.3 (*R*-selective)	1.05 (*R*-selective)
pH=6.7	25.9	2.5 (*R*-selective)	1.05 (*R*-selective)

However, since the separation of benzoylformic acid from R-mandelic acid was not easier than chiral separation of racemic mandelic acid, it would be meaningless to perform the ultrafiltrations at these pH values.

4.3.2.1.2 Ligand Ratio Effect on Total Retention, Enantiomeric Excess and Enantioselectivity

Mandelic acid concentration was increased to 0.25 g/L, enzyme activity was kept constant in the range of 0.18 U/L and 0.54 U/L and ligand ratio effect on total mandelic acid retention, enantiomeric excess and enantioselectivity were investigated. The results of ligand ratio effect were presented in Table 4.7.

According to the results in Table 4.7, when concentration increased two times, total retention, enantiomeric excess and enantioselectivity slightly increased.

It could be understood from these results that only diafiltrating the produced enyzme solution was not enough to remove the cofactor FMN from the medium. The reason is the existence of the cofactor in the structure of MDH and not being separate from its apoenzyme when it is produced by the microorganism. Because, as explained in Section 2.5, FMN interacts selectively and non-covalently with the enzyme due to hydrogen bonding and hydrophobic interactions. MDH enzyme has seven active site residues and five of them are in interaction with FMN, one of the hydrogen bonds is between FMN and the side chain of one active site residue and the flavin ring is in close contact with four active site residues.

Therefore, to remove the cofactor from the enzyme structure or to inhibit it while it is on the structure and to create the apoenzyme effect, following three methodologies were attempted.

Table 4.7 Ligand ratio effect on total retention, enantiomeric excess and enantioselectivity (Regenerated cellulose membrane of 10 kDa, P = 3 bars, N= 300 rpm, Feed pH=5.0)

	Total retention %	**Enantiomeric Excess %**	**Enantioselectivity**
0.125 g/L Mandelic acid	24.9	2.3 (*R*-selective)	1.05 (*R*-selective)
0.25 g/L Mandelic acid	37.0	8.8 (*R*-selective)	1.19 (*R*-selective)

4.3.2.1.3 Sodium Sulfite Inhibition

Sodium sulfite was reported as a reversible inhibitor for MDH enzyme (Lehoux and Mitra, 1999b). It was stated that sodium sulfite binds covalently with cofactor FMN. Therefore, sodium sulfite was tried to inhibit the cofactor and prevent *S*-mandelic acid from converting to benzoylformic acid. Sodium sulfite was added to enzyme solution (40 mM sodium sulfite) and feed solution was prepared with this enzyme solution and racemic mandelic acid solution. Final mandelic acid concentration in the feed solution was 0.125 g/L, i.e. 0.0625 g/L *S*-mandelic acid, 0.0625 g/L *R*-mandelic acid. Ultrafiltration was conducted with regenerated cellulose membrane of 10 kDa, at 3 bar pressure with stirring rate of 300 rpm and unadjusted pH 8.5, pH 6.2 and pH 4.0 were studied. Permeate was collected at every 2.5 ml for each experiment. In Table 4.8, permeate concentrations and total retention values of ultrafiltration with feed solution of unadjusted pH 8.5 were given.

According to the results in Table 4.8, produced benzoylformic acid concentration significantly decreased when compared to ultrafiltration experiments without sodium sulfite. However, retained and bound *S*-mandelic acid amounts also strongly decreased which means that in that case enzyme did not retain mandelic acid significantly. Therefore, total mandelic acid retention was 13.6% which was a very low value.

Table 4.8 Ultrafiltration results with 0.125 g/L mandelic acid and MDH processed with sodium sulfite, Feed solution unadjusted pH 8.5, Regenerated cellulose membrane of 10 kDa, P = 3 bars, N= 300 rpm)

		Concentration* (g/L)	**Total retention %**
Permeate 1	Benzoylformic acid	0.003	1.9
	S-mandelic acid	0.059	
	R-mandelic acid	0.061	
Permeate 2	Benzoylformic acid	0.006	8.6
	S-mandelic acid	0.048	
	R-mandelic acid	0.061	
Permeate 3	Benzoylformic acid	0.008	13.6
	S-mandelic acid	0.044	
	R-mandelic acid	0.061	

* 10% error due to HPLC analysis

Table 4.9 Ultrafiltration results with 0.125 g/L mandelic acid and MDH processed with sodium sulfite, Feed solution pH 6.2, Regenerated cellulose membrane of 10 kDa, P = 3 bars, N= 300 rpm)

		Concentration* (g/L)	**Total retention %**
Permeate 1	Benzoylformic acid	0.001	-
	S-mandelic acid	0.062	
	R-mandelic acid	0.064	
Permeate 2	Benzoylformic acid	0.001	-
	S-mandelic acid	0.062	
	R-mandelic acid	0.064	
Permeate 3	Benzoylformic acid	0.002	-
	S-mandelic acid	0.062	
	R-mandelic acid	0.063	

* 10% error due to HPLC analysis

pH value of the feed solution was changed to see the effect of pH on ultrafiltrations with MDH which were processed with sodium sulfite. In Table 4.9, the results of ultrafiltration of feed solution at pH 6.2 with 0.125 g/L mandelic acid and MDH processed with sodium sulfite were given.

According to the results in Table 4.9, mandelic acid was not retained by the enzyme at pH 6.2. In Table 4.10 the results of ultrafiltration of feed solution at pH 4.0 with 0.125 g/L mandelic acid and MDH processed with sodium sulfite were given. At pH 4.0, mandelic acid was not retained by the enzyme significantly.

Table 4.10 Ultrafiltration results with 0.125 g/L mandelic acid and MDH processed with sodium sulfite, Feed solution pH 4.0, Regenerated cellulose membrane of 10 kDa, P = 3 bars, N= 300 rpm)

		Concentration* (g/L)	**Total retention %**
Permeate 1	Benzoylformic acid	0.001	4.8
	S-mandelic acid	0.060	
	R-mandelic acid	0.058	
Permeate 2	Benzoylformic acid	0.001	3.1
	S-mandelic acid	0.062	
	R-mandelic acid	0.060	
Permeate 3	Benzoylformic acid	0.001	3.4
	S-mandelic acid	0.063	
	R-mandelic acid	0.060	

* 10% error due to HPLC analysis

Consequently, processing MDH enzyme with sodium sulfite before adding to the feed solution did not provide the desired apoenzyme effect since sodium sulfite prevented the conversion but also prevented the binding of the substrate. This may be due to having interaction between both substrate binding sites and active sites with sodium sulfite when it covalently interacts with FMN. This result may be because of having a highly important place of FMN in the enzyme structure for both substrate binding and activity.

4.3.2.1.4 Diafiltration of MDH Solution at Different pH Values

Obstructing the cofactor by binding it with an inhibitor was tried with sodium sulfite. However, conversion was only prevented, at the cost of not binding to target enantiomer, nullifying our aim. In this section, the binding between the cofactor and the apoenyzme site of the enzyme was tried to be broken by diafiltration of MDH solution at different pH values. The enzyme was diafiltrated at two pH values as pH 3.0 and 10.0 considering the pH limits of the ultrafiltration membrane as pH 3.0 and 13.0 and the maximum enzyme activity between pH 5.5 and 9.5. Firstly, the enzyme was diafiltrated at pH 3.0. Ultrafiltration was performed with regenerated cellulose membrane of 10 kDa, at 3 bar pressure with stirring rate of 300 rpm for the feed solution at pH 6.7 which includes 0.125 g/L mandelic acid. Permeate was collected at every 2.5 ml for each experiment. In Table 4.11, permeate concentrations for this experiment were given.

In this run, total mandelic acid retention, enantiomeric excess and enantioselectivity (*R*-selective) were calculated as 55.3%, 14.0%, 1.32, respectively. When compared to the ultrafiltration results which were performed with MDH diafiltrated at unadjusted pH and feed solution at pH 6.7, it can be concluded that at this experiment significantly less benzoylformic acid was produced whereas less *S*-mandelic acid was bound to the enzyme which showed that conversion was prevented. Also, the enzyme selectivity was reversed as R-selective.

Table 4.11 Ultrafiltration results with 0.125 g/L mandelic acid and MDH diafiltrated at pH 3.0, Feed solution pH 6.7, Regenerated cellulose membrane of 10 kDa, P = 3 bars, N= 300 rpm)

		Concentration* (g/L)
Permeate 1	Benzoylformic acid	9.85×10^{-4}
	S-mandelic acid	0.037
	R-mandelic acid	0.030
Permeate 2	Benzoylformic acid	1.45×10^{-3}
	S-mandelic acid	0.041
	R-mandelic acid	0.030
Permeate 3	Benzoylformic acid	2.11×10^{-3}
	S-mandelic acid	0.044
	R-mandelic acid	0.034

* 10% error due to HPLC analysis

Moreover, total mandelic acid retention increased from 25.9% to 55.3% and enantiomeric excess increased from 2.5% to 14.0% when enzyme was diafiltrated at pH=3.0. Therefore, although enantiomeric excess value was low, the aim of higher total retention value of mandelic acid was achieved.

Secondly, the enzyme was diafiltrated at pH 10.0. Ultrafiltration was performed with regenerated cellulose membrane of 10 kDa, at 3 bar pressure with stirring rate of 300 rpm for the feed solution at pH 6.7 which includes 0.125 g/L mandelic acid. Permeate was collected at every 2.5 ml for each experiment. Permeate concentrations for this experiment were given in Table 4.12.

Table 4.12 Ultrafiltration results with 0.125 g/L mandelic acid and MDH diafiltrated at pH 10.0, Feed solution pH 6.7, Regenerated cellulose membrane of 10 kDa, P = 3 bars, N= 300 rpm)

		Concentration* (g/L)
Permeate 1	Benzoylformic acid	0.014
	S-mandelic acid	-
	R-mandelic acid	0.012
Permeate 2	Benzoylformic acid	0.021
	S-mandelic acid	-
	R-mandelic acid	0.013
Permeate 3	Benzoylformic acid	0.035
	S-mandelic acid	-
	R-mandelic acid	0.015

* 10% error due to HPLC analysis

In this ultrafiltration with MDH diafiltrated at pH 10.0, total mandelic acid retention, enantiomeric excess and enantioselectivity was calculated as 77.2%, 38.9%, 2.27, respectively. As a result of comparison with to the ultrafiltration results which were performed with MDH diafiltrated at pH 3.0, at this experiment more benzoylformic acid was produced and all *S*-mandelic acid was bound to the enzyme and consumed. Also, total mandelic acid retention increased from 55.3% to 77.2% and enantiomeric excess and enantioselectivity showed an important increase to 38.9% and 2.27, respectively when enzyme was diafiltrated at pH=10.0. Moreover, the

enzyme selectivity was reversed as R-selective. However, since an important amount of benzoylformic acid was produced, a significant enantioseparation of mandelic acid was not achieved.

By changing the pH of enzyme solution hydrogen bonds between the cofactor FMN and the apoenyzme site of the enzyme was tried to be broken. With diafiltration at a low pH value of 3.0, hydrogen bonds could be broken which provided the removal of cofactor FMN to a certain degree, so conversion could be prevented. The reason may be the breakage of hydrogen bonds of the enzyme at a low pH value which causes the denaturation i.e. the change of the shape of the enzyme, especially at its active site. Because hydrogen bonding is one of the main factors for conformation of the enzyme. However, reaction continued since FMN could not be removed completely because of its hydrophobic interactions and the strength of hydrophobic interactions is not appreciably affected by changes in pH. Also, since the substrate binding sites might not be effected significantly, binding of substrate might occur or non-specific bindings might be occur for substrate. With diafiltration at a high pH value of pH=10.0, conversion could not be prevented as with diafiltration at pH=3.0. Because breakage of hydrogen bonds was not expected at that high pH value. Also, obtained higher total retention value may be because of not effected conformation and less effected substrate binding sites than in the case of diafiltration at pH=3.0.

4.3.2.1.5 Oxygen Saturation

To keep the cofactor FMN in the reduced form, MDH enzyme and mandelic acid was saturated with oxygen before the experiment and ultrafiltration system was presurized with oxygen instead of nitrogen for that experiment. Other parameters membrane type (Regenerated

cellulose membrane of 10 kDa), operating pressure (3 bar), stirring rate (300 rpm) for the feed solution at unadjusted pH 3.5 which includes 0.125 g/L mandelic acid. Permeate was collected at every 2.5 ml for each experiment. Permeate concentrations for this experiment were given in Table 4.13.

In this ultrafiltration with oxygen saturated mandelic acid and MDH, total mandelic acid retention, enantiomeric excess and enantioselectivity were calculated as 52.0%, 3.6%, 1.07, respectively. If this experiment is compared with to the experiment which was performed without oxygen saturation at unadjusted pH 3.5, at this experiment less benzoylformic acid was produced and less *S*-mandelic acid was bound to the enzyme and consumed. Conversion was prevented but enzyme showed no enantioselectivity. Also, total mandelic acid retention increased from 29.5% to 52.0% wheras enantiomeric excess and enantioselectivity showed a decrease to 3.6% and 1.07 (*R*-selective), respectively.

In this developed methodology of oxygen saturation, the FMN was kept in the reduced form which prevents the reaction. As expected, since binding sites for substrate did not effected like in the case of sodium sulfite inhibition and were available, binding of the substrate occured. However, since the enzyme could not react with its cofactor in the reduced form, the conversion prevented.

Table 4.13 Ultrafiltration results with 0.125 g/L mandelic acid and MDH saturated with oxygen, Feed solution unadjusted pH 3.5, Regenerated cellulose membrane of 10 kDa, P = 3 bars, N= 300 rpm)

		Concentration* (g/L)
Permeate 1	Benzoylformic acid	6.44×10^{-3}
	S-mandelic acid	0.030
	R-mandelic acid	0.034
Permeate 2	Benzoylformic acid	7.35×10^{-3}
	S-mandelic acid	0.034^{-3}
	R-mandelic acid	0.038
Permeate 3	Benzoylformic acid	8.7×10^{-3}
	S-mandelic acid	0.035
	R-mandelic acid	0.041

* 10% error due to HPLC analysis

CHAPTER 5

CONCLUSIONS

In this study, resolution of mandelic acid by enyzme enhanced ultrafiltration (EEUF) was studied.

- Membrane did not retain mandelic acid significantly and a diafiltration procedure was applied for BSA solutions before PEUF experiments.

- In PEUF experiments, pH was an important parameter for mandelic acid retention by ligand BSA and total retention of mandelic acid decreased while pH increased. At the unadjusted pH of 4.3, total retention was 74.4% which was a maximum. However, enantiomeric excess values were less than 10% and pH had no effect on enantioseparation of mandelic acid. Also, mandelic acid concentration had no significant effect on total mandelic acid retention.

- The enzyme *S*-mandelate dehydrogenase was produced to conduct the enantioseparation of racemic mandelic acid by EEUF. For this production, the gene which is responsible from the production of *S*-mandelate dehydrogenase was isolated from microorganism *Pseudomonas putida* and expressed in

microorganism *Escherichia coli.* During the enzyme production by recombinant microorganism *E.coli* BL21 (DE3) pLySs p*RSET A::mdIB,* the highest cell concentration was achieved as 2.4 g dry cell/L at t=8 h where the cell growth was reached to the stationary phase. Also, at t=8h maximum enzyme activity was obtained and after that hour enzyme activity started to decrease significantly.

- In EEUF experiments, due to the existence of cofactor in the enzyme structure, the product benzoylformic acid was formed and existed in permeate solutions which obstructed the enantioseparation of mandelic acid. Increasing the pH of the feed solution increased the reaction efficiency.

- Enzyme conversion was prevented by sodium sulfite inhibition, but enzyme did not retain mandelic acid. By oxygen saturation of enzyme, conversion was prevented, binding was achieved but enzyme showed no enantioselectivity. By diafiltration of enzyme at pH=3.0, conversion was prevented, binding was achieved but enantioselectivity values were very low. By diafiltration of enzyme at pH=10.0, conversion was only prevented to a certain degree but binding was achieved and enantioselectivity increased. When MDH was diafiltrated at pH=10, the highest total mandelic acid retention, enantiomeric excess and enantioselectivity were obtained as 77.2%, 38.9%, 2.27, respectively and enzyme selectivity was reversed as *R*-selective.

CHAPTER 6

RECOMMENDATIONS

Following suggestions should be considered to improve this study for the resolution of racemic mandelic acid by enzyme enhanced ultrafiltration:

- For PEUF experiments, ultrafiltrations at adjusted pH values less than 4.3 should be tried since they will be below the pI value of BSA. However, the pH limit of the membrane which is 3.0 should be considered.

- The enzyme *S*-mandelate dehydrogenase should be purified after taken from the production medium since the production medium may contain cell products which can decrease the enzyme activity.

- Pilot-scale bioreactor experiments should be perfomed and the bioreactor parameters for cell growth and enzyme production should be optimized to obtain higher enzyme activity.

- The interactions between the apoenzyme, the cofactor, the substrate and the inhibitors should be investigated in detail.

- If cofactor FMN concentration can be checked, the observation of the removal or inhibition of the cofactor can be easier.

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APPENDIX A

Preparation of Buffers and Solutions Used in Metabolic Engineering Experiments

Table A-1 Alkaline lysis solution I

Glucose	50 mM
Tris-HCl (pH=8.0)	25 mM
EDTA	10 mM

Table A-2 Alkaline lysis solution II

NaOH	0.2 N
SDS	% 1

Table A-3 Alkaline lysis solution III

Potassium Acetate	5 M
Acetic Acid	11.5 (v/v)

Table A-4 10 X TBE buffer

Tris-base	108 kg m-3
Boric Acid	55 kg m-3
EDTA	9.3 kg m-3

Table A-5 SET buffer

NaCI	75 mM
EDTA	25 mM

Table A-6 TEN buffer

Tris-HCl (pH=7.6)	10 mM
NaCl	10 mM
EDTA	1 mM

Table A-7 TE buffer

Tris-HCl (pH=8)	10 mM
EDTA	1 mM

APPENDIX B

Gene Sequence of MDH Gene

(Accession No: AY143338.1)

atgagccagaatctctttaacgttgaggactatcgcaagcttcggcaaaagcgcttgccg
aagatggtctacgactatctggaaggtggggctgaagacgaatacggggtgaaacacaaccgc
gacgtcttccagcaatggcgattcaaaccgaagcggctagtagacgtcagccgccgcagcctcc
aagcggaagtacttggaaagaggcagtcgatgcctctcttgattgggcctactgggctgaacggt
gcgctgtggcctaaggggatctcgctttagctcgagcagcaaccaaggccggaatcccgttcgt
gctgtcgaccgcctccaacatgtccattgaagacctcgcacgtcagtgtgatggcgatctgtggttc
cagctctatgtgatccaccgagagattgcgcaggggatggtgctcaaagccctgcacactggtta
cacgacactggtgcttactacggatgtggcggttaacggctatcgcgagcgcgacctgcataacc
gattcaagataccaatgagctactccgcaaaggtggtgctggacggatgcctgcatccgcgctgg
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gatggctgcgtgacctctggccgcacaaactcctcgtaaaggggttgctcagtgctgaggacgcc
gatcgtgcatcgctgaaggtgcagacggcgtaatcctatcaaaccacggcggtcgccaactcgat
tgcgcgatatcgccaatggaagttttggctcaatcggtagcgaaaactggaaaaccagtgcttat
cgatagcggcttccgacggggttcggacatcgttaaagcacttgcgctaggtgctgaggctgtact
cctgggtcgtgcaactttgtatggccttgcagcacgaggtgaaacgggtgttgacgaggtgctaa
ccctcctaaaagcggatatcgaccgcacccttgcccagattggatgccctgacatcacctccctttc
tcctgattacctccaaaacgagggagtgactaacaccgctccagtcgatcacctcattggtaaagg
aacacacgcatga

APPENDIX C

Thermodynamic Properties of Designed Primers Together with Dimer and Self-Complimentary Formation Affinities

Table C-1 Thermodynamic properties of designed primers

Name	Length (nt)	GC %	Tm (salt) (ºC)	ΔG (kcal/mol)
Forward primer	31	45,2	71,0	-59,9
Reverse primer	37	43.2	74,5	-67,7

Forward primer

Dimer formation

```
                    5'-CGAATTCCTCATGCGTGTGTTCCTTTACCAA-3'
                       ||||||
3'-AACCATTTCCTTGTGTGCGTACTCCTTAAGC-5'

                                dG: -5,31 kcal/mol
   5'-CGAATTCCTCATGCGTGTGTTCCTTTACCAA-3'
          :    : |||| :    :
3'-AACCATTTCCTTGTGTGCGTACTCCTTAAGC-5'
 dG: -2,17 kcal/mol

       5'-CGAATTCCTCATGCGTGTGTTCCTTTACCAA-3'
          |||  :        :  :::
3'-AACCATTTCCTTGTGTGCGTACTCCTTAAGC-5'
 dG: -0,31 kcal/mol
```

Self-complementarity

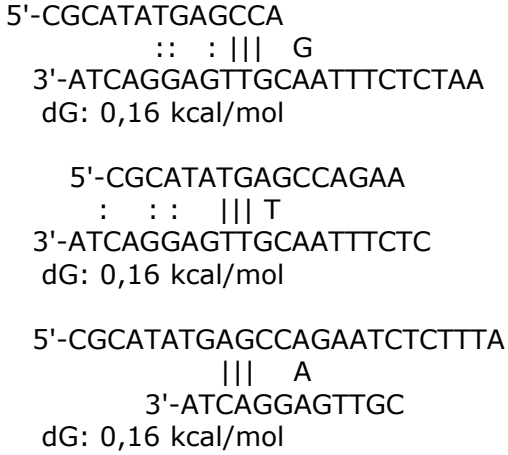
```
5'-CGAATTCCTCA
         |||  :    T
3'-AACCATTTCCTTGTGTGCG
 dG: -0,19 kcal/mol
```

Reverse primer

Dimer formation

```
5'-CGCATATGAGCCAGAATCTCTTTAACGTTGAGGACTA-3'
                :: : |||||| : ::
           3'-ATCAGGAGTTGCAATTTCTCTAAGACCGAGTATACGC-5'
 dG: -6,98 kcal/mol

                     5'-CGCATATGAGCCAGAATCTCTTTAACGTTGAGGACTA-3'
                        : ||||||  :
3'-ATCAGGAGTTGCAATTTCTCTAAGACCGAGTATACGC-5'
 dG: -4,61 kcal/mol

5'-CGCATATGAGCCAGAATCTCTTTAACGTTGAGGACTA-3'
                 : ||||  :
       3'-ATCAGGAGTTGCAATTTCTCTAAGACCGAGTATACGC-5'
 dG: -1,64 kcal/mol

        5'-CGCATATGAGCCAGAATCTCTTTAACGTTGAGGACTA-3'
          ::   : |||      ::: :   ::
3'-ATCAGGAGTTGCAATTTCTCTAAGACCGAGTATACGC-5'
 dG: 0,04 kcal/mol

     5'-CGCATATGAGCCAGAATCTCTTTAACGTTGAGGACTA-3'
         :  : :   ||| :::   : :  :
3'-ATCAGGAGTTGCAATTTCTCTAAGACCGAGTATACGC-5'
 dG: 0,04 kcal/mol

5'-CGCATATGAGCCAGAATCTCTTTAACGTTGAGGACTA-3'
               |||        :::
         3'-ATCAGGAGTTGCAATTTCTCTAAGACCGAGTATACGC-5'
 dG: 0,04 kcal/mol

   5'-CGCATATGAGCCAGAATCTCTTTAACGTTGAGGACTA-3'
     :    : :    |||   :::    : :    :
3'-ATCAGGAGTTGCAATTTCTCTAAGACCGAGTATACGC-5'
 dG: 0,04 kcal/mol
```

Self-complementarity



APPENDIX D

Discrete DNA Fragments of Marker

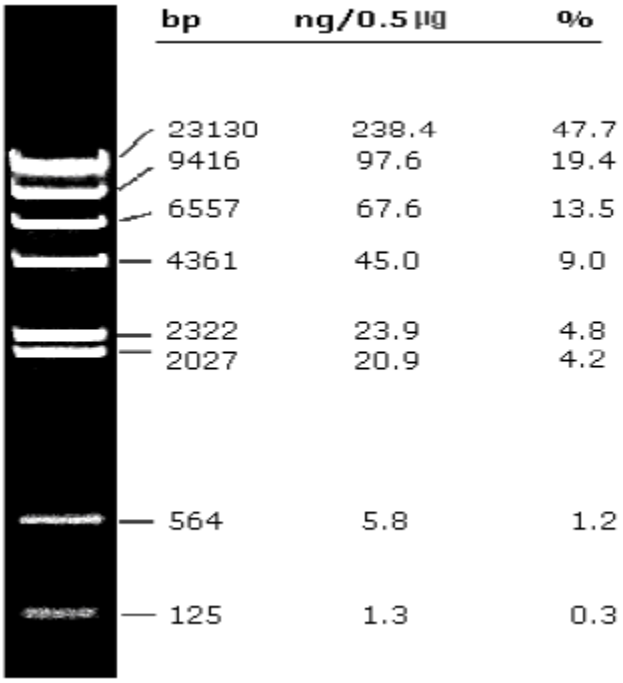


Figure D-1 Discrete DNA fragments of λDNA HindIII Marker

APPENDIX E

Calibration Curves for Spectrophotometric Mandelic Acid and BSA Analysis

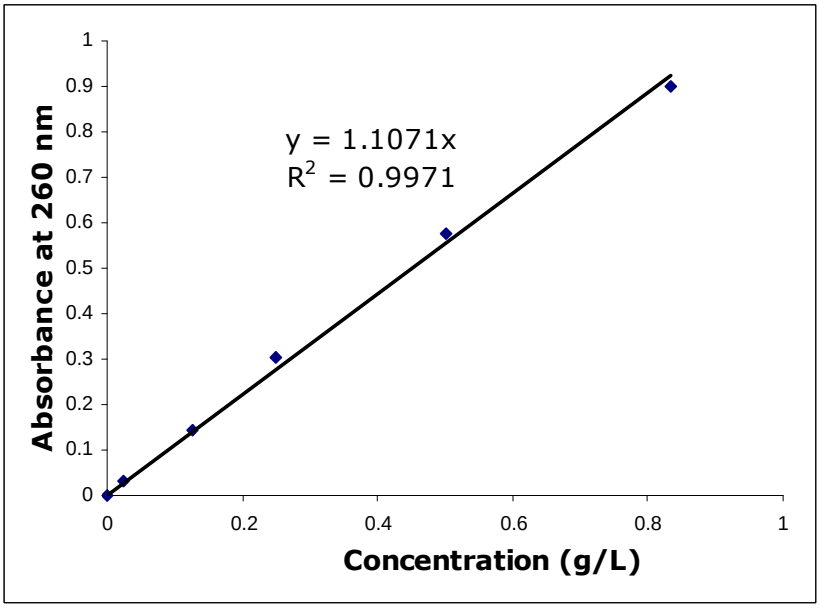


Figure E-1 Calibration curve of mandelic acid for spectrophotometric analysis

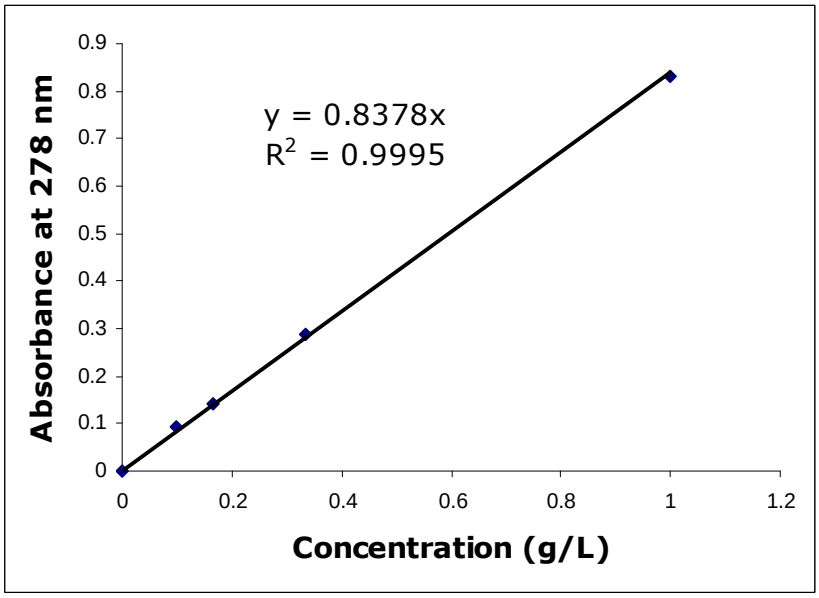


Figure E-2 Calibration curve of BSA for spectrophotometric analysis

APPENDIX F

Calibration Curves for *S*-Mandelic acid, *R*-mandelic acid and BSA Analysis in HPLC for PEUF Experiments

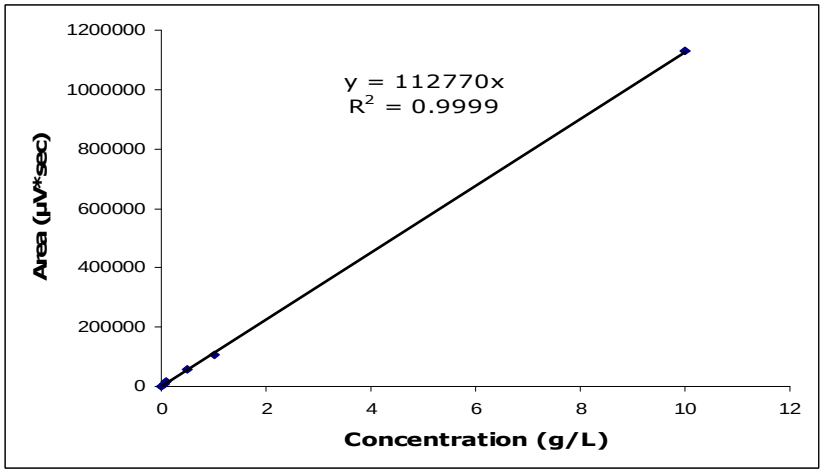


Figure F-1 Calibration curve of BSA for HPLC analysis for PEUF experiments

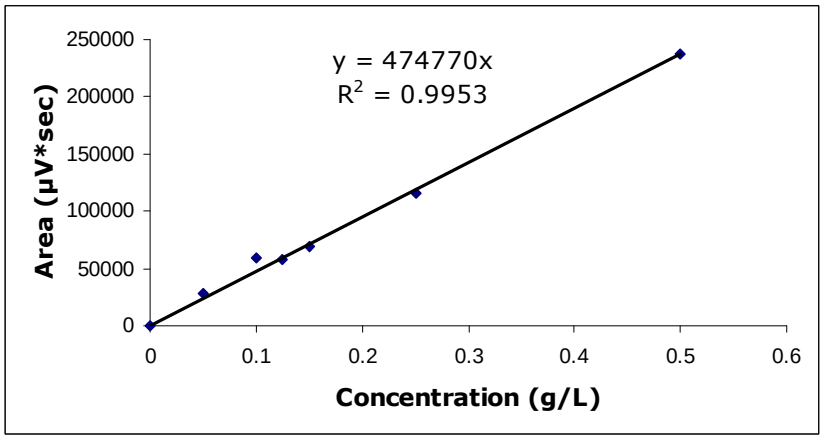


Figure F-2 Calibration curve of *S*-mandelic acid for PEUF experiments (at unadjusted pH)

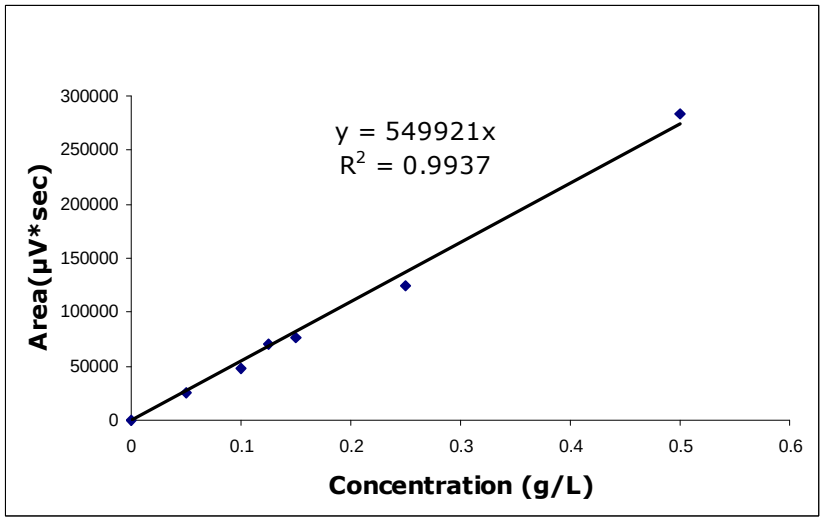


Figure F-3 Calibration curve of *S*-mandelic acid for PEUF experiments (at pH 4.6)

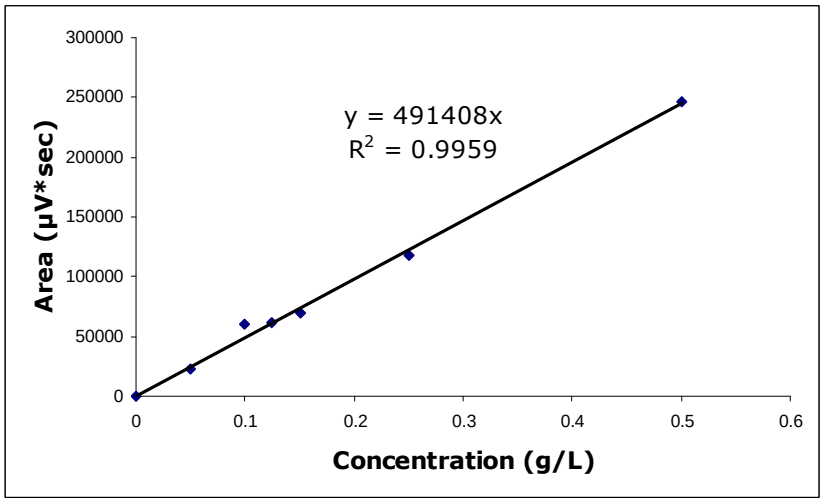


Figure F-4 Calibration curve of *S*-mandelic acid for PEUF experiments (at pH 7.0)

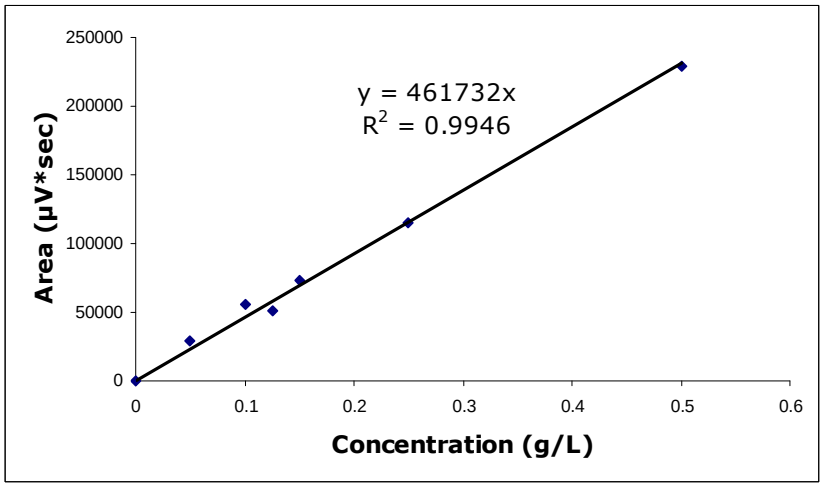


Figure F-5 Calibration curve of *R*-mandelic acid for PEUF experiments (at unadjusted pH)

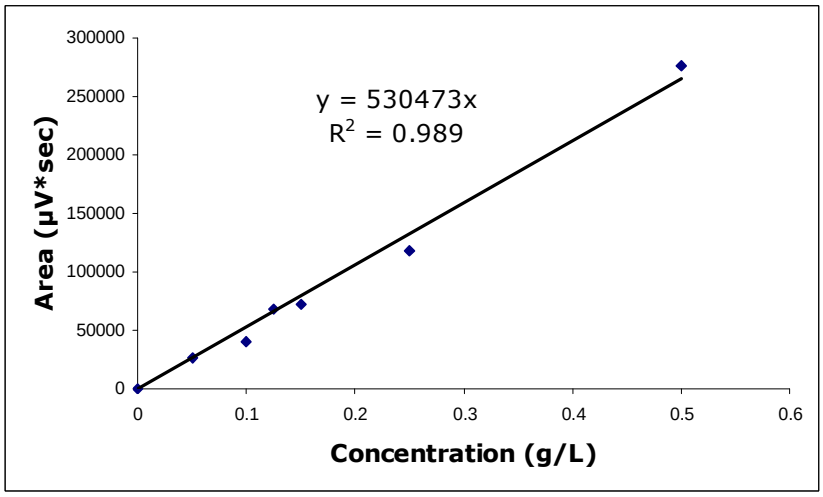


Figure F-6 Calibration curve of *R*-mandelic acid for PEUF experiments (at pH 4.6)

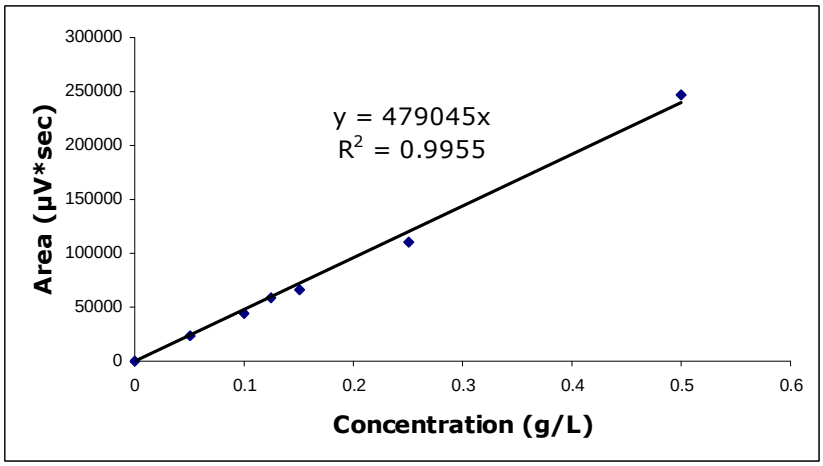


Figure F-7 Calibration curve of *R*-mandelic acid for PEUF experiments (at pH 7.0)

APPENDIX G

Calibration Curves for S-Mandelic acid, R-mandelic acid and Benzoylformic acid in HPLC for EEUF Experiments

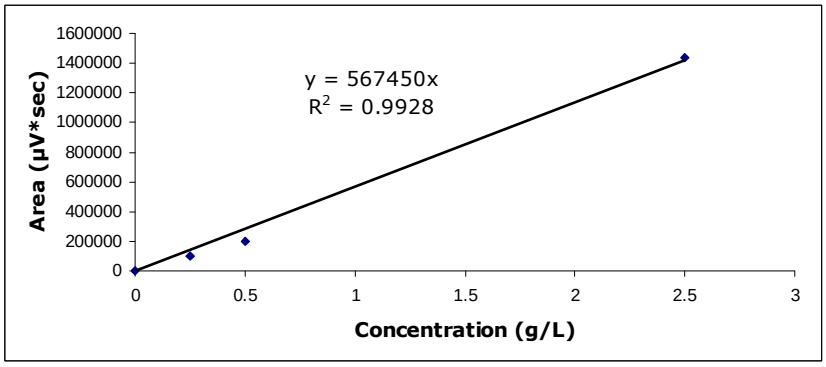


Figure G-1 Calibration curve of *R*-mandelic acid for EEUF experiments

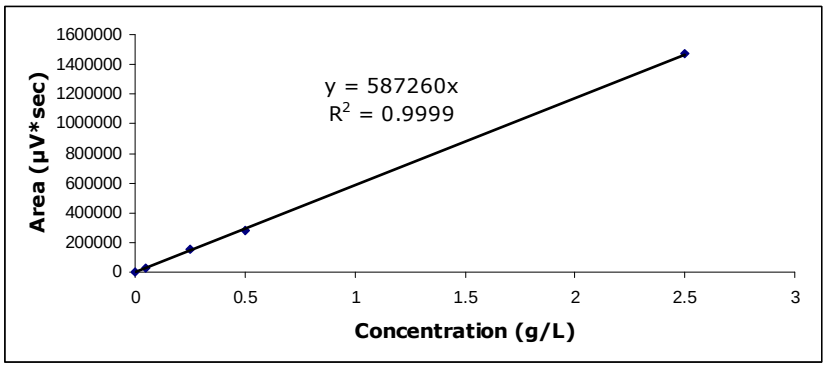


Figure G-2 Calibration curve of S-mandelic acid for EEUF experiments

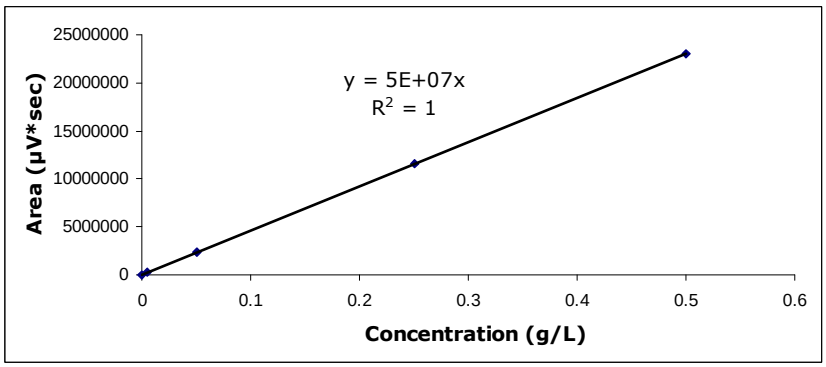


Figure G-3 Calibration curve of benzoylformic acid for EEUF experiments

APPENDIX H

Calibration Curve for Cell Concentration

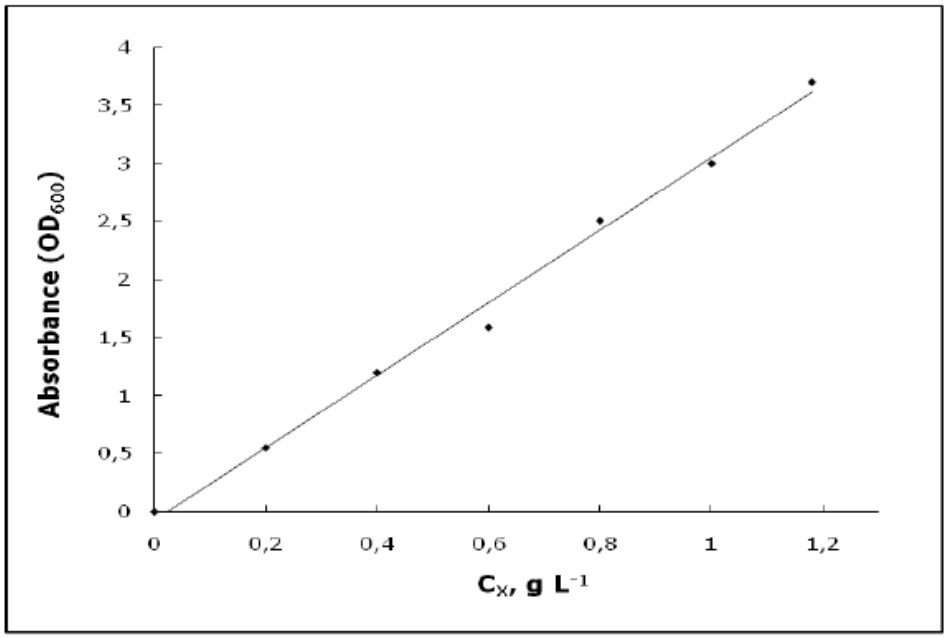


Figure H-1 Calibration curve for *Escherichia coli* cell concentration

$$Slope = 2.8782 \ 1/(\text{g CDW L}^{-1})$$

$$Cell\ concentration\ (C_x) = \frac{OD_{600}}{2.8782}\ x\ Dilution\ ratio$$

APPENDIX I

Calibration Curve for *S*-Mandelate Deyhdrogenase Activity

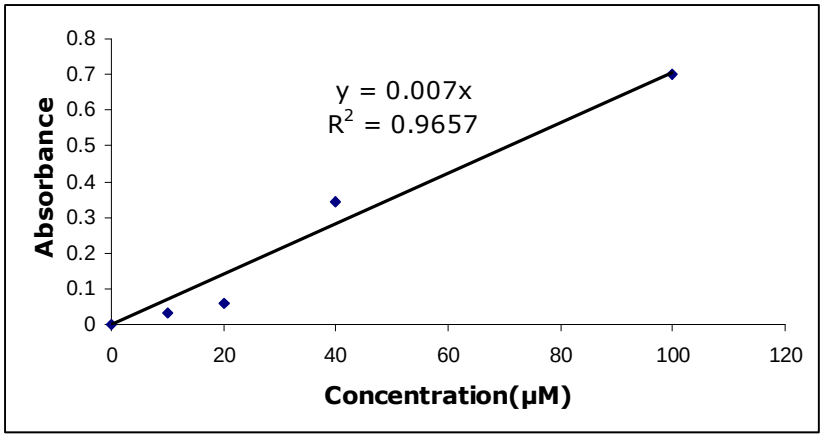


Figure I-1 Calibration curve of *S*-mandelate dehydogenase activity

APPENDIX J

HPLC Chromatograms for EEUF Experiments

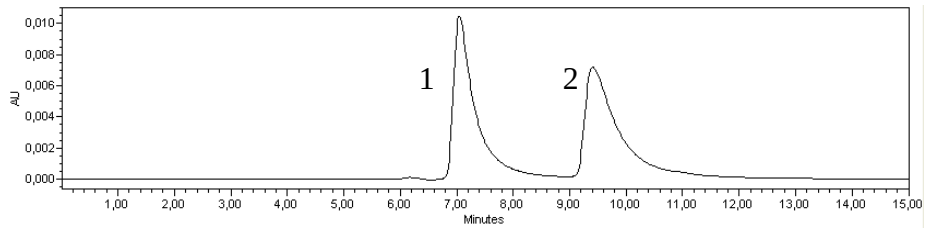


Figure J-1 HPLC Chromatogram of mandelic acid standard for mobile phase 30/70; Methanol / 1% TEAA for the analysis of *S*-mandelic acid (1: *S*-mandelic acid; 2:*R*-mandelic acid)

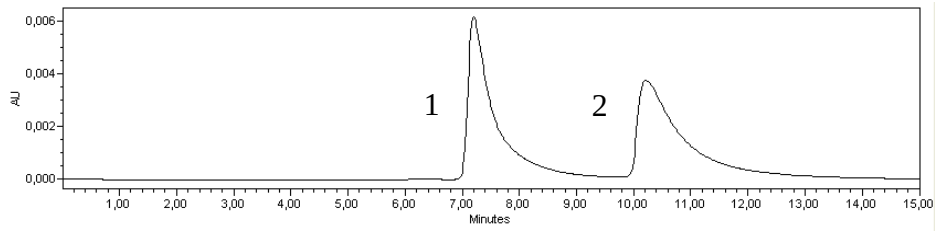


Figure J-2 HPLC Chromatogram of mandelic acid standard for mobile phase 10/90; Methanol / 1% TEAA for the analysis of *R*-mandelic acid (1: *S*-mandelic acid; 2:*R*-mandelic acid)

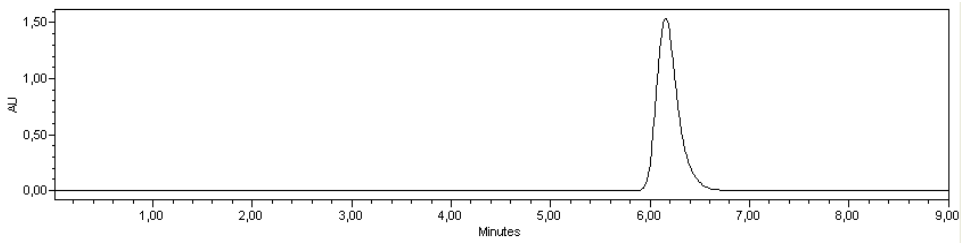


Figure J-3 HPLC Chromatogram of benzoylformic acid standard for mobile phase 30/70; Methanol / 1% TEAA

APPENDIX K

Sample Enzyme Activity Calculations

Production medium of 30 ml is transfered to a centrifuge tube and centrifuged at 13200 rpm at 4°C for 10 minutes. After removing its supernatant, the precipitated cells are stored in -55°C. Before each ultrafiltration experiment, these precipitated cells are dissolved in 36 ml distilled water and separated to 30 eppendorf tubes, each of them contains 1.2 ml from solution which contains the cells. The cells in each eppendorf tube are lysed at f=10 s^{-1} for 10 minutes in agitator bead mill with 30% suspension glass beads. Then, all supernatants which contain the produced enzyme can be collected to one tube to be stored. To calculate the activity of the enzyme produced in this set, for 3 ml assay mixture, 5 µl of this collected enzyme solution is taken. The absorbances at t=0, t=15s and t=30 s are measured at 600 nm with UV-spectrophotometer. A sample result set is given in Table K-1.

Table K-1 Sample results for activity calculation

	Absorbance
t=0	0.415
t=15 s	0.370
t=30 s	0.329

Using the equation in Figure I-1, concentrations are calculated.

At t=0 Concentration=59.3 μM

At t=30s Concentration= 47 μM

$$Activity = \frac{(59.3 - 47)\mu M}{30s} = 0.41\ \mu M / s = 0.41\ U / L$$

Enzyme preparation flowsheet was given in Figure K-1.

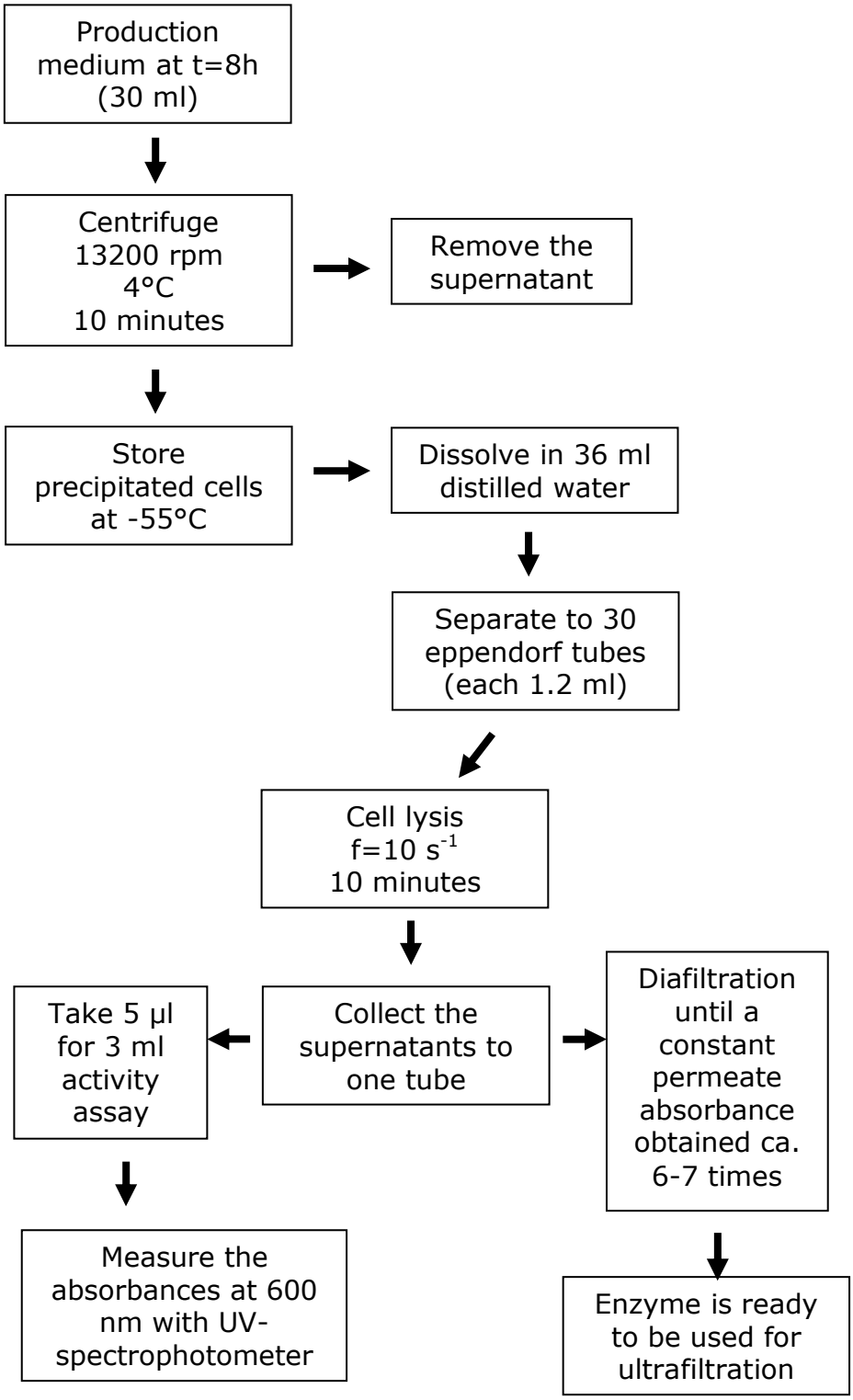


Figure K-1 Enzyme preparation flowsheet