MICROORGANISM MEDIATED STEREOSELECTIVE BIO-OXIDATION AND BIO-HYDROGENATION REACTIONS AND THIAMINE PYROPHOSPHATE DEPENDENT ENZYME CATALYZED ENANTIOSELECTIVE ACYLOIN REACTIONS

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ABSTRACT

MICROORGANISM MEDIATED STEREOSELECTIVE BIO-OXIDATION AND BIO-HYDROGENATION REACTIONS AND THIAMINE PYROPHOSPHATE DEPENDENT ENZYME CATALYZED ENANTIOSELECTIVE ACYLOIN REACTIONS

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In this study various microbial and enzymatic methods developed for enantioselective acyloin synthesis for preparation of some pharmaceutically important intermediates. By performing *Aspergillus flavus* (MAM 200120) mediated biotransformation, enantioselective bio-oxidation of *meso*-hydrobenzoin was achieved with a high ee value (76%). Racemic form of hydrobenzoin was also employed for the same bio-oxidation process and this bioconversion was resulted in accumulation of *meso* form (>90% yield) confirming the suggested mechanism of oxidation-reduction sequence of hydrobenzoin.

Wieland-Miescher ketone (3,4,8,8a-tetrahydro-8a-methylnaphthalene-1,6(2H,7H)-dione) is an important starting material for bioactive compounds like

steroids and terpenoids. Many synthetic approaches include enantioselective reduction of this compound. In this study *Aspergillus niger* (MAM 200909) mediated reduction of Wieland-Miescher ketone was achieved with a high yield (80%), de (79%) and ee (94%) value and these results were found much more superior than previously reported studies.

Carboligating enzymes benzaldehyde lyase (BAL) (EC 4.1.2.38) and benzoiyl formate decarboxilase (BFD) (E.C. 4.1.1.7) are used for biocatalytic acyloin synthesis. These enzymes are immobilized to surface modified superparamagnetic silica coated nanoparticles by using metal ion affinity technique. With this system recombinant histidine tagged BAL and BFD purified and immobilized to magnetic particles by one-pot purification-immobilization procedure. SDS page analysis showed that our surface modified magnetic particles were eligible for specific binding of histidine tagged proteins. Conventional BAL and BFD catalyzed benzoin condenzation reactions and some representative acyloin reactions were performed with this system with a high enantioselectivity (99-92%) and yield. Results obtained with magnetic particle-enzyme system were also found comparable with that of free enzyme catalyzed reactions.

Keywords: *Aspergillus,* alpha-hydroxyketones, BAL, BFD, enzyme immobilization

ÖZ

MİKROORGANİZMALAR İLE STEREOSEÇİCİ BİYO-HİDROJENASYON VE BİYO-OKSİDASYON REAKSİYONLARI VE TİAMİN PİROFOSFAT BAĞIMLI ENZİMLERLE ENANTİOSEÇİCİ AÇİLOİN REAKSİYONLARI

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Bu çalışmada farmasotik açıdan önemli bazı bileşiklerin sentezine yönelik mikrobial ve enzimatik acyloin sentez metotları geliştirilmiştir. *Aspergillus flavus* küfü (MAM 200120) kullanılarak, *mezo*-hidrobenzoinin biyo-oksidasyonu ile yüksek enantioseçicilikte benzoin sentezi gerçekleştirilmiştir (%76). % 13 lük bir verimle oluşan bu dönüşüm rasemik hidrobenzoin için uygulandığında çalışmamızda önerilen ardışık oksidasyon–hidrogenasyon mekanizmasını doğrulayacak şekilde yüksek verimle (>%90) *mezo*-hidrobenzoine dönüşümü ile sonuçlanmıştır.

Wieland-Miescher keton (3,4,8,8a-tetrahidro-8a-metilnaftalen-1,6(2H,7H)-dion) steroid ve terpenoid gibi önemli biyoaktif maddelerin yapıtaşıdır. Birçok organik sentez yöntemi bu temel yapının enantioseçici olarak indirgenmesini de içermektedir. Bu çalışmada *Aspergillus niger* (MAM 200909) kullanılarak

gerçekleştirilen biyotransformasyon ile Wieland-Miescher keton biyokatalitik olarak indirgenebilmiş, optimize edilen koşullarda bu biyodönüşüm literatürdeki sonuçlara kıyasla çok daha yüksek verim (% 80), de (%79) ve ee (% 94) değerleri ile elde edilmiştir.

Mikrobial biyotransformasyonlara ek olarak benzaldehit liyaz (BAL) (EC 4.1.2.38) ve benzoil format dekarboksilaz (BFD) (E.C. 4.1.1.7) enzimleri ile biyo-katalitik açiloin sentezi gerçekleştirilmiştir. Yüksek seçicilik ve verimde C-C bağı oluşum tepkimeleri veren bu enzimler yüzey modifiye silika kaplı manyetik parçacıklar üzerine metal afinite tekniği kullanılarak tutuklanmıştır. Bu yöntem ile rekombinant histidin etiketli BAL ve BFD enzimleri tek basamakta saflaştırılılıp tutuklanabilmiştir. SDS jel elektroforezi sonuçlarına göre yüzey modifiye manyetik parçacıklara histidin etiketli rekombinant proteinler seçici olarak bağlanabilmiştir. BAL ve BFD'nin katalizlediği bilinen benzoin kondenzasyonu ve bazı örnek açiloin tepkimeleri bu yeni tutuklanmış sistem ile yüksek enantioseçicilik (99-92%) ve verim ile gerçekleştirilebilmiş ve elde edilen sonuçlar, serbest enzim reaksiyonlarına ait değerler ile kıyaslanabilir bulunmuştur.

Anahtar kelimeler: *Aspergillus*, α-hidroksiketon, BAL ,BFD, enzim tutuklama

To My Mother

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CHAPTER 1

INTRODUCTION

1.1. Chirality

Optically active molecules are chiral molecules in the way that they are not superimposable as their mirror image like left hand is not superimposable to its mirror image right hand (figure 1.1). This type of central atoms in such atomic constellations is known as asymmetric center or chiral centers and molecules contains such asymmetric center are called as 'chiral molecules' [1]. These stereoisomers that can not be superimposed on each other are called enantiomers of the molecule. Enantiomeric molecules are phisically indistinguishable. But with reactants which are also chiral and selective can be reacted with those molecules to distinguish them. Another method to distinguish the enantiomers is exposing plain-polarized light [1]. Enantiomers rotate polarized light in reverse directions.

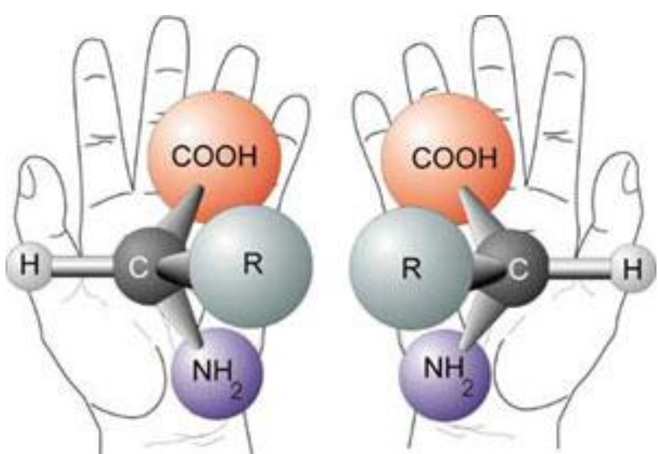

Figure 1.1 Schematic representation of a two enantiomer which are nonsuperimposable mirror image [2]

Classification of a stereoisomer is an important and sometimes ambigious issue. Commonly used nomenclature system was introduced by Robert Cahn, Cristopher Ingold and Vladimir Preog in 1956 [2]. In this system the four groups surrounding the chiral center are ranked according to a certain and arbitrary priorty scheme. If the order of the groups through the lowest priority is seen from this side as clockwise , than the asymmetric center is designated as (*R*), if the order is counterclockwise, the asymmetric center is then designated as (*S*). Major advatage of so-called Cahn-Ingold-Prelog or (*RS*) system is that the chirality of compounds with multiple asymmetric center is described unambiguously.

The enantiopurity of a chiral compound is a very impotant issue especially for their bioactivity and is used as one of the indicators of the success of an asymmetric synthesis.The degree of enantiomeric purity of a solution is measured by its enantiomeric excess. It is defined as the absolute difference between the mole fractions of each enantiomer. In practice, it is most often expressed as a percent enantiomeric excess % ee. For mixtures of diastereomers, there are analogous definitions and uses for diastereomeric excess and percent diastereomeric excess.

Ideally, the contribution of each component of the mixture to the total optical rotation is directly proportional to its mole fraction, and as a result the numerical value of the optical purity is identical to the enantiomeric excess. This has led to informal use the two terms as interchangeable, especially because optical purity was the traditional way of measuring enantiomeric excess. However, other methods such as chiral column chromatography and NMR spectroscopy can now be used for measuring the amount of each enantiomer individually.

1.1.1. Importance of Chirality in Synthesis of Bioactive Molecules

In organic chemistry enantiomerism is genarally coused by the presence of C atom bound to four different groups. Such property of a carbon atom in a molecule is very important in living systems since all biochemical events taking place in an organism are governed by enzymes and enzymes are made from chiral elements L-amino acids. Therefore enantiomers may have different or advers effect on biologicial function in which many diffferent enzymes involve.

The production of single enantiomers of chiral intermediates one of the most expanding fields in organic chemistry since it was recognized in the 1960s that one enantiomer of a chiral compound (thalidomide, a drug prescribed for pregnant women) can be potent drug, whereas the other can be strongly poisonous (Figure 1.2). Both enantiomers of this substance have the desired sedative effect, but the (-)-enantiomer also caused fetal deformities.

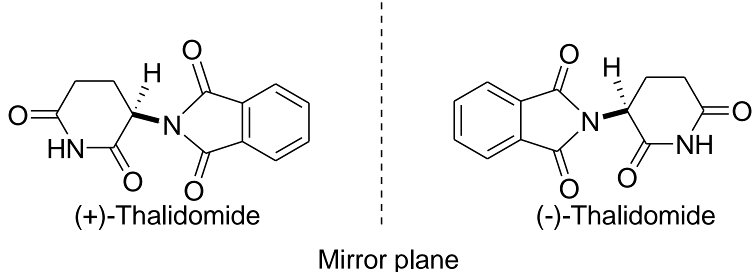

Figure 1.2. The structure of thalidomide

The targets of pharmaceutical compounds such as cell-surface receptors or enzymes are chiral biomolecules. As in the case of thalidomide one of the given compounds can be strongly poisonous, or in some cases does not exert the desired effect. The isomer with highest biological activity is denoted as “entomer” whereas its counterpart possessing less or even undesired effect is termed as the “distomer” [3]. The range of effects derived from the distomer can extend from lower activity, no response or toxic events (figure 1.3)

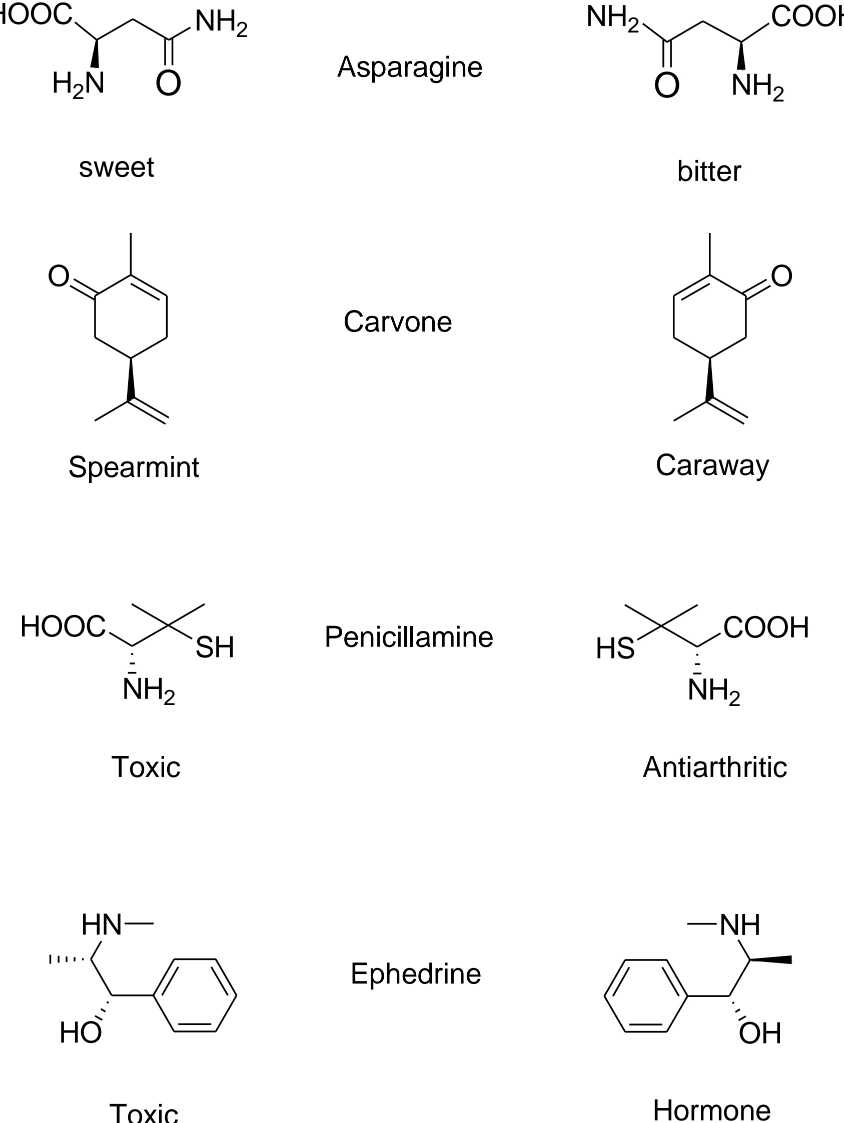

Figure 1.3. Enantiomers of some chiral molecules having different biological effects [3]

The demand for new chiral pharmaceutical reagents is increased since the discovery of potential poisonous effects of stereoisomers. This also forced the FDA (USA food and drug administration) to require the evaluation of both enantiomeric forms. Some examples are amoxicillin (an antibiotic), captopril (an inhibitor of the angiotensin-converting enzyme [ACE]) (figure 1.4). The worldwide sales volume for single enantiomer drugs already exceeds $100 billion [4]. In

2001, 35 % of the intermediates were chiral and this number expected to be 70% by 2010 [5]

Captopril Amoxicillin

Figure 1.4. Structure captopril and amoxicillin

1.2. Biotransformation

The industrial or ‘White Biotechnology’ comprises the production of a variety of different chemical compounds using living systems generally microorganisms. The tools of a white biotechnology are whole cells and enzymes that catalyze the specific reactions. Using of biological sources in synthesis/conversion of substances of industrial interests is also called as ‘Biotransformation’.

The ‘green chemistry’ concept as proposed by P.T Anastas in 1991 includes the design, development, and application of chemical processes and products in such way that are not hazardous to human health and environment [6]. Biotransformations that employ either enzymes or whole cells are well suited for green chemistry because mild reaction conditions applied in these reactions by lowering the energy requirements; fewer by-products are formed as they are highly chemo-, regio-, and stereoselective, and multistep chemical synthese can be achieved within just a few steps via biocatalysis [6]. Use of biocatalysts also minimizes the problems of isomerization, racemization, epimerization and rearrangement that are common in chemical processes [3]. Furthermore, immobilized microbial cells or enzymes can be recycled and reused during a biotransformation process.

Obtaining enantiomerically pure intermediates and products efficiently and economically is of great importance in the pharmaceutical and chemical industries. Esterases, lipases and proteases have been widely applied in the preparation of enantiopure compounds from racemic pairs, prochiral or meso compounds, or diastereomeric mixtures [7]. The biggest role for biocatalysis still remains in the pharmaceutical sector, because its exquisite regioselective and stereoselective properties enable difficult syntheses (often requiring multiple protection and deprotection steps) to be circumvented [8].

The most important issues when developing a biotransformation process are (in order of importance) achieving high enatiomeric excess, high yield and product titer. There are two major biocatalytic procedure to be employ for chemical transformations which are whole cell biotransformations and free enzyme reactions. Depending on the biocatalytic method used different approaches can be applied to increase those above mentioned parameters.

1.2.1. Microbial Biotransformation Using Growing or Resting Cells

The number of organisms available from one various collections of microorganisms drastically exceeds the number of commercially available enzymes. Even very useful enzyme activities present in the cells may not be characterized or isolated. Isolation of an enzyme can be much more difficult if an isolation procedure has not been published. Moreover some enzymes can be rather labile in an isolated form. This is especially true for enzymes introduce an oxygen function into molecules such as mono- and dioxygenases. These are of special interest since they catalyze reactions that usually cannot be carried out by simple chemical means.

On a preparative scale enzyme reactions require coenzyme, such as oxydoreductions needs coenzyme regeneration. Using whole cells usually solves this problem. Co factors like NAD(P)H regenerated by coupling of other

oxidoreductive enzymes involve in metabolic functions during growth. This phenomenon is acountered as the major advantage of growing cell biotransformations.

A microbial transformation is different than fermentation. For microbial transformations often the cells have not to be alive. It may even be an advantage to work with resting instead of growing cells. Often optimal growth conditions are different from the optimal conditions of bioconversion. Therefore, growth conditions and bioconversion conditions can be optimized independently. In addition to that simple bioconversion medium of resting cell biotransformations makes product isolation is much easier. But regeneration of co-enzymes can be necessary with this system. Reduction with yeast are often conducted as co-fermentation to regenerate NAD(P)H. Disadvantages of this method are the dominant use of NADH for the normal catabolism, forming the byproducts i.e. ethanol in high amounts [9].

With many type of bacteria and fungi species it is possible to perform whole cell biotransformaion studies. Due to their vide verity of enzymatic activity many different conversion can be applied for production of bioactive compounds. They can also be used as lyophilized powder. This is especially recognized as practical techniques for researchers not familiar with advanced microbiological procedures

1.2.1.1. Fungi in Biotransformation

Fungi are well known with the economical losses that they have caused since the beginning of the history as they may give damage to the timber, fuel, food and many manufactured goods. Also their pathogenic activity on plants, animals, and humans are well known. On the other hand, they have also been used beneficially for fermentation purposes in food and beverage industry for many years. As the fermentation technology was improved in the last century an

increasing range of valuable products, including antibiotics and other drugs of great pharmaceutical value, agricultural fungicides and plant growth regulators, vitamins and enzymes fungal biotechnology was started to be an important item of the industry. One of the most important uses of fungal biotechnology is the use of whole cells or isolated enzymes of fungi for bioconversions reactions with high regio- and stereo-selectivity. First uses of fungi as biocatalysts were performed in 1950's and that studies were mostly on the synthesis of steroids by hydroxylation reactions. Since that time the use of fungi for bioconversion purposes has been improved and many reactions have started to be conducted by the use of a variety of fungi. Some recent examples of fungal bioconversions still include hydroxylation of steroids [9], among with the oxidation of sulfur compounds [10] [11], oxidation of alcohols [12], reduction of ketones, deracemization of alcohols [13], and Baeyer-Villeger oxidation [14].

Figure 1.5.. Hydroxylation of androst-4-en-3,17-dione by *Mucor racemosus* [8]

Figure 1.6. Examples of fungi mediated bioconversions (a) Oxdation of sulfur compounds, (b) Oxidation of alcohols, (c) Reduction of ketones, (d) Deracemiziation of racemic alcohols, (e) Baeyer-Villiger oxidation of ketones

1.2.2. Enzymes in Biotransformation Studies:

Mankind has utilized enzymes empirically since ancient times for the production or preservation of foods. Today over 500 commercial products are made using enzymes [16]

In nature as catalysts they evolved to speed up and co-ordinate the multitude of chemical reactions necessary to develop and maintain life. Chemical reactions are by far too slow to be effective under conditions prevalent in normal living systems. Even catalysts developed in the chemical industry fall short; enzymes in comparison achieve up to 10^{19} fold faster reaction rates [17].

As catalysts enzymes alter the rate in which a thermodynamic equilibrium is reached, but do not change that equilibrium. This implies that enzymes work reversibly. The acceleration in reaction rate is achieved by lowering the activation energy.

Enzymes bind their substrates by multiple non-covalent interactions on specific surface. This way, a micro-heterogenization occurs and the local concentration

of substrates is increased relative to the bulk solution. Enzymes often bind the substrate in the transition state better than the ground state, which lowers the activation energy. In addition, the chemical potential of specific groups may be drastically changed temporarily compared to aqueous solutions by exclusion of water in the reactive site upon binding of substrate. Both aspects contribute to the observed phenomenon of high acceleration in reaction rate.

Enzymes works chemo-, regio-, and enantio- specific manner. Substrate binding in a defined manner is a prerequisite for enzyme catalysis. It exposes a chemical compound long enough to a unique chemical potential build in to the system, which defines the type of reaction that will proceed: e.g. hydrolysis, oxidation/reduction, or C-C bond formation. The mechanism most often is the same as known from solution chemistry e.g. acid-base catalysis [3]. Since enzymes act on single type of functional group, the other functional groups will remain untouched. The groups positioned differently will be distinguished due to the three dimensional structure of the enzyme. Furthermore recognition of any chirality in the substrate molecule leads chiral molecules which can be used for the synthesis of chiral molecules from prochiral substrates or kinetic resolution of a racemic substrate [3].

Selectivity and excellarated reaction rates of enzymes are the major advantages in biotransformation studies but they are also recognized as green procedures due to their mild reaction conditions. They work in aquous environment with moderate temperature and pH values. But there are enzymes that can be employed for reactions proceeded under extreme conditions as well. These types of enzymes are convenient for chemoenzymatic reactions in which biocatalysts and chemical reagents are used in a collaborative sequence.

1.2.2.1. Common Enzymatic Reactions Used in Organic Synthesis

Almost all type of reactions that can be catalyzed by enzymes has been applied in asymmetric synthesis such as hydrolysis of amide and ester bonds, reduction of ketones, oxidation of alcohols and aldehydes, formation of C-C bond, addition, elimination and de-halogenation reactions. The common enzymatic conversions and the correspondent enzyme classes are summarized in Table 1.1.

Table 1.1 Common enzymatic conversion and corresponded reaction in biotransformation.

E.C. Class	Reaction type
1 Oxidoreductases	Oxidation-reduction: oxigenation of C-H, C-C, C=C bonds, or overall removal or addition of hydrogen atom equivalents.
2 Transferases	Transfer of groups: aldehydic, ketonic, acyl, sugar, phosphoryl or methyl.
3 Hydrolases	Hydrolysis-formation of esters, amides, lactones, lactams, epoxides, nitriles, anhydrides, glycosides.
4 Lyases	Addition-elimination of small molecules on C=C, C=N, C=O bonds.
5 Isomerases	Isomerizations such as racemization, epimerization.
6 Ligases	Formation-clevage of C-O, C-S, C-N, C-C bonds with concominant triphophate-clevage.

In each type of reactions the general goals of bioconversions may be considered to be as fallows: resolution of racemates, selective conversion of functional groups among several groups of similar reactivities, introduction of chiral center, and functionalization of certain non activated carbon.

1.2.2.2. Limitations of Enzyme Catalysed Reactions

Enzymes have limitations as any other specilized catalyst. Most notably in one consequence arising from the selectivity of enzymes with regard to substrates bound and the type of the reaction. The price for such selectivity is the requirement for many special enzymes to cover the diversity of chemical desired in organic chemistry. The enzyme needed in a specific case may not be readily available.

Other limitations with regard to reaction conditions, pH and temperature tolerated enzymes are to some extend predictable by their chemical nature. Many chemicals and polymers are insoluble in water. This makes such compounds uncompetable for enzymatic reactions. But still application of enzymes in several industrial bioconversions has been broadened by the use of organic solvents replacing water as an important developement in enzyme engineering [18].

1.3. Redox Reactions in Biotransformation:

The enzymes are used in redox reactions are classified into three categories: dehydrogenases, oxygenases and oxidases. The former group of the enzymes has been widely used for the reduction of carbonyl groups of aldehydes or ketones and C-C double bonds. Depending on the substitution pattern of the substrate both of these reactions offers an asymmetrization of prochiral substrate leading to a chiral product. The reverse process (e.g. alcohol oxidations or dehydrogenation reactions) usually go in hand with the destruction of a chiral center, and therefore of limited use.

The major crucial disadvantage of redox reactions with purified enzyme system is the necessity of co-factor recycling since those enzymes are naturally assisted by a co-factor as an acceptor or donor of the electron as mentioned earlier. For

the majority of the co-enzymes, nicotinamide adenine dinucleotide [NAD (H)] and its respective phosphate [NADP(H)] are required by about 80% and 10% of redox enzymes, respectively [3]. Flavines (FMN, FAD) and pyrroloquinone (PQQ) are encountered more rarely. These co-factors are cannot be replaced by more economical synthetic substitutes and they are prohibitively expensive if used in stoichiometric amounts. Enzymatic methods for NADH-or NADPH-recycling has been shown to be efficient and can be subdivided in to coupled –substrate and coupled enzyme types.

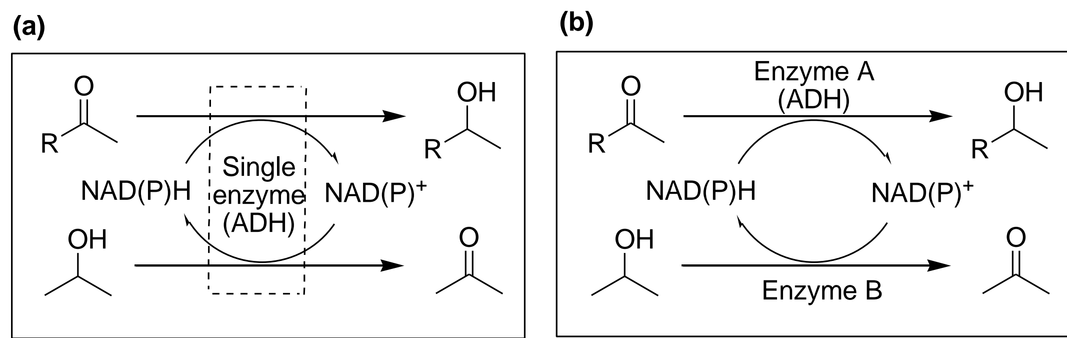

Figure 1.7. Co-factor recycling for purified redox enzymes[3]. (a) coupled substrate (b) coupled enzyme

In the coupled substrate process the co- factor required for the transformation of the main substrate is constantly regenerated by addition of a second auxiliary substrate which is transformed by the same enzyme, but in to the opposite direction. In the case of coupled enzyme approach two parallel redox reactions-conversion of the main substrate and cofactor-recycling- are catalysed by two different enzymes. Both of these methods have pros and cons against to each other mainly related with kinetic properties of enzymatic reactions.

On the other hand co factor recycling is no problem when whole microbial cells are used as biocatalysts for redox reactions. In this case, cheap sources of redox equivalents such as carbohydrates can be used since the microorganisms

possesse all enzymes and co-factors which are required for metabolism. But some disadvantages have to be taken into consideration alongside some significant drawbacks such as low productivity due to toxicity of the non-natural substrates that can be tolerated only in low concentrations (0,1-0,3 %) and low stereo-selectivity as a result of multiple enzyme system in the cell. Chiral transport phenomena into and out of the cell may also influence the specificitity of the reaction, particularly when racemic substrates are used.

As a result of inefficient chiral recognition of a single oxidoreduction enzyme in a whole cell or isolated enzyme system allows an alternative fit of the substrate. In whole cell system moreover, enzymes with high but opposite stereochemical preference compete for the same substrate, the optical purity of the product is determined by the relative rates of the individual reactions. Thus when two or more enzymes are involved in the transformation of enantiomeric substrates, the optical purity of the product can be governed via substrate concentration, because the Michaelis constant (Km's) and turnover number (kcat's) for the substrate enantiomers are different for both competing enzymes. To enhance the selectivity of redox reactions against these phenomena, following general techniques can be applied:

- Substrate modification e.g. by variation of protecting groups which can be removed after the transformation [19-20]
- Variation of substrate concentration. Decreasing substrate concentration gives higher enatiopurity in yeast [21]
- Variation of metabolic parameters by immobilization [22]
- Using cells of different age [23]
- Variation of the fermentation condition [24-25]
- Screening of the microorganisms to obtain strains with the optimum properties [26]
- Selective inhibition of the competing enzyme.

1.3.1. Reduction Reactions:

Microbial reductions can be examplified by many groups of reactions:

- Reduction of monocarbonyl compounds: Cycloalkanones , bi- and polycyclic cylo-alkanones, aliphatic alkanones, sulphur containing molecules, α-heterocyclic substituted ketones, nitrocarbonyl compounds and masked amino ketones.
- Reduction of dicarbonyl compounds: Cyclic di-ketones, acyclic di-ketones
- Reduction of keto esters: α-keto esters, β-keto esters, α,γ-di-keto esters, keto α,γ- di-esters
- Reduction of δ- and γ- keto acids and esters
- Reduction of organometallic compounds
- Reduction of florine- containing compounds

Among the biocatalytic processes mentioned above bioreduction of mono or di carbonyl compounds has much attention and common in this field because chiral alcohols are very important precursors for large number of pharmaceuticals. Their production by asymmetric bioreduction of a prochiral carbonyl precursor is becoming well-established in the field of biocatalysis

1.3.2. Oxidation Reactions

Oxygenases- named for using molecular oxygen as co-substrate have been shown to be particularly useful for oxidation reactions since they are able to catalyze the functionalization of non-activated C-H or C=C bonds (figure 1.8 a,b) affording the hydroxylation and epoxidation procedure respectively. In addition to these bond activations oxigenase type catalysis offers Baeyer-Villiger oxidations of ketones and hydroxilations reactions by dioxygenases (figure 1.8 c,d). Oxidases, which are responsible for the transfer of electrons, play minor role in the biotransformation of non-natural organic compounds.

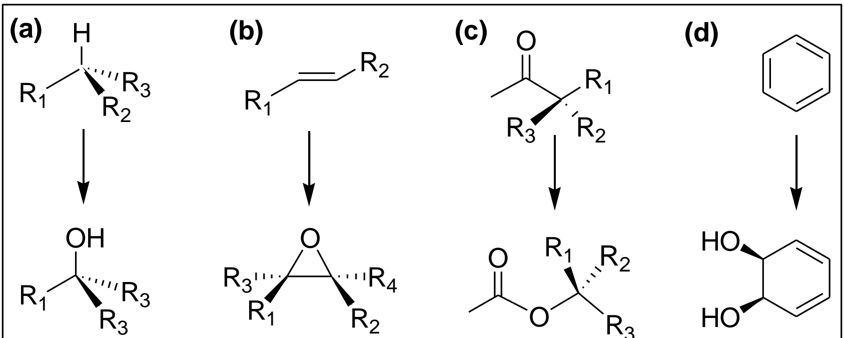

Figure 1.8. Enzymatic oxidative reactions (a) C-H bond functionalization by hydroxylation rection of monooxygenases (b) C=C bond by epoxidation reaction of monooxygenases; Baeyer Villiger oxidations of ketones by monooxygenases (c), dihydrogenation reaction of dioxygenases [27]

Among these biocatalytic reactions hydroxylation of non activated centers in hydrocarbons is one of the most usefull biotransformations due to the fact that this process has only very few counterparts in traditional organic synthesis. Research on the stereoselective hydroxylation of alkanes started late 1940s e.g. 9α- and 16-β hydroxylation of steroids. Nowadays any center in a steroid can be hydroxylated by choosing the appropriate microorganism (figure 1.5) [10].

Use of isolated dehydrogenase reactions for oxidation process is seldom reported due to unfavorable reaction conditions (pH: 8-9) for co-factor and product (particularly aldehyde) and product inhibition as a common phenomenon to such reactions. But still regio selective oxidation of polyols, resolution of alcohols (by selective oxidation of one enantiomer and asymmetrisation of prochiral or meso-diols by HLADH (horse liver alcohol dehydrogenase) are applicable for dehydrogenase mediated oxidative bio-transformation.

Reactions proceeded in oxidative directions with dehydrogenases is also find usage in oxydo-reductive biocatalytic sequences. These processes which are results in deracemization of biotransformation are employed to produce optically active alcohols from their racemic forms.

1.3.3. Oxydo-reductive Biocatalytic Deracemication:

Kinetic resolution is a powerfull technique to obtain single enantiomer starting from a racemate. In these reactions enantiomers of racemic substrate reacts in different rate affording a kinetic resolution. But with kinetic resolution theoretical yield of each enantiomer can never exceed the limit of 50% (Figure 1.9). Moreover in majority of the process only one stereoisomer is desired. In recent years alternatives of kinetic resolution techniques that can deliver a single isomer from a racemate are developed. Some of these methods are created by improvement of classical kinetic resolution techniques for instance reracemization followed by kinetic resolution (Figure 1.9). But it is essential that the starting material racemizes under the reaction condition.

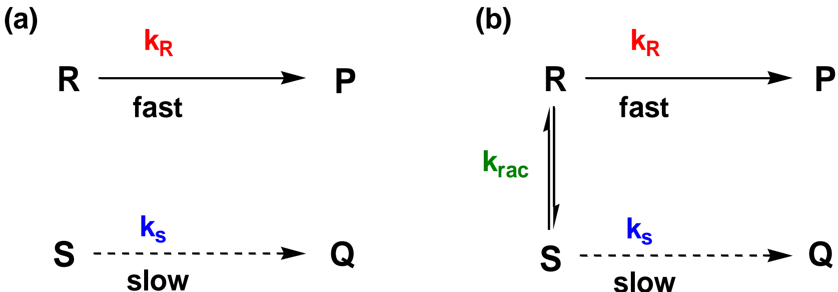

Figure 1.9. Kinetic resolution of racemic mixtures (a) Kinetic resolution (b) Dinamic kinetic resolution; R,S= Substrate enantiomer, P,Q=product enantiomers, k_R,k_S= individual rate constants ($k_R >> k_S$), k_{rac}=racemization constants($k_{rac} \geq k_R$) [28]

The most straightforward method for production of single enantiomer with 100% yield and 100% ee is the conversion of racemic mixture to optically active form without any change in composition of the molecule. With this elegant method the target molecule can be synthesized in its racemic form and than deracemized to obtain the desired single enantiomer [28].

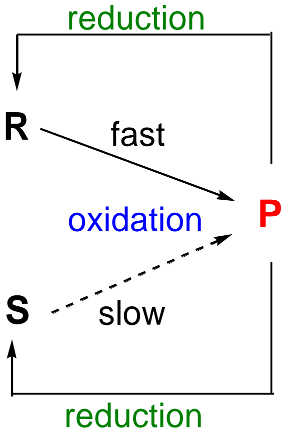

Figure 1.10: Deracemization of sec- alcohols and -amines through cyclic oxidation-reduction [28]

Deracemization reactions tend to involve redox processes, for example the interconversion of chiral secondary alcohols via the ketone or alternatively the interconversion of amino acids/amines via the corresponding imine [29].

It is well known that various microbial systems are able to deracemise racemic secondary alcohols via a process that generally involves two different alcohol dehydrogenases with complementary enantiospecificity [29]. Several fungi mediated deracemization reactions have been reported so far Some recents examples can be listed as *Aspergillus terreus* is able to mediated deracemization of ortho- and meta- flouropenthyl-1-ethanol [30], *Candida parapsilosis* [31] mediated deracemizaiton of α-hydroxyesters and *Rhizopus oryzae* mediated deracemization of benzoin [32-33] (figure 1.11).

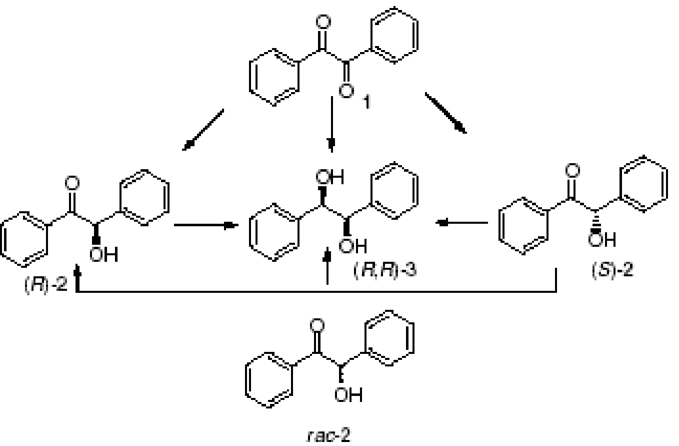

Figure 1.11. Reduction of benzil to benzoin and deracemisation of product by *R.oryzae* [32]

With *R. oryzae* mediated biotransformation it was reported that enantioselective reduction of benzil to benzoin and hydrobenzoin was observed and deracimization of racemic benzoin was obtained with a high yield and enantioselectivity [32-33]. Interestingly *R. oryzae* mediated deracemization of benzoin in a manner that by changing the pH of the medium it was possible to control the absolute configuration of the enantiomer.

1.4. Wieland-Miescher ketone as an Important Synthon for Bioactive Compounds

Wieland-Miescher ketone [34] (3,4,8,8a-tetrahydro-8a-methylnaphthalene-1,6(2H,7H)-dione) and its derivatives are important key synthons for various natural products. Enantiomerically enriched form of this compound has so far been utilized as the starting materials for the synthesies of naturally occurring products, including terpenoids and steroids [35].

For the efficient preparation of enantioenriched Wieland-Miescher ketone different approaches, including asymmetric synthesis [36] various biocataltic

procedures have been established. Asymmetric cyclization of chiral precursor , 2-methyl-2-(3-oxobutyl)-1,3-cyclohexanedione in the presence of (S)-proline was one of the recent and most efficient asymmetric synthesis method [36]. But the optical purity of the product is frequently variable and stays between 40-70%. A biochemical cyclization method with catalytic antibodies can furnishes optically active Wieland-Miescher ketone but this method is yet to be applied to a large scale production [37]. Kinetic resolution of racemate with enzymatic and microbial biotransformation on the other hand offers high enantioselectivity and high yield and these methods are also applicable for large scale preperations. Enzymatic kinetic resolution of butanoate esters [38] and naphtyl acetates [39] to obtain enantioenriched Wieland-Miescher ketone has been reported. In addition yeast mediated (*Saccharomyces cerevisiae, Torulaspora delbruchei Culvullaria falcate*) reduction are also employed for kinetic resolution [40]. Reduction products of these kinetic resolution procedures are also valuable compounds since first step in many synthetic applications often involve regio- and diastereoselective reduction with $NaBH_4$ to give enantiopure product [41].

Figure 1.12. Synthesis and yeast mediated kinetic resolution of Wieland-Miescher ketone

1.5. The Importance of Hydrobenzoin in Chiral Synthesis

Vicinal diols are important structures for synthetic organic chemistry. For example in addition to cleavage reactions to obtain aldehydes and ketones [42], in recent years the oxidation of 1,2-diols to corresponding 1,2-diketons and α-ketoalcohols and hydrolysis to aldehydes has attracted increasing interest and many synthetic approaches are applied [43]. Enantiomerically pure hydrobenzoins (figure 1.13) (1,2-diphenyl-1,2-ethanediol) has also been proven to be very useful structures for variety of synthetic application. In addition to oxidation reaction to obtain benzils [44], and benzoins [45] they are also used as chiral auxiliaries and ligands [46].

meso- hydrobenzoin *R,R*- hydrobenzoin *S,S*- hydrobenzoin

Figure 1.13. Different forms of enantiomeric structures of hydrobenzoin

These diols are accessible through resolution [47], dihydroxilation of olefins [48], reduction of benzils and through carbon-carbon bond formation [49]. Whole-cell biotransformations were also reported. Enantioselective reduction of benzil to benzoin and hydrobenzoin was also obtained with fungi mediated (*R. oryzae*) bioconversion and *R,R*-hydrobenzoin was produced with a high enantiomeric excess and d,l/meso ratio [32].

1.6. α-Hydroxy Ketones as Important Building Blocks

Optically active α-oxyfunctionalized compounds, in particular, carboxylic acids, aldehydes, and ketones are indispensable building blocks for asymmetric synthesis due to their versatile functional groups, which can be easily transformed to other functionalities, e.g.,diols, halo or amino derivatives, and epoxides [50]. A structurally related motif that also fulfills all the criteria of a privileged synthon is the α-hydroxy ketone (acyloin). Several selective transformations of these compounds have been developed and indicate that this motif is ideal for the creation of structural diversity and complexity as examplified in figure 1.14

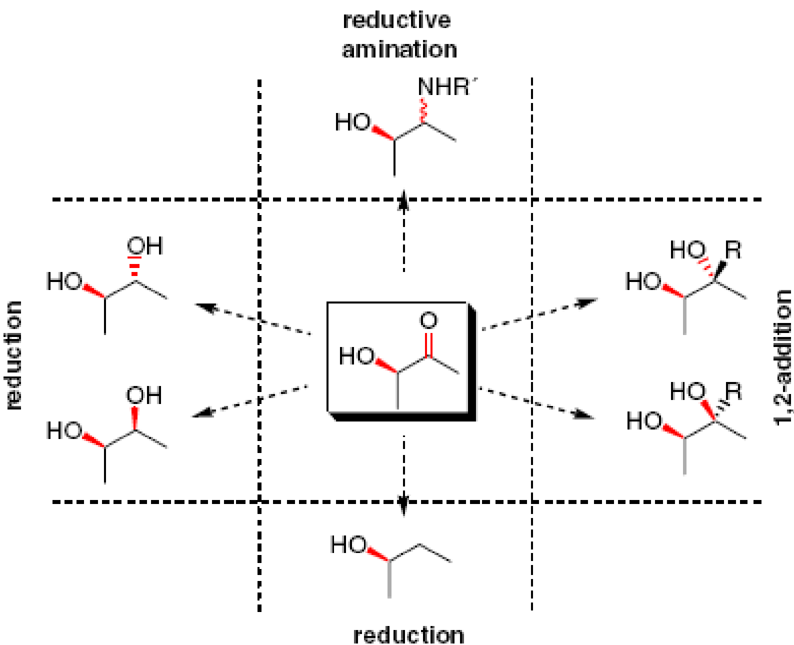

Figure 1.14. α-Hydroxy ketones as building blocks for several active compounds [50].

Acyloins are also important structural units in many biologically active compound [51]. Therefore preperation of optically active acyloins is desirable and many

different synthetic [50] and biocatalytic approaches has been introduced [51]. In a classical acyloin condensation, the C,C-bond is formed, a process, which has been elaborated in an asymmetric fashion via organo- or biocatalysts (see section 1.7)

1.7. TPP dependent Enzyme Catalyzed Carboligation Reactions:

Enzyme catalyzed enantioselective C–C bond formation reactions are rather important aspects of synthetic organic chemistry. Thiamine pyrophosphate (TPP) dependent enzymes, especially, have an important role in the stereoselective formation of C–C bonds.

The classical methodology for the synthesis of α-hydroxyketones involves acyloin and benzoin condensation. The former includes C–C bond formation under reductive conditions while the latter involves C–C bond breaking, followed by C–C bond formation. Benzoin condensation is achieved by cyanide ion catalysis, in which the reaction lacks stereoselectivity,i. e., the resultant benzoin is in racemic form. Both acyloin and benzoin condensation can be accomplished by a number of enzymes under relatively mild conditions via a polarity change (umpolung) on the carbonyl carbon in order to form an acylanion equivalent synthon. Several enzymes, such as acetohydroxyacid synthase (AHAS), benzaldehyde lyase (BAL), benzoylformate decarboxylase (BFD), phenylpyruvate decarboxylase (PhPDC), and pyruvate decarboxylase (PDC) catalyze C–C bond formation. These enzymes all rely on a cofactor, namely thiamine pyrophosphate (TPP). The cofactor thiamine pyrophosphate (TPP) or thiamine diphosphate (TDP), a natural thiazolium salt, has three distinctive units, which include a pyrophosphate part, a thiazolium core, and a pyrimidine unit, (Fig. 1.15) for the TPP (1) structure. It acts by a covalent interaction with the substrate. TPP is mainly engaged in a variety of carbon-carbon bond forming reactions, in which each unit has a special role in enzymatic catalysis [51-52].

Figure 1.15. Thiamine pyrophosphate (TPP) structure.

1.7.1 Benzoylformate Decarboxylase Mediated Carboligation Reactions

Benzoylformate Decarboxylase (E.C. 4.1.1.7) (BFD) is one of the well studied TPP dependent enzyme. This enzyme is found in several closely related microorganisms as part of a pathway for degredation of mandelate [53]. These bacteria are able to use mandelate as sole carbon source by converting it to benzoic acid by non-oxidative decarboxilation, then benzoic metabolized by the β-ketoadipate pathway and citric acid cycle.

Figure 1.16. Mandelate pathway

Wilcocks and coworkers [54] described the carboligation activity of BFD from *Pseudomonas putita*. Since then BFD is used as an efficient catalyst for the enantioselective formation of α-hydroxyketones.

Figure 1.17. Benzoylformate decarboxylase mediated asymmetric C–C bond formation reaction.

The main reaction of BFD- decarboxylation of benzoyl formate to benzaldehyde – is proceeded with a similar mechanism as pyruvate decarboxylases [55] but BFD does not require the previous decarboxylation step for a carboligation reaction such as PDC [56]. Due to the binding geometry the product of BFD, (*S*)-2-hydroxypropiophenone (2-HPP) is an isomeric form of PDC mediated carboligation product- (*R*)-phenyl acetyl carbinol ((*R*)-PAC).

Benzaldehyde + Pyruvic acid —PDC, $-CO_2$→ (*R*)-PAC

Benzaldehyde + Acetaldehyde —BFD→ (*S*)-HPP

Figure 1.18. Carboligation reactions catalysed by PDC and BFD

First general synthesis of enantiomerically pure benzoin and substituted benzoin derivatives from aromatic aldehydes via benzoylformate decarboxylase-mediated C–C bond formation was reported by Demir *et.al.* [57]. By performing the carboligation with benzaldehyde as a sole substrate, (*R*)-benzoin was obtained in 20% yield and 99% ee. The optimization of the reaction conditions (time, amount of enzyme, cofactor, and medium) resulted in a high yield (70%) of (*R*)-benzoin with 99% ee. While self condensation reaction of benzaldehyde furnishing benzoin with (*R*) configuration, the product of cross condensation reaction between benzaldehyde and acetaldehyde is (*S*)-2-HPP).

The reaction, which was carried out with different aromatic and heteroaromatic aldehydes, resulted in the corresponding benzoin derivatives in enantiomerically pure form. The production of various aromatic and even aliphatic (*S*)-2-hydroxy

ketones by the stereoselective cross coupling of various aldehydes instead of 2-keto acids as sole substrates is possible with wild type BFD [58, 59].

1.7.2. Benzaldehyde Lyase Mediated Carboligation Reactions

Benzaldehyde lyase (EC 4.1.2.38) from *Pseudomonas florescens* Biovar I was first reported by Gonzales and Vicuna [60]. The purified enzyme cleaves the α-hydroxy ketones benzoin and anisoin, in a reaction that requires TPP and a divalent cation. BAL catalyzed enantioselective C–C bond forming reaction that afforded (*R*)-benzoin and (*R*)-2-hydroxypropiophenone derivatives was firstly reported in 2001 [61].

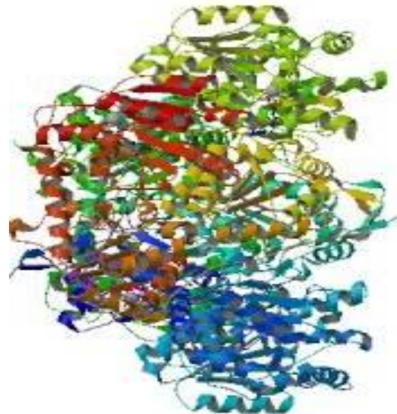

Figure 1.19. Tetrameric structure of benzaldehyde lyase from *Pseudomonas florescens* [62]

Figure 1.20. General carboligation scheme for benzaldehyde lyase.

Since then, the carboligation activity of this enzyme has been extensively studied with a wide substrate spectrum. Mechanism of BAL is in accordance with other TPP-dependent enzymes. It was shown that in contrast to BFD, BAL accepted aromatic aldehydes substituted at the ortho position. Several symmetric benzoin derivatives were synthesized by BAL mediated self-condensation reactions in 96–99% ee [63]. With heterocyclic aldehydes, the enantiomeric excess values were slightly lower; 92% for 2-furaldehyde and 91% for thiophene-2-carbaldehyde. Only a few aromatic aldehydes, such as pyridine-3-and-4-carbaldehyde, as well as sterically demanding aldehydes, gave either a very low yield or no benzoin condensation at all. Moreover, different aromatic and hetero aromatic benzoin, such as acyloins, are accepted as a substrate for kinetic racemic resolution via C–C bond cleavage [63]. Unlike BFD, BAL also has a tolerance for longer chain aliphatic aldehydes (e. g.,propionaldehyde and butyraldehyde) as an acyloin acceptor. Assuming that aldehydes, which are not accepted as donor substrates, still might be suitable substrates as acceptors, and vice versa, a mixed enzyme substrate screening was performed in order to identify a biocatalytic system for the asymmetric cross-carboligation of aromatic aldehydes [64]. Meta- and para-substituted benzaldehydes selectively acted as donors, whereas ortho-substituted analogs served as acceptors in cross-benzoin condensation reactions catalyzed by BAL, cross-benzoin products were obtained with >99% ee. The reaction of aromatic aldehydes with methoxy and dimethoxy acetaldehyde furnished (*R*)-2-hydroxy-3-methoxy-1-arylpropan-1-one and (*R*)-2-hydroxy-3,3-dimethoxy-1-aryl propan-1-one derivatives in high yields and with 89

to 98% ee via acyloin linkage. These 2,3-dioxygenated aryl propanones and are important building blocks for the synthesis of cytoxazone – a novel cytokine modulator [65], (figure 1.21)

Figure 1.21. BAL mediated syntheses of 2,3-dioxygenated aryl propanones.

Enantiopure 2,3-dioxygenated aryl propanones are highly valuable chiral synthons which are useful for the synthesis of various active molecules, such as 1,4-benzodioxane framework found in biologically active lignans, silybin and americanin A. They are antihepatotoxic as haedoxan A and have insecticidal activity. In another study, hydroxyacetophenones were obtained by the direct hydroxymethylation of aromatic aldehydes via the carboligation of aromatic aldehydes with formaldehyde [66]. Several studies have been conducted to improve BAL as a biocatalyst applicable in industry. For this purpose, the bioprocess parameters were optimized by means of reaction engineering [67]. Another approach was to immobilize BAL either by polyvinyl alcohol (PVA) or with metal ion affinity binding to a nickel(II)-nitrilotriacetic acid derivatized carrier [68-69]. BAL entrapped in polyvinyl alcohol enabled a 3-fold better productivity for the synthesis of 2-furoin while it was also used to synthesize symmetric novel benzoin derivatives in hexane with an entrapment efficiency of >90% [68].

1.8. Immobilized Metal Ion Affinity Chromatography (IMAC)

Affinity chromatography is a chromatographic method of separating biochemical mixtures, based on a highly specific biologic interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand.

In mid 1970s, by Porath *et al.* [70] a new type of affinity chromatography technique was introduced which was first called as ‘‘metal chelate chromatography’’, but was later termed ‘‘immobilized metal (ion) affinity chromatography’’ (IMAC). This method is based on the specific coordinate covalent binding of amino acids to metals particularly histidine Since many naturally occurring proteins do not have an affinity for metal ions, therefore recombinant DNA techniques can be used to introduce this property into a protein of interest. Therefore this method improved for recovering of recombinant proteins from cell lysate of overexpressing host cells like *E. coli* [71]

1.8.1. Protein-Metal Binding in IMAC Applications

In IMAC the adsorption of proteins is based on the coordination between an immobilized metal-ion and an electron donor groups from the protein surface. In this kind of chromatography, use is made of a sorbent, or matrix, to which some metal-chelating groups are covalently attached. When the metal-ions are added (loaded), the multidentate chelating groups and metal-ions form complexes in which the metal-ions are secured for subsequent interaction with the compounds to be resolved. To this end, these metal-ions in the complexes must have free coordination sites in order to allow the interaction to the proteins. These interactions between metal-ions and proteins are extremely complex in nature. They can associate the combined effect of electrostatic (or ionic), hydrophobic, and/or donor-acceptor (coordination) interactions .

Electron-donor atoms (N, S, O) present in the chelating compounds that are attached to the chromatographic support are capable of coordinating metal-ions(electron-pair acceptors) forming metal chelates, which can be bidentate, tridentate, etc., depending on the number of occupied coordination bonds. The remaining metal coordination sites are normally occupied by water molecules and can be exchanged with suitable electron-donor groups from the protein.

As a chromatographic technique, the IMAC procedure relies on the ability of immobilized metal-ion chelate complexes to interact with the side-chain moieties of specific amino acids accessible at the surface of proteins via coordinative interactions. For chelating agent, multidentates are most popularly used in research works and commercial chromatographic products. Four different types of dentates, bidentate (e.g., aminohydroxamic acid, salicylaldehyde, 8-hydroxyquinoline (8-HQ), etc.), tridentate (e.g., iminodiacetic acid (IDA), dipicolylamine (DPA), ortho-phosphoserine (OPS), N-(2-pyridylmethyl) aminoacetate, 2,6-diamino methylpyridine, etc.), tetradentate (e.g., nitrilotriacetic acid (NTA), carboxymethylated aspartic acid (CM-Asp), etc.), and pentadentate (e.g., N, N, N0-tris-carboximethyl ethylene diamine (TED), etc.), have been thoroughly investigated since immobilized metal affinity chromatography was exploited. Among those NTA is the common chelating multidentates used for commercially available affinity resins for recombinant histidine tagged proteins.

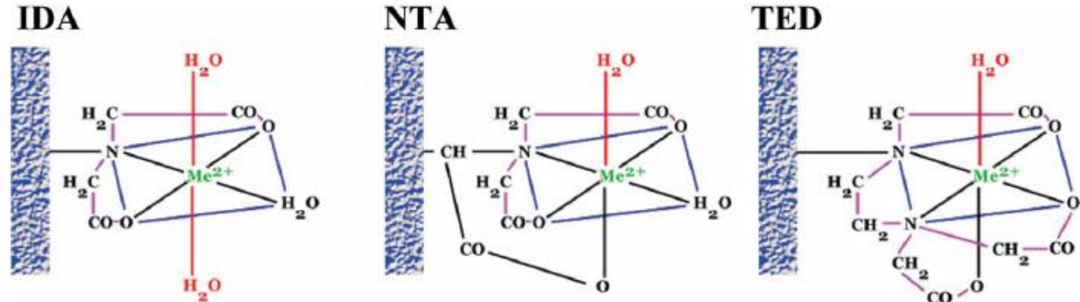

Figure 1.22. Putative structures of some representative dendate chelators in complex with metal-ions for the immobilized metal affinity method.

In addition to purification of recombinant proteins, metal affinity binding techniques can be used for purification of native or un-modified proteins, matrix assisted protein refolding and for analytical purposes. Using metal affinity binding properties of proteins for immobilization of enzymes is also an application area. In these studies different types of matrices like micron or nano scale magnetic particles can be used.

1.9. Magnetic Particles for Biological Applications

Magnetic particles, such as superparamagnetic iron oxide particles, have been extensively studied [72] and used for in vivo applications, like hyperthermia treatment, magnetic resonans imaging (MRI) contrast enrichment and gene and drug delivery [73]. Another application are of magnetic particles are seperation and purification of cells and biomolecules [75]

There are several advantages of using magnetically seperable particles as supports for biological molecules such as enzymes, antibodies and other bioaffinity adsorbents. When magnetic particles are used as solid phase supports for immobilized enzyme systems, the enzyme may be selectively recovered from the media, allowing recycling in enzyme reactors. Centrifugal seperations are time consuming require expensive and energy consuming equipment. Magnetic seperations on the other hand are relatively rapid and easy, requiring simple equipment. The use of non-porosous adsorbent-coupled magnetic particles in affinity chromatography systems allows better mass transfer and results in less fouling than in conventional affinity chromatography. When they are used for enzyme immobilization, particularly in batch reactors and continuous-flow stirred-tank reactors (CSTR), this method facilitates the separation of enzymes from the product after the completion of the reaction in order to permit, reuse, and recycle the enzyme

The surfaces of magnetic particles can be modified by coating with a suitable polymer which provides a matrix for binding of the functional groups [75]. Some of these particles are coated and functionalized with nitrilotriacetic acid with metal ion coordination. These micron- or nano-scale particles selectively bind to histidine-tagged recombinant proteins and combine the advantages of magnetically responsive particles and the metal affinity ligand within enzyme purification and immobilization studies.

1.10. Aim of the Study:

In this study it is aimed to develop new biocatalytic approaches for the enantioselective synthesis of α-hydoxy ketones. For this purpose firstly it was planned to employ fungi mediated biotransformations. Various fungi species which were reported or detected as eligible for oxidoreduction reactions for desired biotransformations aimed to screen. Various fungal species had been selected by literature survey and obtained from TUBITAK MAM and OSAKA University Culture Collection. Bioconversion process for each type of reaction planned to be optimized for desired product - mainly α-hydroxy ketones- enantiomeric excess and yield. Additional products of those bioconversions were also planned to be analyzed for other biologically active compounds.

It was also our aim to develop a new biocatalyst system for carboligation reactions which are one of the important approaches for enantioselective preparation of α-hydoxy ketones. For this purpose carboligation enzymes BAL and BFD planed to be immobilized to surface modified superparamagnetic solid support by making use of histidine tagged metal affinity chromatographic techniques.

CHAPTER 2

RESULTS AND DISCUSSION

2.1. Perspective of the Study:

Many studies have been reported for preparation of optically active benzoin [76]. Biocatalytic synthesis comprises considerable portion of those methods as an environmentally friendly alternative. Enzymatic reduction of α-diketones and kinetic resolution of racemic α-hydroxy and α-acetoxy ketones are reported for preparation of optically active benzoin [77]. As it is discussed in section 1 BAL and BFD mediated carboligation reactions are offers preparation of enantiomerically pure benzoin and substituted benzoins from aromatic aldehydes with a high enanioselectivity and yield (figure 2.1 A). Demir *et.al.* reported the fungi mediated whole cell bio-conversion of benzil to benzoin (figure 2.1 B) [78]. Among those methods in literature a detailed study on biocatalytic oxidation-reduction sequence for hydrobenzoin is still missing.

Figure 2.1. Biocatalytic reactions for the enantioselective synthesis of benzoin

Due to its absolute symmetrical conformation, C-H oxidation reactions furnishing enantiopure product with *meso*-1,2-diols is a challenging task for preparation of optically active compounds. Numerous methods has been developed for the asymmetric desymmetrization of *meso*-1,2-diols [79] but methods that could be developed for enantioselective C-H oxidation of these compounds is restricted. Jakka and co-workers reported the first catalytic C-H oxidation of 1,2-diol mediated by ketone catalysts Shi's oxazolidinone dioxiranes [80]. Even though racemic hydrobenzoins gave better enantioselectivities they could obtain benzoin from *meso*-hydrobenzoin with 70-87% ee and 60-85% yield. But yet there have been no biocatalytic example of enantioselective vicinal *meso*-diol oxidation reported in literature. Therefore it was desirable to develop a biocatalytic method for enantioselective synthesis of benzoin starting from *meso*-hydrobenzoin.

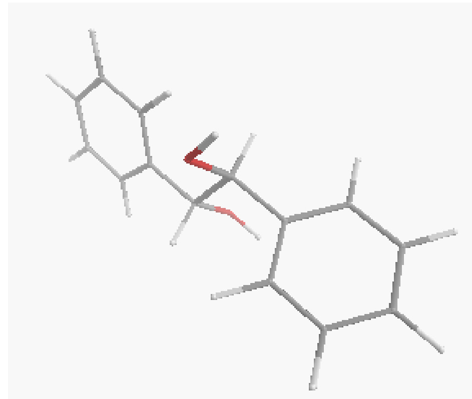

Figure 2.2. Three-dimensional representation of symmetrical structure of *meso*-hydrobenzoin

From the knowledge available from previous experiences which are biocatalytic deracemisation of racemic mixture of benzoin [32,33] and reduction to hydrobenzoin during the biocatalytic process of reduction of benzil [78] it was tought that with a successive deracemization process it was possible to prepare benzoin starting from, *meso*-hydrobenzoin enantioselectively.

Figure 2.3. Enantioselective conversion of *meso*-hydrobenzoin to benzoin

In addition to *Rhizopus oryzae* various *Aspergillus species* was screened throughout this study for oxidation of *meso*-hydrobenzoin and *A. flavus* mediated enantioselective oxidation achieved with a low conversion rate. Enantioselective conversion of racemic *d,l*-hydrobenzoin was also found to be interesting if *A. falvus* performs the oxidation reaction enantioselectively or convert the absolute configuration of *d,l*-hydrobenzoin substrate as a result of an oxidation-reduction

sequence (figure 2.4).Therefore racemic mixture of this compound is also thought to be an interesting substrate for *A. flavus* mediated bioconversion.

Figure 2.4. Conversion of racemic *d,l*-hydrobenzoin to *meso*-hydrobenzoin

Wieland-Miescher ketone (3,4,8,8a-tetrahydro-8a-methylnaphthalene-1,6(2H,7H)-dione) and its derivatives are important key synthons for various natural products like steroids and terpenoids [81][82]. Fungi mediated bioconversions are one of the common method for biocatalytic enantioselective hydroxylation of cyclic compounds. *Aspergillus niger* was employed to enantioselective hydroxylation and cyclic alpha-hydoxyketones (tetralone and indanone) are obtained in medium containing starch as carbon source [83]. With this knowledge available to us, first it was our aim to obtain α-hydroxylation product of B ring of Wieland-Miescher ketone by employing *A. niger* mediated biotransformation. In addition to an oxidative route it was also expected the carbonyl group of B ring in the substrate could be reduced. But in this case diastereoselectivity of the reaction is prerequist.

As well as preparation of enantiopure Wieland-Miescher ketone, stereoselective functionalization of B ring by reduction of carbonyl group is a key step for many

of synthetic approaches which are designed to obtain steroidal compounds [84] Various enzymatic methods have been developed to obtain reduction product (4,4a,5,6,7,8-hexahydro-5-hydroxy-4a-methylnaphthalen-2(3H)-one Enzymatic kinetic resolution of butonoate esters and naphtyl acetates [85] [86] has been reported. To obtain enantiomerically enriched form of Wieland-miescher ketone microbial kinetic resolution of this substance furnished reduction product by yeast mediated (*Saccharomyces cerevisiae, Torulaspora delbruchei Culvullaria falcate*) biotransformation [87]. But as the major drawback of kinetic resolution the theoretical yield of each enantiomer can never exceed a limit of 50% in these conversions. Moreover this process requires additional labor to separate the starting material and the product. Enzymatic hydroxylation of hydronanaphtalenon also furnished functionalized B ring but it was necessary to start with enantiopure Wieland-Miescher ketone to obtain enantiopure product [88].

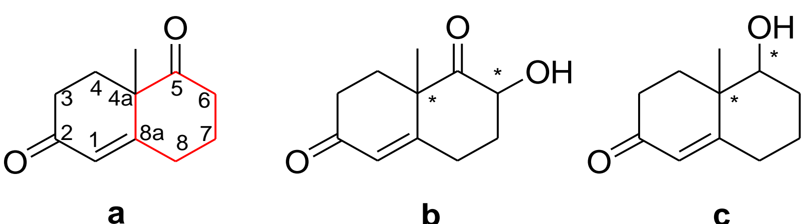

Figure 2.5. Wieland-Miescher ketone and its hydroxilation and reduction products **(a)** Wieland-Miescher ketone (3,4,8,8a-tetrahydro-8a-methylnaphthalene-1,6(2H,7H)-dione)**, (b)** hydroxylation and **(c)** B ring reduction product, of Wieland-Miescher ketone

Many reactions of the native end recombinant BAL and BFD have been reported and the applicability of BAL has already been extended through a wide range of substrate spectrum [51]. Not just because of their versatility but also having an industrial interest [89], immobilization of these enzymes, has became important achievement. Asymmetric C-C coupling is indispencible reactions in organic chemistry and development of novel reaction procedures is highly desirable for

research purposes as well. Obtaining reusable handy heterocatalysts systems by immobilization offers many advantages in screening of novel reactions and optimization studies.

Carboligation reactions that are catalyzed by immobilized BAL have been reported. In addition to increase the cost effectiveness of BAL catalyzed reactions, it was aimed to design heterocatalyst systems for continuous processes [90] and to stabilize the enzyme to be used in organic media.[91] Since recombinant histidine tagged BAL and BFD can be produced by overexpressing a recombinant *E.coli* strain, it is eligible to make use of metal affinity ligands within immobilization studies. Using superparamagnetic particles as solid support in these studies is advantageous because these particles are highly responsive to magnetic field and easily dispersed after the magnetic field is removed. Moreover since superparamagnetic particles do not form aggregates, surface of the particles covered by the immobilized enzyme stays free and available for substrate.

Considering above mentioned advantages of both metal affinity chromatography and superparamagnetic particles introduction of a heterocatalyst system that offers the ease of immobilization along with the separation steps of metal affinity ligand (Co^{2+}-NTA) coated magnetic particles was found to be highly desirable for BAL and BFD catalyzed carboligation reactions.

2.2. *Aspergillus flavus* Mediated Oxidation of *meso*-Hydrobenzoin for Preparation of Benzoin

To obtain an enantioselective oxidation product of *meso*-hydrobenzoin, in addition to candidate species *Rhizopus oryzae* various *Aspergillus species* were also screened for the ability to perform this desired reaction (table 2,1). Aspergillus species are reported as eligible microorganisms for many types of oxidoreductive bioconversions in literature [92]. *A.niger, A.flavus, A. oryzae* had been obtained from TUBİTAK MAM, isolated strain *A. nidulans* was kindly

supplied by Prof. Dr. Zümrüt Ögel and Osaka University culture collection (OUT) to be employed for oxidoreductive bioconversion studies and those species were also subjected to enantioselective enzymatic production of benzoin from. hydrobenzoin.

Figure 2.6. Enantioselective conversion of *meso*-hydrobenzoin to benzoin

Table 2.1. Fungi species screened for enantioselective oxidation of *meso*-hydrobenzoin. (In 100 mL potato dextrose medium (PDB) with 10^5 spore/mL and 0,4 mg/mL substrate, 30°C, 160 rpm. Dry weights were measured at the end of 12 day period).

Fungi	**7 day ee**	**12 day ee**	**Dry weight**
	%	**%**	**mg**
A.niger MAM 200909	Racemic	Racemic	271±32
A.nidulans (isolate)	8±3 (*S*)	5±2 (*S*)	262±21
Aspergillus oryzae OUT5302	8±2 (*S*)	7±4 (*S*)	312±12
Aspergillus oryzae OUT5048	5±2(*S*)	35±6(*S*)	264±34
Aspergillus oryzae MAM 200919	11±3 (*S*)	37±5 (*R*)	324±23
A.flavus MAM 200120	14,2±3 (*S*)	57±7 (*S*)	282±31
Rhizopus oryzae ATCC 9363	-	-	254±41

Bioconversions with these fungi were performed in whole cell biocatalytic sytem. in 100 mL potato dextrose medium (PDB) in which all the fungi species that used in screening studies showed a sufficient growth for 2 days cultivation (30°C, 160 rpm). This medium selected according because it is a recommended medium in

literature for growth of fungi species and *Aspergillus* as well. Inoculation of media was done at the same concentration of fungi spores (10^5 spore/mL). and biotransformation was induced by adding 40 mg substrate, in common water miscible solvent dimethyl sulphoxide (DMSO, 1%). To avoid the inhibition of growth of microorganism induction was performed at the end of the 2th day. Experiments were also performed with fungi and substrate free media and no conversion was detected from both control experiments. For 3-4 day period of fungi mediated conversion, benzoin production was observed with thin layer chromatography (TLC). At the end of bioconversion culture medium filtered and and reaction terminated by adding equal amount of ethylacetate and standart work-up procedure was applied to extract the product. Structure of the product was confirmed by ^{1}H-NMR analysis. Calculation of ee values by HPLC analysis at the end of 7 and 12 day showed that *A. flavus* was able to furnish oxidation product benzoin with 57±7 % ee favoring the (*S*) enantiomer. Eventhought *R. oryzae* have already known that they perform reduction of benzyl to hydrobenzoin and deracemisation of benzoin from the previous works, under experimental condition there were no benzoin detected from the fermentation medium of this fungus. *A.oryzae* (MAM 200919) was the only species furnishing the (*R*) enantiomer under experimental conditions. But at first 7 days of bioconversion while the (*S*) enantiomer was produced, after 15 day the other enantiomer (*R*) became predominant with 37±5 % ee. It was thought that change in absolute configuration of product at prolonged period of conversion was possibly due to the involvement of other competing enzymes in *A. oryzae* mediated whole cell bioconversion. *A.nidulans and A.niger* mediated biotransformations also furnished benzoin but with low ee values or as racemic mixture. Eventhough the inoculums size was the same, dry weights of the cultures at the and of the 12 day for different fungi species were different This is most probably due to the differeneces in their growth curves or in such some may enter the lag phase earlier than the others. But still the ee values obtained with *A. oryzae* (~40%) and *A. flavus* (~60 %) was much more superior than the other species employed biotransformation while the difference in their dry weight was not that much significantly different than the conversions having low ee values .

Figure labels: A.oryzae; A.flavus; oxidation; OH; OH; O; OH; (*R*)-benzoin; O; OH; (*S*)-benzoin

Figure 2.7. Oxidation of hydrobenzoin to benzoin by *A. flavus* and *A. oryzae* mediated bioconversions.

According to former studies performed in our laboratory *A. flavus* is able to perform deracemization, reduction and hydroliysis reactions to produce benzoin enantioselectively (figure 2.8). To further investigate this multi bioconversion ability of this fungus and also having highest ee value *A.flavus* is chosen for further optimization studies for oxidation of hydrobenzoin.

Figure 2.8.. Multi bioconversion reactions for production of benzoin from different substrates by *A.flavus.*

Various reaction conditions were examined to increase ee value and conversion rate of benzoin production from hydrobenzoin as it is summarized in table 2.2 and 2.3. The effect of the different medium composition, substrate concentration, inoculum size (spore concentration), and initial pH values on the ee and conversion was investigated. Both ee and conversion values are calculated by HPLC analysis using chiral coloumn (Chiralpak AD-H). Reactions that are performed with whole-cell *A.flavus* were performed for 12-21 days in until the ee value and conversions reach at a plateu. These values remained constant after 12 days possibly due to the cellular decomposition which was observed at that prolonged time of conversion.

Table 2.2. Effects of different medium composition on ee of *A. flavus* mediated conversion of *meso*-hydrobenzoin to benzoin at the end of 12 day. (In 100 mL medium, 0,4 mg/mL substrate, 160 rpm, 30°C).

Medium type	ee (%)	Conversion (%)
(A): 2% glucose, 2% yeast extract, 0,5% peptone, minerals	21±3	5±2
(B): PDB (8% potato extract, 2%glucose)	64±4	2±1,5
(C): 2%malt extract, 2%glucose	67±3	1±0,7
(D): 0,4%yeast extract, 1,5% starch, minerals	61±2	2±1

Different type of complex medium compositions were used for *A.flavus* mediated bioconversion at same reaction condition previously descried for screening studies. These media was sellected according to the literature in which used for growth of *Aspergillus* species. In all condition other than medium (A) ee values were moderately high (63-69%) but unfortunately neither of medium composition was able to yield high conversion rates (at most 5±2%). With medium (C) eventhough the ee value reached up to 67±2,5 conversion was only around 1%. Since it is difficult to understand the effects of carbon and nitogen sources from the results obtained from complex media, defined medium composition described for *Aspergillus* species was also employed for hydrobenzoin bioconversion. Probably due to low growth rate of microorganism in this medium there were no benzoin production observed. But still if we assume medium (A) as the standart composition, we can interpret the effects of different complex C and N sources added to the other media. For example using 1,5% starch as C source in the medium D instead 2% glucose results in positive effect on ee value – increase from 21±3% to 61±2%. This might be concluded as the effect of increase in C/N ratio of the medium or change in metabolism of microorganism upon using a polysaccharide instead a readily utilizable sugar. Using potato and malt extract has the similar effect on ee and these comlexes also contains

polysaccharides as C source in addition to N containing compounds which may also influence the positive effect by stimulating the growth.

Influences of other factors on *meso*-hydrobenzoin conversion in PDB medium are summarized in table 2.3. During these experiments conditions were the same as previous experiments (30°C and rotation 160 rpm). Entry 1, 2, 3 shows the effect of inoculum size (spore concentration-number of spores/mL) on ee and conversion rate of benzoin production. When we increase the inoculum size enantiopurity of the benzoin was also increased and reached to 68±4% for the (*S*) enantiomer at 10^6 spore/ml concentration. Above this concentration, saturation of substrate by biocatalysis – whole cell fungi – was observed and ee value remained around 70% at the same period of bioconversion. Eventhough inoculums size has also a positive effect on conversion of the reaction this value was rised only up to 4±0,3% at highest spore concentrations.

Table 2.3. Effects of different experimental conditions on whole cell *A.flavus* mediated enantioselective oxidation of *meso*- hydrobenzoin to benzoin. (In 100 mL PDB at 160 rpm and 30°C. Products were obtained at the end of 12 days of biotransformation). *Dry weights measured at 12th day of transformation for different inoculums size are 211 mg for 10^3 mg/mL, 254 mg for 10^5 mg/mL, and 284 mg/mL for 10^6 mg/mL.

Entry	Inoculum size	Initial pH	Substrate concentration	ee	Conversion
	Spore/mL		(mg/mL)	(%)	(%)
1	*10^3	6	0,4	46±5(*S*)	1,9±0,7
2	*10^5	6	0,4	61±6(*S*)	3,2±0,6
3	*10^6	6	0,4	68±4(*S*)	4±0,3
4	10^6	4	0,4	30±4(*R*)	2,2±0,4
5	10^6	5	0,4	65±4(*S*)	1,8±0,3
6	10^6	8	0,4	21±5(*S*)	3,2±0,5
7	10^6	6	0,4	64±3(*S*)	6,8±0,7
8	10^6	6	0,4	70±4(*S*)	2,4±0,8
9	10^6	6	0,4	75±4(*S*)	0,9±0,3

Whole cell bioconversion of *meso*-hydrobenzoin was also performed at different initial pH (entry 4-6). But in 3-4 days in all cases pH of the culture medium settled to 5 and increased throughout the converversion. At initial pH 9 there were no conversion observed and pH 4 and 8 ee values were low (30±4,-21±5%) at pH 4 bioconversion favored (*R*) enantiomer. The highest ee values were obtained pH 6 and 5 (70±4 and 65±4%). Since morphology of the mycelia cells are significantly affected from initial pH results are summarized in table 2.3 are possibly occurred as a result of this morphological adaptation. To stabilize the pH during the conversion using a buffering system is necessary but with wresting cell bioconversion the desired product couldn't be produced.

When we used different substrate concentrations at the optimum inoculums size (10^6 spore/mL) the highest ee value (75±4%) was obtained with 0,8 mg/mL substrate and increase in substrate concentration resulted in increased ee value but contraversially caused a decrease in conversion rate. Indeed the factors - other than the inoculums size- which has a positive effect on ee, caused a decrease in conversion rate. With the increase in inoculums size dry weight of mycelia was also increased according to the values obtained at the end of transformation. But in this range of inoculums size morphology of the mycelia were not significantle changed (no disperse growth was observed) other than size of the myceles. So the results obtained with increased inoculum size mostly the result of increased amount of biocatalyst. Eventhought moderately high ee values (entry 9) were obtained, conversion of substrate was reached only up to 6,8±0,7% at the best condition (entry 7). It was thought that this might be the result of a mechanism proceeded as equilibrium controlled oxidation re-reduction sequence in which reduction of benzoin is favored over the oxidation of hydrobenzoin. In this system the rate of the reduction of (*R*)- benzoin (k_r) should be higher than that of (*S*)-benzoin (k_s) to furnish the (*S*) enantiomer.

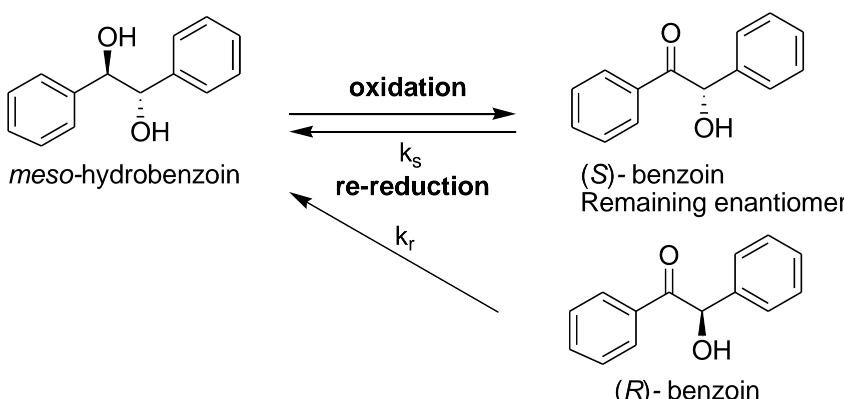

Figure 2.9. Proposed mechanism for oxidation re-reduction sequence of *A. flavus* mediated bioconversion of *meso*-hydrobenzoin to benzoin.

2.2.1 *A. flavus* Mediated Conversion of Racemic *d,l*-Hydrobenzoin to *meso*-hydrobenzoin

Upon low conversion rate of benzoin production from *meso*-hydrobenzoin we proposed a mechanism based on whole-cell oxidation-re-reduction sequence. To investigate this mechanism racemic mixture of *d,l*-hydobenzoin was also employed to *A.flavus* mediated bioconversion. Reaction conditions were choisen as described for the optimum conditions for *meso*-hydrobenzoin conversion. As a result of this experiment it was expected that racemic mixture of hydrobenzoin could be inverted to *meso* counterpart (figure 2.10.). Racemic mixture of *d,l*-hydrobenzoin used as starting material for *A.flavus* mediated reaction at the same experimental conditions that of *meso*-hydrobenzoin experiments. HPLC analysis was performed from the following day of the induction to monitor the conversion. For this a chiral HPLC column was used which resolves tree different diastereomers of hydrobenzoin and enantiomers of benzoin (Chiralpac AD-H). According to the HPLC results racemic hydrobenzoin was converted to meso-hydrobenzoin while there was no or a small amount of benzoin (<0,1%) produced at the first day of reaction. In 3 to 4 days benzoin was started to be produced. But at the end of the 15 days the conversion rates and ee values of those reactions was not higher than that of *meso*-hydrobenzoin reactions. Throughout the reaction period conformation of the *meso*-hydrobenzoin didn't changed and ratio of *meso* form in hydrobenzoin produced remained between 87-96%.

As a result, starting with racemic hydrobenzoin first we could produce *meso*-hydrobenzoin with a high yield (>90%) and diasteroselectivity and then following period of time benzoin was started to accumulated in the bioconversion medium. With these results it was also supported that a re-reduction reaction occures resulting in change in absolute configuration of racemic hydrobenzoin and low conversion rate of benzoin.

Figure 2.10. *A.flavus* mediated stereoinversion of racemic hydrobenzoin to *meso*-hydrobenzoin.

2.2.2. Effects of Different Co-solvents on ee and Conversion Values of *A. flavus* Mediated Bioconversion of *meso*-Hydrobenzoin to Benzoin

Alcohols and ketones are usually employed as hydrogen donors and hydrogen acceptors respectively to increase the rate by shifting equilibrium [3]. In oxidoreductive biotransformation studies, acetone was reported as hydrogen acceptor for oxidation/reduction sequence for deracemization of secondary alcohols [93] In our case we added acetone and isopropyl methyl ketone (IPMK) as co-solvent for whole-cell bioconversion medium to direct the reaction sequence through oxidation of hydrobenzoin. Control experiments were done with dimethyl sulphoxide (DMSO) to dissolve the substrate and no co-substrate added. In all cases 10% co-substrate is added but substrate first dissolved in DMSO when IPMK was used. Experimental conditions were the same as the optimized conditions in the previous experiments (100 mL PDB medium, 0,4 mg/mL substrate, 10^6 spore/mL, 30°C, 160 rpm). According to the results summarized in table 2.4 IPMK did not show any effect on conversion and ee value. So it seemed that IPMK was not accepted as hydrogen acceptor for reaction sequence of hydrobenzoin oxidation. In the case of acetone conversion was increased up to 13±3% while ee value was not affected. Eventhough

conversion is still low for en efficient bioconversion process acetone showed the expected effect which was increase in conversion with a high ee value.

Table 2.4. Effects of different co-solvents on ee and conversion of *A. flavus* mediated bioconversion of *meso*-hydrobenzoin to benzoin. (In PDB medium under optimised conditions)

	ee (%)	**Conversion (%)**
DMSO	75±4(S)	5±2
IPMK	73±5(S)	5±2
Acetone	76±4(S)	13±3

2.3 *Aspergillus niger* Mediated Enantioselective and Diasteroselective Reduction of Wieland-Miescher Ketone:

Fungi mediated whole-cell biotransformation was employed for the desired α-hydroxilation product from Wieland-Miescher ketone. For this reaction, previously defined bioconversion conditions were based on to the informations obtained from *A. niger* mediated hydroxylation reactions of cyclic ketones [83]. Knowing the fact that this fungus has the potential for many different oxydoreductive reactions, including an oxidative path for hydroxylation reaction, reduction of the carbonyl group of B ring of Wieland-Miescer ketone was also expected to be observed.

In the complex medium previously described as medium D (0,4%yeast extract, 1,5% starch, minerals), whole cell biotransformation was induced by adding 40 mg racemic substrate in 1% DMSO to 2 days old *A. niger* culture. At the end of 12 days reaction when there were no further change occurred in the reaction media, single product that was detected by TLC, isolated and analyzed. ^{1}H-NMR spectrum of the product showed that substrate was reduced at the carbonyl group of B ring. NMR spectrum of the product exhibited two different proton shifts for -CH proton of reduced carbon at 5th position of B ring and for the

olefinic proton adjacent to the carbonyl group at A ring for two different diastereomer. The ratio of the proton intensity of these shifts belonging to (*4aS,5S*) and (*4aR,5S*) [40], isomers were calculated from the NMR spectrum and de value was obtained as 82% for (*4aR,5S*) isomer.

Figure 2.11. Reduction of Wieland-Miescher ketone via *A. niger* mediated bioconversion.

So far in literature reduction product of Wieland-Miescher ketone is obtained as a result of a kinetic resolution with 50% theroretical yield or the substrate is enantioenriched up to 80% ee before it is subjected to the reduction [87]. Therefore conversion of both enantiomer of this compound to single reduction product with a high diastereo-, and enantioselectivity (80±2%, 93±1%) was found to be rather interesting and this promted us to further investigate this reaction at different reaction condition.

Different medium composition, reaction time and substrate concentration were employed for the *A. niger* mediated reduction and these results are summarized in table 2.5.

Table 2. 5 Effects of different reaction conditions employed for *A. niger* mediated reduction of Wieland-Miescher ketone (de: diastereomeric excess, ee: enantiomeric excess, c: conversion)

	Medium type	Time (day)	Substrate (mg/mL)	de (%)	ee (%)	c (%)
1	A	5	0,5	43±2 (*4aR,5S*)	93±2	50±2
2	A	12	0,5	47±4 (*4aR,5S*)	93±2	54±3
3	D	5	0,5	58±3 (*4aR,5S*)	91±1,3	93±2
4	D	5	1	54±2 (*4aR,5S*)	93±1	78±2
5	D	12	0,5	79±2 (*4aR,5S*)	93±1	>99
6	D	15	1	67±3 (*4aR,5S*)	90±0,8	80±3
7	PDB	5	0,5	60±2 (*4aS,5S*)	97±0,7	59±3
8	PDB	5	1	55±2 (*4aS,5S*)	95±0,5	49±3
9	PDB	12	0,5	56±2 (*4aR,5S*)	97±1,4	64±2
10	PDB	15	1	49±2 (*4aR,5S*)	96±1	51±3

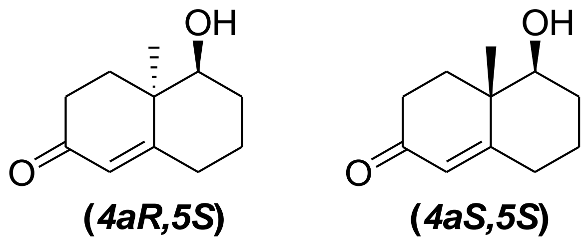

Figure 2.12. Izomers of reduction product of Wieland-Miescher ketone

Conversions with medium A (2 % glucose, 2% yeast extract, 0,5% peptone minerals) and potato dextrose broth (8% potato extract, 2% glucose) resulted in corelatively low de values (43±2-60±2%) and conversions (50±2-59±3%) than medium D employed reactions. In addition, ^{1}H-NMR spectrum results showed an interesting results that intensity of the protons which are typical for (*4aS,5S*) diastereomer became predominat (61%) over proton shifts of (*4aR,5S*). This means that diasteroselectivity shifted to (*4aS,5S*). and bioconversion is strongly influenced by medium composition (entry 5). To obtain better de value, conversion time prolonged to 12 day and diasteroselectivity turned to (*4aR,5S*)(entry 7) most probably due to change in rate of reacton catalyse by different competing enzymes with the change of metabolism of growing cells. To confirm the diastereoselectivity shifts during the conversion period, de values from the first day of the PDB employed conversion at the same experimental condition summarized in the table as entry 8. As it can be seen from the table 2.6 until 6th day of the bioconversion the biocatalyst favored the (*4aS,5S*) diastereomer with an increasing de value and at 7th day it was observed from the NMR data that the diasteroselectivity is shifted to (*4aR,5S*) with 35±4 % de and increase to 51±2 % at 12th day.

Table 2.6. Change in de values during the *A. niger* mediated bioconversion of Wieland-Miescher ketone in PDB medium. (In 100 mL medium, 0,5 mg/mL substrate, 30°C,160 rpm)

Time (day)	Diastereomer	de (%),
1	(*4aS,5S*)	34±2
3	(*4aS,5S*)	50±3
5	(*4aS,5S*)	55±2
7	(*4aR,5S*)	35±4
12	(*4aR,5S*)	51±2

Reaction period in the case of PDB employed bioconversions effect the diasteroselectivity while with other medium types this only observed as increase in de value of same diastereomer. 12 day conversion period in medium D of was sufficient for total conversion of 0,5 mg/mL substrate to reduction product (entry 5). Capacity of the biocatalytic system was also tested for high amount substrate and 1mg/mL substrate was used at an extended period of conversion (15 day) and 67±3 % de for (*4aR,5S*) with a 80±3% conversion (entry 6) was achieved. For PDB de value was droped to 49±2% with relatively low yield 51±3% (entry 10)

As it is summarized in table 2.5 while total conversion of substrate was accheieved with medium D, medium A and PDB employed transformations at same experimental conditions conversion of reduction product was only around 51% (entry 1) and 64% (entry 10). This corelatively low conversion rates was thought to be the result of kinetic resolution of the substrate**.** To investigate this phenomenon, remaining substrate which is recovered from the media at 5 th day of conversions was also analysed by HPLC to determine its ee values. table 2.7 shows the ee values of recovered substrate for different medium composition.

Table 2.7. ee values of recovered substrate Wieland-Miescher ketone obtained with *A. niger* mediated bioconversion in different media. (With 0,5mg/mL substrate and under optimized conditions).

Medium type	ee of recovered substrate (%)
Medium A	93±2 (*S*)
Medium D	72±3 (*S*)
PDB	58±3 (*S*)

The highest ee value was obtained with medium A employed experiment (93%) with 48% yield of recovered substrate. For medium D 72±1 % ee was obtained while the ee value was moderately high for PDB. These results shows that biocatalysis favors (*R*) enantiomer of the substrate for reduction while (*S*) enantiomer remains in medium resulting in a kinetic resolution of racemic mixture and medium A yields a better resolution (93%) than other mediums (73-59%)(table 2.7). The ee value of substrate during the conversion period was increased to 93% after 4 day and remained as 91% until the 7th day (figure 2.13).

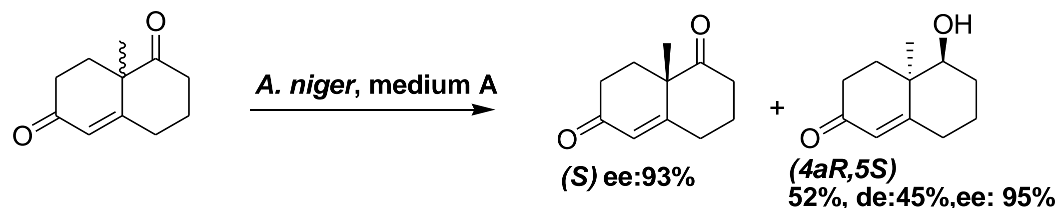

Figure 2.13. ee values of recovered substrate obtained with *A. niger* mediated kinetic resolution of Wieland-Miescher ketone in medium A.

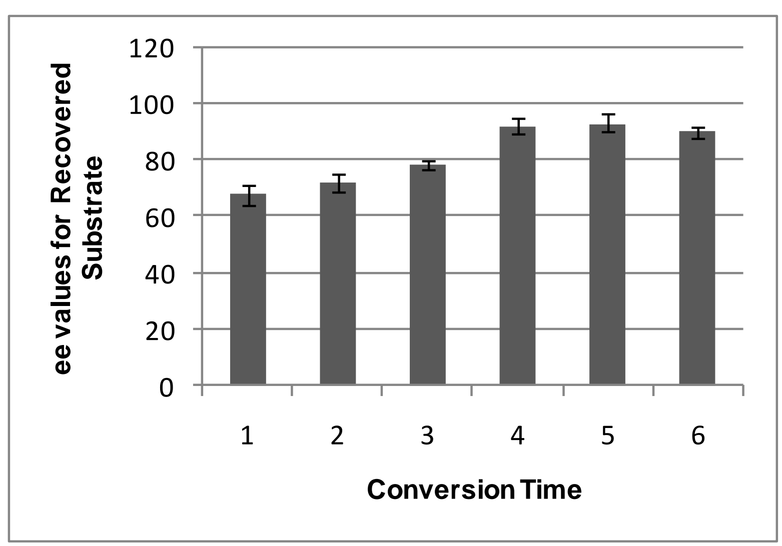

Figure 2.14. Change in ee values of recovered substrate for *A. niger* mediated kinetic resolution of Wieland-Miescher ketone in medium A. (With 0,5 mg/mL substrate, under optimized conditions).

For the bioconversion performed with medium D which furnishes a single diastereomer (*4aR,5S*) with 82% de and 99 % first we focused on a reaction mechanism which is called as dynamic kinetic resolution where it is possible to convert a racemate to a product with 100% chemical yield and 100% enantiomeric excess [28] . This is possible only if an equilibrium controlled racemization of substrate proceeded at the same time with conversion of substrate (see section). But, the ee value of our substrate obtained was far from being racemic (72±1% for (*S*) enantiomer). Therefore this case might be explained by crossing over the symmetry plane by inversion of configuration at for the resulting diastereomer. As it is shown in figure 2.13 while the (*R*) enantiomer of Wieland-Miescher ketone reduced to reduction product which has (*4aR,5S*) configuration remaining the configuration *R* configuration, the configuration of (*S*) enantiomer inverted when it is reduced to (*4aR,5S*) configuration at the substrate or product level.

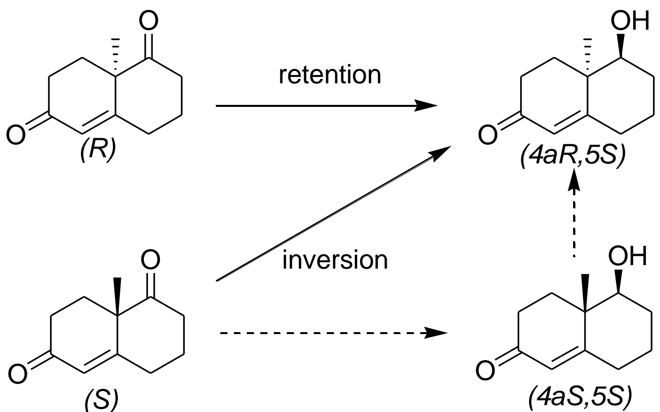

Figure 2.15. Retention and inversion of enantiomers of Wieland-Miescher ketone throughout the *A. niger* mediated bioconversion in medium D.

A. niger mediated reduction of Wieland-Miescher ketone is one of the earliest reports related to microbial reduction of this compound. With his pioneering work Prelog and co-workers reported the *A. niger* and *Rhizopus nigricans* [94] mediated reduction with 99% conversion. However reaction that catalyzed by these fungal enzymes was produced racemic mixture of reduction product. In our case it's we were able to achieve the 99 % conversion of racemic mixture of substrate to the product with a high (79±2 %) diasteroselectivty. With these results we re-investigated the *A. niger* mediated reduction of Wieland-Miescher ketone and succeeded to introduce a bioreduction process of this compound with high de and especially high conversion rate which is not possible with kinetic resolution process. It's also noteworthy to mention that we could also control the conversion mechanism and absolute configuration of product by changing the medium composition. In the case of medium C and medium D product has (*4aR,5S*) configuration while for PDB it was (*4aS,5S*).

2.4. Preperation of Enzyme Immobilized Magnetic Nanoparaticle Biocatalyst for Carboligation Reactions for Preperation of α-Hydoxyketones:

The immobilized biocatalyst sytem for carboligation reaction that we created is based on a technique that can be also called as high gradient magnetic fishing (HGMF) since in this method surface modified magnetic particles are used as solid support [95]. In our immobilization experiments nitrilotriacetic acid (NTA) attached silica coated superparamagnetic nanoparticles were used. Hexahistidine tagged recombinant BAL is immobilized on particle via coordination with cobalt (Co^{2+}) ions that are previously charced on to the NTA molecules.

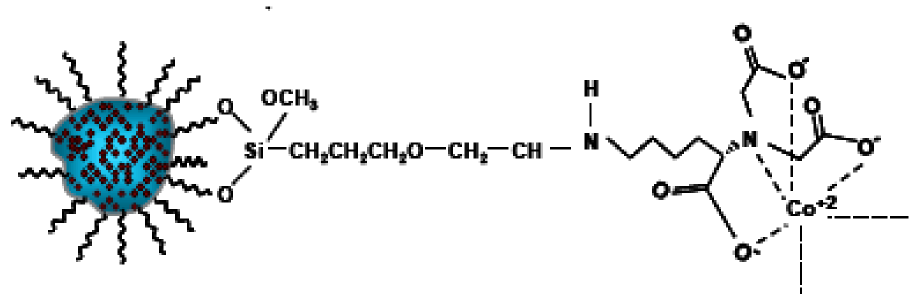

Figure 2.16. Schematic illustration Co^{+2} charged γ-Fe_2O_3 – silica – nanocomposite particles used for BAL and BFD adsorption [96]

The specific surface area (i.e. the surface area per unit volume or unit mass of particles) of the γ-Fe_2O_3-silica nanocomposite particles that are used in this study was calculated as 0.95 m^2/cm^3 [95].

2.4.1 Production of Carboligating Enzymes BAL and BFD:

Production of histidine tagged recombinant BAL was performed in autolytic *E. coli* host strain (BL21 PLysS (DE3)-invitrogen). The plasmid pUC19 carrying the hexahistidine tagged BAL gene transformed to competent *E.coli* cells. For BFD production the plasmid pKK322 containing hexahistidine tagged BFD gene was transformed *E. coli* SG13009 cells. Enzymes were expressed in these host cells. Expression was analyzed by SDS-Page electrophoresis with induced and non-induced *E. coli* cells (figure 2.16). Over expressed recombinant BAL and BFD was observed as correspondent bands in the induced cell lane (56 and 59 kDa). Benzoin condensation reaction (self condensation of benzaldehyde) was also performed with the crude with BAL and BFD containing crude cell lysate was performed to analyze the expression. The product benzoin was analyzed by HPLC with a chiral column (Chiralpak OJ-H) to calculate the ee values. In both cases the reactions yielded benzoin with high ee value (99%). Conversion of reaction performed with lyophilized crude cell lysate as (>90%). Since benzaldehyde is not a natural substrate for BFD the self condensation activity of this enzyme was performed with crude cell lysate containing total protein 10 fold higher than crude cell lysate containing BAL to able to observe benzoin production.

Large scale production of BAL and BFD was also optimized to able to obtain enzyme in bulky amount for long term storage and utilization. For this, production was performed in 2L fermentor (New Brunswick BioFlo110). Expression of the enzymes was performed for 12 h for BL21 and 5 h for SG13009 *E. coli* cells. Crude cell lysate obtained and lyophilized to be used in purification and immobilization studies. Initial rate of BAL catalysed benzoin formation is determined by HPLC analysis with C-18 (Phenomenex) column. 100μL sample was withdrawn from the reaction medium and transferred to equal amount of DMSO to stop the reaction and than analysed by HPLC. Concentration of benzoin was calculated according to tha standart curve prepared with commercial benzoin. In the soluble fraction of 10 mg lyophilized crude cell lysate 3-7U (μmol/min^{-1}) enzyme was detected (1U carbolgase activity

equals the amounts of enzyme that catalyzes the formation of 1 µmol of benzoin per minute under standart condition (BAL carboligase reaction was performed at 30 °C, pH:7,8; BFD activity was performed at 25 °C, pH:7,5). For BFD this value was estimated as 0,25-0,5 U. Protein determination was performed by Bradford assay using BSA as standart.

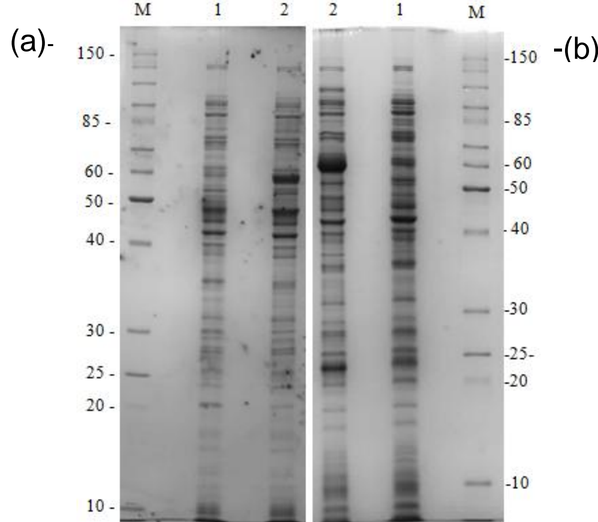

Figure 2.17. SDS-page analysis of expression of BAL and BFD. Expression of 6XHis-Tagged BAL and BFD induced with 1 mM IPTG. Aliquots were removed before and after induction (after 12h for BAL and 5h for BFD). 12% agarose was used for electrophoresis and proteins were visualized by Coomassie staining **M:** Molecular weight marker (5 µL, Fermentas-PageRuller^{TM} 10-100kDa) **(a)** BFD Lane1: Crude extract before induction (10 µg), Lane 2: Crude extract from 4 h after induction (10 µg) **(b)** BAL Crude extract before induction (10 µg), Lane 2: Crude extract from 12 h after induction (10 µg)

2.4.2 Immobilization of BAL and BFD to Surface Modified Fe_2O_3 Nanoparticles

For immobilization of histidine tagged BAL metal affinity strategy was used. For this surface modified silica coated nanoparticles (γ-Fe2O3 – silica - GLYMO-NTA-Co^{2+}) [96] were employed for the one-pot purification-immobilization steps.

Crude cell lysate obtained from BAL and BFD overexpressing *E. coli* cells was used for immobilization with the standart method that was described for commercially available methal affinity resins. The cell free extract HIS-tagged enzymes were incubated with resin, after excessive washing, the protein was eluted the quantification of the protein was subsequently performed by BSA standart assay [97]. Figure 2.18 shows the protein amount eluted from the resin from different total protein loaded batchs. Different amount of protein loaded to the resin from crude extract obtained from BL21(pLySs) overexpressing BAL. Optimum protein amount that was attached to the resin was estimated as 3,02±0.31 mg/g for BAL and 3.18±0.22 mg/g for BFD.

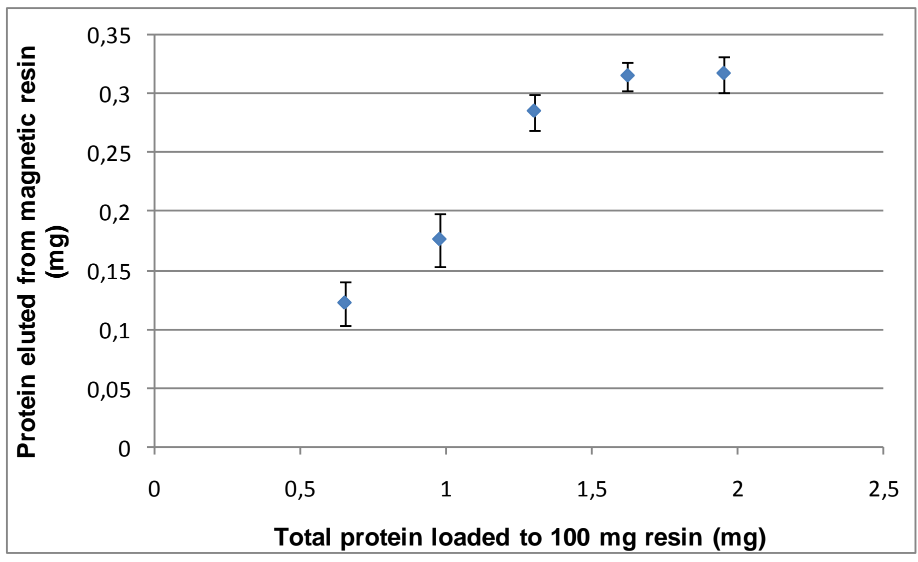

Figure 2.18. Amount of BAL eluted from 100 mg surface modified magnetic resin at different amount of total protein employed.

Theoretical binding capacity of surface modified particles are calculated from the absorption equilibria which was described by the Langmuir (1918) [98] and for 60 kDa protein capacity of the resin was estimated as ~3 mg g^{-1}. This confirms that our result was consistent with theoretical binding capacity of the resin.

SDS-Page analysis was performed in METU Central Laboratory with the protein eluted from magnetic resin. Protein bands are belong to the target proteins were obtained as single band on the gel and correspondent to their expected molecular weight, which are 59,8 kDa homotetameric subunit for BAL [99] and 56 kDa that of BFD [100] in the literature. With these results it was shown that surface modified magnetic nanoparticles were eligible for the selective binding of HIS –tagged enzymes BAL and BFD (figure 2.19).

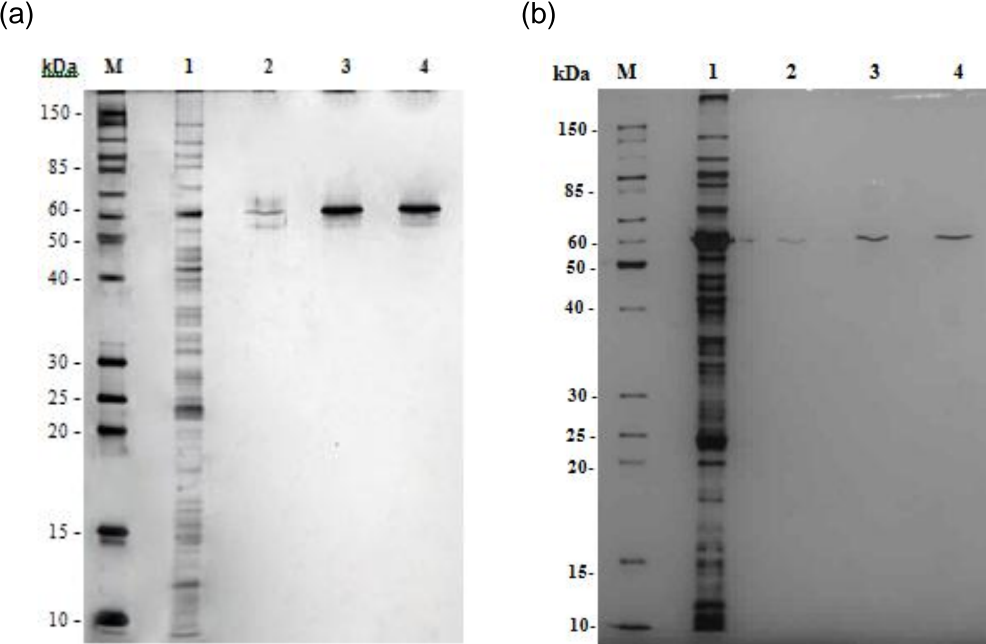

Figure 2.19. SDS-PAGE analysis of the purifiedBAL and BFD. 12% SDS-polyacrylamide gel was used and proteins were visualized by silver staining method. (a) **M**: Molecular weight marker (5 µL, Fermentas-PageRuler^{TM} 10-200kDa), **1**: Crude extract of BAL expressing BL21 *E.coli* cells (5 µg) ; **2-4**: protein eluted from 0,96, 1,6 and 1,97 mg total protein loaded resin (30 µL sample from 3 mL eluents of 100 mg resin, 0,66 µg, 3,2 µg and 4. µg) (b) BFD, M: SDS-PAGE molecular weight marker;**1**: crude extract of BFD expressing SG13009 *E.coli* cells (10 µg); **2-4**: protein eluted from 0,82, 1,5 and 1,75 mg total protein loaded resin (20 µL sample from eluents, 5mL eluents of 100 mg resin 0,2, 0,5, 0,5 µg)

Since metal affinity resins are designed for purification of tagged reacombinant proteins we also purified BAL and BFD with 1 g of surface modified resin. BAL purification was also performed with 1 mL commercial Co^{2+} charged TALON® resin. To remove the imidazole 5 mL Hi-Trap^{TM} sephadex G-25 superfine column (Ammersham) was used as desalting column. The yield and purification folds for BAL and BFD purification with superparamagnetic particles and TALON resin is summarized in figure 2.8. and 2.9.

Table 2.8: Purification table for BAL. The enzyme was purified with surface modified magnetic nanoparticles (1g) and commercial metal affinity resin (1 mL of 20% solution). Activity was calculated according to benzoin condensation reaction in 50 mM potassium phosphate buffer (0,25 mM TPP, 2,5 mM $MgSO_4$, 25% DMSO, 100 µM Benzaldehyde at pH:7.8) at 30°C.

Step	Volume	Protein	Activity	Total activity	Specific activity	Yield	Fold
	ml	mg/mL	U/mL	U	U/mg	%	
Crude extract (centrifuged and filtered)	10	38	1,7	17	0,045	100	1
Purified enzyme with magnetic resin (After desalting with sephadex G-25 Hi-Trap coloumn)	1	3,2	11	11	3,5	63	74
Purifed enzyme with Talon resin (After desalting with sephadex G-25 Hi-Trap coloumn)	1	4	14	14	3,55	82	76

Table 2.9. Purification table for BFD. The enzyme was purified with surface modified magnetic nanoparticles (1g). Activity was calculated according to initial rate of benzoin condensation reaction in 50 mM potassium phosphate buffer (0.5 mM TPP, 2.5 mM $MgSO_4$, 25% DMSO 50µM benzaldehyde, pH:7.5) at 25°C.

Step	**Volume**	**Protein**	**Activity**	**Total activity**	**Specific activity**	**Yield**	**Fold**
	ml	**mg/mL**	**U/mL**	**U**	**U/mg**	**%**	
Crude extract (centrifuged and filtered)	10	36	0,25	2,5	0,007	100	1
Purified enzyme magnetic resin (After desalting with sephadex G-25 Hi-Trap coloumn)	1	3,4	1,7	1,7	0,51	68	73

The enzyme-magnetic resin heterocatalyst system was highly responsive to a magnetic field, where the slurry was able to be clarified in 30 seconds by using a regular magnet. Resin was immediately re-suspended with gentle shaking in the solution after magnetic field released. These properties were typical behaviors for superparamagnetic resins. The advantage of using paramagnetic particles in enzyme immobilization in this sense are easy sampling procedure due to fast response to magnetic field and homogeneous reaction media as a result of low self aggregation.

Figure 2.20. Response of BAL-magnetic resin heterocatalyst to the magnetic field.

2.4.3. BAL Catalyzed Benzoin Condenzation Reactions with Immobilized System

The benzoin condensation reaction of immobilized BAL was conducted in a batch synthesis (figure. 2.20). The reaction conditions were chosen as previously reported by Drager et al.[90] and kinetic analysis was performed by following an increase in benzoin concentration (figure 2.21). 185 mg resin was used for immobilization of BAL.

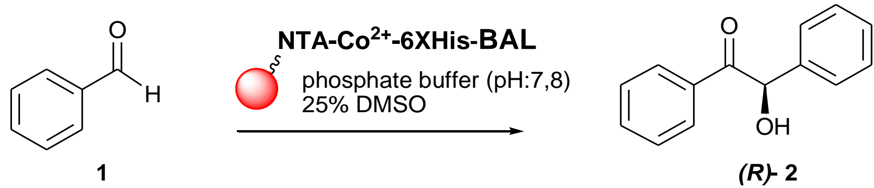

Figure 2.21. Immobilized BAL mediated self condensation reaction of benzaldehyde

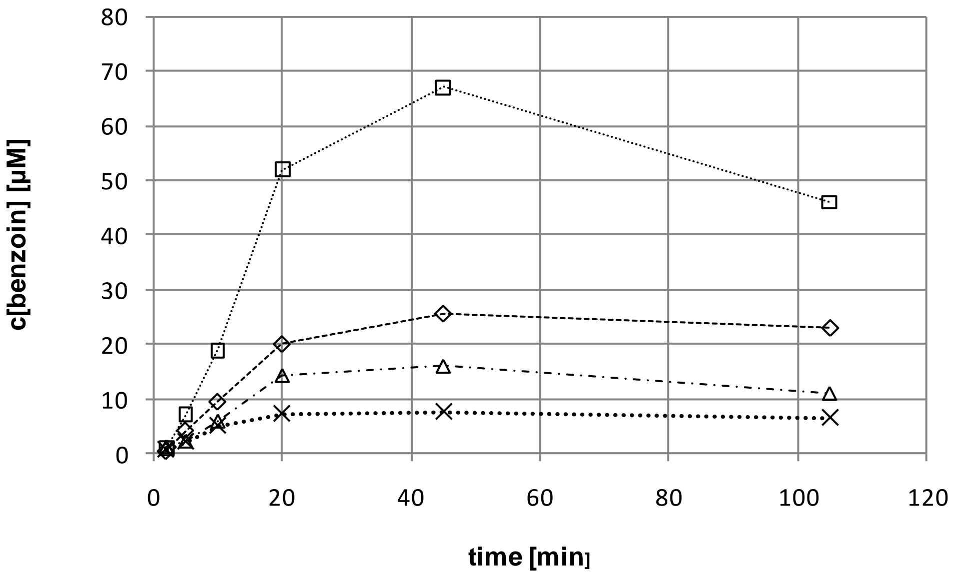

Figure 2.22. Time course of the change in benzoin concentration during the reaction of BAL immobilized on γ-Fe2O3 nanoparticles at a different benzaldehyde concentration (Δ:1 mg/ mL, ◊: 4 mg/mL, □ : 10 mg/mL, x: 50 mg/mL), all reactions were monitored by HPLC.

Specific activities was calculated from initial recation rates as 0,85±0,1 U (for 1 mg mL^{-1} benzaldehyde), 1,65±0,2 U (for 4 mg mL^{-1}) and 0,23±0,1 U (for 50 mg mL^{-1}) and there was no activity detected at higher benzaldehyde concentrations (100 mg mL^{-1}). But the reaction conducted with 10 mg mL^{-1} benzaldehyde resulted in a >90% conversion at the 45th min as a result of higher catalytic

activity of this reaction (2,4±0,3 U) and a decline in benzoin concentration occurred at the subsequent time points due to product inhibition. Reactions were fallowed up to 200 min and a fluctuation in benzoin concentration was observed at high concentration of benzaldehyde (50 mg/mL). Similar observation was also reported by Drager et. al. [90] with benzaldehyde concentration during BAL immobilized batch reaction and this phenomenon was also explained by product inhibition of ligase reaction. The lyase activity of BAL is reported as 3 fold less than lyase activity 14 therefore this fluctuation may also explained by the cleavage of benzoin when the benzoin concentration reached at saturation concentrations for the lyase reaction. One thing should also be noted that benzaldehyde was less soluble at high concentration (50 mg/mL and 100 mg/mL) and partially stays as small droplets in reaction media for longer time. This may explains the low or no activity at these higher benzaldehyde concentrations. As it reported by Pohl et. al. [99] during their kinetic analysis of BAL with free enzyme, at higher concentration of benzaldehyde activity was increased with the increase in co-solvent (DMSO) amount (30%).

Reactions with free enzyme and were also performed to compare the activity of immobilized enzyme. First we purified BAL with commercial Co^{2+}-NTA affinity resin and successive desalting process. Eluent obtained from desalting colomn was directly used for activity studies. Equal amount of protein (3 mg) was used to be able to compare the activity of free enzyme and immobilized one. Reactions were performed with free enzyme was showed same pattern with immobilized enzyme system

Repeated experiments were performed with the same batch of immobilized enzyme after washing with 50% DMSO in lysis buffer. The resin was able to be reused without any loss of activity for the three sets of experiments, in which the respective activity decreased at the 4th repetition and the remaining activity was calculated as 96 and 86.4% for the 4th and 5th repetition (Figure 2.22).

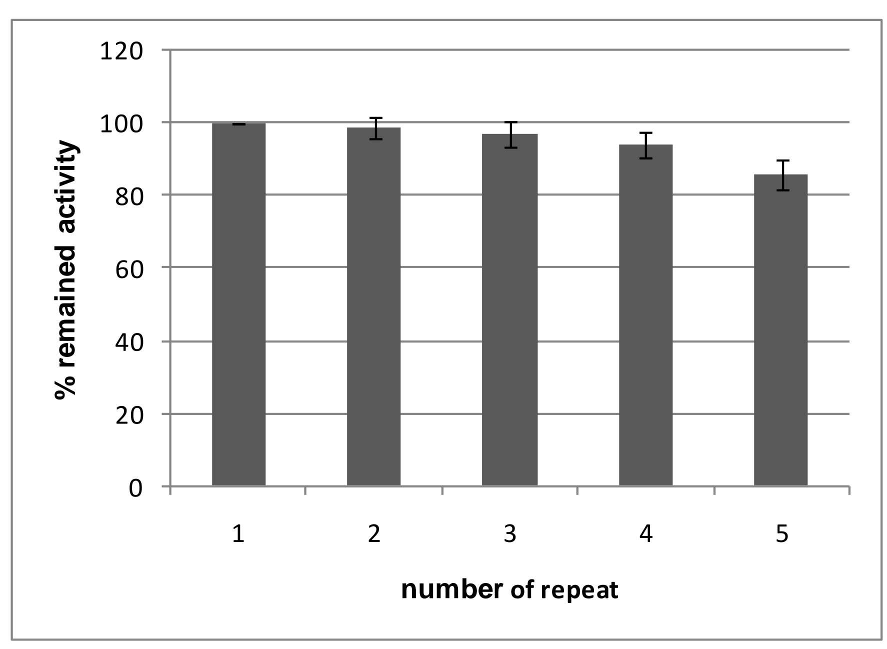

Figure 2.23. Remained activity of immobilized BAL with a repeated set of immobilization enzyme reaction. All reactions were monitored by HPLC.

2.4.4 Synthesis of Pharmaceutically Important HPP Derivatives with BAL Catalyzed Immobilized System:

In previous reports, it was achieved the BAL mediated synthesis of (*R*)-2-hydroxy-1-phenylpropanone [(*R*)-2 HPP] **(*R*)-3** [100] (starting from racemic benzoin and acetaldehyde) and 2-hydroxy-3,3-dimethoxy-1-(4-methoxyphenyl)propan-1-one [101]. In the former case, the reaction was first performed by lyase activity, which results in the kinetic resolution of racemic benzoin and ligase activity promoting both cross and self condensation products (Figure 2.21.-a). In the latter case, by the ligase activity of BAL, an important building block in the synthesis of cytoxazone was synthesized as a cross condensation product of *p*-anisaldehyde and dimethoxyacetaldehyde (figure 2.21-b). In both cases, reactions were carried out by adding excess aliphatic aldehydes in order to promote the cross condensation reaction. Reactions were followed by TLC and products that were produced were analysed by ^{1}H-NMR.

For determination of the enantiomerc excess HPLC analysis was performed with chiral coloumns (Chiralpak OJ for benzoin and chiralpak AD-H for HPP, Chiralpak OD for (*R*)-2-hydroxy-3,3-dimethoxy-1-(4-metoxyphenyl) propan-1-one)

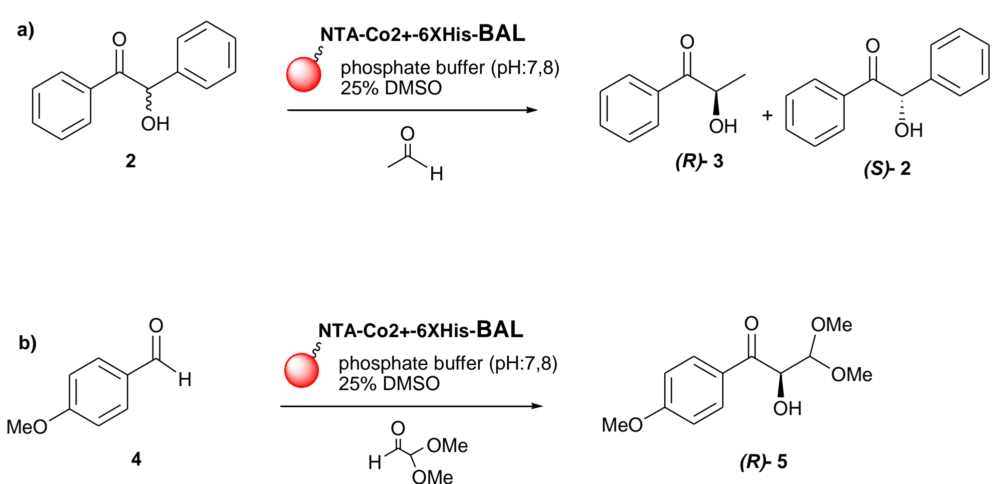

Figure 2.24. Immobilized BAL mediated reactions, a) Kinetic resolution of racemic benzoin with the lyase activity of the BAL in turn resulting in cross condensation with acetaldehyde b) Synthesis of (*R*)-5, which is a cross condensation product of 4.

The reactions were carried out for 2 to 4 days in order to obtain the desired conversions. Therefore, it was necessary to add fresh enzyme every 24 hrs to maintain the reaction that was proceeding. In the present study, it was our aim to observe the behavior of BAL-magnetic nanoparticles hybrid within long period processes (2-4 days) that requires the involvement of stable catalysts. For this, we performed reactions through free and immobilized enzymes based on the standard conditions that have been mentioned in the present study. We obtained results for the 24 and 48h from the reactions that were carried out both with free BAL with an enzyme addition (where the enzyme was added at fixed time intervals) and immobilized BAL as listed in table 2.8.

Table 2.10 Yield and ee values of BAL catalyzed condensation reaction with free and immobilized enzyme

Reaction type	Reaction mode*	Yield (%)	ee (%)	Recovery [(*S*)-benzoin] Yield (%)	Recovery [(*S*)-benzoin] ee (%)	Time
	Free (40U)	89±5	> 99			40 min
	Immobilized (40U)	85±6	> 99			40 min
	Free (80U)	47±5	> 99	44±4	>99	48 hrs
	Immobilized (80U)	44±5	> 99	43±5	>99	48 hrs
MeO, H, OMe, OMe, OMe, OH	Free (80U)	85±7	> 99			24 hrs
	Immobilized (80U)	83±7	> 99			24 hrs

As it is shown in table 2.8.with respect to their ee vales and yields reactions that were performed with our heterocatalyst system was comerable to reactions performed with free enzyme (10 mg per day, ee>99%), even though no additional enzyme was used other than immobilized on 300 mg resin. At the same reaction period BAL mediated conventional carboligation reaction and representative conversions with immobilized system furnished high yield and ee value. The ee values obtained from benzoin condensation reaction and representative carboligation reactions were as high as (99%) the ones obtained from free enzyme reactions. For kinetic resolution of benzoin and cross condensation reaction furnishing 2-hydroxy-3,3-dimethoxy-1-(4-methoxyphenyl) propan-1-one yields were slightly lower than free enzyme rection (44%-73%).

2.4.5. BFD Catalyzed Carboligation Reactions with Immoilized System:

The benzoin condensation reaction of BFD was performed with immobilized enzyme in a batch synthesis. The reaction conditions were choicen according to literature (0.5 mM TPP, 2.5 mM $MgSO_4$, 25% DMSO in 50 mM potassium phosphate buffer at pH:7.5, 25°C) [58 c]. Since the capacity of BFD for self condensation of benzaldehde is lower than BAL catalysed reaction 1 g of resin was used for the immobilization to increase the amount of BFD. Different amount of benzaldehyde was used for this reaction (50, 100, 200 and μM) and increase in benzoin concentration was determined by HPLC analysis as it is mentioned for BAL catalyzed reaction. Time course of increase in benzoin concentration is shown in figure 2.26.

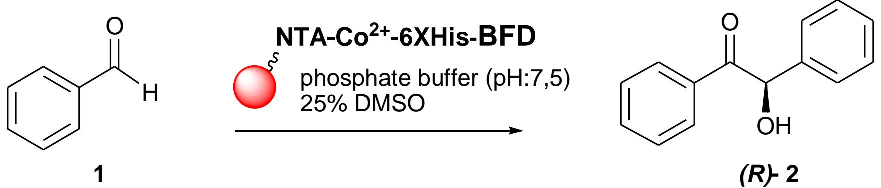

Figure 2.25. BFD mediated self condensation reaction of benzaldehyde with the immobilized enzyme

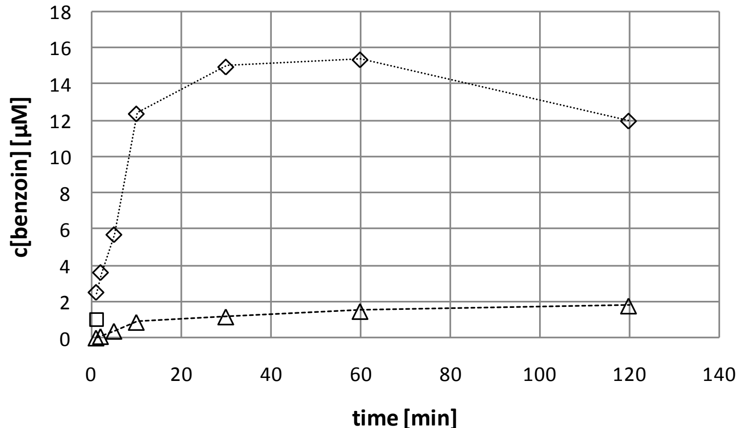

Figure 2.26. Time course of the benzoin concentration during the reaction of BFD immobilized on γ-Fe_2O_3 nanoparticles at a different benzaldehyde concentration (◊: 50 μM and Δ: 100 μM), all reactions were monitored by HPLC.

As it was indicated the specific activity with respect to self condensation of benzaldehyde is lower than that of formation of HPP in the presence of benzoiylformate and acetaldehyde. Specific activity of BFD was calculated for the self condensation reaction performed with different amount of benzldehyde and best results were obtained from the reaction performed with 50 μM benzaldehyde (1,4±0,6 U). With the higher amount of benzaldehyde the rate of the reactions droped significantly and the activity was too low (0,13±0,4 U) yielding 0,6% benzoin with 200 μM benzaldehyde. As in the case of BAL catalysed benzoin condensation benzoin concentration start to decrease after reaction reached at the plateu. With BFD this phenomenon was observed after

60 min. With lower rate of reaction this decline is delayed. This decline cab be explained by increase in activity for reverse reaction resulting in increase of benzaldehyde and decrease of benzoin.

We also performed cross condensation reaction between benzaldehyde and acetaldehyde which furnishes (*S*)-2-HPP. For ease of calculation of conversions the magnetic 2 g resin holding 5U BFD was used for immobilized system. For the comparison of free enzyme catalysed reaction was also performed and for this crude extract containing 160 mg total protein and 5U BFD was used. Reactions are performed in a reaction buffer (0.5 mM TPP, 2.5 mM $MgSO_4$, 25% DMSO in 50 mM potassium phosphate buffer) which contains 10 mmol benzaldehyde and 20 mmol acetaldehyde.

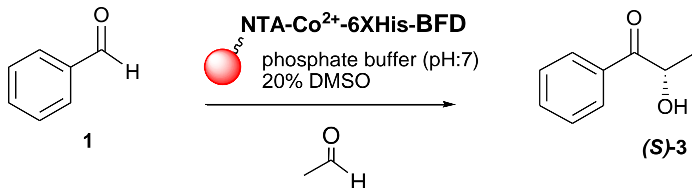

Figure 2.27. Cross condensation reaction between benzaldehyde and acetaldehyde with immobilized BFD.

In accordance with literature this cross condensation reaction resulted in comperatively low ee (92%) than that of BAL catalysed cross condensation reaction which furnishes (*R*)-2-HPP [58]. The ee values and conversions with immobilized and free enzyme catalysed reaction with BFD are summarized in Table 2.9. For determination of the enantiomeric excess HPLC analysis was performed with chiral coloumns (Chiralpak OJ for benzoin and chiralpak AD for HPP). Yield for self condensation reaction were calculated from kinetic analysis from the time point in which the reaction yields the maximum product concentration. Crude extract containing 3U enzyme was used for free enzyme catalysed reaction under the same experimental condition.

Table 2.11. Yields and enantiomeric excess (ee) values for immobilized and free BFD catalyzed carboligation reactions.

Reaction	Reaction mode	ee%	Yield (%)
Benzaldehyde ⇌ benzoin	Free (3U)	>99 (R)	30±4
	Immobilized (3U)	>99 (R)	36±7
Benzaldehyde + acetaldehyde → 2-HPP	Free (5U)	92±4 (S)	96±5
	Immobilized (5U)	92±3 (S)	94±4

As it is seen from the table 2.9 ee values and yields obtained from immobilized system were comparable with that of crude extract reaction. In accordance with literature [58] BFD catalysed cross condensation reaction yields (*S*)-2-HPP instead (*R*) enantiomer and with a correlatively lower ee value. As it is mentioned BFD has lower activity for self condensation of benzaldehyde than cross condenzation reaction. Therefore the yield of benzoin formation was only up to 30% while 96% was accieved for HPP production.

These representative reactions performed with two important carboligating enzyme which are immobilized to magnetic nanoparticles were resulted with correlative yield and ee values. This system allows us to one pot purification /immobilization since it is designed for metal affinity ligant binding of histidine tagged reacombinat protein. Therefore it can also be used for purification of these proteins. Moreover magnetically responsive system was found to be rather convenient due to the ease of separation and this immobilized biocatalyst system could be reused without significant drop of activity and easily recovered from the reaction medium with almost high recovery.

CHAPTER 3

EXPERIMENTAL

3.1. Materials and Methods

Microorganisms used in this study were generous gifts of Osaka University Department of Biotechnology (OUT) and *A. flavus* and *A. niger* was purchased from TUBİTAK MAM culture collections.

Column chromatographies were performed for the purification of the products by using Merck Silica Gel 60 (partical size 40–63 am).

All the substrates and the products synthesized were identified by 1H-NMR and spectra recorded by BRUKER DPX 400MHz using tetramethylsilane (TMS) as an internal standard and deutero chloroform as solvent.

Enantiomeric excesses of all the products were determined by Agilent 1100 series HPLC device using appropriate optically active columns.

3.2. Preparation of Stock Cultures

Microbial cultures were inoculated onto sterile petri dishes containing potato dextrose agar (PDA) After incubation at 25°C for 12 -14 days complete growth was observed. The cultures were stored at 4°C for maximum 3 months.

3.3 Medium Compositions used in Fungi Mediated Whole-Cell Biotransformation Studies:

Medium compositions for 1 L medium

Medium A: 20g glucose, 20g yeast extract, 5g peptone, 1g KH_2PO_4, 2g K_2HPO_4, 2g$NaNO_3$, 0,5gKCl, 0,05g $MgSO_4.7H_2O$, 0,02g $FeSO_4.7H_2O$

Medium B: 8 g potato extract, 20 g glucose

Medium C: 20 g malt extract, 20 g glucose

Medium D: 4 g yeast extract, 15 g starch, 1g K_2HPO_4 and 0.5g $MgSO_4.7H_2O$ minerals

3.4. Cultivation of *Aspergillus flavus* for Bio-oxydation of *meso*-Hydrobenzoin and Analysis of the Products:

Bio-oxydation of meso-hydrobenzoin was performed in different mediums which were suggested for Aspergillus species in literature After inoculation microorganism from petri dishes to 100 mL medium in 250 mL flask microorganism was incubated for 2 days and induced with hydrobenzoin in 1 mL DMSO. Samples were taken periodically and the conversion was monitored with TLC with 1:3 ethyl acetate: hexane under 254 nm UV light. After the reaction is stopped microorganism was filtered off the supernatant, washed with distilled water and the combined aqueous phases were extracted with 1/1 (V/V) ethyl acetate two times. The organic extract was dried, filtered and concentrated and reaction products and remaining substrate was isolated as a colorless solid.

Products were analysed by HPLC with chiral coloumn (Chiralpak AD-H, 90:10 hexane: isopropanol, 1 mL/min, 254 nm)

3.5. Cultivation of *A. flavus* for the Bioconversion of Wieland-Miescher Ketone and Analysis of the Products:

Bioreduction of Wieland Miescher Ketone was performed in different media. A. niger strains were inoculated into 250 mL sterile erlenmeyer which contains 100mL growth medium with a sterile inoculation loop by rubbing of spores grown on the surface of a PDA petri dish. The organism was grown on rotary shaker at 37°C, 160rpm for 48 hours. The biotransformation was initiated by the induction of Wieland-Miescher Ketone dissolved in 1mL DMSO and added to the medium. Bioconversion of Wieland-Miescher Ketone to the target molecule was monitored via TLC. Conversion was followed by NMR and HPLC analysis of the samples which were taken at days of biotransformation in order to identify the course of reaction**.** The reaction was stopped at 12th day of bioconversion by filtering of microorganisms from the supernatant. After washing of microorganisms, aqueous phases were extracted three times with 100mL ethyl acetate (3x100mL). Combined organic phases washed with saturated NaCl solution were dried over $MgSO_4$, and concentrated under reduced pressure to afford the target compound as a yellow liquid which was isolated via preparative TLC (eluent 3:1 ethylacetate/ hexane). Product were analysed by HPLC with a chiral coloumn to determine the ee value (Chiralpak OD-H, 9:1 Hexane/ 2-propanol, 0.5 mL/min)

3.6. Preperation of Immobilized Biocatalyst for Carboligation Reactions

3.6.1. Preparation of a Cell Free Extract from Recombinant *E.coli* strain Overexpressing BAL and BFD

Transformation of pUC19-BAL_{HIS} Construct to competent *E. coli* BL21(DE3) PLysS Cells:

E. coli cells were grown in LB agar containing 35 µg/mL chloramphenicol 100 ml LB medium containing was inoculated with a single colonie from LB agar plate. Then E. coli cells were incubated until the optical density of the medium reaches to 0,5 at 600 nm. Cells were collected by centrifugation and resuspended in 10 mL ice cold 50 mM $CaCl_2$ solution after centrifugation at 4000 rpm for 10 min pelleted cells were resuspended in 2 mL 50 mM $CaCl_2$ solution and dispensed in aliquots, freezed in dry ice-ethanol mixture and keept at -80 °C until it is used. pUC19-BAL_{His} plasmid construct was transformed to autolytic *E. coli* BL21(DE3) PLysS cells by heat shock. Transformed cells were steaked out to LB plates containing 100 µg/mL ampicilline and 35 µg/mL chloramphenicol and incubated for overnight at 37°C.

3.6.1.1. Expression of *E. coli* BL21(DE3) PLysS containing the pUC19-BAL_{HIS} Construct

E. coli BL21(DE3) PLysS containing the pUC19-BAL_{HIS} construct grown in LB plates was transferred to 10 ml Luria broth (LB) containing containing 100 µg/mL ampicilline and 35 µg/mL chloramphenicol incubated for overnight. Cells were transferred to 100mL LB with same compositon for approximately 1 hrs until the OD_{600} reaches to 0,5 and transferred to in 1.65 L LB medium with antibiotics in fermentor (New Brunswick BioFlo110) and incubated for 2 hrs at 37°C. Expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranosid IPTG. 6 hrs later from the induction cells were harvested by centrifugation at 5000rpm at 10 min at 4°C. Pelleted cells were transferred to a petri dish and freezed in dry ice-ethanol to allow the autolytic cells disintegrated. For long term preservation frozed cells were öelted at 4°C, transferred to a petri dish and lyophilized for 36 hrs at -80°C.

3.6.1.2. Purification of BAL with Co^{2+}-NTA Coloumn

Purification of BAL was performed with FPLC system (ÄKTA FPLC GE Healthcare Life Sciences) with column (5X80 cm) prepared with 100 mL Talon

metal affinity resin for affinity chromatography and 100 mL sephadex G-25 superfine for desalting of eluted sample from affinity coloumn. 5 g lyophilized crude cell lysate was dissolved in 100 ml 50 mM potassium phosphate buffer at pH:7,8. After sonication (60 ampere 10X10 second) crude cell lysate was centrifuged at 20.000 rpm for 30 min at 4 °C. The supernatant filtered through 0,45 µm membrane and 100 mL sample was applied by using pump from buffer valve 8 with 2 mL/min to Co^{2+}-NTA affinity coloumn (Invitrogen). Protein was eluted by the elution buffer (50 mM potassium phosphate, pH:7,8, 150 mM imidazole) at 2 mL/min. 20 mL eluted samle was applied to 100 mL desalting column. Fragments containing protein fraction was collected and directly used for activity assay or lyophilized for long term preservation. During the process columns and samples was always kept at 4°C.

3.6.1.3. Expression of *E. coli* SG13009 Containing pKK-$BFDL_{HIS}$ Construct

Recombinant *E. coli* SG13009 strain used in containing HIS-tagged BFD_{HIS} plasmid. Cells were grown in 100 ml LB medium until the optical density reaches to 0,5 at 600 nm, and they were induced by adding 1 mM isopropyl-β-D-thiogalactopyranosid (IPTG). After 4 hrs cells were collected by centrifugation and sonicated in 10 mL 50 mM potassium phosphate buffer (pH:7,5, 10 mM NaCl and 10 mM imidazole) for crude cell lysate preparation.

3.6.2. Determination of Protein Concentration:

Protein amount in crude cell extract and purified enzyme solutions was performed with Bradford assay. 1 mL solution of Bradford reagent (SIGMA) was (prewarmed at room temperature) was transferred to 1,5 mL eppendorf tube cuvet and 100 µL sample was added to this solution and mixed well with vortex and incubated for 10min. The solution transferred to 1,5 mL disposable spectrophotometer cuvet. Absorbance of the samples was measured at 595 nm. Protein amount was calculated by using BSA (Bovine serum albumin) as

standart. Purified enzyme solutions contain imidazole was measured carefully. Since imidazole influence the absorbance samples were diluted until the imidazole concentration drop to less than 50 mM.

3.6.3. Enzyme Activity Assays

Activity Assay for BAL:

BAL activity was determined by calculating the initial rate of benzoin formation. 10 mg lyophilized crude extract of *E. coli* BL21(DE3) PLysS containing the pUC19-BAL_{HIS} was dissolved in 5 mL reaction buffer (0.25 mM TPP, 2.5 mM $MgSO_4$, 25% DMSO in 50 mM potassium phosphate buffer at pH:7.8. Reaction was initiated by adding 50 mg benzaldehyde. Before reaction is initiated reaction mixture is pre-incubated at 30°C for 5 min. Reaction was performed at 30°C with gentle shaking (90 rpm) in 15 mL Falcone tube. At 1 min time intervals 100 µL samle was withdrawn from the reaction mixture and transferred to 100 µL DMSO to terminate the reaction. Samples are analyzed by HPLC (1,1 mL/min, 45% acetonitrile, 0.5% aceticacid and 54.5% water was used as mobile phase, C-18 coloumn (Phenomenex)) to determine the benzoin concentration (Benzoin R_t: 12 min). Benzoin concentration was calculated according to the standart curve prepared with commercial benzoin (SIGMA).

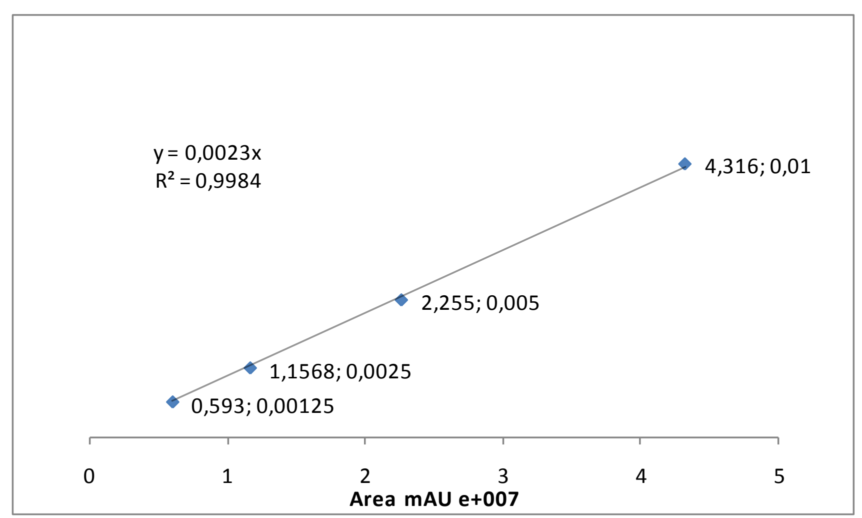

Figure 3.1: Standart curve for benzaldehyde

Activity Assay for BFD:

BFD activity was determined by calculating the initial rate of benzoin formation. 10 mg lyophilized crude extract obtained from recombinant *E. coli* SG13009 containing HIS-tagged BFD_{HIS} plasmid was dissolved in 5 mL reaction buffer (0.5 mM TPP, 2.5 mM $MgSO_4$, 25% DMSO in 50 mM potassium phosphate buffer at pH:7.5). 5 mg/mL benzaldehyde was added to the reactiom media to initiate the reaction. Reaction buffer was pre-incubated at 25°C for 5 min. Reaction was performed at 30°C with gentle shaking (90 rpm) in 15 mL Falcone® tube. At 1 min time intervals 100 µL samle was withdrawn from the reaction mixture and transferred to 100 µL DMSO to terminate the reaction. Samples are analyzed by HPLC (1,1 mL/min, 45% acetonitrile, 0.5% aceticacid and 54.5% water was used as mobile phase, C-18 coloumn (Phenomenex)) to determine the benzoin concentration (Benzoin R_t: 12 min). Benzoin concentration was calculated according to the standart curve prepared with commercial benzoin (SIGMA).

3.6.4. Purification of BAL and BFD with Surface Modified Magnetic nanoparticles:

1g surface modified magnetic nanoparticles were used to purify BAL and BFD. Nanoparticles first suspended in 10 mL potassium phosphate buffer (pH:7,8, 50 mM, 10 mM imidazole, 100 mM NaCl). Resin was washed in same buffer twice and 10 mL crude extract containing overexpressed BAL or BFD was added to the equilibrated resin. Crude extracts were prepared as it is described in section 3.6.1. This slurry incubated at 4°C with gentle shaking for 30 min and centrifuged for 5 min at 5000 rpm. The supernatant was discarded and resin was washed twice with the same buffrer. Protein was eluted with 5 mL elution buffer (potassium phosphate buffer, 50mM, pH:7,8, 200 mM imidazole, 10 mM NaCl). Eluted sample was concentrated with spin column concentrator (Millipore, Amicon 5K ultra) to 800 µL. Eluted and concentrated protein was applied to Hi-Trap^{TM} sephadex G-25 desalting column and desalted according to the default purification procedure template for the column. Eluted sample was used for activity assay and protein determination.

3.6.5. Immobilization of BAL and BFD to Superparamagnetic Particles

Superparamagnetic γ-Fe_2O_3 (maghemite)-silica nanocomposite particles were synthesized using the sol–gel method, in which the surface modification method was also applied for the immobilization of 6Xhistidine tagged recombinant BAL and BFD. γ-Fe_2O_3 nanoparticles were washed with a lysis buffer (10 mM imidazole, 100 mM NaCl in 50 mM potassium phosphate buffer) and incubated for a couple of hours at 4°C in order to enable the silica coat of nanoparticles to swell. Equilibrated resin was settled for 1-2 min by the aid of a magnet and the supernatant was removed by pipeting. Lyophilized crude extract was dissolved in a lysis buffer. After sonication, the slurry was centrifugated and the supernatant was filtered through a 0.45 µm filter and crude extract incubated with gentle mixing (90 rpm) at 4°C with magnetic nanoparticles for 20 min to

immobilize histidine tagged enzyme. The amount of crude loaded was estimated according to the saturation concentration for the resin. After the protein was immobilized, the resin was settled as described before and washed with a lysis buffer twice.

The amount of immobilized enzyme was determined by measuring the protein content in the eluted enzyme solution (200 mM imidazole, 100 mM NaCl, eluted protein in 50 mM potassium phosphate buffer pH:7,8) and by the colorimetric method at 595 nm using the SIGMA Bradford reagent with bovine serum albumin as a standard. SDS page analysis was performed in METU Central laboratory to confirm the selective binding of his-tagged BAL by the method Leammly 1970.

3.6.6. Benzoin Condensation Reaction with BAL Immobilized Superparamagnetic Particles

BAL immobilized resin was equilibrated with a reaction buffer containing 0.25 mM TPP, 2.5 mM $MgSO_4$, 25% DMSO in 50 mM potassium phosphate buffer at pH:7.8. The mixture was incubated via gentle shaking (90 rpm), at 25°C for 1 min and the reaction was started by adding benzaldehyde. The increase in benzoin concentration was followed by HPLC analysis. (Nucleodur C18, 1 mL/min, 254 nm, retention time 12 min). For this, a 100μL sample was withdrawn from the reaction medium, transferred to 200μL DMSO, and before the HPLC analysis, samples were diluted with 200μL mobile phase (45% acetonitrile, 0.5% aceticacid and 54.5% water). For determining the optimal conditions, reactions were repeated under identical conditions with different benzaldehyde concentrations. Reaction with free enzyme was performed under identical conditions.

3.6.7. Synthesis of (*R*)- 2-hydroxy-1-phenylpropanone [(*R*)-2 HPP] from Racemic Benzoin and Acetaldehyde

For synthesizing (*R*)- 2-hydroxy-1-phenylpropanone, 0,3 mmol benzaldehyde and 20 mmol acetaldehyde were added to the 5 mL reaction medium that was prepared as described for the benzoin condensation reaction. The reaction was performed at 25°C. To direct the reaction to cross condensation between benzaldehyde (resulting from enantioselective hydrolysis of benzoin) and acetaldehyde, 4 mM additional acetaldehyde was added at the 30th and 120th minutes. The reaction was followed by TLC and stopped at 48 hrs by adding an equal amount of chloroform. The mixture was extracted twice with chloroform and after the standard work up procedure the products were analyzed by NMR. Determination of enantiomeric excess of benzoin was performed with HPLC analysis (Chiralpak AD, 90:10 hexane: isopropanol, 1 mL/min, 254 nm, retention time for (*R*)-2-hydroxy-1-phenylpropanone:12,45, for (*S*)- benzoin: 33,35).

3.6.8. Synthesis of (*R*)-2-hydroxy-3,3-dimethoxy-1-(4-methoxyphenyl) propan-1-one

For synthesizing (*R*)-2-hydroxy-3,3-dimethoxy-1-(4-methoxyphenyl) propan-1-one to the reaction mixture, 2 mM *p*-anisaldehyde and 4mM dimethoxy acetaldehyde were added and incubated at 30°C via gentle shaking (90 rpm). After 2 hrs, 5 mM dimethoxy acetaldehyde was added to the medium. The reaction was followed by TLC and stopped at 48 hrs. The product was analyzed by NMR and enantiomeric excess was determined by HPLC analysis (Chiralpak OD, 0,8 mL/min, 97:3 hexane : isopropanol, 254 nm, retention time for (*R*)-2-hydroxy-3,3-dimethoxy-1-(4-methoxyphenyl)propan-1-one 38,4 and for (*S*)- form 43,6 min.)

3.6.9. Benzoin condensation reaction with BFD Immobilized Superparamagnetic particles

BFD immobilized resin was equilibrated with a reaction buffer containing 0.5 mM TPP, 2.5 mM $MgSO_4$, 25% DMSO in 50 mM potassium phosphate buffer at pH:7.5. The mixture was incubated via gentle shaking (90 rpm), at 25°C for 1 min and the reaction was started by adding benzaldehyde. The increase in benzoin concentration was followed by HPLC analysis. (Nucleodur C18, 1 mL /min, 254 nm, retention time 12 min). For this, a 100 µL sample was withdrawn from the reaction medium, transferred to 200µL DMSO, and before the HPLC analysis, samples were diluted with 200 µL mobile phase (45% acetonitrile, 0.5% aceticacid and 54.5% water). For determining the optimal conditions, reactions were repeated under identical conditions with different benzaldehyde concentrations. Reactions performed under identical conditions with crude extract.

3.6.10. Synthesis of (*S*)-2-hydroxy-1-phenylpropanone [(*S*)-2 HPP] from benzaldehyde and acetaldehyde

For synthesizing (*S*)-2-hydroxy-1-phenylpropanone, 10 mmol benzaldehyde and 20mmol acetaldehyde were added to the 5 mL reaction medium that was prepared as described for the benzoin condenzation reaction. The mixture was incubated at 25°C via gentle shaking (90 rpm). The reaction was followed by TLC and stopped at 48 hrs by adding an equal amount of chloroform. The mixture was extracted twice with chloroform and after the standard work up procedure, the products were analyzed by NMR. Determination of enantiomeric excess of benzoin was performed with HPLC analysis (Chiralpak AD-H, 90:10 hexane: isopropanol, 1 mL min^{-1}, 254 nm, retention time for (S)-2-hydroxy-1-phenylpropanone: 14,33)

3.7. Identification Biotransformation Products:

NMR, HPLC, and optical rotation data of all derivatives are given below.

3.7.1. 1,2-diphenyletane-1,2-diol

Figure 3.1. 1,2-diphenyletane-1,2-diol

^{1}H NMR (400 MHz, $CDCl_3/CCl_4$):HPLC: δ= 2,05(2H,S), 4,7(2H,S), 7,1-7,3 (10H) Chiralpak AD-H column, UV detection at 254 nm, eluent: n-hexane/2-propanol = 90:10, flow 0,7 mL min-1, 20°C; Rt = 21.2 and 22,1 min

3.7.2. (1R, 2S)-1,2-diphenyletane-1,2-diol

Figure 3.2. (1R, 2S)-1,2-diphenyletane-1,2-diol

^{1}H NMR (400 MHz, $CDCl_3/CCl_4$): δ= 2.0(2H, b), 4,7(2H,S), 7,1-7,3(10H); HPLC: Chiralpak AD-H column, UV detection at 254 nm, eluent: n-hexane/2-propanol = 90:10, flow 0,7 mL min-1, 20°C; Rt = 23.2 min

3.7.3. (S)-2-Hydroxy-1,2-diphenylethan-1-one

Figure 3.3. (*S*)-2-Hydroxy-1,2-diphenylethan-1-one

^{1}H NMR (400 MHz, $CDCl_3/CCl_4$): δ= 7.82 (d, J=7,8 Hz, 2H), 7.44 (t, J=7.5, 1H), 7.30 (t, J=7.6), 7.16–7.23 (m, 5H), 5.73 (d, J=5.9 Hz, 1H), 4.42 (d, J=5.9 Hz, 1H); ^{13}C NMR (100 MHz, CDCl3/CCl4): 198.7, 139.1, 133.8, 133.6, 129.1, 129.0, 128.6, 128.5, 127.7, 76.2 HPLC: Chiralpak OD column, UV detection at 254 nm, eluent: n-hexane/2-propanol = 90:10, flow 1.0 mL min^{-1}, 20°C; Rt (S)=13.0 min; Rt (R)=19.0 min. $[\alpha]^{20}_{D}$: solvent CHCl3, c 0.5.

3.7.4. (*4aR,5R*)-4,4a,5,6,7,8-hexahydro-5-hydroxy-4a-methylnaphthalen-2(3H)- one:

Figure 3.4. (*4aR,5R*)-4,4a,5,6,7,8-hexahydro-5-hydroxy-4a-methylnaphthalen-2(3H)- one:

^{1}H NMR (400 MHz, $CDCl_3/CCl_4$): δ= 1.19 (3H, s), 1.31-1.49 (1H, m), 1.62 -1.93 (5H, m), 3.35 (1H, dd, J= 4.3, 11.59 Hz (4aS 5S), 3.55 (1H, dd, J= 2.5, 2.6 Hz (4aR 5S), 5.65 (1H, d, J=1.35 Hz) which were in accordance with the literature and via HPLC: Chiralpak OD-H, 9:1 Hexane/ 2-propanol, 0.5 mL/min, UV detection at 254 nm, R_t (4aS 5S) =21.4, R_t (4aR 5S) =24.8

3.7.5. 2-hydroxy-1-phenylpropanone

Figure 3.5. (*R*)- 2-hydroxy-1-phenylpropanone

^1H NMR (400 MHz, $CDCl_3/CCl_4$): δ=1.45(d, J= 6.8 Hz, 3H), 3.7 (br, 1H), 5.15 (q, J=6.8 Hz, 1H), 7.5-7.9(m,Ar,5H); HPLC: Chiralpak AD-H, 90:10 Hexane/ 2-propanol, 0.8 mL/min, UV detection at 254 nm, R_t (S) =10.3, R_t (R) =13.1

3.7.6. 2-hydroxy-3,3-dimethoxy-1-(4-methoxyphenyl)propan-1-one

O
OMe
OMe
OH
MeO

Figure 3.6. 2-hydroxy-3,3-dimethoxy-1-(4-methoxyphenyl)propan-1-one

^1HNMR(400 MHz, $CDCl_3/CCl_4$): δ=3.30 (s, 3H), 3.39 (s, 3H), 3.62 (d, J= 7.2, 1H), 3.82 (s, 3H), 4.33 (d, J= 3.5, 1H), 5.00(dd, J= 3.5, 7.2, 1H), 6.92 (d, J= 8.7, 2H), 7.97 (d, J= 8.7, 2H); HPLC: Chiralpak OD, 97:3 Hexane/ 2-propanol, 0.8 mL/min, UV detection at 254 nm, R_t (R) =38,4, R_t (S) =43,6

CHAPTER 4

CONCLUSION

In this study we aimed to develop oxidoreductive bioconversions using whole cell fungi mediated biotransformation and immobilized enzyme mediated carboligation reactions for the synthesis of α-hydroxyketone which are indispensible building block for preparation of bioactive compounds. In the scope of whole cell bioconversions *A. flavus* and *A. niger* mediated biooxidation and bioreduction reactions were performed. Biooxidation of *meso*-hydrobenzoin to obtain enantiopure benzoin *via A.flavus* mediated biotransformation furnished moderately high ee (78%) values but the yield of this reaction was low 7% due to the proposed mechanism for oxidation reduction sequence of hydrobenzoin with *Aspergillus* mediated conversion. With the addition of acetone as hydrogen acceptor directed the reaction through the oxidation state showing the expected effect by increasing the yield up to 13%.

To obtain an alpha hydroxylation product, Wieland-Miescher ketone, an important synthon for steroid synthesis, was subjected to *A. niger* mediated bioconversion. Surprisingly this fungus converted this compound to its reduction product with a high diastereoselectivity (82%) starting from the racemic mixture of the substrate. This result was much more superior then the results that have been published for microbial bioreduction of this compound. This conversion was found interesting with respect to inversion of one enantiomer of the substrate to the other during the reduction process.

In the enzymatic part of this study BAL and BFD mediated acyloin condensation reactions were performed with an immobilized system. For this we used surface modified magnetic nanoparticles to immobilize histidine tagged recombinant BAL and BFD. With this particles one pot purification/immobilization procedure was applied and BAL and BFD mediated conventional and some representative acyloin condensations were performed. The results were found to be comparable with the reaction were performed with free enzyme. In addition to this enzyme-magnetic particle hybrit system offered some advantages such as reusability and ease of operation during reaction procedure.

REFERENCES

1. Voet, D.; Voet, J.G. Biochemistry, John Willwy and Sons, New York, **1995**

2. Cahn, R. S.; Ingold, C. K.; Prelog, V. *Experientia,* **1956**, 12, 81

3. Faber, K. Biotransformations in Organic Chemistry, Springer-Verlag, Berlin, **1997**

4. Arnold, L.; Demain, J.; Adrio,L. *Mol. Biotechnol*. **2008**, 38,41

5. Polard, D. J; and Woodly, J.M. *Trends. Biotechnol*. **2006**, 41, 356

6. (a) Anastas, P.T.; Warner, J.C.; Green Chemistry: Theory and Practise, Oxford University Press, Oxford, **1998**. (b) Anastas, P.T.; Kirchoff, M. M. *Acc. Chem. Res.* **2002,** 35,686

7. Kirchner, G.; Scollar, M. P.; Klibanov, A. *J. Am. Chem. Soc*. **1995,** 107, 7072

8. Patel, R. N. *Curr. Opin. Drug. Discov. Devel*. **2003,** 6, 902

9. Simon, H.; Bader, J.; Günther, H.;. Naumann, P. S.; Thanos, J*. Angew. Chem. Int. Engl.* **1985**, 24, 539

10. Faramarzi, M.A.; Badiee, M.; Yazdi, M. T.; Amini, M.; Torshabi, M. *J. Mol.Catal. B: Enzym.* **2008**, 50, 7

11. Holland,H. L.; Allen, L. J.; Chernishenko, M. J.; Diez, M.; Kohl, A.; Ozog, J.;Gu, J. X. *J. Mol. Catal. B: Enzym*. **1997**, 3, 311

12. Pinedo-Rivilla, C.; Aleu, J.; Collado, I.G.; *J. Mol. Catal. B: Enzym*. **2007**, 49,18

13. Garciıa-Burgos, C.; Carballeira, J. D.; Sinisterra, J. V. J. *Mol. Catal. B: Enzym.* **2006**, 41, 16

14. Comasseto, J. V.; Assis, L.F.; Andrade, L. H.; Schoenlein-Crusius, I. H.;Porto, A. L.M. *J. Mol. Catal. B: Enzym*. **2006**, 39, 24

15. Carballeira, J. D.; Álvarez,E.; Sinisterra, J. V. *J. Mol. Catal. B: Enzym.* **2004**, 28, 25

16. Demain, A. L.; Adrio, J. L. *Mol. Biotechnol*. **2008**, 38, 41

17. Frey, P. A.; Hegeman, A. D. Oxford University Press , New York, **2007**

18. Klibanov, A. *Nature*, **2001**, 409, 241

19. Hoffmann, R. W.; Ladner, W.; Helbig. W. Liebigs. *Ann. Hem.Soc*. **1984**, 107, 2993

20. Fuganti, C.; Grasselli, P.; Casati P.; Carmeno, M. *Tetrahedron Lett.* **1985**, 26, 101

21. Nakamura, K.; Kawai, Y.; Oka, S.; Ohno, A. *Tetrahedron Lett*. **1989**, 30, 2245

22. Buisson, D.; Azerad, R.; Sanner, C.; Larcheveque, M. *Tetrahedron Asymmetry* **1991**, 2,987

23. Ushio, K.; Inoue, K.; Nakamura, K.; Oka, S.; Ohno, A. *Tetrahedron Lett.* **1986**, 27, 2657

24. Kometani, T.; Kitatsuji, E.; Matsuno, R. *Agric. Biol. Chem*. **1991**, 55, 867

25. Buisson, D.; Azerad, R. *Tetrahedron Lett*. **1986**, 27, 2631

26. Seebach, D.; Züger, M. F.; Giovanni, F.; Sonnleitner, B.; Fiechter. A. *Angew. Chem., Int. Ed. Engl*. **1984**, 23,151

27. Urlacher, B.; Schimit, D. *Curr. Oppin. Chem. Biol*. **2006**, 10, 156

28. Strauss, U. T.; Felfer U.; Faber, K. Tetrahedron:Asymmetry **1999**, 10,107

29. Turner, J. N. *Curr. Oppin. Chem. Biol*. **2004**, 8,114

30. Comasseto, J. V.; Omori, A. T.; Andrade, L. H.; Porto, A. L. M. *Tetrahedron: Asymmetry*, **2003,** 14, 711

31. Chadha, A.; Baskar, B. *Tetrahedron:Asymmetry*, **2002**,13, 1461

32. Demir, A. S.; Hamamci, H.; Ayhan, P.; Duygu, A .N.; Igdir, A. C.; Capanoglu, D. *Tetrahedron: Assymetry* **2004**,15, 2579

33. Demir, A. S.; Hamamci, H.; Sesnoglu, O.; Neslihanoglu, R.; Asikoglu, B.; Capanoglu, D. *Tetrahedron Lett*. **2002**, 43, 6447

34. Wieland, P.; Miescher, K. *Helv. Chim. Acta*. **1950**, 279, 2215

35. (a)Yajima, A.; Mori, K. *Chem. Eur. J.* **2002**, 24, 4079. (b) Ling, T.; Chowdhuri, C.; Kramer, B. A.; Wong, B. G.; Palladino, M. A.; Thedoraskis, E. A. *J. Org. Chem*. **2001**, 66, 8843. (c) Harada, N.; Kohori, J.; Uda, H.; Nakanishi, K.; Takeda, R. *J. Am. Chem. Soc.* **1985**, 107,423. (d) Harada, N.; Kohori, J.; Uda, H.; Torumi, K. *J. Org. Chem*. **1989**, 54,1820. (e) Harada,N.; Sugioka, T.; Ando, H.; Uda, H.; Kuriki, H. *J. Am. Chem. Soc*. **1988**, 110,8483

36. (a) Eder, U.; Sauer, G.; Wiechert, R. *Angew. Chem. Int. Ed. Engl*. **1971**, 10, 469. (b) Hajos, Z. G.; Parrish, D. R. *J. Org. Chem.* **1974**,39,1615. (c) Gutzwiller, J.; Buchschacher, P.; Fürst, A. *Synthesis* **1977**, 167 (d) Buchschacher, P.; Fürst, A. *Org. Synth*. **1985**, 63, 37

37. (a) Zong, G.; Hoffmann, T.; Lerner, R. A.; Danishefsky, S.; Barbas, C. F. *J. Am. Chem. Soc.* **1997**,119, 8131. (b) Hoffmmann, T.; Zhong, G.; List, B.; Shabat, D.; Anderson, J.; Grama-Tikova, S.; Lerner, R. A.; Barbas, C.F.; Shimizu, N.; Akita, H.; Kawamata, T. *Tetrahedron: Asymmetry*, **2002**, 13, 2123

38. Shimizu, N.; Akita, H.; Kawamata, T. *Tetrahedron: Asymmetry* **2002**, 13, 2123

39. Lo, L. C.; Shie, J. J.; Chou, T.C. *J Org Chem.* **2002**, 67,282

40. (a) Fuhsuhuku, K.; Funa, N. A.; H.; Hosomi, H.; Ohba, S.; Sugai, T. *J. Org. Chem*. **2000**,65, 129. (b) Hioki, H.; Hashimoto, T.; Kodama, M. *Tetrahedron: Asymmetry*, **2000**, 11,143

41. (a) Yeo, S. K.; Hatae, N.; Kanematsu, K. *Tetrahedron* **1995**, 51, 3499. (b) Cheung, W. S.; Wong, H. N. C. *Tetrahedron* **1999**, 55, 1101

42. Riano, S.; Fernandez, D.; Fadini, L. *Catal. Lett*. **2008**, 9,1282

43. (a) Ravindran, R.; Bulasubramaniam, T. T. *Indian J. Chem. Sect. B* **1986**, 25B, 1093. (b) Iwahama, T.; Sakaguchi, S.; Nishiyama, Y.; Ishii, Y. *Tetrahedron Lett*., **1995**, 36, 1523. (c) Khurana, J. M.; Kandpal, B. M. Tetrahedron Lett. **2003**, 44, 4909.(d) Sayama, S.; Onami, T. *Synlett* **2004**, 2369.(e) Jain, S. L.; Sharma, V. B.; Sain, B. *Synth. Commun*. **2005**, 35, 465. (f) Jain, S. L.; Sharma, V. B.; Sain, B. *Tetrahedron Lett.* **2004,** 45,1233. (g) Joseph, J. K.; Jain, S. L.; Sain, B. *Eur. J. Org. Chem*. **2006**, 590

44. Li, J.T.; Sun, X.L. *Lett. Org. Chem.* **2006**, 3, 842

45. Onomura, O.; Arimoto, H.; Matsumura, Y.; Demizu, Y. Tetrahedron Lett. **2007**, 48, 8668

46. (a) Ray, C. A.; Wallance, T. W.; Ward, R. A. *Tetrahedron Lett.* **2000**, 41, 3501. (b) Ishimaru, K.; Monda, K.; Yamamoto, Y.; Akiba, K. *Tetrahedron* **1998**, 54, 724. (c) Gaertner, P.; Schuster, C.; Knollmuller, M. *Lett. Org. Chem.* **2004**, 1,249

47. (a) Dietl, F.; Merz, A.; Tomahogh, R. *Tetrahedron Lett.* **1982**, 23,5255. (b) Kawashima, M.; Hirayama, A. *Chem. Lett.* **1991**, 763

48. (a) Kolb, H.C.; Vanieuwenhze, M. S.; Sharpless, K. B. Chem. Review. **1994**, 94, 2483. (b) Becker, H.; Sharpless, K.B. *Angewanthe Chem. Int. Ed.* **1996**, 35,448

49. Chatterjee, A.; Bennur, T. H.; Joshi, N. N. *J. Org. Chem.* **2003**, 68, 5668

50. Adam, W.; Lazarus, M.; Saha-Miller, C. R.; Schreier, P. *Acc. Chem. Res.* **1999**, 32, 837

51. Demir, A. S.; Ayhan, P.; Sopacı, S. B. *Clean*, **2007**, 5, 406

52. (a) Pohl, M.; Sprenger, G. A. Müller M. *Curr. Opin. Biotechnol.* **2004**, 15,335. (b) Sukuraman J., Hanefeld, U. *Chem. Soc. Rev.* **2005**, 34, 530.

53. Halpin, R. A.; Hegeman, G. D.; Kenyon, G. L. *Biochem.* **1981**, 20,1525

54. (a) Wilcoks, R.; Ward, O. P.; Collins, S.; Dewney, N. J.; Hong, Y.; Prosen, E. *Appl. Environ. Microbiol.* **1992**, 58, 1699. (c) Prosen, E.; Ward, O. P. *J. Ind. Microbiol.* **1994**, 13, 287

55. (a) Weiss, P. M.; Garcia, G. A.; Kenyon, G. L.; Cleland, W. W.; Cook, P. F. *Biochemistry-US* **1988**, 27, 2197; (b) Reynolds, L. J.; Garcia, G. A.; Kozarich, J. W.; Kenyon, G. L. *Biochemistry-US* **1988**, 27, 5530

56. Maria de, P. D.; Pohl, M.; Gocke, D.; Gröger, H.; Trautwein, H.; Stillger, T.; Walter, L.; Müller, M. *Eur. J. Org. Chem.* **2007**, 2940

57. Demir, A. S.; Dünnwald, T.; Iding, H.; Pohl, M.; Müller, M.; *Tetrahedron: Asymmetry* **1999**, 10, 4769

58. (a) Dünnwald, T.; Demir, A. S.; Siegert, P.; Pohl, M.; Müller, M. *Eur. J. Org.Chem.* **2000**, 2161; (b) Dünnwald, T.; Müller M. *J. Org. Chem.* **2000**, 65, 8608. (c) Iding, H.; Dünnwald, T.; Greiner, L.; Liese, A.; Müller, M.; Siegert, P.; Grötzinger, J.; Demir, A. S.; Pohl, M. *Chem. Eur. J.* **2000**, 6,1483

59. Lingen, B.; Kolter-Jung, D.; Dünkelmann, P.; Feldmann, R.; Grötzinger, J.; Pohl, M.; Müller, M. *ChemBioChem* **2003**, 4, 721

60. Gonzalez, B.; Vicuna, R. *J. Bacteriol.* **1989**, 171, 2401

61. Demir, A. S. Pohl, M. Janzen, E. Müller, M. *J. Chem. Soc., Perkin Trans. 1* **2001**, 633

62. Mosbacher, T.G.; Mueller, M.; Schulz, G.E. *Febs J.* **2005**, 272, 6067

63. Demir, A. S.; Sesenoglu, Ö.; Eren, E.; Hosrik, B.; Pohl, M.; Janzen, E.; Kolter, D. *Adv. Synth. Catal.* **2002**, 344, 96

64. Dünkelmann, P.; Kolter-Jung, D.; Nitsche, A.; Demir, A. S.; Siegert, P.; Lingen, B.; Baumann, M.; Pohl, M.; Müller, M. *J. Am. Chem. Soc.* **2002**, 124,12084

65. Demir, A. S.; Sesenoglu, Ö.; Dünkelmann, P.; Müller, M. *Org. Lett*. **2003**, 5, 204.

66. Demir, A. S. Ayhan, P. Igdir, A. Ç. Duygu, A. N. *Tetrahedron* **2004**, 60,6509

67. (a) Maria de, P. D.; Stillger, T.; Pohl, M.; Wallert, S.; Drauz, K.; Gröger, H.; Trauthwein, H.; Liese, A. *J. Mol. Catal. B: Enzym*. **2006**, 38, 43. (b) Stillger, T.; Pohl, M.; Wandrey, C.; Liese, A.; *Org. Process Res. Dev*. **2006**, 10,1172. (c) Çalik, P.; Yilgör, P.; Ayhan, P.; Demir, A. S. *Chem.Eng. Sci.* **2004**, 59, 5075 (d) Çalik, P.; Yilgör, P.; Demir, A. S. *Enzyme Microb. Technol.* **2006**, 38, 617

68. Ansorge-Schumacher, M.B.; Lasse, G.; Schroeper, F.; Mirtschin, S.; Hischier, T. *Biotechnology Journal* **2006,**1,564

69. (a) Kurlemann, N.; Liese, A. *Tetrahedron: Asymmetry* **2004**, 15, 2955. (b) Adam, W.; Lazarus, M.; Saha-Müller, C. R.; Schreier, P. *Acc. Chem. Res.***1999**, 32, 837

70. Porath, J.; Carlsson, I.; Olsson, G.; Belfrage, N. *Nature*,**1975**, 258, 598

71. (a) Oswald, T.; Wende, W.; Pingound, A.; Rinas, U. *Appl. Microbiol. Biotechnol.* **1994** 42, 73. (b) Enzelberger, M.M.; Minning, S.; Schmid, R.D. *J. Chromatogr. A* **2000**, 83, 898. (c) Chaga, G.; Widersten, M.; Andersson, L.; Porath, J.; Danielson, U.H.; Mannervik, B. *Protein Eng*. **1994**, 7, 1115. (d) Chaouk, H.;. Hearn, M.T. *J. Biochem. Biophys. Methods* **1999**, 39, 161

72. Park J.; An, K.; Hwang, Y.; Park, J.-G.; Noh,H,.-J.; Kim, J.-K.; Park, J.-H.; N.-M; Hyeon, T. *Nat. Mater*. **2004**, 3, 891

73. (a) Pankhurst, Q. A.; Conolly, J.; Jones, S. K.; Dobson, J. J. Phys. D: *Appl. Phys.* **2003**, 36, R167. (b) Weisleder, R.; Kelly, K.;Sun, E. Y.; Shtatland, T.; Josephson, L. *Nat. Biotechnol.* **2005**, 23, 1428

74. (a) Gu, H.; Xu, K.; Xu, C.; and Xu, B. *Chem. Comun.* **2006**, 941. (b) Campo, A.D.; Sen, T.; Lellouche, J.P.; Bruce, I.*J. J. Magn. Magn. Mater.* **2005**, 293, 33. (c) Gupta, A.K.; Gupta, M. Biomaterials **2005**, 26, 3995. (d) Tartaj, P., Morales, M.P.; González-Carreño, T.; Veintemillas-Verdaguer, S.; Serna, C. *J. J. Mag. Mag. Mater.* **2005**,1, 28. (e) Zaitsev, V.S.; Filimonov, D.S.; Presnyakov, I.A.; Gambino, R.J.; Chu, B. *J. Colloid Inter.Sci.* **1999**, 212, 49.

75. Wilson, J. L.; Poddar, P.; Frey, N. A.; Srikanth, H.; Mohomed, K.; Harmon J. P.; Kotha, S.; Wachsmuth, J. *J. Appl. Phys.* **2004,** 95, 1439

76. (a) Adam, W.; Mueller, M.; Prechtl, F. *J. Org. Chem.* **1994**, 59, 2358. (b) Adam, W.; Fell, R. T.; Stegmann, V. R.; Saha-Möller, C. R. *J. Am. Chem. Soc.* **1998**, 120, 708. (c) Adam, W.; Saha-Moeller, C. R.; Zhao, C. G. *Tetrahedron:Asymmetry* **1998**, 9, 4117; (d) Adam, W.; Fell, R.T.; Saha-Mö ller, C. R.; Zhao, C. G. *Tetrahedron: Asymmetry* **1998**, 9, 397. (e) Adam, W.; Dı´az, M. T.; Fell, R. T.; Saha-Mo¨ ller, C. R. *Tetrahedron: Asymmetry* **1996**, 7, 2207

77. Demir, A. S.; Hamamcı, H.; Sesenoğlu, O.; Aydoğan, F.; Capanoğlu, D.; Neslihanoğlu, R. *Tetrahedron: Asy*mmetry **2001**, 12, 1953

78. Demir, A. S.; Hamamci, H.; Sesnoglu, O.; Neslihanoglu, R.; Asikoglu, B.; Capanoglu, D. Tetrahedron Lett. **2002**, 43, 6447

79. (a) Demizu, Y.; Matsumoto, K.; Onomura, O.; Matsumura, Y. *Tetrahedron Lett.* **2007**, 51, 67. (b) Matsumoto, K.; Mitsuda, M.; Ushijima, N.; Demizu, Y.; Onomura, O.; Matsumura, Y. *Tetrahedron Lett.* **2006** , 47, 48. (c) Maezaki, N.;

Soejima, M.; Takeda, M.; Sakamoto, A.; Kinugasa, M.; Harada, T.; Oku, A. *J. Am. Chem. Soc* **1997**, 119, 38. (d) Matsumori, Y; Tanaka, T.; Iwata, C. *Tetrahedron* **1996**, 52,19. (e) Nicolosi, G.; Patti, A.; Piattelli, M.; Sanfilippo, C. *Tetrahedron:Asymmetry* **1995**, 3, 753

80. Jakka, K.; Zhao, G. *Organic Letters* **2006**, 8, 3013

81. Wieland, P.; Miescher, K. *Helv. Chim. Acta* **1950**, 279, 2215

82. Yajima, A.; Mori, K. *Eur. J. Chem*. **2002**, 24, 4079. (b) Ling, T.; Chowdhuri, C.; Kramer, B.A.; Wong, B.G.; Palladino, M. A.; Thedoraskis E. A. *J. Org. Chem*. **2001**, 66, 8843. (c) Harada, N.; Kohori, J.; Uda, H.; Nakanishi, K.; Takeda, R. *J. Am. Chem. Soc*. **1985**, 107, 423. (d) Harada, N.; Kohori, J.; Uda, H.; Torumi, K. *J. Org. Chem*. **1989**, 54,1820. (e) Harada,N.; Sugioka, T.; Ando, H.; Uda, H.; Kuriki, .T. *J. Am. Chem. Soc*. **1988**, 110, 8483

83. Karabacak, E. Ö. Ms Thesis, Middle East Technical University, **2006**, Ankara

84. (a) Yeo, S- K.; Hatae, N.; Kanematsu, K. *Tetrahedron* **1995**, 51, 3499. (b) Cheung, W. S.; Wong, H. N. C.; *Tetrahedron* **1999**, 55, 11001

85. Shimizu, N.; Akita, H.; Kawamata, T. *Tetrahedron: Asymmetry*, **2002**, 13, 2123

86. Lo, LC.;Shie, J. J.; Chou, T. C. *Org. Chem*. **2002**, 67

87. (a) Fuhshuku K., Tomita, M.; Sugai, T. *Adv. Synth. Catal*. **2004**, 345,766. (b) Fuhshuku K.; Funa, N. A.; Ohta H.; Hosomi, H.; Ohba, S.; Sugai, T. *J. Org. Chem.* **2000**, 65, 129. (b) Hioki, H.; Hashimoto, T.; Kodama, M. *Tetrahedron:Asymmetry*, **2000,** 11, 829

88. Parvez, A.; Hanson, J.R. *J. Chem. Res-S* **2004**, September,647

89. Demir, A.S.; Müller, M.; Pohl, M. WO02002753, **2003**.

90. (a) Kurlemann, N.; Liese, A. *Tetrahedron: Asymmetry* **2004**, 15, 2955. (b) Drager, G.; Kiss, C.; Kunz U. Kirschning, A. *Org. Biomol. Chem*. **2007**, 5, 3657

91. A-Schumacher, M. B.; Greiner, L.; Schroeper, F.; Mirtschin S. and Hischer,T. *Biotechnol. J.* **2006**, 1, 564

92. (a) Comasseto, J. V.; Assis, L. F.; Andrade, L.H.; Schoelein-Crusius, I. H.; Porto, A. L. M. *J. Mol. Catal.* **2006**, 39, 24. (b) Keppler, A. F.; Porto, A. L. M.; Schoelein-Crusius, I. H.; Comasseto, J. V.; Andrade, L. H. *Enzyme Microb. Tech.* **2005**, 36, 967. (c) Sichard, R.; Chen, L.S.; Marsaioli, A.J.; Reymond, J.L. *Adv. Synth. Catal*. **2005**, 347,1041

93. Voss, C.V.; Gruber, C.C; Kroutil, W. *Tetrahedron: Asymmetry* **2007**, 18, 276

94. (a) Acklin , W.; Dütting, D.; Prelog, V. *Helvetica Chimica Acta* **1958**, 159,1424 (b) Acklin , W.; Prelog, V.; Zach, D. *Helv. Chim. Acta* **1958**, 160, 1428

95. Franzreb, M.; Sienmann-Herzberg, M.; Hobley, T. J.; Thomas, O. R. T. *Appl Microbiol. Biot*. **2006**, 70, 505

96. Tural, B.; Özenbaş, M.; Atalay S.; Volkan, M. *J. Nanosci. Nanotech.* **2008**, 8, 861

97. Bradford, M. M. *Anal. Biochem*. **1976**, 72, 248

98. Langmuir, I. *J Am. Chem. Soc*. **1918**, 40,1361

99. Jansen, E.; Müller, M.; Kolter-Jung, D.; Kneen, M.M.; McLeish, M.J.;Pohl, M. *Bioorg. Chem.* **2006,** 34, 345

100. Demir, A.S.; Şeşenoglu, Ö.; Dünkelmann, P.; Müller, M. *Org. Lett.* **2003**, 5, 2047

101. Demir, A.S.; Pohl, M.; Janzen, E. and Müller, M. *J. Chem. Soc. Perkin Trans.* **2001**, 1, 633

APPENDIX

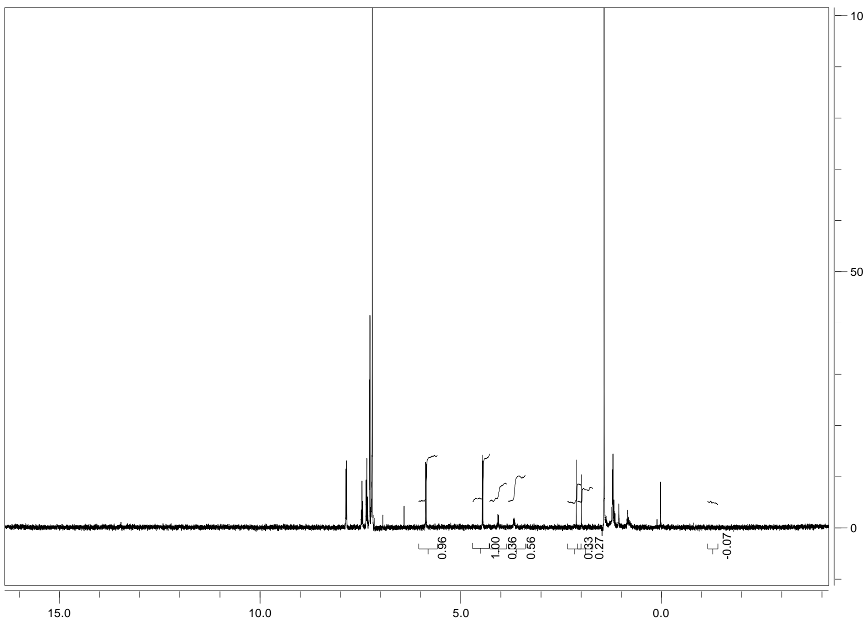

Figure A.1. ^{1}H-NMR spectrum of 2-Hydroxy-1,2-diphenylethan-1-one

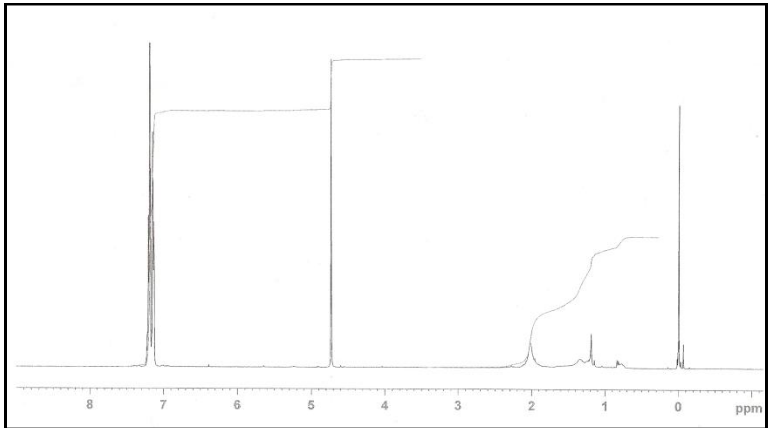

Figure A.2. ^{1}H-NMR spectrum of *meso*-1,2 diphenyletane-1,2-diol

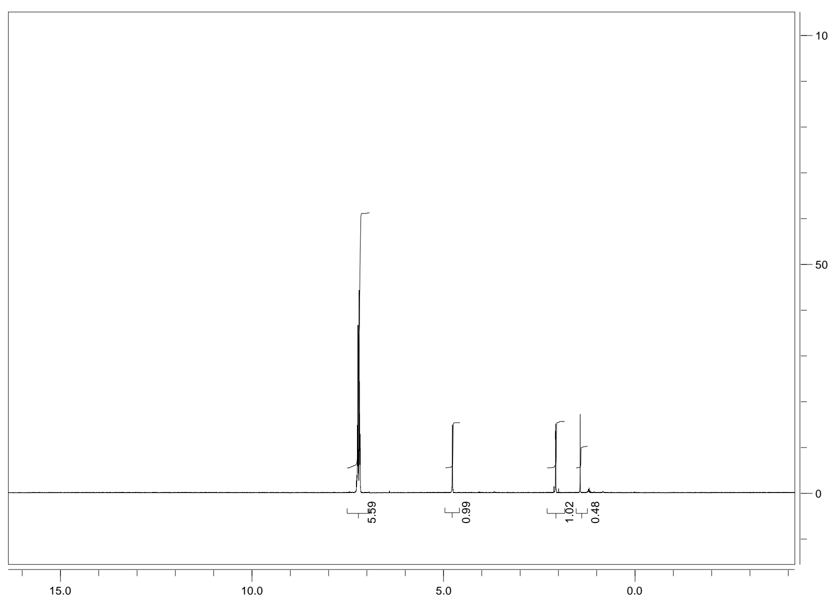

Figure A.3. ^{1}H-NMR spectrum of 1,2 diphenyletane-1,2-diol

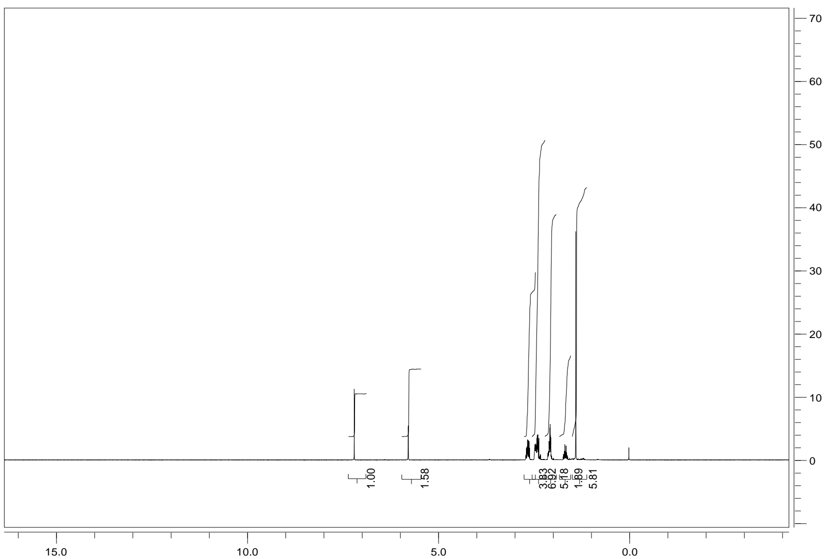

Figure A.4. ^{1}H-NMR spectrum of 3,4,8,8a-tetrahydro-8a-methylnaphthalene-1,6(2H,7H)-dione

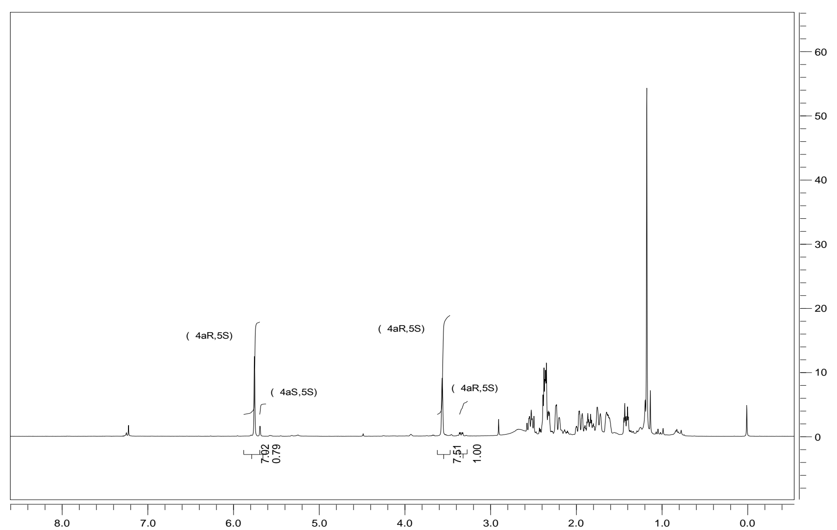

Figure A.5. ^{1}H-NMR spectrum of 4,4a,5,6,7,8-hexahydro-5-hydroxy-4a-methylnaphthalen-2(3H)-biotransformation *A. niger* mediated biotransformation in medium D at 12 day

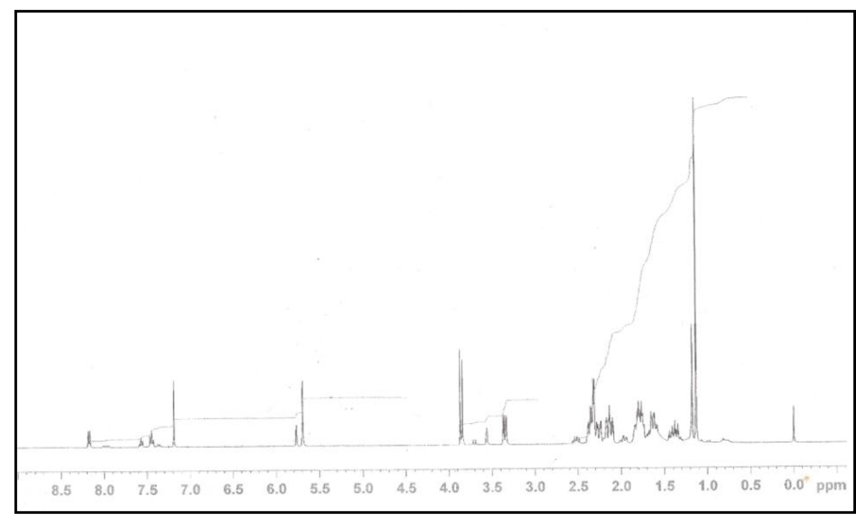

Figure A.6. ^{1}H-NMR spectrum of 4,4a,5,6,7,8-hexahydro-5-hydroxy-4a-methylnaphthalen-2(3H)-biotransformation *A. niger* mediated biotransformation in PDM 12 day

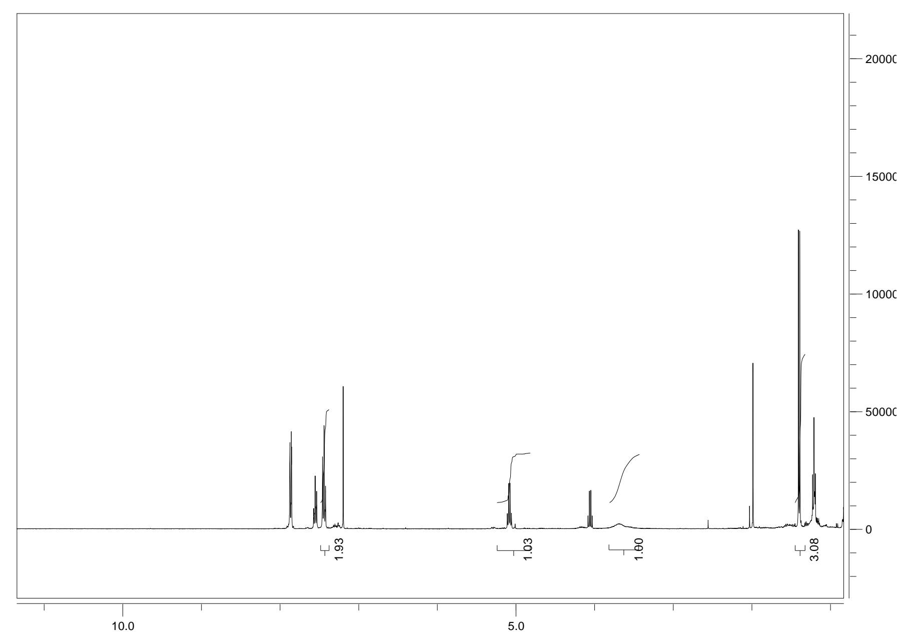

Figure A 7. ^{1}H-NMR spectrum of 2-hydroxy-1-phenylpropanone

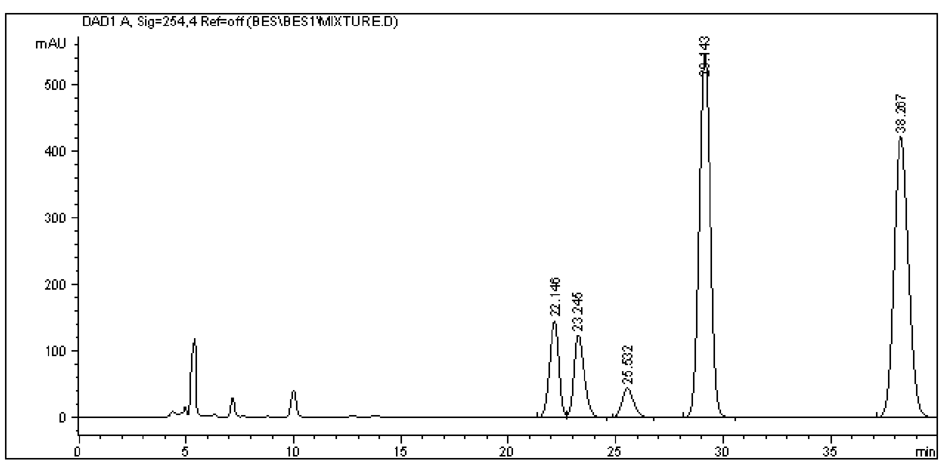

Figure A. 8. HPLC analysis of isomers of 1,2 diphenyletane-1,2-diol and 2-Hydroxy-1,2-diphenylethan-1-one as standart

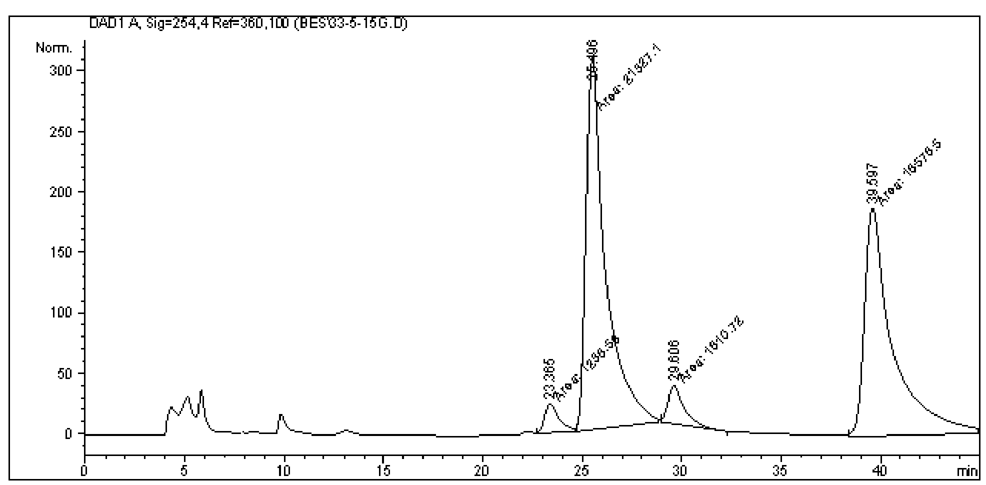

Figure A.9. HPLC analysis of reaction medium of *A. flavus* mediated biotransformation of *meso*-1,2 diphenyletane-1,2-diol

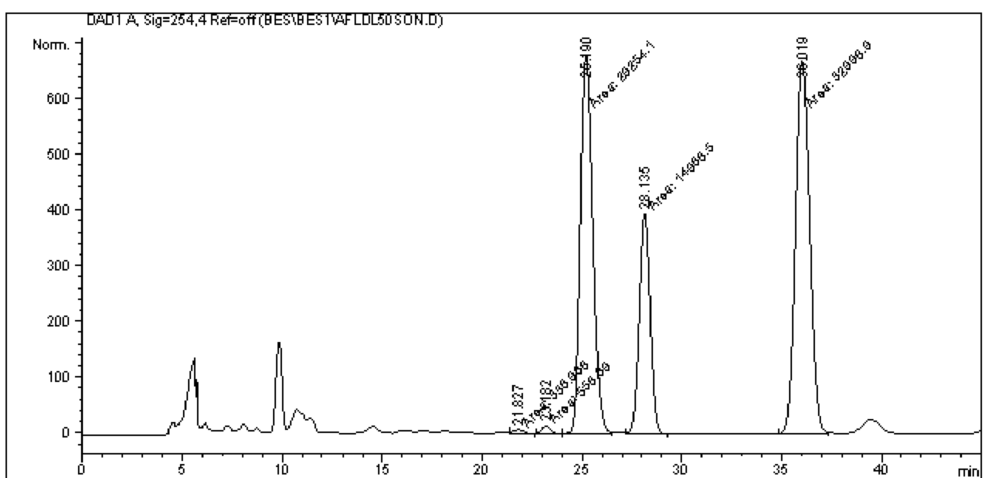

Figure A.10. HPLC analysis of reaction medium of *A. flavus* mediated biotransformation of *D,L*-1,2 diphenyletane-1,2-diol

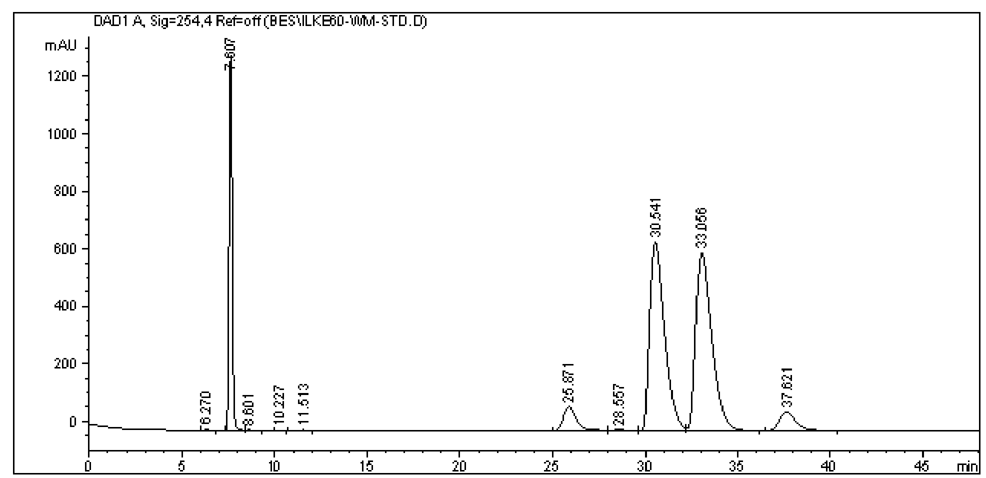

Figure A.11: HPLC analysis of 3,4,8,8a-tetrahydro-8a-methylnaphthalene-1,6(2H,7H)-dione

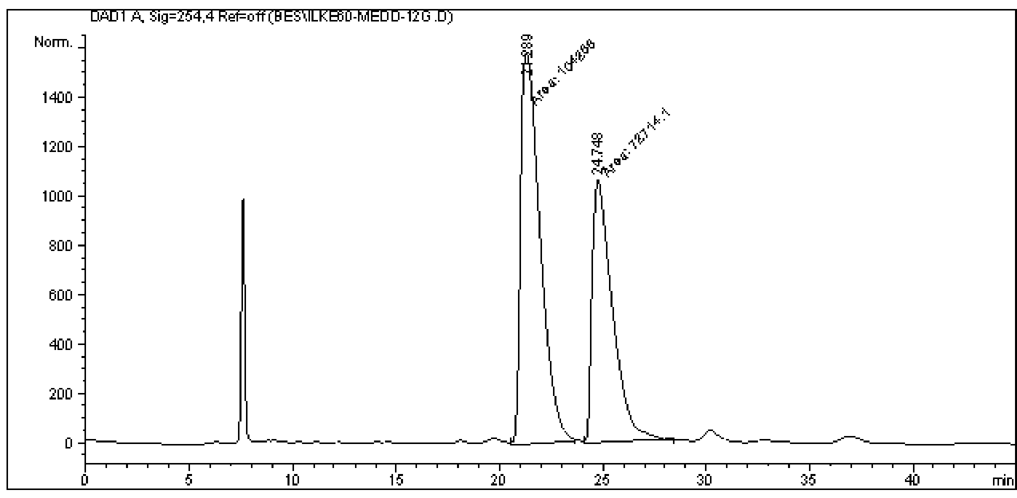

Figure A. 12. HPLC analysis or reaction medium of A. niger mediated biotransformation of 3,4,8,8a-tetrahydro-8a-methylnaphthalene-1,6(2H,7H)-dione in medium D

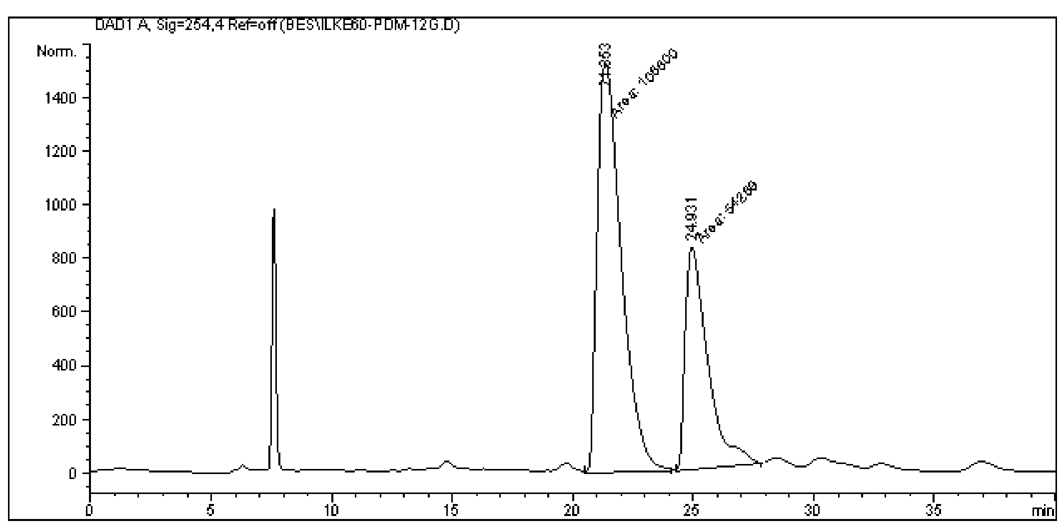

Figure A.13. HPLC analysis or reaction medium of *A. niger* mediated biotransformation of 3,4,8,8a-tetrahydro-8a-methylnaphthalene-1,6(2H,7H)-dione in PDM

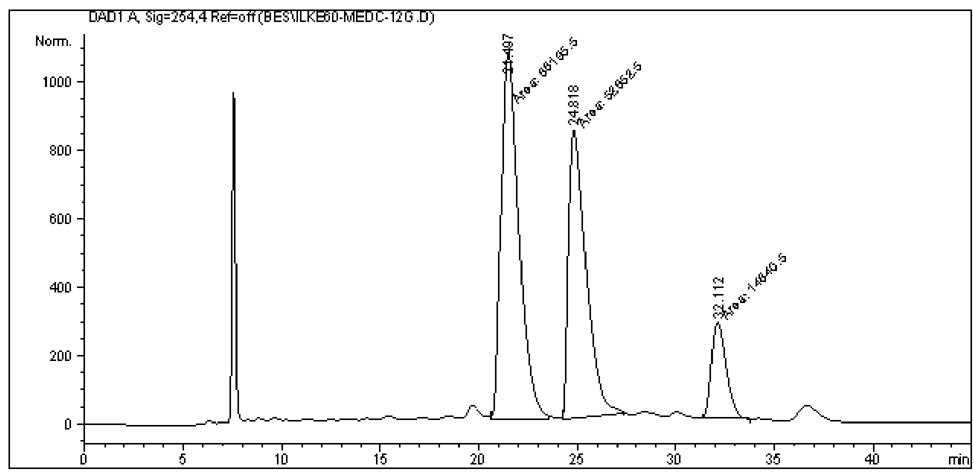

Figure A.14. HPLC analysis or reaction medium of *A. niger* mediated biotransformation of 3,4,8,8a-tetrahydro-8a-methylnaphthalene-1,6(2H,7H)-dione in medium C

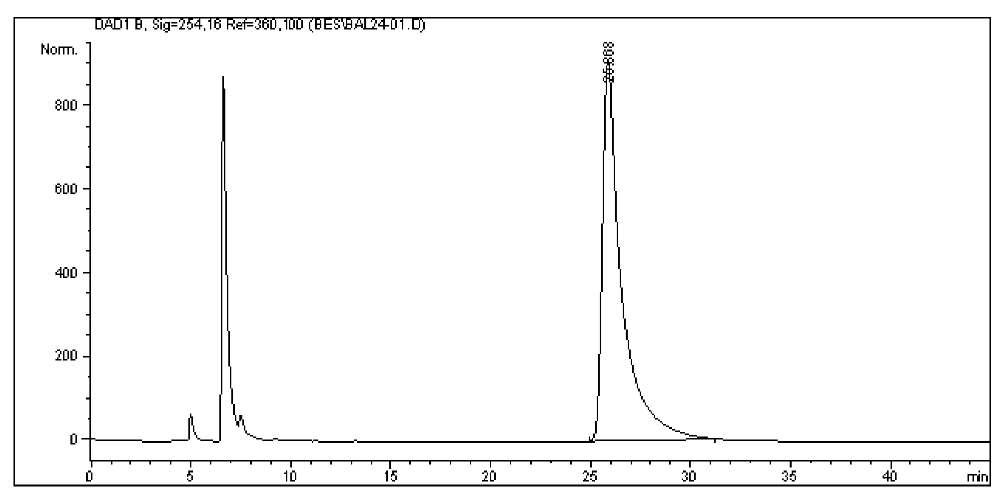

Figure A.15. HPLC analysis of BAL-magnetic particle heterocatalyst mediated carboligation reaction of benzaldehyde

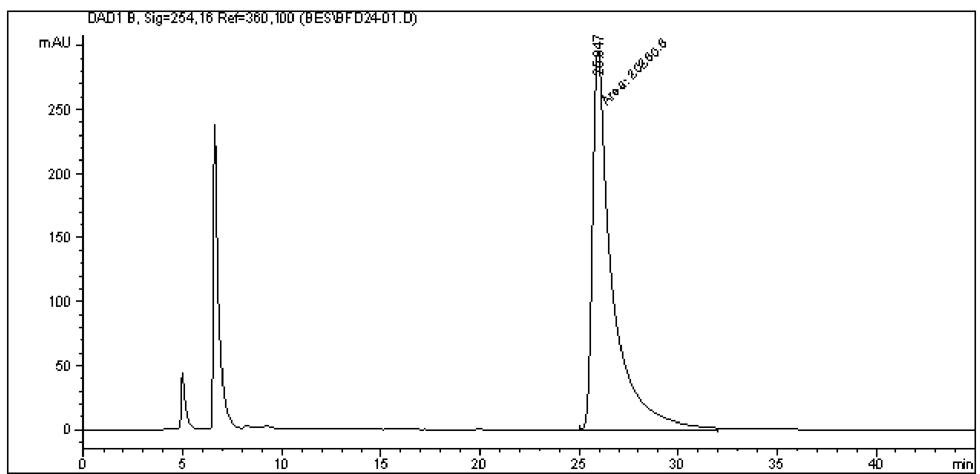

Figure A.16. HPLC analysis of BFD-magnetic particle heterocatalyst mediated carboligation reaction of benzaldehyde

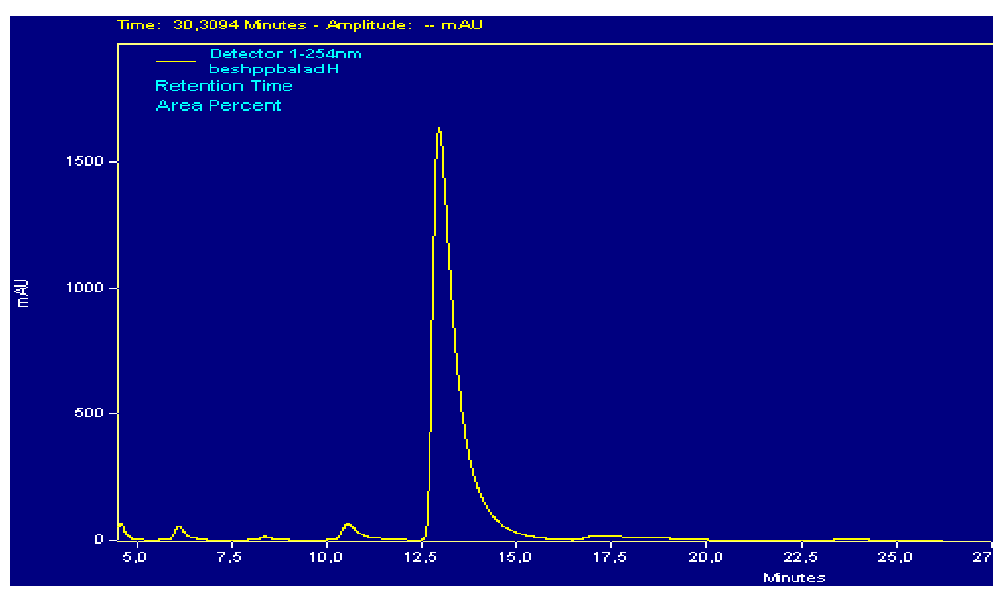

Figure A.17: HPLC analysis of BALcatalysed crosscondensation of benzaldehyde and acetaldehyde

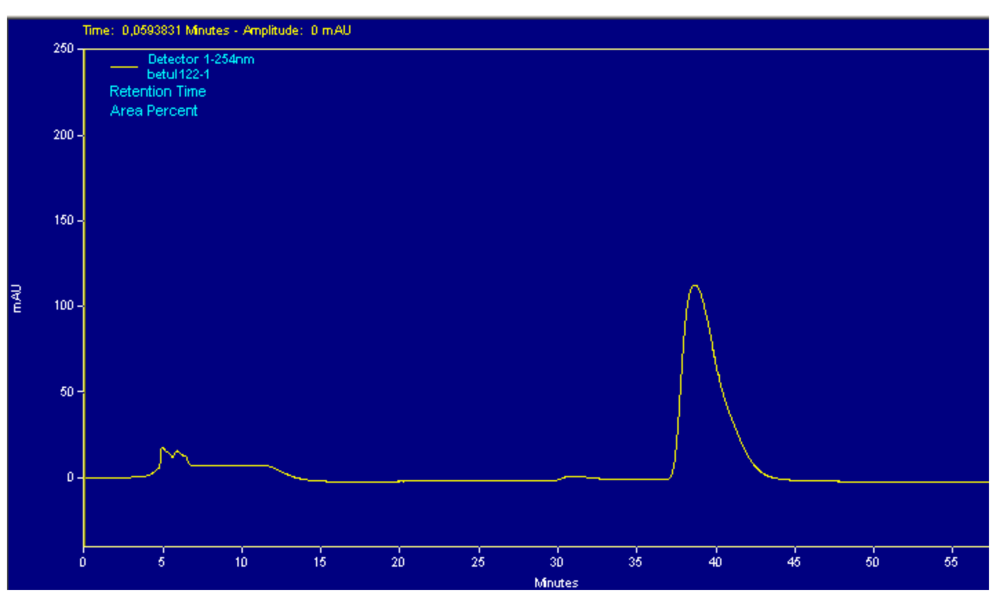

Figure A.18: HPLC analysis of BAL-magnetic particle heterocatalyst mediated carboligation reaction of p-anisaldehyde and dimethoxyacetaldehyde

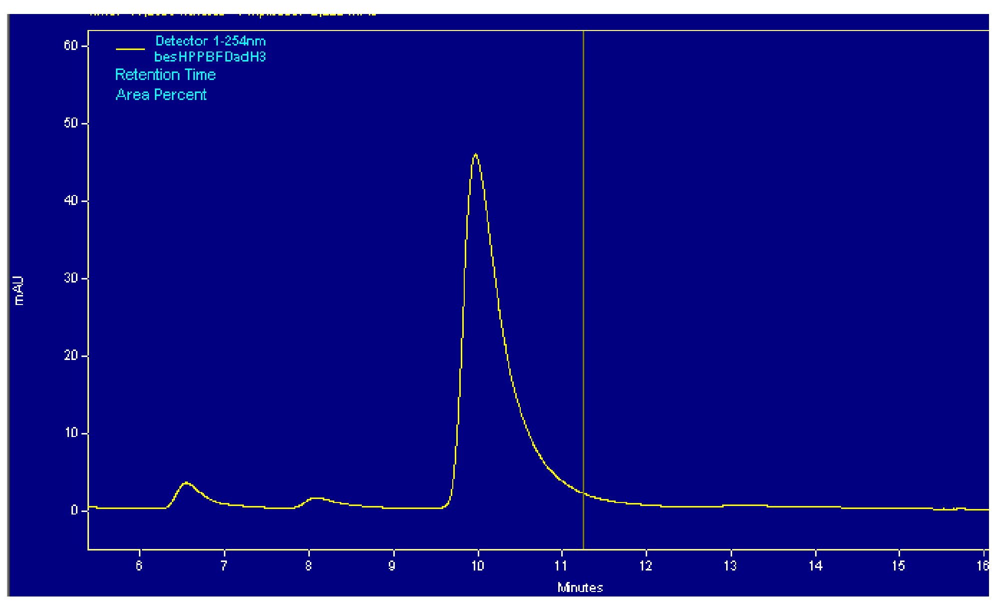

Figure A.19. HPLC analysis of BFD-magnetic particle heterocatalyst mediated crosscondenzation of benzaldehyde and acetaldehyde

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FOREIGN LANGUAGES

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PUBLICATIONS

1. "Thiamine Pyrophosphate Dependent Enzyme Catalyzed Reactions : Stereoselective C-C Bond Formations in Water" Demir, A. S.; Ayhan, P.; Sopacı, S. B. *Clean*, **2007**, 5, 406

2. "Carboligation Reactions with Benzaldehyde Lyase Immobilized on Superparamagnetic Solid Support" Sopaci, Ş. B.; Şimek, İ.; Tural, B.; Volkan, M. and Demir, A. S. 2009, **DOI:** 10.1039/b819722a