NOVEL BIOCONVERSION REACTIONS FOR THE SYNTHESES OF A-HYDROXY KETONES

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

JANUARY 2009

Approval of the thesis:

NOVEL BIOCONVERSION REACTIONS FOR THE SYNTHESES OF A-HYDROXY KETONES

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ABSTRACT

NOVEL BIOCONVERSION REACTIONS FOR THE SYNTHESES OF A-HYDROXY KETONES

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January 2009, 125 pages

The objective of the study presented here was to develop either enzymatic or whole cell mediated green procedures for the syntheses of a-hydroxy ketones. Production of optically active synthons is crucial for the preparation of fine chemicals. Enzymes and whole-cell biocatalysts have proven to be excellent vehicles with their chiral nature for the biotransformations. Under the light of this discussion, firstly benzaldehyde lyase [BAL, (EC 4.1.2.38)] was used in novel C-C bond formation reactions to obtain interesting and biologically important precursors; 2-Hydroxy-1-arylethan-1-ones and functionalized aliphatic acyloin derivatives. All the compounds were obtained with high yields and in the case of aliphatic acyloin derivatives with high enantiomeric excesses (ee's). Another strategy was to use whole cell biocatalysis. *A.flavus* 200120 was found to be a promising biocatalyst with the ability to catalyze a broad range of reactions; reduction, hydrolysis and deracemization, while another fungus; *A. oryzae* 5048 was utilized in bioreduction reactions of benzil and its derivatives. Each reaction was investigated, optimized and thus enhanced via medium design. Products were obtained with high yields and ee's. To sum up, in this study novel efficient green procedures were developed to synthesize various a-hydroxy ketones with high yield and stereoselectivity. These newly established methods present promising alternatives to classical chemical methodologies.

Keywords: A-hydroxy ketone, Benzaldehyde lyase, Whole-cell biocatalysis, Biotransformation.

ÖZ

A-HİDROKSİ KETONLARIN SENTEZİ İÇİN YENİ BİYODÖNÜŞÜM REAKSİYONLARI

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Ocak 2009, 125 sayfa

Bu çalışmanın amacı, a-hidroksi ketonların sentezi için enzimatik veya tüm hücrelerle gerçekleştirilen yeşil prosedürlerin geliştirilmesidir. Özellikli kimyasalların hazırlanmasında optikçe aktif başlangıç maddelerin üretimi gereklidir. Kiral doğaları gereği enzimler ve tüm-hücre katalizörlerinin biyodönüşüm reaksiyonları için mükemmel birer araç oldukları kanıtlanmıştır. Bu tartışmaların ışığında öncelikli olarak benzaldehit liyaz enzimi [BAL, (EC 4.1.2.38)] 2-hidroksi-1-ariletan-1-on ve fonksiyonlandırılmış alifatik açiloin türevleri gibi ilginç ve biyolojik olarak önemli yapı taşlarının eldesi için yeni C-C bağ oluşum reaksiyonlarında kullanılmıştır. Bütün bileşikler yüksek verimde ve alifatik açiloin türevleri için yüksek enansiyomerik fazlalıkla (ee'de) elde edilmiştir. Uygulanan başka bir strateji ise tüm hücre biyokatalizörlerinin kullanılmasıdır. *A.flavus* 200120 indirgeme, hidroliz ve derasemizasyon gibi birçok reaksiyonu katalizleyebilme özelliğiyle ümit vadeden bir biyokatalizör olarak bulunmuşken, başka bir küf olan *A. oryzae* 5048 benzil ve türevlerinin biyoindirgeme reaksiyonlarında kullanılmıştır. Her bir reaksiyon ayrı ayrı incelenmiş, ortam tasarımıyla optimize edilmiş ve geliştirilmiştir. Ürünler yüksek verimlilikte ve ee'de elde edilmiştir. Özet olarak bu çalışmada, a-hidroksi ketonların yüksek verimlilik ve steryoseçicilikte sentezlenmesi için yeni etkin yeşil prosedürler geliştirilmiştir. Geliştirilen bu yeni yöntemler klasik kimyasal yöntembilimlere ümit vadeden alternatifler olmuşlardır.

Anahtar Kelimeler: Alfa-hidroksi keton, Benzaldehit liyaz, Tüm-hücre biyokatalizörü, Biyodönüşüm.

To My Family and Beloved Friends

ACKNOWLEDGEMENT

Firstly, I would like to thank to my supervisor Prof. Dr. Ayhan S. Demir for giving me the chance to work in several interesting projects covering several hot topics thus, enabling me to enlarge my scope and for his continuous support, encouragement and guidance in all manners throughout this study. I am really happy and lucky to work with him.

My gratitude also goes to my co-supervisor Prof. Dr. Pınar Çalık for allowing me to work in her lab and teaching me the principles of biochemical reaction engineering.

I would like to express my deep feelings to all the wonderful people that happen to be labmates as well as friends throughout this thesis; Zuhal, Şehriban, Devrim, Elif, Hacı, Umut Demirtaş and Umut Erkılıç. I am also grateful to my recent labmates Betül, Ilke and Naime for their friendships.

I find myself lucky to be a part of ASD group and I would like to thank to all former and present ASD group members. I am thankful to my seniors; Çiğdem, Neşe and Ömer for helping me throughout my adaptation and for their patience and guidance.

I present my sincere gratitude to Fatoş Doğanel Polat, M. Seda Karayılan and Zehra Uzunoğlu for NMR spectra.

Being a part of an interdisciplinary program gave me the opportunity to meet great people such as Pınar Yılgör, Ceylan, Arda, Oğuz, Şeyda, Serdar. I had a wonderful time with them both professionally and personally.

Lastly but not leastly I would like to thank to my beautiful and big family for supporting me throughout my life and loving me without any cause.

Thank you all…

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INTRODUCTION

Biotechnology is defined as any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use as implied by the United Nations Convention on Biological Diversity [1].

Biotechnology is the use of microorganisms, such as bacteria or yeasts, or biological substances, such as enzymes, to perform specific industrial or manufacturing processes. Applications include the production of certain drugs, synthetic hormones, and bulk foodstuffs as well as the bioconversion of organic waste and the use of genetically altered bacteria in the cleanup of oil spills. Biotechnology is divided into several subfields;

- *Red biotechnology* is related with medical processes. It is used to create substances for medical use or to aid directly to the body in fighting a disease or illness as in the case of genetically modified yeasts and bacteria used for production of drugs which are hard to synthesize via classical methodologies.

- *White biotechnology* which is also sometimes referred as *grey biotechnology* is a biotechnology applied to industrial processes in which biological organisms are used to produce or manipulate things in a way that is beneficial for industry. Bioremediation can be given as an example.

- *Green biotechnology*, also known as agricultural biotechnology, deals with applications related to agriculture. Genetically modified organisms (GMOs) have wide applications in this area.

- *Blue biotechnology* includes the marine and aquatic applications of biotechnology, but its use is relatively rare.
- *Bioinformatics* also known as *computational biology* is an interdisciplinary field which includes the analysis of biological systems using computers by applying statistical techniques. Bioinformatics plays a key role in various areas, such as functional genomics, structural genomics, and proteomics, and forms a key component in the biotechnology and pharmaceutical sector.

Biotransformations which deal with use of natural or recombinant microorganisms (e.g., yeast, fungi, bacteria), enzymes, whole cells, etc., as catalysts in organic synthesis are enzymatic or microbial conversions of non-natural substrates to products. It plays a key role in the area of foodstuff, chiral drug industry, vitamins, specialty chemicals, and animal feed stock. Because of all its aforementioned properties, biotransformation lies in the heart of biotechnology.

1.1. Biotransformations

The use of enzymes and microorganisms, which nature has developed, is undoubtedly an ideal choice toward *greening* chemical reactions. *Biotransformations* (also referred as bioconversions) have been developed alternatively instead the use of classical chemical transformations. They have a number of advantages when viewed alongside the corresponding chemical methods. Many biotransformations are not only regio- and stereospecific but are also enantiospecific allowing the production of chiral products from racemic mixtures. The conditions for biotransformations are mild and in the majority of cases do not require in the protection of other functional groups. Furthermore, the features governing their regiospecificity differ from those controlling chemical specificity and indeed it is possible to obtain biotransformations at centres that are chemically unreactive transformed to chemically reactive.

From a commercial point of view some biotransformations can be cheaper and more direct than their chemical analogues whilst the transformations proceed under conditions that are normally regarded as environmentally friendly. However, many of the ground rules for applying biotransformations are not yet well-understood or well-defined and there are many chemical reactions for

which there is no equivalent biotransformation. Biotransformation is still the most promising technology to take over the humble classical chemical methodologies.

1.1.1. Syntheses of Optically Pure Compounds

Enzyme-catalyzed reactions are often highly enantio- and regio- selective making these approaches an effective tool for the production of chiral intermediates and fine chemicals. This fact enables biotransformations to become an accepted method for generating optically pure compounds and for developing efficient routes to target molecules [2].

Biotransformation is a widely used strategy for the production of single enantiomers of chiral intermediates which has become increasingly important in the pharmaceutical industry [3]. The efficiency of drugs depends on chirality, since often only one enantiomer of a racemic mixture has the required activity. Generally, microorganisms and their enzymes have been most frequently used to functionalize nonactivated carbon atoms, to introduce centers of chirality into optically inactive substrates, and to carry out optical resolutions of racemic mixtures. For many metabolites, chemical synthesis can be quite difficult or even impossible, especially for drugs with complex molecular structures. Microbial biocatalytic systems are easy to prepare at a low cost, and they can be readily screened in large numbers to identify those most suitable for the production of metabolites. Typical metabolic enzyme transformations that could be chemically difficult, such as hydroxylations and stereospecific conversions, can be mimicked by microbial enzymes.

Several examples are presented in the literature dealing with syntheses of pharmaceutically important compounds; Figure (Fig.) 1 and Fig. 2 illustrate biocatalytic synthesis of *nelfinavir* **3** which is the Active Pharmaceutical Ingredient (API) of the anti-HIV drug Viracept® and *rosuvastatin* **7**, the API of Crestar® and Atorvastatin®, respectively [4].

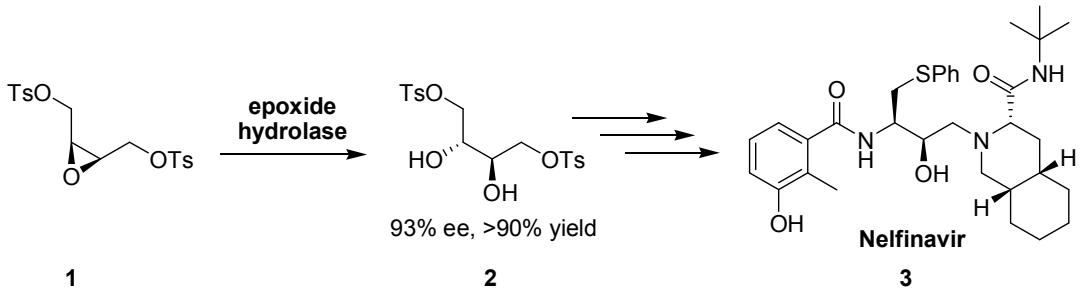


Figure 1 Biocatalytic synthesis of *nelfinavir*

The production of optically active intermediates is an area of growing demand in the fine chemical industry also and here biocatalysis has developed from a niche technology to a widely used manufacturing method. There are several established procedures for the biocatalytic synthesis of α- and β-amino acids, amines, amides, peptides, nitriles, alcohols, organic acids, epoxides and complex multifunctional molecules [5].

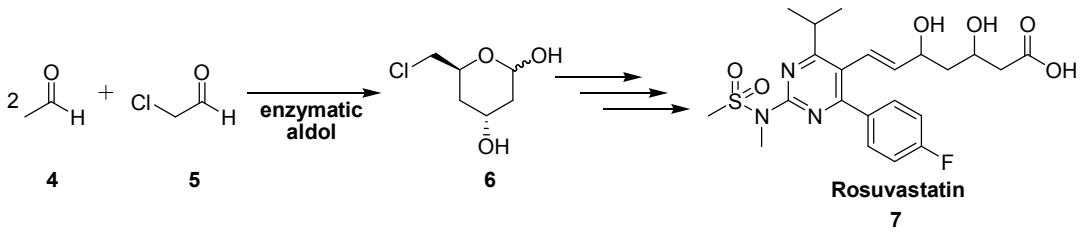


Figure 2 Biocatalytic synthesis of *rosuvastatin*

Great Lakes process for the combined utilization of α-amino transferases with *L*-ornithine-d-amino transferase to produce α-amino acids is represented in Fig. 3 [6].

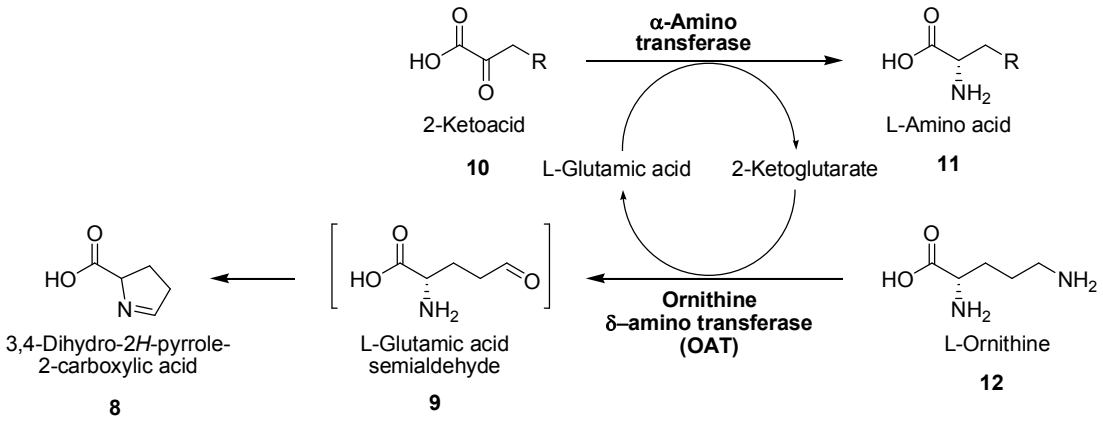


Figure 3 A commercial synthetic route for the production of coupled enzymatic synthesis of α-amino acids

1.1.1.1. α-Hydroxy Ketones

Acyloins (α-hydroxy ketones) are relevant in organic synthesis owing to their bifunctional nature, mainly due to one chiral centre which is amenable to further modification as shown in Fig. 4.

Enantiomerically pure α-hydroxyketones are useful building blocks in the synthesis of different pharmaceuticals and fine chemicals, such as antitumoral antibiotics (*Olivomycin A* and *Chromomycin* A_3), inhibitors of farnesyl transferase (*Kurasoin A* and *B*), inhibitors of amiloid-β protein production or antidepressant drugs (*bupropion* and its metabolites)[7].

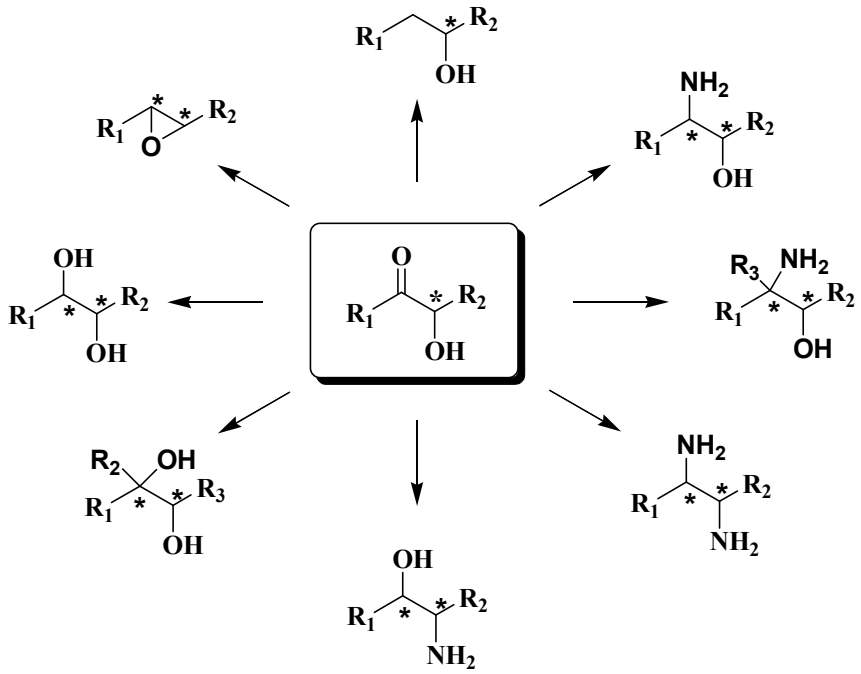


Figure 4 α-Hydroxy ketones as important synthons

There are several approaches for obtaining optically active α-hydroxy ketones biologically and chemically. A few methodologies are listed below:

- By $Mn(OAc)_3$-mediated direct acetoxylation and acyloxylation of enones and aromatic ketones followed by the enzymatic- and fungus-mediated resolution of acyloxy enones which is shown in Fig. 5 [8].

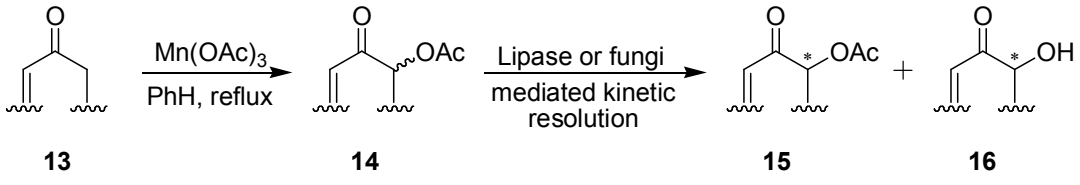


Figure 5 $Mn(OAc)_3$ oxidation of enones coupled with lipase mediated kinetic resolution [8]

- By silylation or silylation/alkylation from ketones using the SAMP-/RAMP-hydrazone method which is then oxidized to yield optically active hydroxy ketones [9], Fig. 6.

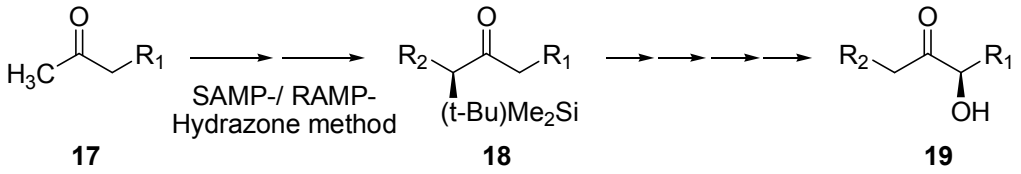


Figure 6 A route to optically active α-hydroxy ketones through silylation [9]

- A-hydroxy ketones can be obtained chemically by treatment of their enolate forms with a molybdenum peroxide reagent in THF-hexane at –70^{o}C [10].

- By direct α-hydroxylation of ketones given in Fig. 7,

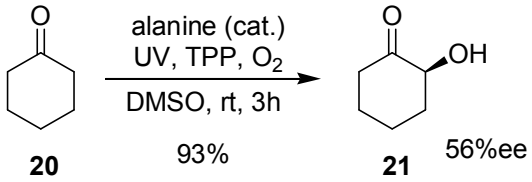


Figure 7 The alanine-catalyzed aerobic asymmetric α-oxygenation [11]

- Via C-C bond formation reactions catalyzed by enzymes such as benzaldehyde lyase (BAL), benzoylformate decarboxylase (BFD) and pyruvate decarboxylase (PDC).

1.1.2. Biocatalysts

Biocatalysis (also referred as bioorganic catalysis) can be defined as the use of biological systems (whole cells or pure enzymes) to produce organic compounds.

Biocatalysts are currently used to produce a wide range of products in the fields of food manufacture (such as bread, cheese, beer), fine chemicals (e.g., amino acids, vitamins), and pharmaceuticals (e.g., derivatives of antibiotics). They not only provide access to innovative products and processes, but also meet criteria of sustainability by reducing energy and raw material consumption and generating less waste and toxic side-products. In organic synthesis, recombinant technologies and biocatalysts have greatly widened the scope of application.

Biocatalysis are used in a number of forms. Enzymes can be wild-type, recombinant, or genetically modified to increase their specificity or activity. Cell-free enzymes may be used in solution, in a membrane reactor, as a suspension, cross-linked, or immobilized. Alternatively, one or more enzymes that carry out the required steps may be present in whole cells, which may be growing, resting or immobilized. The medium for the reaction may be aqueous, organic or two-phase.

Enzymes are classified according to the nomenclature (EC numbers) developed by the International Union of Biochemistry and Molecular Biology. The enzyme classes are grouped based on its mechanism:

- **EC 1 - Oxidoreductases:** These enzymes achieve electron transfer in oxidation and reduction reactions.
- **EC 2 - Transferases:** These catalyze the transfer of a functional group (*e.g.* a methyl or phosphate group).
- **EC 3 - Hydrolases:** These catalyze the hydrolysis of various bonds by transferring of functional groups to water.
- **EC 4 - Lyases:** These cleave various bonds by means other than hydrolysis and oxidation.
- **EC 5 - Isomerases:** These catalyze isomerization changes within a single molecule.

- **EC 6 - Ligases:** These enzymes join two molecules with covalent bonds via formation of C-C, C-S, C-O and C-N bonds.

1.1.2.1. Advantages of Biocatalysts

Enzymes display three major types of selectivities:

- *Chemoselectivity*: Since the purpose of an enzyme is to act on a single type of functional group, other sensitive functionalities, which would normally react to a certain extent under chemical catalysis, survive. As a result, biocatalytic reactions tend to be cleaner and laborious purification of product(s) from impurities emerging through side-reactions can largely be omitted.

- *Regioselectivity and Diastereoselectivity*: Due to their complex three-dimensional structure, enzymes may distinguish between functional groups which are chemically situated in different regions of the substrate molecule.

- *Enantioselectivity*: Since almost all enzymes are made from *L*-amino acids, enzymes are chiral catalysts. As a consequence, any type of chirality present in the substrate molecule is recognized upon the formation of the enzyme-substrate complex. Thus a prochiral substrate may be transformed into an optically active product and both enantiomers of a racemic substrate may react at different rates.

These reasons, and especially the latter, imply that why synthetic chemists have become interested in biocatalysis. This interest in turn is mainly due to the need to synthesize enantiopure compounds as chiral building blocks for drugs and agrochemicals.

Another important advantage of biocatalysts are that they are environmentally acceptable, being completely degraded in the environment. Furthermore, the enzymes act under mild conditions, which minimize problems of undesired side-reactions such as decomposition, isomerization, racemization and rearrangement, which often plague traditional methodology.

1.1.2.2. Disadvantages of Biocatalysts

Although the use of biocatalysts has several advantages, synthetic chemists have been reluctant to employ them for a number of reasons [12]:

- Enzymes are provided in Nature in only one enantiomeric form, and there is limited availability of biocatalysts with the desired activity and substrate specificity,
- Enzymes require narrow operation parameters,
- Enzymes display their highest catalytic activity in water, while most organic compounds are water-insoluble, and the water removal is tedious and expensive,
- Biocatalysts are prone to inhibition phenomena,
- Enzymes mostly have deficiency in stability,
- Biocatalysts are at relatively high cost.

1.1.2.3. Biocatalyst Selection

After selecting an appropriate starting material to be converted into the product, it is necessary to select the appropriate biocatalyst with suitable activity, selectivity and stability to work under required operational conditions (temperature, salt concentration, pH, organic solvents, substrate and product concentrations).

Several strategies can be followed to obtain the biocatalyst for the pertinent biotransformation: (a) screening for novel biocatalysts, (b) use of existing biocatalysts.

1.1.2.3.1. Screening for Novel Biocatalysts

Selection of new microorganisms with novel activities is still worth-while taking into account the overwhelming biochemical diversity present in nature. The screening of large numbers of organisms requires that cheap, simple, rapid and selective detection methods, preferably capable of some automation, should be available to facilitate this usually tedious process.

Selective methods for colonies on plates can be very useful, as shown for the isolation of microorganisms able to hydroxylate *L*-tyrosine to *L*-DOPA, a drug used in the treatment of Parkinsons' disease. The colonies that produce *L*-DOPA turn violet-black as a result of the reaction of *L*-DOPA with ferrous irons added to the agar plates.

Microbial selection has also been performed in the presence of high concentrations of the target compound. This approach was used to isolate benzoic-acid-assimilating strains for the production of *cis,cis*-muconic acid from benzoic acid. Similar approaches have been followed to isolate nitrile-hydrolyzing enzymes, such as nitrile hydratase, nitrilase and amylase, which have great potential as catalysts for producing high-value amides and acids from corresponding nitriles.

The resistance to organic solvents is often an important criterion in the selection of a suitable biocatalyst. *Pseudomonas* strains have been isolated with the ability to grow in the presence of toluene, aromatic, aliphatic hydrocarbons and long chain alcohols. These strains and their enzymatic activities are therefore important biocatalytic sources for the degradation of harmful compounds as well as for the synthesis of important chiral compounds. Extremophiles have been receiving a great deal of attention. These microorganisms that thrive in extreme environments are likely to provide biocatalysts able to cope with the often harsh industrial reaction conditions.

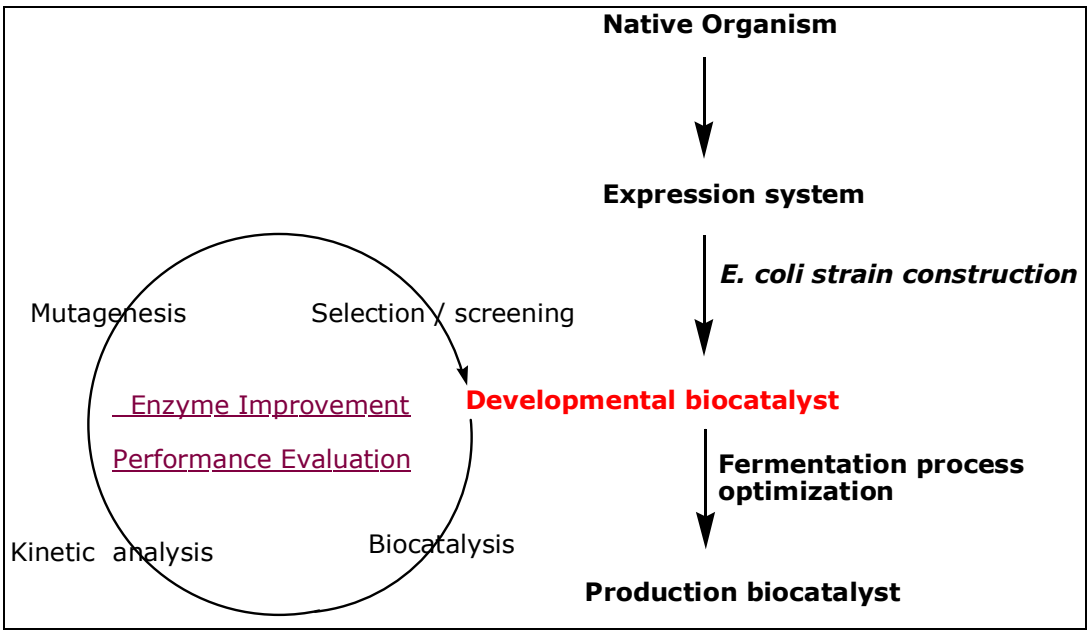


Figure 8 Biocatalyst Improvement Process

After the biocatalyst is chosen, improvement of the biocatalysis for obtaining a superior reaction takes place. The biocatalyst improvement process is presented in Fig. 8.

1.1.2.3.2. Use of Existing Biocatalysts

A well-known way to accomplish a desired biotransformation is the use of existing biocatalysts (e.g. commercial enzymes) on natural and unnatural substrates. The substrate specificities of lipases and proteases are currently under intense investigations. The hydrolytic capacity of lipases is not restricted only to triacylglycerols. This type of enzymes is also able to hydrolyse mono-, di- and tri-acyl esters with different chain lengths of various acyl groups.

The exploitation of existing enzymes under different reaction conditions or employing genetical modification could lead to attaining of proper biocatalyst for the desired biotransformation.

Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of

seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer [13a].

1.1.2.4. Isolated Enzymes vs. Whole Cells

Isolated enzymes or whole organisms may be used for biotransformations as biocatalysts. Each approach has its advantages and disadvantages, Table 1. Many isolated enzyme systems are commercially available or are relatively easy to isolate, at least in a crude form. They can be stable and easy to use, often giving clean, single products. For many hydrolytic reactions, no cofactor is needed. However, for redox reactions in which a co-factor is used, the need to regenerate this can add a complication. Whole organisms do not have this disadvantage. They do tend to give more than one product, which may or may not be an advantage. They are often cheaper to use than isolated enzyme systems.

1.2. Enantioselective Enzymatic C–C Bond Forming Reactions

The classical methodology for the synthesis of α-hydroxy ketones 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.

The stereoselective formation of C–C bonds is a central aspect of synthetic organic chemistry. Much effort has been directed to find chemical methods that catalyze asymmetric C-C bond forming reactions. When the stereoselectivity of biocatalysts is taken into consideration, enzymes exhibit promising alternatives for C–C coupling [13b,c].

Table 1 Advantages and disadvantages of whole cell and isolated enzyme techniques

Technique	Advantages	Disadvantages
Whole cells	* Inexpensive * Enzyme co-factors are present	* Workup can be difficult * Large glassware are required * Side reactions can interfere * Co-solvents can distrupt membrane bound proteins
Isolated enzymes	* Simple apparatus * Simplified workup * Highly specific for desired reaction * More tolerant of co-solvents	* Expensive * Co-factors must be supplied * Co-factor recycling may be necessary

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) [14]. Thiamine diphosphate (ThDP)-dependent enzymes are involved in many different pathways and catalyze broad range of reactions.

Acetohydroxyacid synthase (AHAS) [15] and pyruvate decarboxylase (PDC) [16] are especially important from an industrial point of view as they catalyze the decarboxylation of pyruvate to an activated acetaldehyde followed by the enzymatic combination with resulting in the formation of (*R*)-phenylacetylcarbinol [(*R*)-PAC] which is a key intermediate in ephedrine and pseudoephedrine production, Fig. 9.

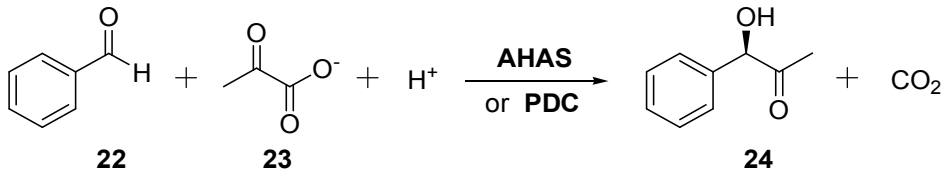


Figure 9 AHAS and PDC catalyzed decarboxylation coupled with carboligation

1.2.1. Benzaldehyde Lyase [BAL, (EC 4.1.2.38)] and Benzoylformate Decarboxylase [BFD, (E.C. 4.1.1.7)] for the Syntheses of α-Hydroxy Ketones

Benzoylformate decarboxylase (BFD) from *Pseudomonas putita* was shown to possess decarboxylase activity first by Wilcocks and coworkers [17a], It has proven to be an efficient catalyst for the enantioselective formation of 2-hydroxy ketones and (*R*)-benzoin. Synthesis of enantiomerically pure benzoin and substituted benzoin derivatives from aromatic aldehydes via benzoylformate decarboxylase-mediated C–C bond formation was performed by Demir *et al.* [17b]. Via carboligation with benzaldehyde as a sole substrate, (*R*)-benzoin was obtained, while carboligation with acetaldehyde resulted in (*S*)-2-hydroxy-1-phenyl propanone [(*S*)-2-HPP]. BFD catalyzed reactions are presented in Fig. 10.

BFD was shown to be not effective with benzaldehyde derivatives substituted at *ortho*-position. The limitation for *o*-substituents was circumvented via directed evolution tools where substrate specificity of BFD was altered and BFD mutant L476Q and M365-L461S which accepted *o*-substituents were obtained [18]. Although the catalytic capacity of the BFD was improved with directed evolution, benzaldehyde lyase still represents a better catalyst for C-C bond formation reactions.

BAL is an enzyme, natively found in *Pseudomonas florescens* Biovar I was firstly introduced to the literature by Gonzales and Vicuna in 1989 [19a]. This enzyme was shown to be promising with the ability to grow on benzoin as sole carbon

source. Later, Hinrichsen *et al.* cloned the gene encoding BAL into pUC18 vector and an *Escherichia coli* strain was transformed using this vector [19b].

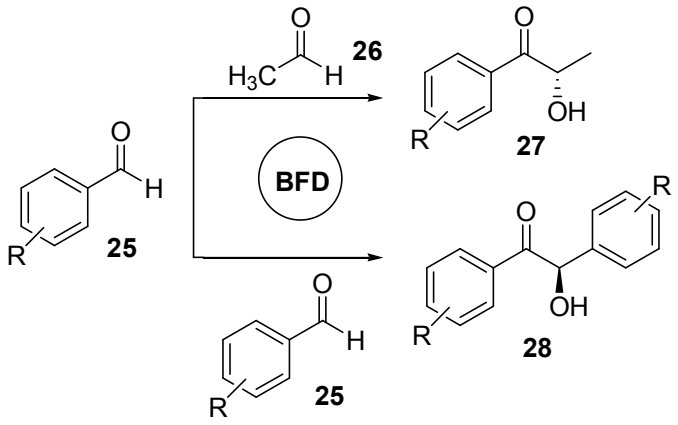


Figure 10 Benzoylformate decarboxylase mediated asymmetric C–C bond formation reactions

Since than its carboligation and C-C bond breaking activities are widely investigated. Its carboligation activity depends on a cofactor *thiamine diphosphate*. Catalytic cycle of ThDP (TPP) for the synthesis of (*R*)-benzoin, Fig. 11, begins with the attack of ylide **30** (ionized TPP formed with the loss of a proton) on the carbonyl carbon of benzaldehyde which results in the adduct **31**. The enamine formed **32** acts as a carbanion **33** (resonance effect) and attacks another benzaldehyde molecule. Thus, C-C bond formation is accomplished. Catalytic cycle is completed with the release of (*R*)-benzoin formed and the regeneration of ylide from the 1,2-diphenyl-1,2-diol ethyl-TPP complex **34**.

Benzaldehyde lyase (BAL) is perhaps the most versatile enzyme concerning the acceptance of aromatic donors. In the presence of TPP and a divalent cation this enzyme is capable of the synthesis of a broad range of heteroaromatic, and *ortho*-, *meta*-, and *para*- substituted aromatic aldehydes, which bear groups with both electron-releasing and electron-withdrawing properties yielding (*R*)-benzoin derivatives with excellent yields and optical purities. BAL is also shown to be capable of cross coupling aromatic aldehydes with acetaldehyde. This

way, the corresponding (*R*)-2-hydroxypropiophenones [(*R*)-HPP] have been obtained in high yields and enantiomeric excess (ee) values [20a,b]. When cross-coupling reaction is performed with mono- and di-methoxy acetaldehyde (*R*)-2-hydroxy-3-methoxy-1-arylpropan-1-one and (*R*)-2-hydroxy-3,3-dimethoxy-1-aryl propan-1-one which are important synthons for cytoxazone (a novel cytokine modulator) the side chain of taxol, and 5'-methoxyhydnocarpin having multidrug pump inhibitor activity, in high yields and ee's [20c]. All these carboligation reactions are presented in Fig. 12.

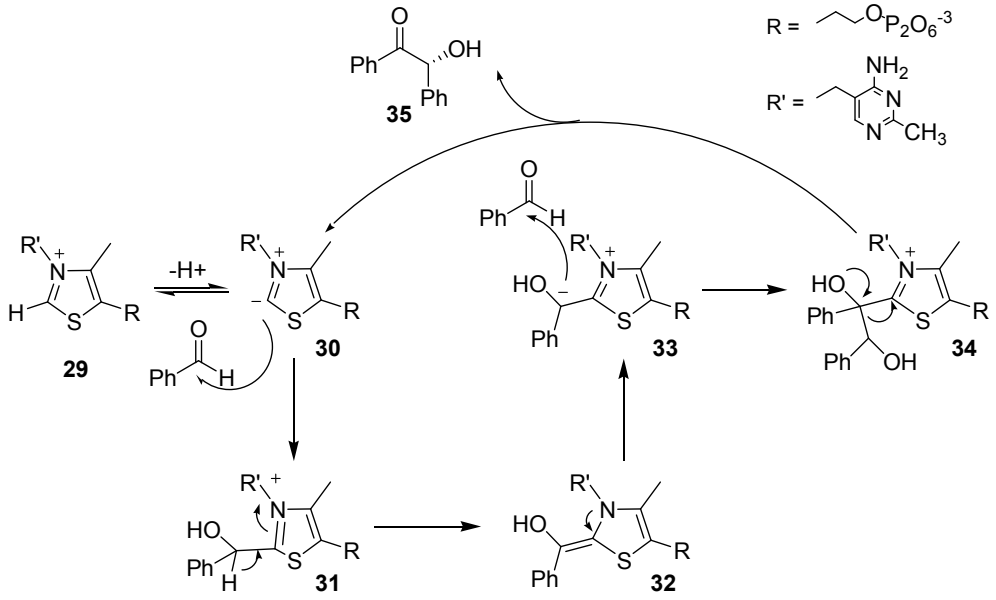


Figure 11 Catalytic cycle of ThDP in BAL mediated C-C bond formation reaction

BAL was improved to an industrial catalysis by bioreaction engineering means. Effect of several physiological parameters were investigated and optimized for obtaining more efficient BAL mediated reactions [21].

Lately, BAL is shown to perform the condensation reactions of aliphatic aldehydes [22], Fig. 13.

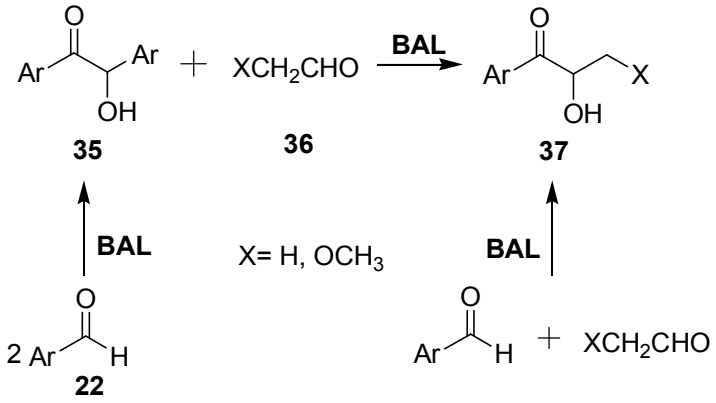


Figure 12 Examples of BAL mediated carboligation reactions

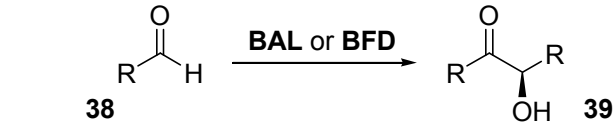


Figure 13 BAL & BFD mediated self-condensation reactions of aliphatic aldehydes [22]

1.3. Fungal Biotransformations

Fungi are heterotrophic eukaryotic organisms which typically grow as filaments, termed hyphae with apical growth. They are widely distributed in nature (air, water, soil, decaying organic debris), nearly 400,000 types exist. Yeasts, molds, mushrooms are all fungi.

The traditional use of fungi in biotechnology is in fermented foods such as bread, wine, etc. Then other industrial applications have been discovered and proceeded. With the prefinding of these microorganisms to be effective in producing secondary metabolities of penicillins and griseofulvin, fungi became preferred biocatalysts for the enantioselective bioconversion reactions.

Baker's yeast (*Saccharomyces cerevisiae*) is the most widely used microorganism and best known fungi to be employed in organic synthesis. This microorganism is commercially available, easy to handle, and able to be used in several types of reactions such as reduction, condensation, cyclization and oxidation [23]. Synthesis of (*R)*-Fenfluramine **42** which is an anorectic drug via Baker's yeast is given in Fig. 14.

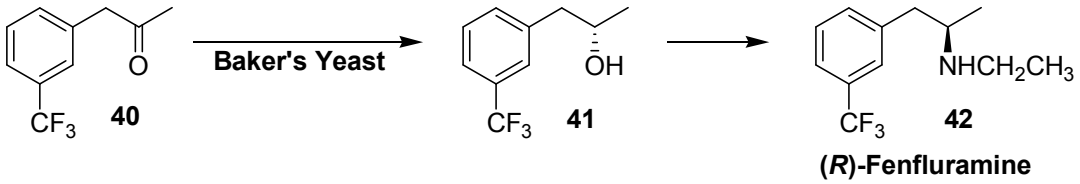


Figure 14 Baker's yeast mediated synthesis of (*R)*-Fenfluramine [24]

After exploitation of Baker's yeast as a catalyst, several other fungi have been investigated for their catalytic properties. Several interesting reactions are found. One application where different types of fungi are useful is the hydroxylation of steroids. In Fig. 15 *Rhizopus arrhizus* mediated conversion of progesterone **43** to 11α-hydroxyprogesterone **44** is illustrated.

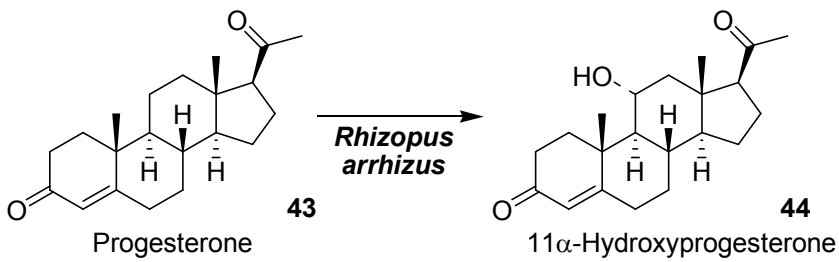


Figure 15 Bioconversion of progesterone to 11α-hydroxyprogesterone catalyzed by *Rhizopus arrhizus* [25]

R. oryzae is known to have a potential for biotransformations. It is shown to hydrolyze acetoxy ketones selectively in high enantiomeric excess yielding (*R*)-hydroxy ketones, aid in the synthesis of pharmacological interesting (*R*)-2-hydroxypropiophenones from propiophenone derivatives and deracemize benzoin in a pH-dependent manner [8, 26].

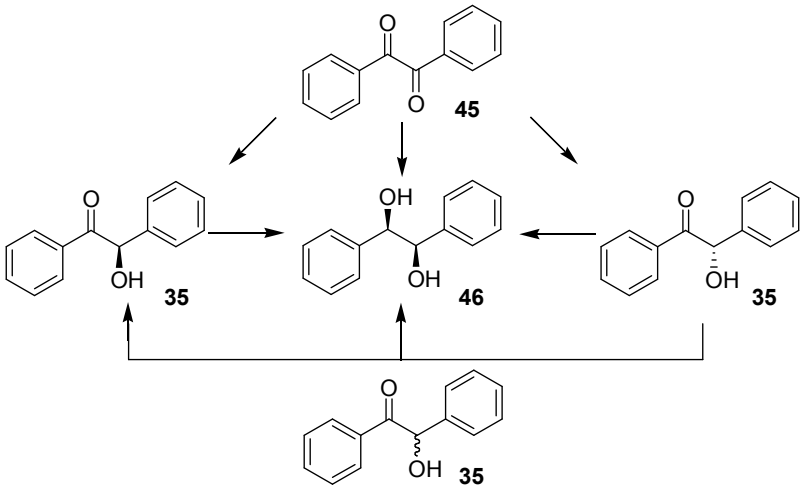


Figure 16 General conversion mechanism of *R.oryzae* mediated bioconversions

Rhizopus oryzae (ATCC 9363) and *Rhizopus oryzae* (72465) as well as *Rhizomucor miehei* (72460), *Rhizomucor pusillus* (72561) catalyzed the reduction of benzil to benzoin, as well as benzoin to hydrobenzoin. Starting from benzil, (*R*)-, (*S*)- and *rac*-benzoin only (*R,R*)-hydrobenzoin **46** was obtained in high ee and chemical yield [27]. These biotransformations are illustrated in Fig. 16.

Aspergillus is a genus containing 200 molds whose species are of great medical and commercial importance. *Aspergillus* species are highly aerobic, they commonly grow as molds on the surface of a substrate, as a result of the high oxygen tension.

Aspergillus species are effective in a series of bioorganic transformations. In a study by Keppler *et al*. some *Aspergillus* strains are shown to possess

interesting properties as sources of enzymes for bioreduction of ketones, alcohol deracemization and Baeyer–Villiger reactions [28], Fig. 17.

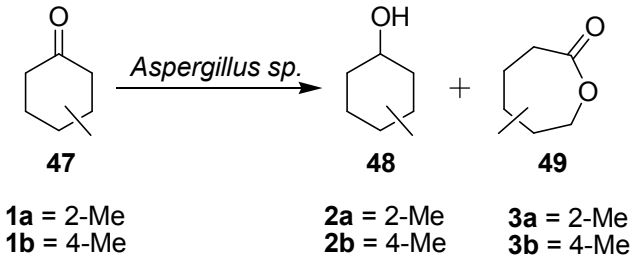


Figure 17 Biotransformation of cyclic ketones by *Aspergillus* strains [28]

Through all the *Aspergillus* species two strains; namely *Aspergillus niger* and *Aspergillus terreus* are the most pronounced ones in the field of biotransformation. In a study by Shen *et al*. *Aspergillus niger* is employed for the transformation of baccatin VI and 1β-hydroxybaccatin I to four new taxane diterpenoids taxumairol S_1, taxumairol T_1 and taxumairol S, taxumairol T, respectively [29]. Flavonoids possessing antihaemolytic, antioxidative, antifungal, estrogenic and antiumor activities are other class of compounds which are also targeted to be transformed by *A. niger* [30]. This transformation is presented in Fig. 18.

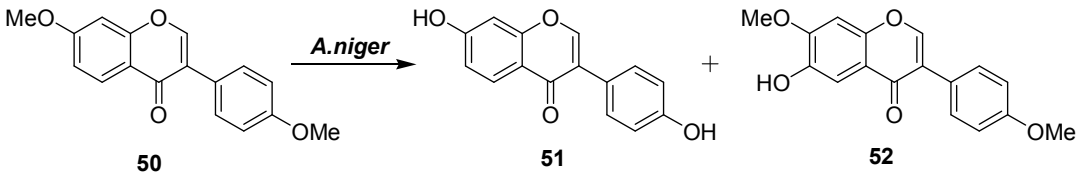


Figure 18 Oxidation of 7,4′-dimethoxydaidzein to daidzein and 6-hydroxy-7,4′-dimethoxyisoflavone by *A. niger* [30a].

A. terreus the other well-known *Aspergillus sp.* is able to perform several biotransformation reactions. Biomethylation reaction of organoselenium compounds [31], reduction of ketones and deracemization of alcohols (Fig. 19) can be given as examples [32].

Aspergillus flavus is a mold fungus mostly related with aspergillosis as *Aspergillus fumigatus*. Although it is pathogenic to humans and plants, this microorganism has a high biocatalytic capacity. Teng *et al.* revealed the ability of *A.flavus* to convert *imperatorin* – a compound possessing several biological activities - to its metabolites [33]. Biosynthesis of *kojic* also is another field of application of this microorganism [34].

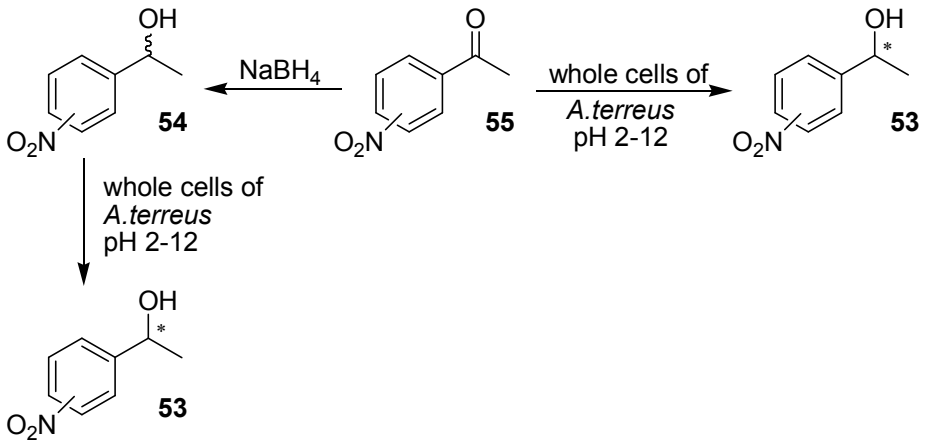


Figure 19 Reduction of nitroacetophenones and deracemization of (*RS*)-1-(nitrophenyl)ethanols with *A. terreus* [32]

Aspergillus oryzae is another promising *Aspergillus* strain to possess catalytic activity. Spizzo *et al*. revealed the ability *A. oryzae* dry cells to resolve (*R,S*)-*flurbiprofen* through esterification in pure organic solvent [35]. This reaction is given in Fig. 20.

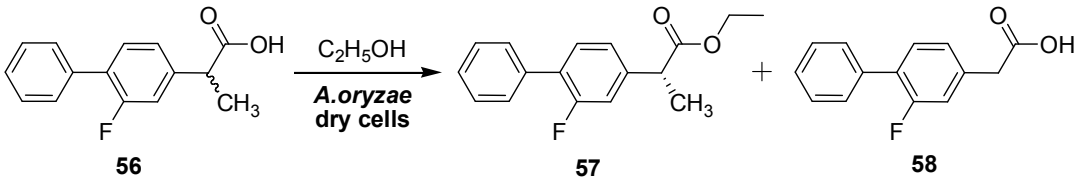


Figure 20 Enantioselective esterification of (*R,S*)-*flurbiprofen* via *A.oryzae* dry cells [35]

1.4. Aim of the Work

This study aimed to develop novel green procedures for the synthesis of optically active α-oxyfunctionalized compounds; namely α-hydroxy ketones which are important building blocks in several biologically active compounds and are also employed as chiral ligands in several synthetic procedures. To achieve this goal both enzyme and whole-cell biocatalysts which are ideal with their high selectivity are used in environmentally friendly reactions.

Benzaldehyde lyase [BAL, (EC 4.1.2.38)] is well-known enzyme to synthesize α-hydroxy ketones. In this study, BAL is used in novel C-C bond formation reactions to synthesize 2-Hydroxy-1-arylethan-1-ones which are valuable synthetic intermediates for various important compounds such as substituted 2-amino-1-arylethanols [36]. This enzyme is also used for the syntheses of polyfunctionalized acyloins via self- and cross-condensation reactions of functionalized acetaldehyde.

Beside these enzymatic approaches whole cells biocatalysts are also employed. *A.flavus*, a fungus which is not so common in bioconversion reactions, is used in several reactions such as reduction, hydrolysis and deracemization while another fungus; *A. oryzae* is utilized in bioreduction reactions of benzil and its derivatives. Each reaction is investigated and optimized in terms of reaction conditions via bioprocess engineering means.

To sum up this study aimed to develop novel green procedures via enzymatic or microbial processes for the synthesis of optically active hydroxy ketones or diols.

CHAPTER 2

RESULTS AND DISCUSSION

2.1. Perspective of the Work

Optically active α-hydroxy carbonyl moieties are a common motif for several important natural products. The enantioselective syntheses of these synthons are important in that sense.

There are several strategies present for the syntheses of these compounds. Enzymes and whole-cell biocatalysts, as a result of their complex chiral constitution, are predominantly suited for the manufacture of optically pure stereoisomers and they are widely used for obtaining α-hydroxy ketones. They are advantageous over classical chemical strategies as they apply mild conditions and have less requirements with the advantage of selectivity.

This study aimed to develop novel green procedures via enzymatic or microbial processes for the synthesis of optically active hydroxy ketones or diols.

For that purpose firstly, benzaldehyde lyase (BAL) mediated C-C bond formation reactions were performed. BAL is a thiamine diphosphate (ThDP)-dependent enzyme which has the ability to catalyze a broad range of symmetric and asymmetric reactions. Its catalytic capacity is better compared to other enzymes that catalyze similar reactions [such as benzoylformate decarboxylase (BFD)]. In this study, BAL was used initially to synthesize hydroxyacetophenone derivatives via cross-condensation of benzaldehyde derivatives with formaldehyde. Hydroxymethylation of aldehydes were achieved with high yields.

BAL was also used for the acyloin reactions of functionalized acetaldehyde where aliphatic aldehydes were used as donors. Aliphatic aldehydes with

various functional groups furnished the corresponding acyloin products with good yield and enantiomeric excesses.

Then microbial processes were achieved. Several readily available fungi were screened for their catalytic ability for the syntheses of α-hydroxy ketones. First promising microorganism was *A.flavus* 200120; a fungus which was not generally employed for the bioconversion reactions. This organism was found to catalyze a broad range of reactions such as hydrolysis, reduction, deracemization, etc. Each of these reactions were investigated and optimized in terms of enantioselectivity and yield.

Also other strains belonging to the genera of *Aspergillus* were screened for the reduction of benzil to benzoin which is a chiral linker and a precursor for several pharmaceutically important compounds. *A.oryzae* 5048 was found to catalyze this reaction with promising enantioselectivity. The enantioselectivity and the yield of the reaction were improved by optimization of the bioprocess parameters, and an efficient bioreduction process was developed for reduction of benzil derivatives to the corresponding benzoins.

2.2. Benzaldehyde Lyase (BAL) Mediated Self- and Cross-Condensation Reactions

Benzaldehyde lyase (BAL; E.C. 4.1.2.38), is a well known thiamine diphosphate (ThDP)- and Mg^{2+}-dependent enzyme from *Pseudomonas fluorescens* Biovar I. It has been used in C-C bond cleavage and C-C bond formation reactions. BAL is able to perform the enantioselective formation of (*R*)- and (*S*)-benzoins and (*R*)-2-hydroxypropiophenone [(*R*)-2-HPP] derivatives. (*R*)-2-HPP derivatives are formed in preparative scale by benzaldehyde lyase (BAL)-catalyzed C–C bond formation from aromatic aldehydes and acetaldehyde, methoxy- and dimethoxyacetaldehyde with remarkable ease in high chemical yields and high optical purity [20].

BAL is used for novel C-C bond formation reactions via self- and cross- coupling for the syntheses of optically active synthons. The reactions can be given under two headings:

- BAL mediated syntheses of hydroxyacetophenones.
- BAL mediated acyloin reactions of functionalized acetaldehyde.

2.2.1 Benzaldehyde Lyase (BAL) Mediated Syntheses of Hydroxyaceto-phenones

2-Hydroxy-1-arylethan-1-ones are valuable synthetic intermediates for the preparation of a range of compounds with biological and pharmaceutical activity and these compounds can be synthesized with selective hydroxymethylation of aromatic aldehydes with formaldehyde leading to terminal hydroxymethyl functionality in which one carbon extension of carbonyls in order to obtain hydroxy ketones is achieved.

The novel thiamin diphosphate (ThDP) dependent enzyme; BAL was natively isolated from *P.fluorescens* Biovar I. Cloning of the gene encoding BAL to an *Escherichia coli* strain using vector pUC18 enabled the bulk production and the wide use of the enzyme [19b]. Later on the activity of this enzyme to enantioselectively form (*R*)- and (*S*)-benzoins and (*R*)-2-hydroxypropiophenone ((*R*)-2-HPP) derivatives via C–C bond cleavage and C–C bond formation reactions were revealed.

With the previously mentioned abilities BAL is thought to be useful in the cross-coupling reactions of benzaldehyde derivatives **25** with formaldehyde **59** for the syntheses of hydroxyacetophenones **60** which is given in Fig. 21.

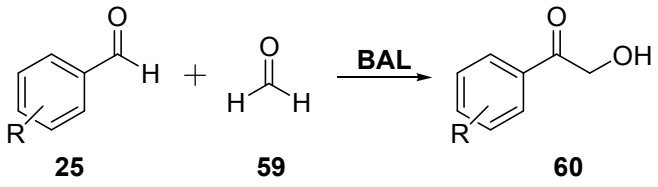


Figure 21 Biocatalytic route to 2-hydroxy-1-arylethan-1-ones

In the carboligation of aromatic aldehydes with formaldehyde shown in Fig.21, benzaldehyde derivatives (**25**) were dissolved in potassium phosphate buffer (pH 7, containing $MgSO_4$ and ThDP) containing 20% DMSO and formaldehyde solution. After the addition of BAL, the reaction was started which was then carried out at 37^{o}C. The reactions were monitored by TLC and GC-MS using a

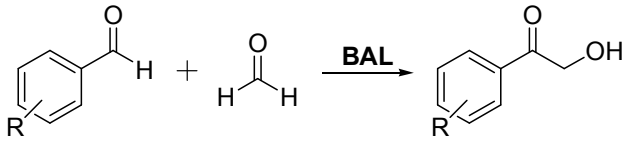
commercially available authentic sample. In the case of benzaldehyde after 3 days, no more change was observed and the purification of the crude product by column chromatography provided 2-hydroxy-1-phenylethan-1-one (entry 1) in 94% yield. The corresponding reaction was carried out using the above described conditions with a wide range of aromatic aldehydes and the corresponding acyloin derivatives were obtained in high yields as summarized in Table 2.

The amount of BAL added and substrate concentrations, reaction time, and benzaldehyde substituents were all found to be effective on the yield and range of products formed during reactions. Maximum yields were obtained with excess amounts of formaldehyde added at fixed time intervals. No acyloin formation was observed in the absence of the enzyme.

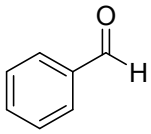
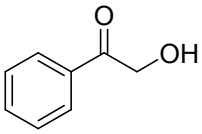
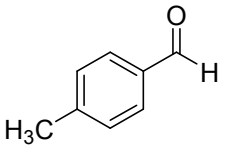
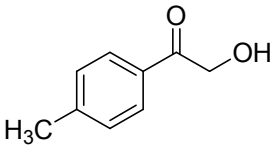
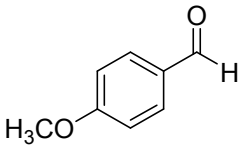
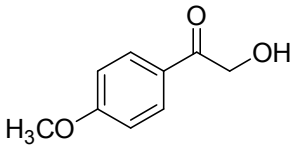
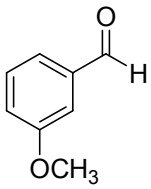
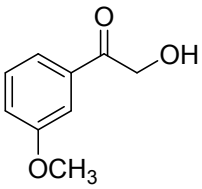
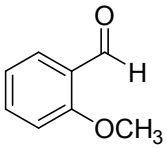
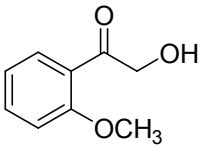
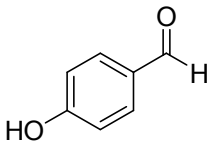
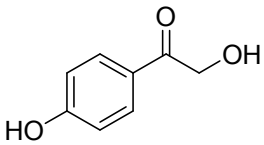
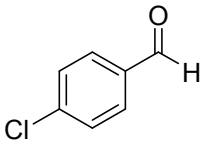
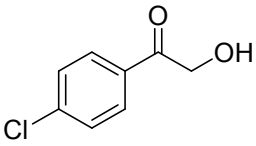
The ratio of benzaldehyde to formaldehyde was found to be very important for the product distribution, since excess formaldehyde resulted in high yield formation of 2-hydroxy-1-arylethan-1-one, whereas a 1:1 ratio of benzaldehyde/formaldehyde gave mixture of (*R*)-benzoin and 2-hydroxy-1-arylethan-1-one. However, temperature had a small influence on the reaction. It is required to state that yields have been shown to increase by passing nitrogen gas through the reaction solution at the onset of the reaction.

^{1}H NMR spectrum (Fig. 50) of 2-hydroxy-1-phenylethanone (Table 2, entry 1) showed a broad singlet at 3.37 ppm that resulted from terminal hydroxy proton (-OH). Singlet at 4.76 ppm was due to the methylene protons (CH_2) at α-position. Signals of methylene protons at α-position and terminal hydroxy proton (-OH) are characteristic to all the 2-hydroxy-1-arylethan-1-one derivatives. The aromatic protons gave varying signals depending on the substitution of the aromatic ring. For 2-hydroxy-1-phenylethanone triplets at 7.41 and 7.53 ppm as well as doublet at 7.83 ppm resulted from aromatic protons.

Table 2 Results of carboligation reactions of aromatic aldehydes with formaldehyde



25 **59** **60**

Entry	ArCHO (25)	HAP Derivative (60)	Yield (%)*
1			94
2			94
3			91
4			92
5			68
6			89
7			88

* Reactions were concluded after 3 days for entry 1. For other derivatives reactions were concluded after 4 days

The formation of 2-hydroxy-1*p*-tolylethanone (Table 2, entry 2) was also justified with NMR analysis. ^{1}H NMR spectrum of this compound is given in Fig. 52. Singlet at 2.35 ppm resulted from the three methyl protons connected at *para*- position to aromatic ring. Characteristic (-OH) at β-position and methylene protons (CH_2) at α-position gave a broad singlet at 3.49 ppm and a singlet at 4.77, respectively. Two aromatic protons near to the methyl group came at 7.22 ppm as methyl group was an electron releasing group so it increased the electron density of these protons and shifted them to the high field. On monosubstituted rings, electron donating groups resonate at high chemical shifts. They increase the electron density by releasing electrons into a reaction center. Other two aromatic protons gave doublet at 7.74 ppm. ^{13}C NMR spectrum of this compound (Fig. 53) showed a signal at 22.7 ppm resulting from carbon of methyl group connected to the aromatic ring and a peak at 65.3 ppm belonging to the methylene carbon at α-position. Signals at 127.8 ppm and 129.7 ppm resulted from the aromatic carbons. Signal at 130.9 ppm came from the quarternary carbon in aromatic ring which was connected to the carbonyl group. Other quarternary carbon connected to the methyl group gave signal at 145.2 ppm. Peak at 198.0 ppm justified the presence of carbonyl carbon.

^{1}H NMR spectrum (Fig. 51) of 2-hydroxy-1-(4-methoxyphenyl)ethanone (Table 2, entry 3) showed the characteristic signals of hydroxy and methylene protons. Three protons of the methyl group connected to the oxygen (-OMe) substituted at the *para*-position gave singlet at 3.81. Two aromatic protons near to the methoxy group came at 6.89 ppm as doublets because of electron donating effect of methoxy group (methoxy group donates electron via resonance) while other two protons gave doublet signals at 7.82 ppm.

The formation of 2-hydroxy-1-(3-methoxyphenyl)ethanone (Table 2, entry 4) was verified with NMR analysis. ^{1}H NMR spectrum of this compound is illustrated in Fig. 54. Characteristic signals of (-OH) at α-position came at 3.36 ppm as broad singlet and methylene protons (CH_2) at α-position at 4.74 ppm as singlet. Three protons of the methoxy group connected to the aromatic ring gave signals at 3.79 ppm. Multiplet peak at 7.07 ppm belonged to the proton which was adjacent to the methoxy group. Other aromatic proton that was near this proton gave multiplet signal at 7.29 ppm. Multiplet signal at 7.36 ppm was due to two remaining aromatic protons at β-position. ^{13}C NMR spectrum of this

compound (Fig. 55) showed a signal at 54.8 ppm resulting from carbon of methoxy group connected to the aromatic ring and a peak at 65.0 ppm belonging to the methylene carbon at α-position. Signals at 111.5, 119.5, 120.1, 129.2 ppm resulted from the aromatic carbons. Signal at 132.8 ppm came from the quaternary carbon in aromatic ring which was connected to the carbonyl group. Other quaternary carbon connected to the methoxy group gave signal at 159.6 ppm. Peak at 197.4 ppm justified the presence of carbonyl carbon.

Fig. 56 illustrated ^{1}H-NMR spectrum of 2-hydroxy-1-(4-hydroxyphenyl) ethanone. Signals of the characteristic protons were as same as the other derivatives. Singlet signal at 4.94 ppm resulted from the second –OH group present in the molecule connected at *para*- position of the ring system. Two aromatic protons adjacent to the hydroxy group gave doublet signal at 6.82 ppm where as other two aromatic protons at β-position gave doublet signals at 7.80 ppm.

Commercial formaldehyde solution, which is an aqueous solution containing 37% formaldehyde and 8–10% methanol is cheap, easy to handle, and stable even at room temperature was used as the source of formaldehyde instead of paraformaldehyde because paraformaldehyde easily self-polymerizes decreasing the available formaldehyde in the reaction medium.

The steric and electronic demands of the substituent played a role in the yield of the reaction. Highest yields were obtained with non-substituted 2-hydroxy-1-arylethan-1-ones (Table 2, entry 1) and methyl substituted derivative at *para*-position [2-hydroxy-1-*p*-tolylethanone, (Table 2, entry 2)] with 94%. Aryl substitutions at *para*- and *meta*- positions were favored over *ortho*- substituted substrate (2-methoxy benzaldehyde). 2-methoxy benzaldehyde was not preferred most probably because of the steric hindrance.

Employing electron donating groups resulted in higher conversion yields compared to the electron withdrawing groups. Synthesis of 1-(4-chlorophenyl)-2-hydroxyethanone (Table 2, entry 7) was achieved with lower yield (88%) compared to the other derivatives substituted with electron donating groups at *para*- position (4-Me, 4-MeO and 4-OH derivatives).

Hydroxymethylation of aromatic aldehydes were achieved via BAL mediated reactions and 2-hydroxy-1-arylethan-1-one derivatives were obtained with high yield.

2.2.2. Acyloin Reactions of Functionalized Acetaldehyde

BAL is believed to need aromatic aldehydes as donors while acceptors can both be aromatic and aliphatic aldehydes. Recently, this enzyme has been used in asymmetric condensation reactions of aliphatic aldehydes in very low yields [22]. However the products formed lack functionality as the groups on the molecule can be hardly converted to other functionalities, so chirality formed can not pass through a more functional molecule.

BAL is used for the acyloin reactions of functionalized acetaldehyde where aliphatic aldehydes are used as donors. Aliphatic aldehydes with various functional groups furnished the corresponding acyloin products with good yield and enantiomeric excesses.

Benzyloxyacetaldehyde and dimethoxyacetaldehyde are highly functionalized acetaldehydes. Their self- and cross- condensation reactions have been performed.

In general protected hydroxyl acetaldehyde is used. For deprotection to obtain hydroxyl acetaldehyde derivative the best group is benzylic group. The self- and cross- condensation products can then be reduced to obtain important synthons which are very valuable.

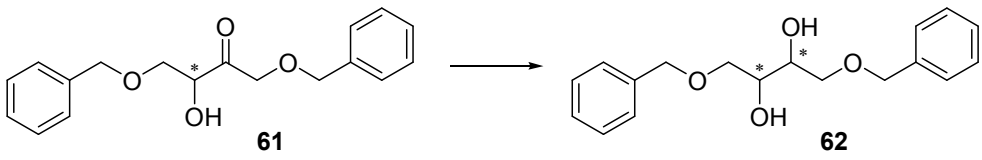


Figure 22 A route to optically active 1,4-bis(benzyloxy)butane-2,3-diol

The products obtained as the result of condensation reactions are valuable intermediates which can further be reduced to optically active diol derivatives with pharmaceutical importance. In Fig. 22, reduction of the 1,4-bis(benzyloxy)-3-hydroxybutan-2-one to 1,4-bis(benzyloxy) butane-2,3-diol is given.

2.2.2.1. Self-Condensation of Benzyloxyacetaldehyde

For the self-condensation reaction benzyloxyacetaldehyde **63** have been reacted in the presence of BAL, Fig. 23.

In the classical BAL reaction to solubilize the unsoluble aldehyde derivatives DMSO was used and it has been shown to increase the yield and ee of the reaction by this way [20a-b]. However, in a recent study by De Maria *et al.* the enantioselectivity of BAL reaction was figured out to be dependent on the solvent used and by employing isopropanol higher selectivities were achieved in some cases [22].

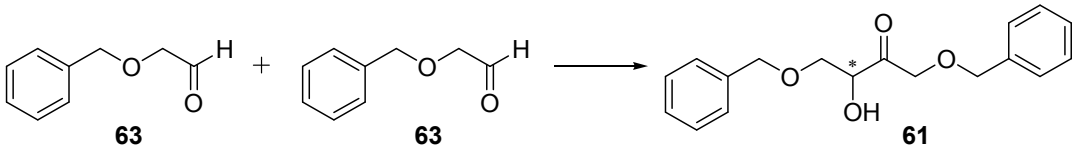


Figure 23 Self-condensation of benzyloxyacetaldehyde

When self-condensation of benzyloxyacetaldehyde was achieved with the classical methodology where substrate (**63**) was dissolved in potassium phosphate buffer (pH 7, containing $MgSO_4$ and ThDP) containing 20% DMSO. After the addition of BAL, the reaction was started which was then carried out at 37 ^{o}C. After 4 days reaction was terminated. The ee of the product **61** was 30%. Various solvents such as dimethyl formamide (DMF), toluene, ether, benzene, acetonitrile and tetrahydrofuran (THF) were tested to increase the selectivity. With acetonitrile and THF product formation could not be achieved.

It was found that when toluene was used with phosphate buffer (two-phase reaction) the condensation product was obtained with 73% ee and 75% yield. The HPLC analysis of **61** is given in Fig. 60.

The product was characterized by NMR analysis. In Fig. 57, ^{1}H NMR spectrum of the 1,4-bis(benzyloxy)-3-hydroxybutan-2-one is shown. Broad singlet at 3.35 ppm belonged to the –OH proton at α-position. Doublet of doublet of doublet at 3.72 ppm was resulted from the methylene proton adjacent to the oxygen atom and to the carbon connected to the hydroxy group. Other methylene protons at α-position and neighbour to the oxygen atom gave quartet at 4.17 ppm. A multiplet at the range of 4.58-4.30 ppm included the –CH proton as well as the two methylene group protons adjacent to phenyl ring and oxygen, so this range contained the signal of five protons. Multiplet signal resulting from ten aromatic protons came at a range of 7.37-7.09 ppm. ^{13}C NMR spectrum of the same compound (Fig. 58) showed four signals resulting from methylene carbons at 70.86, 73.21, 73.53, 73.63 ppm respectively. The peak at 75.07 ppm is due to the sp^3 hybridized carbon at α-position. Aromatic four carbons gave signals at 127.80, 127.93, 128.12, 128.45 ppm. The signals at 132.75 & 134.12 ppm were resulted from two aromatic quaternary carbons. Peak at 204.90 ppm indicated the presence of carbonyl carbon.

The absolute configuration of the product should be (*R*)-configuration according to the results obtained with aliphatic aldehydes.

2.2.2.2. Cross-Condensation of Benzyloxyacetaldehyde with Acetaldehyde

To investigate the ability of BAL to perform cross condensation reactions with other aliphatic aldehydes, it has been reacted with acetaldehyde under the catalysis of BAL. It was aimed to observe which aliphatic aldehyde would be accepted as donor by BAL. As in the synthesis of 2-hydroxypropiophenones (HPP), acetaldehyde was used in excess [20a,b].

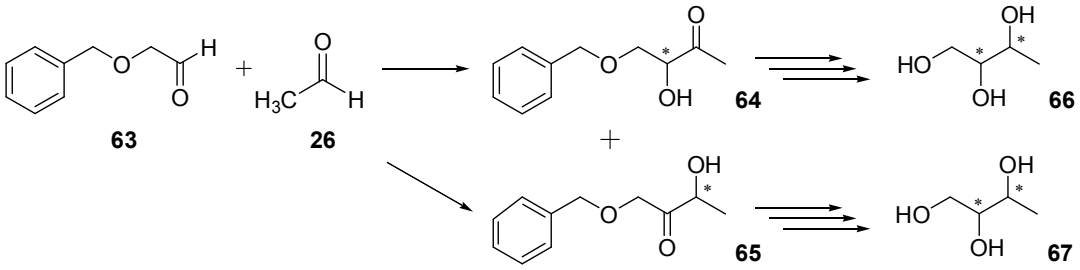


Figure 24 Cross-condensation of benzyloxyacetaldehyde with acetaldehyde

However two main products were observed in the reaction medium, two-cross condensation products **64** & **65**, Fig. 24. Self-condensation product of **63** was not observed in the reaction medium while self-condensation product of benzyloxyacetaldehyde **61** was formed in trace amounts. Cross-condensation products **64** & **65** appeared as one spot in the TLC and could not be separated via column chromatograph. Self-condensation products with acetaldehyde were not formed. These two products were detected and characterized via GC-MS (Fig. 61). The mass spectrum of the compound at 19.09 min. belonged to the condensation product **65**. Molecular ion (M^+) peak can be realized at m/z 194. Base peak in the spectrum was 91 which was the mass of benzyl generated with the removal of 1,3-dihydroxybutan-2-one. The mass spectrum of the compound at m/z 19.32 belonged to the condensation product **64**. Molecular ion peak was observed again at m/z 194 and base peak was at m/z 91 also. In the mass spectrum, the mass peak at m/z 150 enabled to distinguish between **64** & **65**, this mass peak was 149 for **65**.

Acetaldehyde was thought to act only as acceptor as in the case of 2-HPP [2-hydroxypropiophenone] and it was employed in excess amounts (1:5, benzyloxyacetaldehyde:acetaldehyde) to avoid the self-condensation product **61.** That's why **61** was observed in trace amounts in the reaction medium.

2.2.2.3. Self-Condensation of Dimethoxyacetaldehyde

Another functional acetaldehyde is dimethoxyacetaldehyde **68**, previously this acetaldehyde derivative was used in cross-condensation reaction with aromatic aldehydes where it acted as an acceptor [20c]. for the synthesis of various biologically active molecules such as the 1,4-benzodioxane framework, which

has often been found in biologically active natural products. The product is the protected dialdehyde **70** which can be further functionalized to obtain various interesting compounds.

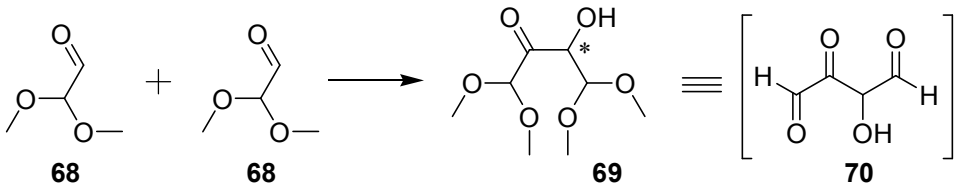


Figure 25 Self-condensation of dimethoxyacetaldehyde

To investigate the capacity of BAL to use this aliphatic aldehyde as donor, self-condensation reaction was performed in the presence of DMSO as cosolvent, Fig. 25. The product was purified and then characterized via ^{1}H-NMR spectroscopy and GC-MS analysis. The ee of the product formed **69** was not determined via HPLC as the product was not detectable with UV detector and HPLC employed had UV or DAD detectors. However the optical rotation of the molecule was measured with polarimeter; $[\alpha]_D^{20}$: +30 (c 0.5, $CHCl_3$).

The product was characterized via ^{1}H-NMR (Fig. 62) and GC-MS (Fig. 63) analyses. In ^{1}H-NMR spectrum, -OH proton yielded doublet at 3.15 ppm while protons of the 4 methoxy groups gave singlets at 3.31, 3.34, 3.37 and 3.40 ppm. Doublet at 4.38 ppm resulted from the –CH proton adjacent to the hydroxyl group. The singlet at 4.50 ppm came from the –CH proton at α-position. Other –CH proton which was also at α-position and affected with two neighboring oxygen atoms gave singlet at 4.82 ppm.

In mass spectrum of **69** molecular ion (M^+) peak was seen at m/z 207 and base peak was observed at m/z 75 which denoted dimethoxymethane. This may be resulted from the stability of this compound or the ease of breaking dimethoxy-methane, two of which were readily present in the molecule **69**.

2.3. *Aspergillus flavus* Mediated Whole Cell Multi Reactions

The idea to use whole microbial cells for the bioconversion reactions is very attractive form environmental and selectivity concerns. Mostly, a certain microorganism is employed in one type of reaction. For example, acetic acid bacteria are generally employed for the oxidation [37].

Few microorganisms however have been shown to be capable of catalyzing several reactions; the famous Baker's yeast was employed in numerous biotransformation reactions, reduction reactions (for the reduction of carbonyls and double bonds), cyclization and condensation reactions [38]. Resting cell and dried-cell preparations of *Geotrichum candidum* were applied in oxidation, reduction, and deracemization reactions [39].

Although the biocatalytic capacity of *A.flavus* has not been widely explored in the literature, it is found to have high bioconversion capacity. It was effective in several reactions as hydrolysis, deracemization, reduction, oxidation, shown in Fig. 26.

There are several ways to obtain non-*racemic* benzoin and conversion of this compound to chiral hydrobenzoin:

1. Enantioselective reduction of benzil (D)
2. Monooxidation of hydrobenzoin (B)
3. Enantioselective hydrolysis of benzoyl benzoin (A)
4. Deracemization of racemic benzoin (C)
5. Reduction of benzoin to the hydrobenzoin (E)

For each of the reactions above, the reaction conditions have been explored and bioprocess parameters have been analyzed and optimized in order to get satisfactory conversion yields and selectivities.

2.3.1. Bioreduction of Benzil to Benzoin via Whole Cells

Benzil is a cheap substrate which is available in high quantities, because of this it represents an interesting and suitable starting material for obtaining optically active benzoin.

Reduction reaction of benzil **45** to benzoin **35** can be performed with whole-cell biocatalysts. There are several examples in the literature where whole-cells from different microorganisms are used such as *Bacillus cereus* [40a], *Rhizopus oryzae* and *Rhizomucor sp.* [40b].

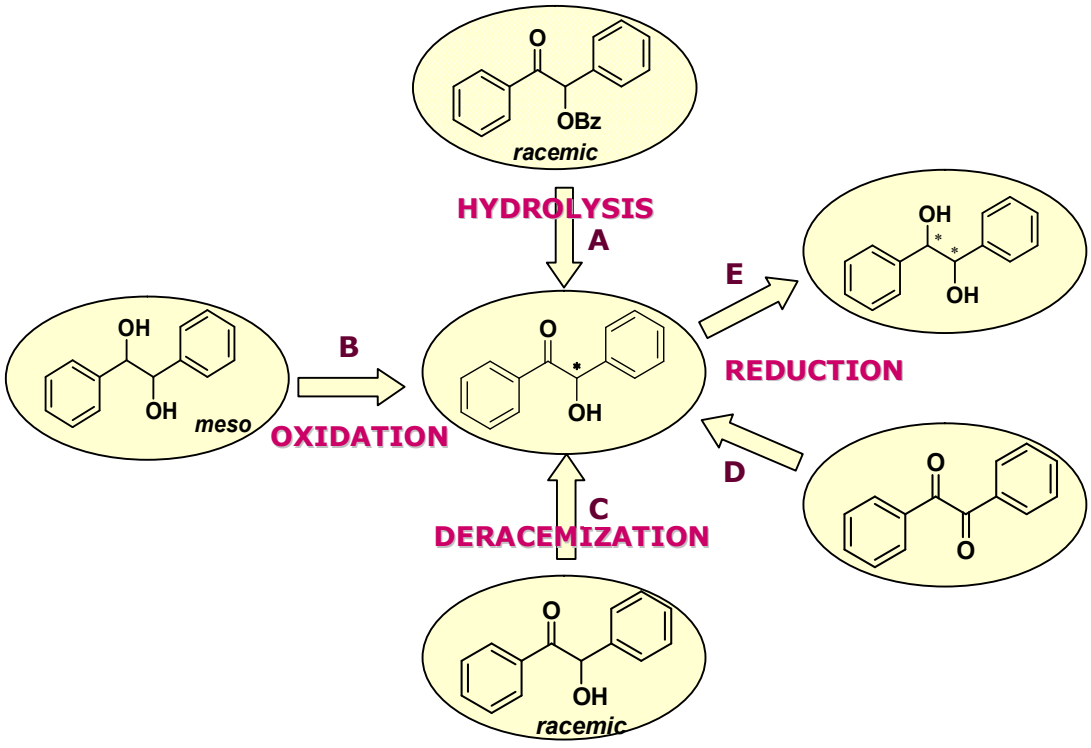


Figure 26 *A.flavus* catalyzed multi reactions for the synthesis of α-hydroxy ketones

The whole cell systems which represent a *packaged enzyme* or *enzyme system* are advantageous over isolated enzymes in oxidation and reduction reactions

from economic point of view by eliminating the need for expensive cofactors such as NADH and NADPH.

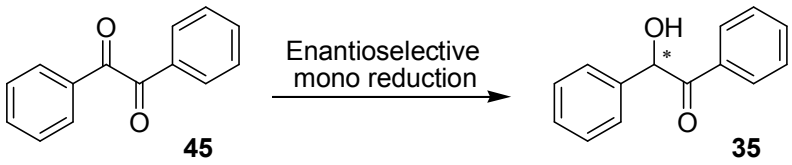


Figure 27 Reduction of benzil to benzoin

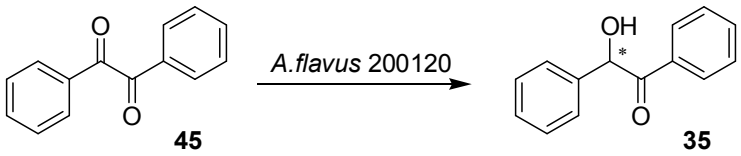
Reduction of benzil to benzoin was tested with *A.flavus* 200120, Fig. 27. Yield of the conversion and ee value of the product formed were determining factors while the reaction conditions were optimized based upon them. The conditions analyzed were as follows;

- Medium for the bioconversion
- Physiological state of the cell (growing cells, stationary cells, broken cells)
- In the case of wet cells (stationary phase cells), pH of the buffer employed
- Additives

2.3.1.1. Selection of the Medium for Bioreduction

Several media which are known to be suitable for the cultivation of *A.flavus* were tested for the optimum conversion and enantioselectivity; Potato Dextrose Broth (PDB), Glucose Peptone Broth (GPB), Medium C (4g/L yeast extract, 15g/L starch, 1g/L K_2HPO_4, 0.5 $MgSO_4.7H_2O$) [41], Malt Extract Broth (MEB), and MGYP (malt extract, glucose, yeast extract, peptone broth). Results are presented in Table 3.

Table 3 Effect of medium employed on the benzil bioreduction reaction



Medium	Reaction time(h)	Yield of conversion(%)	Ee(%)**
PDB (hand-made)	5	98	77
PDB *	4	95	75
GPB	7	80	69
Medium C	7	78	74
MEB	4	91	72
MGYP	3	90	75

* Potato dextrose broth prepared with commercially available potato extract

** In all the cases (*S*) enantiomer is obtained

Microorganism was grown for 2 days in every medium tested then substrate dissolved in DMSO was added. Reactions were concluded till no starting material was observed via TLC.

PDB and MGYP seemed to be the best media. Lowest conversion yield (80%) and enantioselectivity (69%) was obtained in a prolonged reaction time (7h) when GPB was employed as the growth medium. Although hand-made PDB yielded the most efficient reaction with the highest selectivity and conversion yield, there would be a deviation in the results that would have been obtained because of the difference in the physical properties of the potato lots purchased. That's why PDB medium prepared with the commercially available potato extract was preferred over the hand-made PDB.

Also MGYP yielded an efficient reaction with the quickest reaction time (3 hours), however the enantioselectivity and the conversion yield was still low

compared to PDB. So, PDB was chosen as the medium for bioreduction reaction.

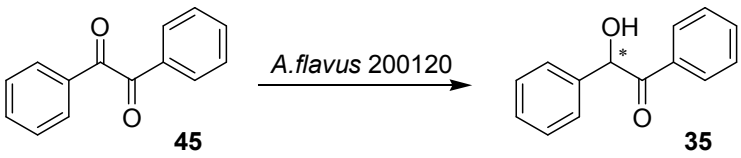
2.3.1.2. Effect of Substrate Concentration

Different substrate concentrations were investigated to find out the best concentration to be applied. In the previous works, it was reported that 0.5 mmol/100mL concentration enabled the desired bioconversion reaction [26,27].

Substrate solution was prepared with different concentrations (0.05mmol, 0.1mmol, 0.2mmol, 0.25mmol & 0.5mmol, all in 1mL DMSO) were introduced into the growth medium. Results obtained are tabulated in Table 4 and presented in Fig. 28.

As can be seen in Table 4 and Fig. 28 as substrate concentration increased enantioselectivity decreased. Highest ee value was obtained with 0.05mmol (85%) substrate within 1.5h while lowest ee value was achieved with 0.5mmol (70%) in 48h. Yield of conversion was high in the substrate range of 0.05mmol-0.2mmol (94%-99%) while it declined at higher concentrations such as 0.25mmol & 0.5mmol (65% & 70%).

Table 4 Effect of substrate concentration on the bioreduction reaction



45 35

	Amount of the substrate added (mmol) for 100mL growth medium				
	0.05	**0.1**	**0.2**	**0.25**	**0.5**
Reaction time(h)	1.5	2.5	6	24	48
Yield of conversion(%)	94	99	96	78	75
Ee(%)	85	83	77	65	70

A bioprocess is expected to be more economical when the highest convertable substrate concentration is inoculated into the growth medium, as same biomass is used for high conversions. That's why 0.2mmol was chosen as the substrate concentration per 100mL growth medium although the enantioselectivity was a little bit lower when compared to lower substrate concentrations (0.05mmol and 0.1mmol).

2.3.1.3. Effect of the Physiological State of the Cells and the Buffer pH

Then physiological state of the cell was tested, i.e., growing cells (cells in the growing medium), wet cells (cells at the stationary phase which are filtered from the growth medium), crude cells (lyophilized and crushed cells).

The idea that employing wet cells in buffer solutions with a precise pH might have resulted in a higher ee value led us to try out a low concentration phosphate buffer (50mM) at pH values of; 5 to 8. As the control experiment wet cells were suspended in sterile distilled water. However employing wet cells in buffer solutions at different pH values did have a negative effect on enantioselectivity (Table 5).

However, when wet cells were transferred to the sterile distilled water instead of phosphate buffers with varying pH's enantioselectivity was increased to 91%. This value was even higher than the control where substrate was directly added to the growth medium, i.e, growing cells were employed while the reaction time was shortest with 3h.

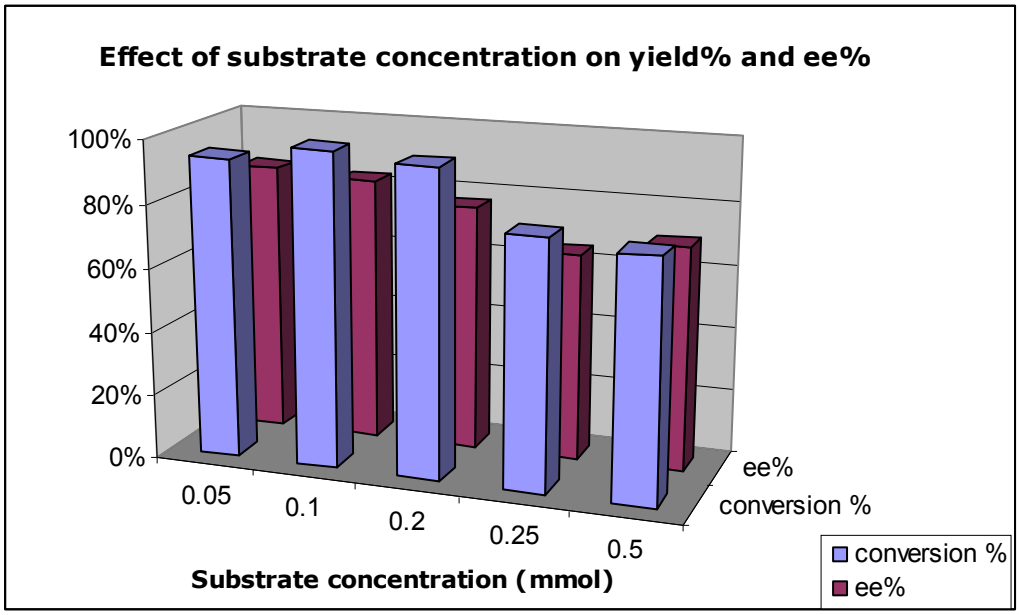
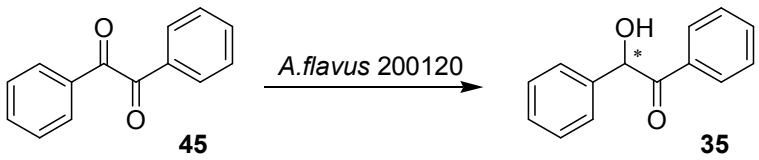


Figure 28 Variation of yield and ee with respect to substrate concentration

Table 5 Effect of physiological state of the cells at different pH values on the bioconversion



Physiological state of the cell – pH of the buffer	Reaction time(h)	Yield of conversion(%)	Ee(%)
Wet cell- pH 5 phosphate buffer	4	96	76
Wet cell- pH 6 phosphate buffer	4	96	74
Wet cell- pH 7 phosphate buffer	4	93	73
Wet cell- pH 8 phosphate buffer	4	97	71
Wet cell- sterile distilled water	3	95	91
Control (Growing cells) *	4	98	78

* Substrate was directly added to the growth medium

From these three cell types, wet cells grown in PDB for 2 days and suspended in sterile distilled water was the most successful one with 85% ee and 97% conversion.

2.3.1.4. Effect of Additives

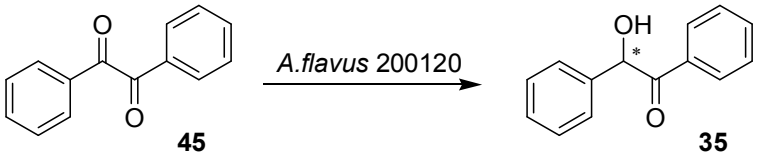
Additives are molecules used to enhance enantioselectivity. They have several mode of action: they act as sources of hydrogen for cofactor regeneration (alcohols) or as enzyme inhibitors (allyl bromide, allyl alcohol); they increase availability of the substrate to the enzyme (surfactants) and they perform chemical modification of the enzyme increasing its activity (sulfur compounds) [42].

Additives such as dimethyl sulphate (DMS), isopropyl methyl ketone (IPMK), isobutyl methyl ketone (IBMK), methanol, ethanol and isopropanol, as potent enantioselectivity enhancers, were tested. Of the additives tried DMS, methanol, isobutyl methyl ketone did not have an effect on enantioselectivity, isopropyl methyl ketone, on the other hand, showed inhibitory effect on the microorganism and cell lysis occurred. In the reduction reaction which was held in the presence of this additive, benzoin formed was racemic and the yield of conversion was lower (85%) compared to the other additives. Both ethanol and IPA increased enantioselectivity while shortening the reaction time. Results are shown in Table 6.

With addition of 1mL ethanol; enantioselectivity was as high as 95% and conversion was 98% within a shorter reaction time (2 hours). All of the values were superior over control with no additives where stationary-phase cells (wet cells) were directly transferred to Erlenmeyer containing sterile distilled water. However, best selectivity (97% ee) and yield (98%) were obtained in the case of IPA with the best bioreduction time (2 hours). So, wet cells employed in the distilled water with the additive IPA was chosen as the condition of bioconversion.

These results imply the fact that there is a hydrogen shortage in the cofactor regeneration, so alcohol derivatives such as ethanol and IPA were effective.

Table 6 Effect of additives on the bioreduction reaction



Additive [a]	Rxn time(h)	Yield of conversion(%)	Ee(%)
DMS [b]	3.5	92	83
IPMK [c]	24	85	-
IBMK [c]	6	90	89
Methanol [d]	2	93	90
Ethanol [d]	2	98	95
Isopropanol [d]	2	98	97
Control – no additive	3	97	92

[a] The amount of additives employed and the method of introduction were taken from the literature [42]

[b] Added in the ratio of 1:4 (DMS: substrate)

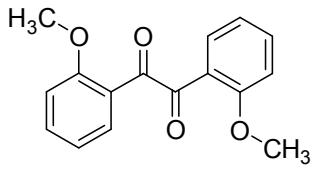
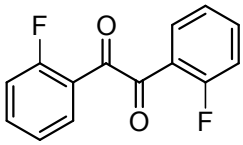
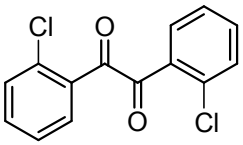
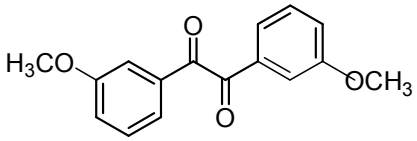
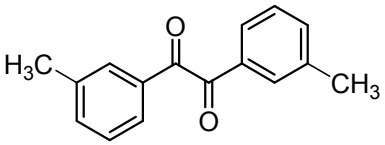
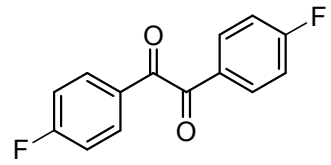
[c] 500μL was added

[d] 1000μL was added (amount added was checked with the optimization)

2.3.1.5. Bioreduction of Different Benzil Derivatives

Many of the benzil derivatives were examined for the bioreduction to corresponding benzoins after determining the proper reaction conditions. Table 7 represents the results. Benzil derivatives where the phenyl rings were substituted with representative donating and electron withdrawing groups were used as starting materials.

Table 7 Bioreduction of different benzil derivatives with *A.flavus*

Benzil derivative	Rxn time(h)	Yield of conversion(%)	Ee(%)
 1,2-bis(2-methoxyphenyl)ethane-1,2-dione, **(71)**	94	41.1	85.4
 1,2-bis(2-florophenyl)ethane-1,2-dione, **(72)**	6	89.5	44.5
 1,2-bis(2-chlorophenyl)ethane-1,2-dione, **(73)**	24	85.5	88.4
 1,2-bis(3-methoxyphenyl)ethane-1,2-dione, **(74)**	7	92.0	84.4
 1,2-di*m*-tolylethane-1,2-dione, **(75)**	72	96.0	69.1
 1,2-bis(4-florophenyl)ethane-1,2-dione, **(76)**	24	91.0	62.6

For all the derivatives microorganism was grown for 2 days, wet cells filtered from the growth medium were transferred to Erlenmeyer containing distilled water and then IPA was added, afterwards the benzil derivative dissolved in DMSO was introduced. Conversions were carried out until the starting material was no longer observed.

With the case of 1,2-bis(2-methoxyphenyl)ethane-1,2-dione **71** complete conversion could not be achieved, and the reaction was continued until there was no more conversion in the reaction medium, 41.1% conversion was the maximum attainable conversion after 94h, HPLC spectrum is given in Fig. 29. In this spectrum the presence of peak at 18.1min. indicated the presence of high amount of unconverted benzil; **71**.

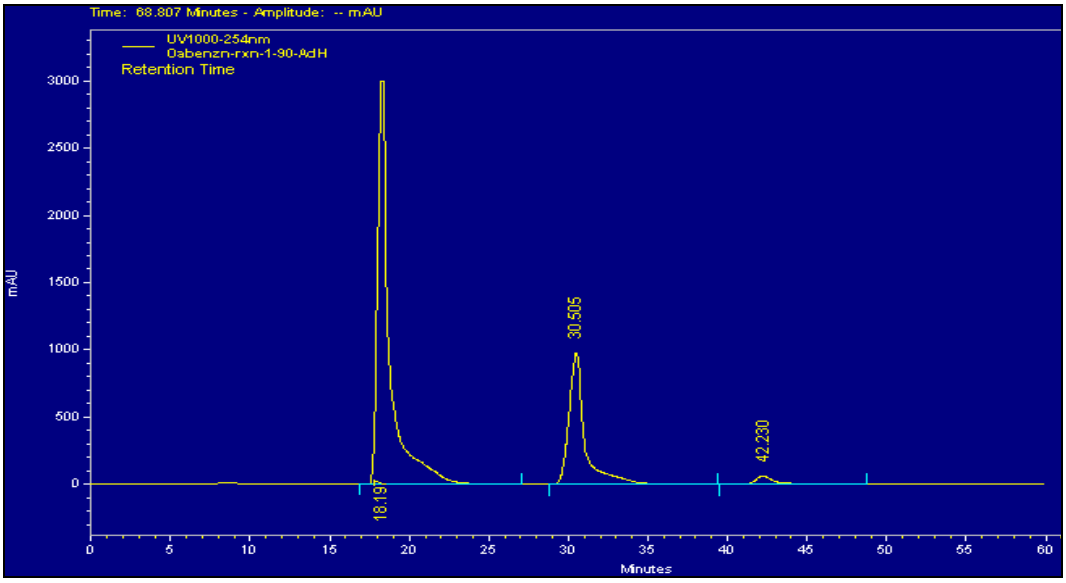


Figure 29 Reduction of 1,2-bis(2-methoxyphenyl)ethane-1,2-dione with *A.flavus*

With the flor- substituted derivatives **76** was favored over **72** with the higher conversion yield and enantioselectivity. In Fig.30 HPLC spectrum of the reduction reaction of **76** is illustrated.

Reductions of symmetrical benzil derivatives substituted with electron donating groups were achieved with higher enantioselectivity and yields compared to the derivatives substituted with electron withdrawing groups.

The additive (IPA) was used as 1mL for 100mL sterile distilled water. The effect of additive amount on the enantioselectivity and the yield of conversion was investigated with a case study where different amount of additives (IPA and ethanol) were introduced in the reduction of 1,2-bis(3-methoxyphenyl)ethane-1,2-dione. The results are tabulated in Table 8.

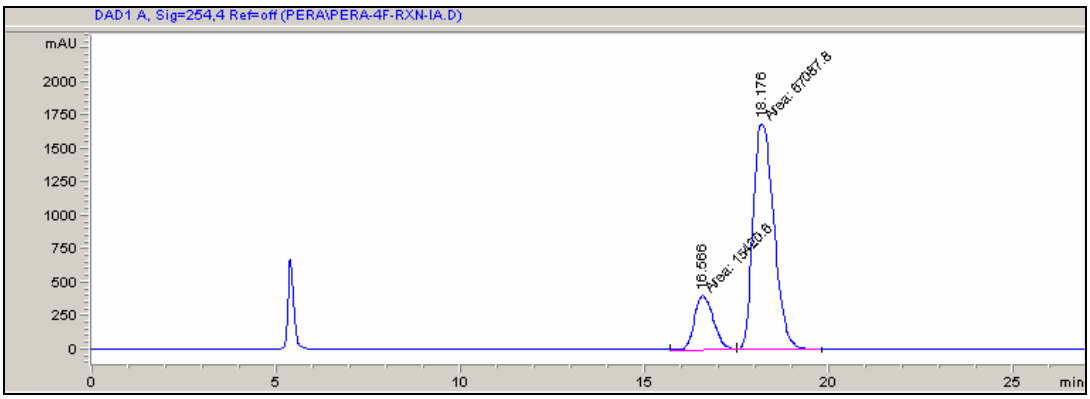
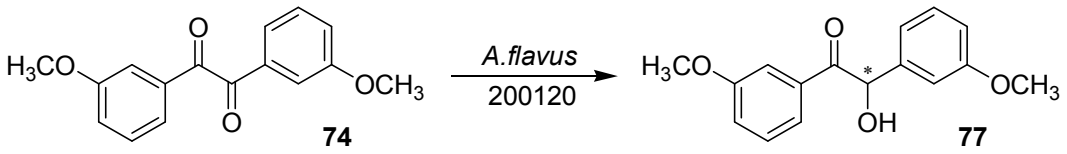


Figure 30 HPLC spectrum of *A.flavus* mediated reduction of **76**

As control experiment wet cells were transferred to sterile distilled water and no additive was added. Best enantioselectivities were obtained with IPA as additive. As the amount of IPA used was increased the ee and conversion yields also increased.

Table 8 The effect of additive amount and additive used on reduction of 1,2-bis(3-methoxyphenyl)ethane-1,2-dione



	Control	IPA 0.5mL	IPA 1mL	IPA 1.5mL	Ethanol 1mL	Ethanol 1.5mL	Ethanol 2mL
Conv %	87.8	86.4	91.6	92.1	89.4	89.2	86.7
Ee %	80.5	83.2	84.4	85	81.1	81.8	82.4

In Fig. 31, the growing cell, resting cell and resting cell with IPA was compared via HPLC spectra. The upper spectrum belonged to the reaction where **75** was directly added to the growth medium (growing cells). The reduction product was obtained with 95.2% conversion and 60.1% ee after 96h. In the middle the spectrum of the reaction catalyzed with resting (wet) cells in sterile distilled water is given. Resting cells without the use of additives were able to convert **75** with a yield of 93.5% and 66.0% ee within 96h. The bottom spectrum in Fig. 23 represents the reduction of **75** with resting cells in the presence of IPA as additive. Highest conversion (97.0%) and ee (69.1%) was achieved with this reaction at 96h. So, Fig. 31 presents a visual evidence for the positive effects of the use of additive (IPA) on enantioselectivity and conversion yields for *A.flavus* mediated reduction of benzils.

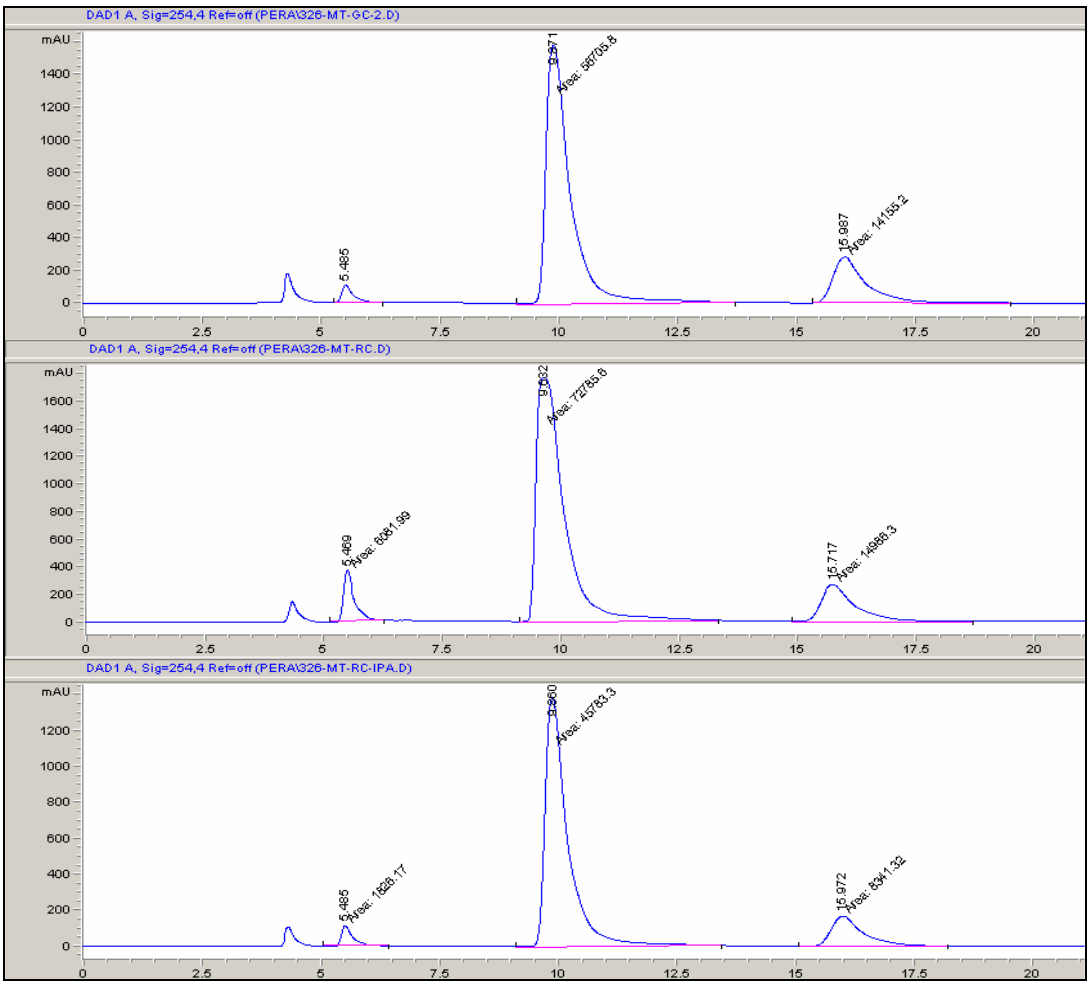


Figure 31 Comparison of the HPLC spectra of growing cell, resting cell and resting cell (+additive) mediated bioreduction of **75**

2.3.2. Hydrolysis of Benzoyl Benzoin Coupled with Deracemization

Unsymmetrical benzoins which are hard to obtain with chemical methodologies can be obtained via the hydrolysis reaction of protected benzoin: i.e, benzoyl benzoin **77**, Fig. 32.

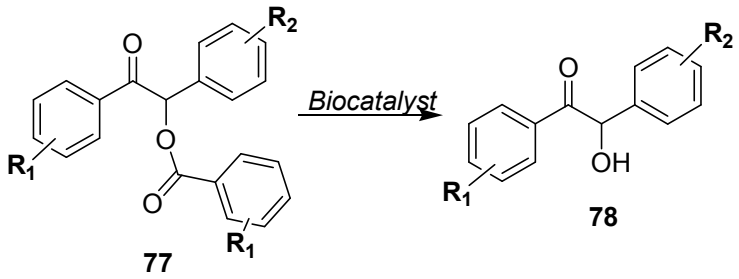


Figure 32 Biocatalytic hydrolysis of benzoyl benzoin

This reaction is especially important as it is easy to obtain unsymmetrical protected benzoins with enzyme-cleavable protecting group (-OBn) via a method developed by Demir *et al.* In this method the cyanide ion-catalyzed cleavage of benzils is used for the generation of various *masked* acyl intermediates. The reaction of these intermediates with various aldehydes furnishes the corresponding esters of unsymmetrical benzoins in very good yields. A variety of unsymmetrical benzoin derivatives are synthesized in this way, including ferrocene derivatives such as 1-phenyl-2-oxo-2-ferrocenylethyl benzoate, 2-(ferrocenyl)-2-oxo-1-(1-bromo-2-naphthyl)-ethyl, 2-naphthoate and 2-(2-methylphenyl)-1-ferrocenyl-2-oxoethyl 2-methylbenzoate [43].

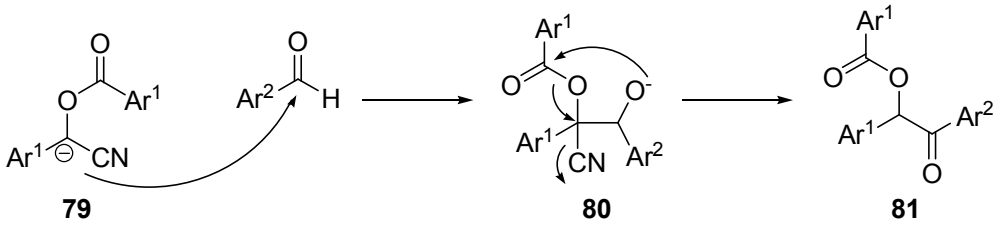


Figure 33 Synthesis methodology of benzoyl benzoin [43]

Several strategies have been employed such as the use of lipases and whole-cell mediated reactions for the targeted hydrolysis reactions which are described in detail in Sections 2.3.2.1 and 2.3.2.2.

2.3.2.1. Hydrolysis with Lipases

If a hydrolysis reaction is aimed, the first choice would be lipases (triacylglycerol hydrolases; EC 3.1.1.3) which are water-soluble enzymes whose natural function is the hydrolysis of triglycerides to partial glycerides and fatty acids during digestion. These enzymes catalyze ester hydrolysis or synthesis, typically of acetates, which can be seen in Fig. 34 [44].

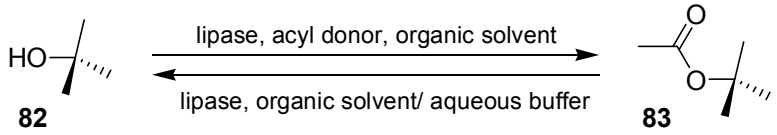
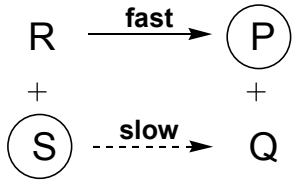


Figure 34 Lipases as catalysts for ester hydrolysis and synthesis

The mechanism of ester-hydrolyzing enzymes is very similar to the chemical hydrolysis by base. A nucleophilic group from the active site of the enzyme attacks the carbonyl group of the substrate ester. This nucleophilic *chemical operator* can be a hydroxy group of a serine (e.g. pig liver esterase, subtilisin and the majority of microbial lipases), a carboxy group of an aspartic acid (e.g. pepsin) or thiol functionality of a cysteine (e.g. papain) [45].

Kinetic resolution can be defined as complete resolution of a racemate by virtue of unequal reaction rates of the enantiomers with a chiral agent, such as a biocatalyst. The attainable maximum theoretical yield for conversion with this approach is 50% (Fig. 35) [46].

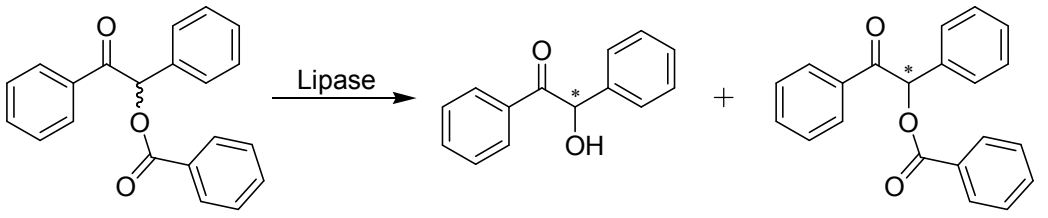


Yield: 50% P + 50% S

R,S: enantiomeric starting material
P,Q: product enantiomers

Figure 35 Kinetic resolution approach

Kinetic resolution of racemate is the most common application for lipases in organic synthesis. To hydrolyze enzyme-cleavable protecting group (-OBn) of benzoyl benzoin to yield benzoin, on the model reaction given in Fig. 32 lipases can be employed with their ester hydrolyzing property. This reaction is expected to proceed in a kinetic resolution manner as given in Fig. 36.



Rac-Benzoyl benzoin | Benzoin - one enantiomer (*R*) or (*S*) | Benzoyl benzoin - one enantiomer

Figure 36 Kinetic resolution of benzoyl benzoin

Several lipases obtained commercially were investigated for this purpose. The lipases screened were as follows:

- Lipase from *Candida cylindracea* (CCL)
- Lipase from *Hug pancreas* (HPL)
- Lipase from *Pseudomonas cepacia* (PCL)

- Lipase from *Aspergillus* (AL)
- Lipase from *Mucor miehei* (MML)
- Lipase from *Pseudomonas fluorescens* (PFL)
- Lipase from *Rhizopus arrhizus* (RAL)
- Lipase from *Rhizopus niveus* (RNL)
- Lipase from *Candida antartica* (CAL)
- Lipase TL
- Lipase SL
- Lipase UL
- Lipase QLM
- Lipase PL
- Lipase AL

The reactions were carried out with the classical one-phase methodology. Firstly, benzoyl benzoin was dissolved in DMSO which was used to solubilize the substrate. Use of DMSO was proper in the perspective that it is miscible with the aqueous phase, as a result one phase occurred. Phosphate buffers at varying pH's (pH 6, pH 7, pH 8) were employed as aqueous phase. Reactions were monitored with TLC and stopped at 50% conversion (when the density of the spot of substrate was same as the density of the product) with the addition of the water immiscible organic phase such as ethyl acetate. In the case of higher conversions (than 50%) the enzyme would have converted the other enantiomer lowering the ee value of the product as well as the ee value of the substrate.

Hydrolysis reactions that took place in the reaction media were revealed to be resulted from the physiological reaction conditions of the medium in prolonged reaction times. This fact was verified with the control experiment where same medium conditions were applied without the addition of the enzyme, hydrolysis reaction was observed. Ee values of the benzoin formed and the benzoyl benzoin remained in the reaction medium were found to be around zero in all reaction media including control. This also proved the self-hydrolysis in the reaction media.

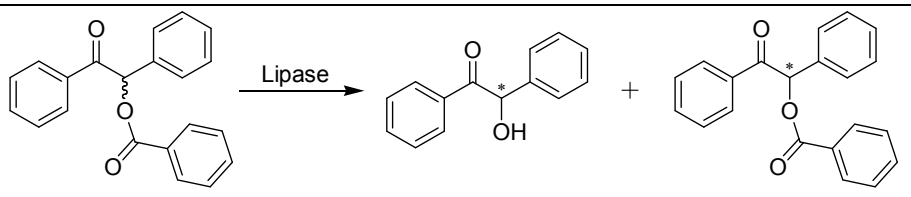
As basic conditions mostly favor ester hydrolysis, more acidic conditions might have prevented the self hydrolysis. To investigate this hypothesis same experiment was performed with more acidic conditions. pH of the phosphate

buffer was set to 5 or 5.5. In addition to the lipases used in previous experiment other lipases were also employed. Total number of enzymes was 23. Enzymes that were newly used in this set of experiment were as follows:

- Lipase from *Aspergillus oryzae* (AOL)
- Lipase form *Candida lipolytica* (CLL)
- Lipase from *Mucor javanicus* (MJL)
- Lipase from *Penicillum roqueforti* (PRL)
- Lipase from *Rhizomucor miehie*, recombinant from *A.oryzae* (RM-rAOL)
- Lipase from wheat germ (WGL)
- Lipoprotein lipase from *Chromobacterium viscosum* (CVLL)
- Lipoprotein lipase from *Pseudomonas sp.* (PLL)

Same reaction conditions were applied with the previous experiment, only pH of the phosphate buffer employed was 5 or 5.5. This time no self-hydrolysis occurred in the reaction media (verified with the control experiment). The pH 5.5 was found to be optimum. At this pH, only two enzymes achieved the desired reaction namely; AL and PLL. The results obtained with these two enzymes are given in Table 9.

Table 9 Enantioselective hydrolysis of benzoyl benzoin via lipases



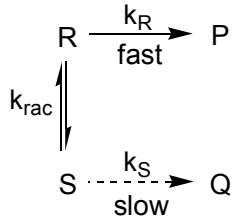
Rac-Benzoyl benzoin | Benzoin - one enantiomer (*R*) or (*S*) | Benzoyl benzoin - one enantiomer

Enzyme	Yield of conversion(%)	Benzoyl benzoin(ee%)	Benzoin (ee%)
AL	40	4.0	6.5
PLL	20	*racemic*	≥99

Although the product was obtained with high optical purity with PLL, the substrate was in racemic form. This was surprising because with kinetic resolution substrate would be expected to have high ee. This revealed the fact that the reaction catalyzed by PLL took place in a dynamic kinetic resolution manner, Fig. 37. Only one enantiomer was turning to the desired product while the substrate racemized. The theoretical maximum yield attainable was 100%.

HPLC analysis result of the PLL catalyzed reaction is given in Fig. 38. At 11.2 and 13.1min. benzoyl benzoin peaks were observed. Areas of these two enantiomers were nearly same so it was clear that the starting material remained in racemic form in the reaction medium, while only one enantiomer of benzoin [(*S)* enantiomer] was formed (R_t = 20min). No peak was detected at 25min. [R_t of (*R*)-benzoin], so ee of the benzoin formed was calculated as ≥99.

However, although large activities of this expensive enzyme (PLL) were employed for increasing the conversion yield to reach higher values, yield could not be increased and the reaction time was also very long for a reaction catalyzed with a pure enzyme. From this point of view, this process was accepted as inefficient with commercial concerns. And new strategies were employed. The next strategy was to employ whole cell systems which are efficient in all kinds of reactions.



R,S: enantiomeric starting material
P,Q: product enantiomers

Figure 37 Dynamic kinetic resolution approach

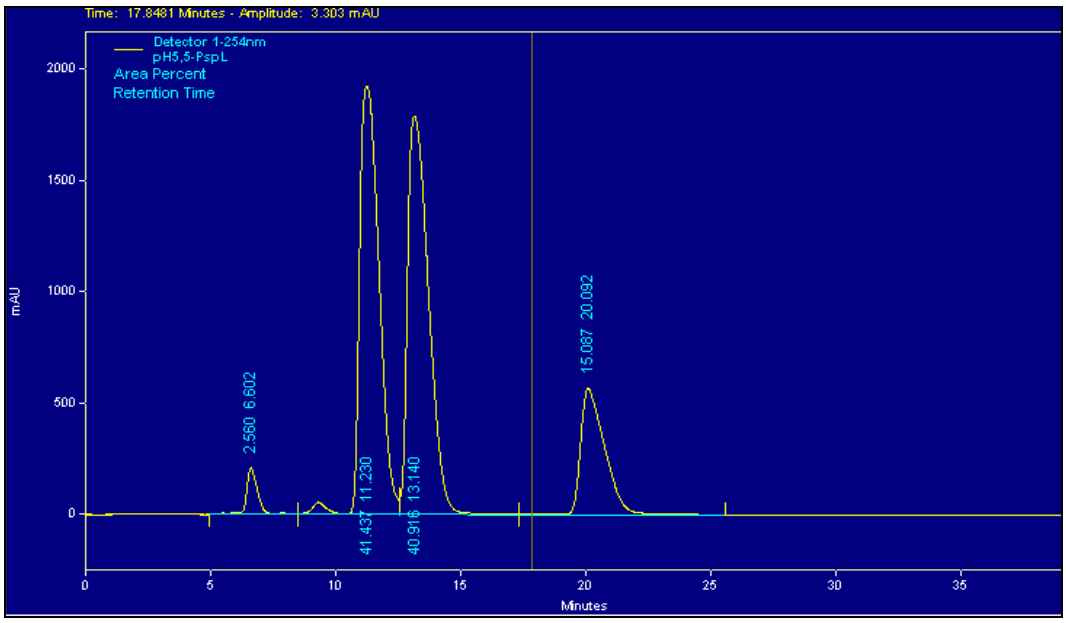


Figure 38 PLL catalyzed hydrolysis of benzoyl benzoin

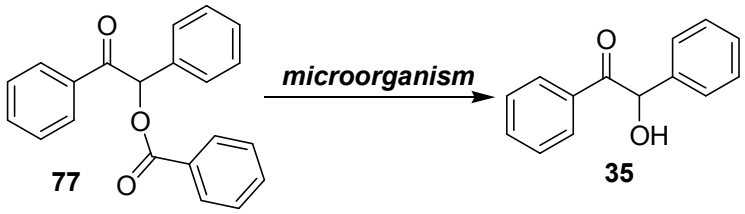
2.3.2.2. *Aspergillus flavus* Mediated Hydrolysis

Complementary to the use of isolated enzymes, whole microbial cells have also been used to catalyze some esterolytic reactions. Both fungi and bacteria were shown to possess this activity.

Although the reaction control becomes more complex on using fermenting microorganisms, the selectivities achieved are sometimes significantly higher when compared to the use of isolated enzymes. *Bacillus* sp. mediated hydrolysis of a secondary alcohol acetate resulting in the resolution of the secondary alcohol can be given as an example. In comparison, other biocatalytic methods to obtain the desired masked chiral hydroxyaldehyde failed [47].

Firstly, some fungi were tested for the hydrolysis reaction. Reaction was expected to occur in a kinetic resolution manner, so reactions were terminated at 50% conversion. The results obtained were as follows, (Table 10).

Table 10 Fungi mediated hydrolysis of benzoyl benzoin



Microorganism	Rxn time(h)	Conversion(%)	Ee(%) (Benzoin)	Ee(%) (Benzoyl benzoin)
A.fumigatus	25	26.82	6	*Racemic*
A.nidulans	9	38.66	*racemic*	*Racemic*
A.oryzae	7	42	4.2	*Racemic*
Rhizopus oryzae (ATCC 9363)	48	trace	*racemic*	*Racemic*
Rhizomucor miehei 72460	48	-	-	*Racemic*
Rhizomucor pusillus 72561	48	trace	*racemic*	*Racemic*
Mucor racuum	48	-	-	*Racemic*
***A.flavus* 200120**	**4**	**52**	**18**	***Racemic***

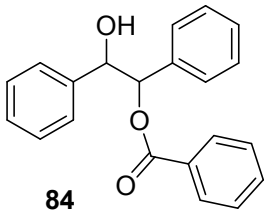
From the results obtained *A.flavus* 200120 was the most promising fungus through all the fungi that have been tested.

As the substrate (benzoyl benzoin) remained in racemic form has led to an opinion that the hydrolysis might have occurred as dynamic kinetic resolution as in the case of PLL mediated hydrolysis stated earlier. In order to prove this idea, reaction was carried for 7 days, and (*S)*-benzoin was obtained with 60% ee and 70% yield.

2.3.2.2.1. Investigation of Occurence of Reduction Side Product

As *A.flavus* was found to be capable of reduction (like bioreduction of benzil to benzoin), an experiment was set to clarify whether a reduction product formed in the medium as a side product or not. If benzoyl benzoin was reduced than 2-

hydroxy-1,2-diphenylethyl benzoate **84** (Fig. 39) would have been expected to be observed in the reaction medium.



84

Figure 39 2-hydroxy-1,2-diphenylethyl benzoate

Reaction was monitored with TLC and although spots were observed other than the hydrolysis product, neither of these belonged to reduction product of benzoyl benzoin which was also verified with characterization methods such as ^{1}H NMR, ^{13}C NMR and GC-MS.

2.3.2.2.2. Hydrolysis of Benzoyl Benzoin Derivatives

With the finding of the ability of *A.flavus* to hydrolyze benzoyl benzoin, some derivatives; both symmetrical and unsymmetrical benzoyl benzoins were tried to be hydrolyzed enantioselectively. The derivatives that have been tested were as follows:

- 1-(naphthalen-2-yl)-2-(naphthalen-6-yl)-2-oxoethyl 1-naphthoate,
- 2-oxo-2-phenyl-1-o-tolylethyl benzoate **85**,
- 1-(2-florophenyl)-2-oxo-2-phenylethyl benzoate.

For all the derivatives, firstly, microorganism was grown for 48 hours in potato dextrose broth at 37°C, and then cells were filtered from the supernatant, washed and transferred to the Erlenmeyers containing pH 5.2 50mM phosphate buffer under aseptic conditions. Then substrate (0.25mmol) dissolved in DMSO (4mL) was inoculated to the wet cells in phosphate buffer.

No hydrolysis product was observed with 1-(naphthalen-2-yl)-2-(naphthalen-6-yl)-2-oxoethyl 1-naphthoate after 12 days, while 2-oxo-2-phenyl-1-o-tolylethyl benzoate and 1-(2-florophenyl)-2-oxo-2-phenylethyl benzoate achieved hydrolysis with 50% and 24%, respectively. Products were characterized with ^{1}H NMR after purification with flash column chromatography.

However, from ^{1}H NMR results it was clear that two isomeric products formed in the hydrolysis of 2-oxo-2-phenyl-1-o-tolylethyl benzoate (**87** & **88**, Fig. 40) and 1-(2-florophenyl)-2-oxo-2-phenylethyl benzoate (**91** & **92**, Fig. 41).

The presence of two isomers was obvious in ^{1}H-NMR spectrum (Fig. 75). Although this fact was not clear in aromatic signals, two doublets at 5.88 ppm and 5.83 ppm result from two different –CH protons at α-position of **91** & **92**. Also two doublets existed at 4.42 ppm and 4.39 ppm for two –OH protons at α-position.

This revealed the fact that hydrolysis occurred not in an enantioselective manner. Firstly, benzoyl benzoin was hydrolyzed to *rac*-benzoin then benzoin formed deracemized via enolate formation (**95**), Fig. 42. With the symmetrical benzoin the deracemization results in one enantiomer, while with the unsymmetrical benzoin as in this case, deracemization resulted in two isomers (**93** & **94**).

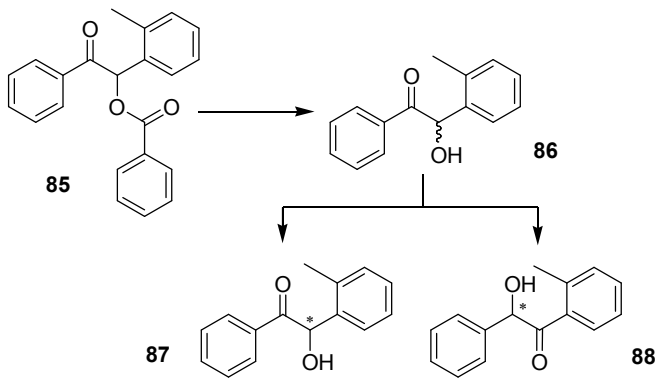


Figure 40 Hydrolysis of 2-oxo-2-phenyl-1-o-tolylethyl benzoate

So, the strategy of using benzoyl benzoin as starting point for obtaining unsymmetrical benzoin derivatives failed. Instead benzoin itself was used for obtaining pure benzoins.

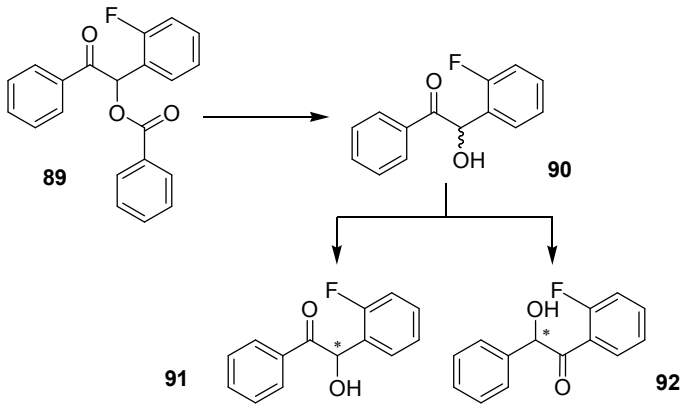


Figure 41 Hydrolysis of 1-(2-florophenyl)-2-oxo-2-phenylethyl benzoate

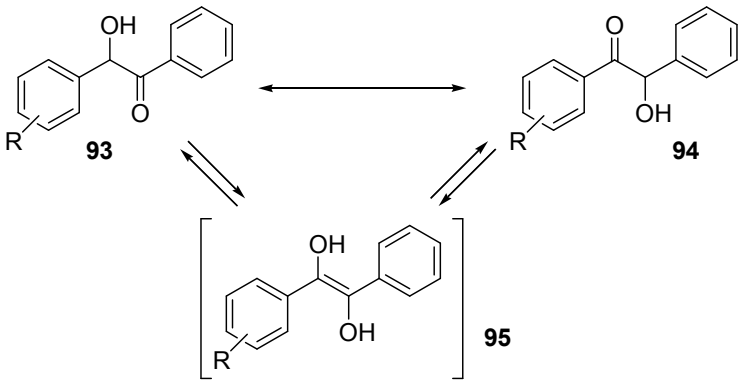


Figure 42 Isomerization of unsymmetrical benzoin

2.3.3. *Aspergillus flavus* Mediated Deracemization of Benzoin Coupled with Reduction

When benzoyl benzoin was introduced to the growth medium of *A.flavus*, after 1 day it was hydrolyzed completely to benzoin, then deracemization occurred, Fig. 43. As the time course of the reaction prolonged, the ee value of the benzoin in the growth medium increased.

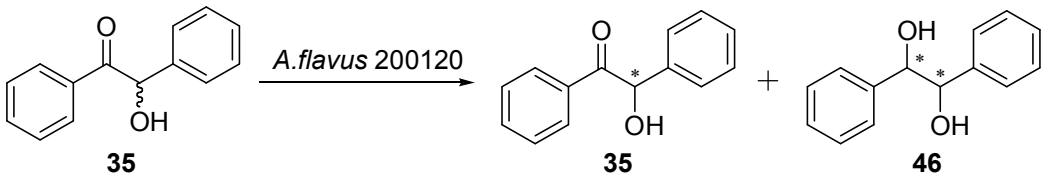


Figure 43 *A.flavus* mediated hydrolysis coupled with deracemization

However, when the reaction time was kept longer, another product; hydrobenzoin was observed by means of TLC.

Several studies were held in order to understand the course of the reaction, effect of medium components and bioprocess parameters.

2.3.3.1. Effect of Potato Extract Concentration on the Deracemization and Reduction of Benzoin

PDB broth was used as the medium of bioconversion. This is composed of potato extract and glucose. Potato extract is a complex ingredient containing 15% starch, 2.2% dietary fiber, 0.1% fat, 2% protein (weight percent each), several vitamins such as thiamine, riboflavin, niacin, Vitamin B_6, Vitamin C, and minerals as calcium, iron, magnesium, phosphorus, potassium and sodium.

These two ingredients affect the metabolism of the microorganism. That's why their effect on enantioselectivity of the deracemization as well as the reduction reaction has been explored.

Different potato extract concentrations were chosen. Hand-made potato extract broth which was prepared by slicing and boiling 200g potatoes per 1L distilled water was found to contain potato extract concentration in the order of 8g/L. So, this value was taken as the central point. PDB media were prepared by keeping glucose concentration constant at 20g/L and changing the potato extract concentrations at an interval of 4g/L to 16g/L.

The effect of potato extract concentration on the ee% of benzoin in the growth medium is given in Fig. 44 and Table 11. Enantioselectivity increased as the potato extract concentration increased. Highest ee value was obtained as 80% at 11th day with 16g/L potato extract concentration. Keeping reaction time longer did not result in higher enantioselectivity as ee value dropped slightly to 78% at 14th day. However with 12g/L potato extract concentration lower ee values not as good as lower potato extract concentration of 8g/L were obtained The lowest ee values were obtained with 4g/L potato extract concentration at every time interval tested. From economical point of view 8g/L potato extract concentration was chosen as optimum for deracemization reaction of benzoin.

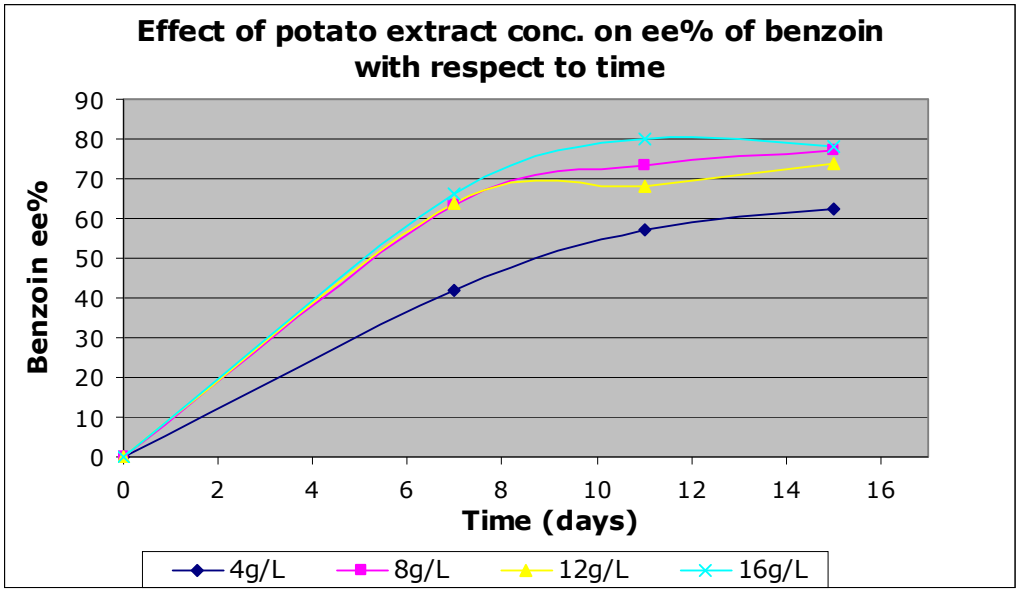
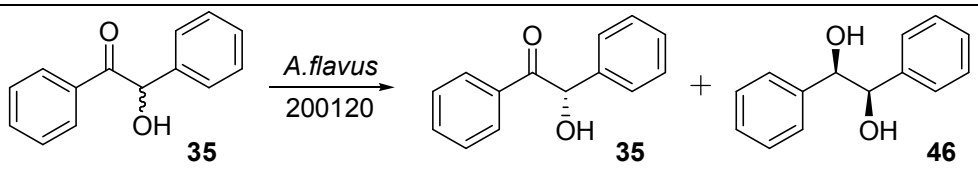


Figure 44 Effect of potato extract concentration on benzoin ee%

The potato extract concentration was also effective on the reduction of benzoin to hydrobenzoin, the yield of conversions with respect to time is given in Table 11. As the time of the reaction prolonged the yield of the hydrobenzoin formed increased for all the potato extract concentrations tested. For low potato extract concentrations it looks longer for the microorganism to convert the benzoin to hydrobenzoin. For example, when 4g/L potato extract was used in the medium hydrobenzoin formation was observed at the 7th day while with the potato extract concentration of 8g/L first hydrobenzoin was observed at 4th day. This time was shortened to 2 days and 1.5 with 12g/L and 16g/L potato extract respectively.

Highest reduction yield was obtained 16g/L potato extract with 80% at 14th day. The yields of hydrobenzoin formed with 8g/L (76% at two weeks) and 12g/L (77% at two weeks) potato extract were similar. Maximum attainable hydrobenzoin yield was 54% after 14 days with 4g/L. Reaction time could not be kept longer than two weeks as complete cell lysis occured.

Table 11 Effect of different potato extract concentrations on the enantioselectivity of benzoin and yield of hydrobenzoin formed



	7th day		11th day		14th day	
Potato extract conc.	**(46) CY %***	**ee_B (%)**	**(46) CY %***	**ee_B (%)**	**(46) CY %***	**ee_B (%)**
4g/L	0	42	33	57	54	62.5
8g/L	28.4	63.1	54	73.3	76	77
12g/L	46	64	63	68.3	77	74
16g/L	55	66	73	80	80	78

***** Conversion yield of compound **46** (hydrobenzoin)

Keeping reaction time shorter provided a possibility to control the reaction at the deracemization state. Longer reaction times could be employed for having the reduction reaction rather than the deracemization. 8g/L was the optimum potato extract concentration for both deracemization and reduction reactions of benzoin. With this potato extract concentration racemic benzoin was deracemized to (*S*)-benzoin (10% yield, 77% ee) and reduced to (*R,R*)-hydrobenzoin (76% yield and 60% ee) with a 45/55 *dl/meso* ratio.

2.3.3.2. Effect of Sugar Concentration on the Deracemization and Reduction of Benzoin

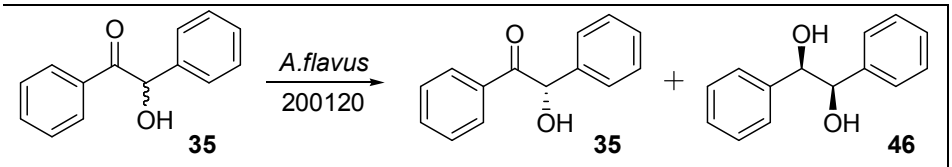
After exploring the effect of potato extract concentration on the course of *A.flavus* mediated deracemization and reduction reactions of benzoin, effect of the other medium component; sugar on the control of the selectivity of the same reactions have been investigated. Concentrations tested were 10g/L, 20g/L, 30g/L, 40g/L.

For this screening, data of the 11^{th} day was not collected as the general trend could have been understood from the 7^{th} day and 14^{th} day. The results are presented in Table 12. The rate of conversion of benzoin to hydrobenzoin was higher for the first week and the conversion reaction slowed down in the second week.

As glucose concentration increased biomass increased. Biomass increase would have been expected to result in high enantioselectivity and high hydrobenzoin formation yield. For low glucose concentration (10g/L) this fact was evident; both the enantioselectivity of the deracemization reaction and the yield of hydrobenzoin formed as a result of the reduction reaction were low. Increasing glucose concentration to 20g/L led to higher enantioselectivities (54% for 7^{th} day & 70% for 14^{th} day) as well as hydrobenzoin yields (60% for 7^{th} day & 75% for 14^{th} day). With 30g/L glucose concentration although ee value of the benzoin deracemized was higher, hydrobenzoin yields were lower compared to 20g/L. The best enantioselectivity and hydrobenzoin yield was obtained with 40g/L glucose concentration. From economical point of view employing 20% glucose concentration was optimum (with 20% benzoin with 70% ee) although slightly better ee results were obtained with 30% glucose (17% benzoin with

76% ee) and 40g/L glucose (11% benzoin with 72.5) at 14th day for deracemization reaction.

Table 12 Effect of different glucose concentrations on the enantiomeric excess of benzoin and yield of hydrobenzoin formed



	7th day		14th day	
Glucose conc.	**(46) CY %**	**ee$_B$ (%)**	**(46) CY %**	**ee$_B$ (%)**
10g/L	25	20	45	32
20g/L	60	54	75	70
30g/L	50	58	62	76
40g/L	67	63	82	72.5

For the reduction of benzoin to hydrobenzoin besides yield of hydrobenzoin (HB) formed, ee value and *dl/meso* ratio of the hydrobenzoin are also important. Higher the ee value and *dl/meso* ratio the more selective is the reaction. Both ee value and *dl/meso* ratio were calculated from HPLC analysis from area percents. The results can be seen in Fig. 45. As glucose concentration increased *dl/meso* ratio decreased. The highest *dl/meso* ratio was obtained as 47/53 for 10g/L glucose while the ratio obtained was 31/69 for 40g/L glucose after 14 days. Ee value increased with the increased glucose concentration, highest value; 79.5% was obtained with 30g/L glucose while a drop in ee occurred at 40g/L glucose (ee was 62.1% at 14th day). Highest ee values for benzoin at 14th day were obtained with 30g/L and 20g/L glucose concentrations.

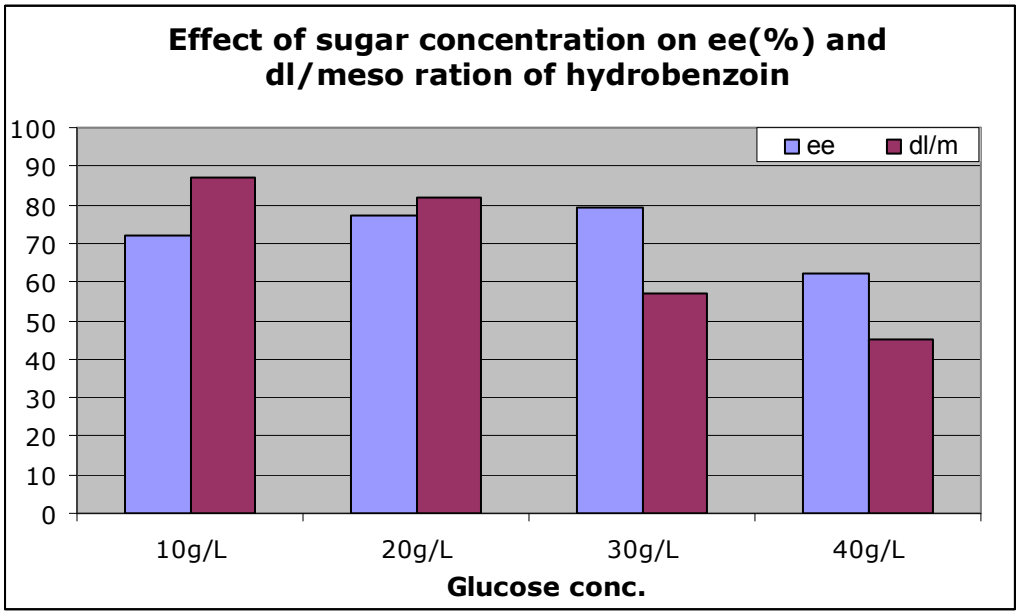


Figure 45 Effect of glucose concentration on hydrobenzoin ee% and *dl/meso* ratio

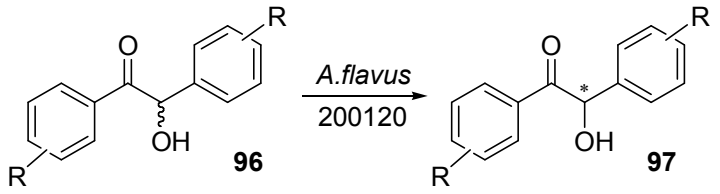
20g/L glucose was chosen as the optimum concentration for *A.flavus* mediated bioreduction of benzoin to hydrobenzoin as this concentration afforded high HB yield, ee_{HB} (%) and *dl/meso* ratio.

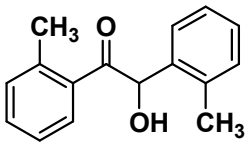
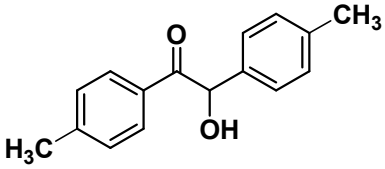
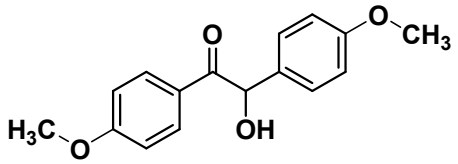
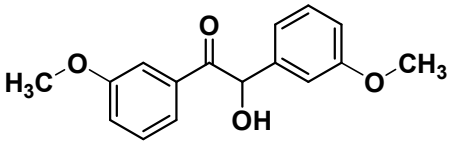
With employing 20g/L glucose in the growth medium after 14 days, (*S)*-benzoin was obtained with 20% yield and 70% ee via deracemization while reduction reaction occurring at the same time yielded (*R,R*)-hydrobenzoin with 77.4% ee and 45/55 *dl/meso* ratio.

2.3.3.3. Deracemization of Different Benzoin Derivatives

Different benzoin derivatives were tested for deracemization with *A.flavus*. For these reactions, microorganism was grown for 2 days in PDB medium with optimized concentrations (8g/L potato extract & 20g/L glucose). To 400mL growth medium 0.4mmol benzoin derivative dissolved in 4mL (1%) DMSO was inoculated. The reaction was carried out until the observation of the hydrobenzoin formation initiation via TLC, results obtained are presented in Table 13.

Table 13 Deracemization of different benzoin derivatives



Entry	Benzoin derivative	Reaction time	ee (%)
A	 2-hydroxy-1,2-diotolylethanone,(98)	12 days	49*
B	 2-hydroxy-1,2-di*p*-tolylethanone, (99)	11 days	19*
C	 2-hydroxy-1,2-bis(4-methoxyphenyl) ethanone, (100)	11 days	21*
D	 2-hydroxy-1,2-bis(3-methoxy phenyl)ethanone, (77)	10 days	47*

* In all cases (*S*)-benzoin was formed

Benzoin derivatives substituted with methoxy and methyl group at *o*-, *m*- and *p*- positions were deracemized. In all the reaction media slight hydrobenzoin (5-10%) formation occurred. Best enantioselectivities were obtained with 2-hydroxy-1,2-di*o*-tolyethanone (*o*-tolu benzoin) with 49% ee, 78% yield (Table 13, entry a) and 2-hydroxy-1,2-bis(3-methoxyphenyl)ethanone (*m*-anisoin)

with 47% ee, 80% yield (Table 13, entry d). Benzoin derivatives substituted at *para*- position yielded lowest enantioselectivities; 19% ee at a yield of 68% for 2-hydroxy-1,2-di*p*-tolyethanone (Table 13, entry b) and 21% ee at a yield of 75% for 2-hydroxy-1,2-bis(4-methoxyphenyl)ethanone (Table 13, entry c).

Enantioselectivities obtained were lower and reaction times were longer compared to the deracemization reaction of benzoin.

2.3.3.4. Effect of the Physiological State of the Cells and the Buffer pH

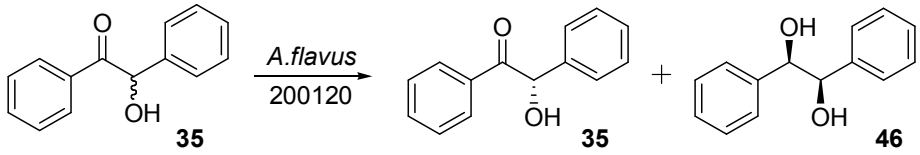
The metabolism of growing cells is totally different from that of resting cells as they are in different growth phases. Metabolic rates also vary greatly. Because of this reason to control the selectivity of deracemization and reduction, an experiment was done to investigate the effect of the physiological state of the cell and buffer pH on the path and selectivity of the reaction.

Reactions were monitored with TLC for two weeks, the wet biomass employed were 1.5 times the biomass employed in previous sections. As control experiments, cells were filtered from the growth medium washed and then transferred to the new growth medium [Control (1)] and wet cells were transferred to the sterile distilled water [Control (2)]. The results that were obtained are given in Table 14.

There was a general trend for all the conditions tested i.e., as time of the reaction prolonged hydrobenzoin conversion increased except for Control (2) where this value dropped from 89% to 88.4% and pH 7 where it was kept constant. In all the pH values tested ee of the hydrobenzoin formed decreased with time while the behaviour of the enantioselectivity of the benzoin did not follow a pattern.

The selectivity of the reactions catalyzed with wet cells in different pH buffers and growing cells in fresh medium were consistent. Benzoin in the media were in (*S*) form while (*R,R*)-hydrobenzoin was obtained as the result of reduction. The mechanism of the *A.flavus* mediated hydrolysis coupled with deracemization is represented in Fig. 46.

Table 14 Effect of physiological state of the cells at different pH values on the deracemization and reduction



	4th day				7th day			
	ee_B (%)	ee_{HB} (%)	*dl/meso*	**(46)** CY %	ee_B (%)	ee_{HB} (%)	*dl/meso*	**(46)** CY %
pH5.1	49.4 (*S*)	99.0 (*R,R*)	0.56	49.0	45.8 (*S*)	80 (*R,R*)	0.58	69.0
pH6	47.2 (*S*)	99.0 (*R,R*)	0.48	47.0	63.0 (*S*)	80 (*R,R*)	0.43	70.6
pH7	54.0 (*S*)	90.0 (*R,R*)	0.98	92.0	43.0 (*S*)	87.5 (*R,R*)	0.93	92.0
pH8	50.8 (*S*)	89.6 (*R,R*)	0.74	80.3	62.0 (*S*)	85.3 (*R,R*)	0.73	84.5
Control (1)	58.0 (*S*)	83.2 (*R,R*)	0.85	65.2	56.5 (*S*)	75.1 (*R,R*)	0.93	69.0
Control (2)	68.7 (*S*)	88.6 (*R,R*)	0.82	89.0	53.6 (*S*)	77.0 (*R,R*)	0.97	88.4

The reaction media were analyzed at other time intervals such as the 2nd day of conversion, *dl/meso* ratio was higher for all media although hydrobenzoin formation was much lower at this time.

Reactions were ended after 14 days, however ee of hydrobenzoin dropped as well as dl/meso ratio while hydrobenzoin formation yield did not increased significantly (data is not shown in here). So, the reactions were carried out for 7 days. In Fig. 47 the spectrum for the HPLC analysis of the reaction medium of Control (1) experiment at the 14th day is given. The HPLC analysis was performed with AD-H column, peaks belonged to (*S,S*)-hydrobenzoin, (*R,R*)-hydrobenzoin, *meso*-hydrobenzoin, (*R*)-benzoin and (*S)*-benzoin respectively.

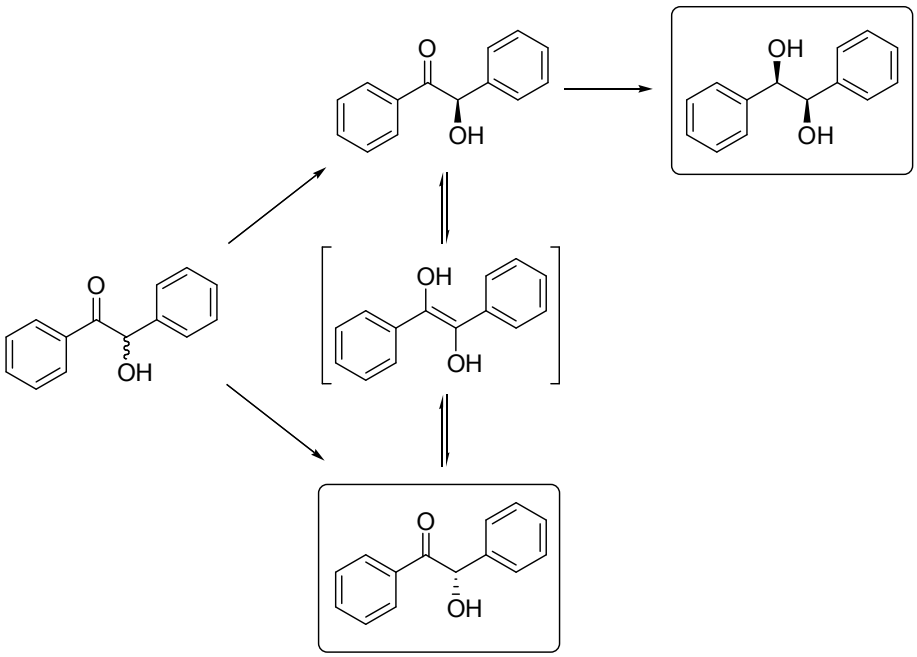


Figure 46 Mechanism of *A.flavus* mediated hydrolysis coupled with deracemization

For obtaining hydrobenzoin as a result of reduction of benzoin employing wet cells in pH 7 phosphate buffer appeared to be the best condition as hydro-benzoin was obtained with 90.0% ee, 92.0% yield and relatively high *dl/meso* (49/50) ratio of 0.98 at the 4^{th} day.

So, it was possible to control the path of reaction by applying the proper biotransformation conditions; for reduction of hydrobenzoin. Employing wet cells in pH 7 50mM phosphate buffer resulted in reduction reaction with the resultant product having high ee, *dl/meso* ratio and yield.

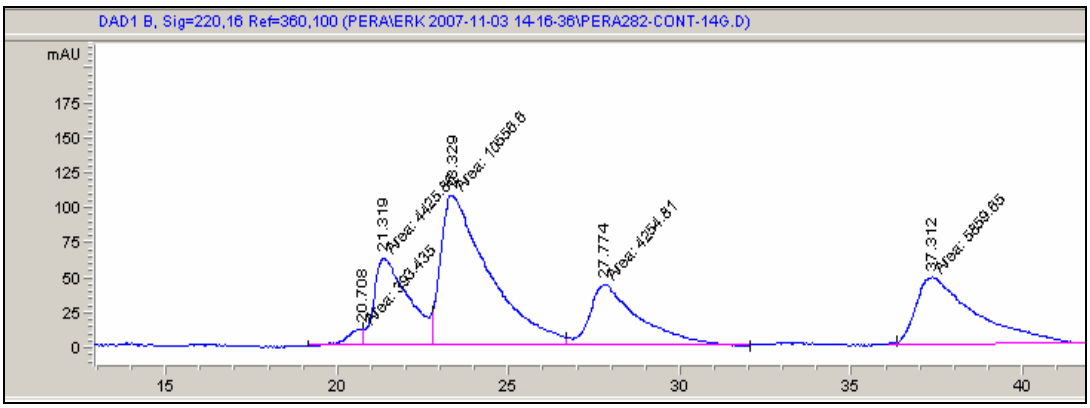


Figure 47 HPLC spectrum of the control medium at 14th day

2.4. *Aspergillus oryzae* 5048 Mediated Reduction of Benzil Derivatives

Several approaches are present for asymmetric biotransformation procedures. One can select a native organism performing the desired biotransformation with a high ee or an organism with a moderate ee can be improved via either site-directed mutagenesis approach or optimization of bioprocess parameters.

A more conventional strategy, i.e., enantioselectivity enhancement via optimization of physiological conditions of culture medium, presents a reliable alternative. A screening among several organisms is performed, and then microorganism with the highest enantioselectivity is selected.

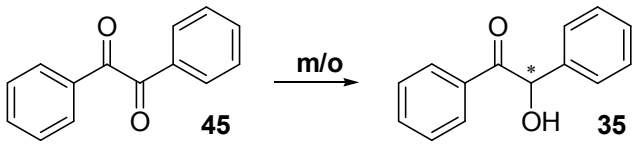
As *A.flavus* 200120 was successful in bioreduction of benzil to benzoin, an idea that *Aspergillus sp.* could be good biocatalysts has arised. The proper strain then was selected and after optimization processes selected strain was used for the reduction of benzil derivatives to the corresponding benzoins.

2.4.1. Reduction of Benzil with Different *Aspergillus* Species

Seventeen different *Aspergillus* species [13 from Osaka University Department of Biotechnology (OUT) and 4 from TUBITAK Marmara Research Center (MRC culture collection)] were screened as shown in Table 15. PDB medium was used as the medium of bioconversion for this screening, as it was suitable for all the strains used.

From the *Aspergillus* species screened, most of them showed (*S)* selectivity, only two of them showed reverse selectivity *A.kawachii* 5308 and *A.terreus* 5112. The reverse of selectivity for two species may be explained with the presence of different reductase enzymes and isoenzymes active within different strains.

Table 15 Screening of different *Aspergillus* species for bioreduction of benzil to benzoin



Microorganism	Growth period(day)	Reaction Time(h)	Yield(%)	Ee(%)	Selectivity
A.oryzae 5300	2	2	81.8	54	(*S*)
A.inuii 5182	2	7.5	74.75	22	(*S*)
A.luchuensis 5183	2	7.5	84	24.8	(*S*)
A.kawachii 5308	2	26	94.5	29	(*R*)
A.niger (MAM)	2	28	79.16	6.2	(*S*)
A.foeditus 5180	2	-	91.5	racemic	-
A.oryzae 5137	3	7	95.31	56.4	(*S*)
A.oryzae 5302	3	5	95.4	68.3	(*S*)
A.oryzae (MAM)	3	5	95.4	67	(*S*)
***A.oryzae* 5048**	**3**	**3.5**	**96.8**	**74**	**(*S*)**
A.tamarii 5071	5	2	83.4	62	(*S*)
A.terreus 5073	5	40	96.6	60.6	(*S*)
A.terreus 5075	3	3.5	98	25	(*S*)
A.terreus 5112	5	24	56	34.6	(*R*)
A.nidulans (MAM)	3	4	91	72	(*S*)
A.fumigatus (MAM)	3	4.5	95	68	(*S*)

Throughout the *Aspergillus sp.* that were screened, the best satisfactory result was obtained with *A.oryzae* 5048. This strain was able to reduce benzil in 3.5 hours with an ee of 74% and conversion yield of 96.8%. Although the rates of reactions were high with *A.tamarii* 5071 and *A.oryzae* 5300, their conversion yields and ee values were lower. Therefore, *A.oryzae* 5048 was chosen as the whole-cell biocatalyst.

2.4.2. Optimization of Fermentation Conditions

Several parameters such as growth medium, temperature, pH, and physiological state of the cell (resting, living (growing) or lyophilized) are known to be effective on the enantioselectivity of a whole cell bioreduction reaction. After the preliminary screening enantioselectivity enhancement studies took place and several optimization reactions were held.

The effects of the parameters investigated were as follows:

- Growth medium formulations
- Initial pH
- Physiological state of the cells and the buffer pH

2.4.2.1. Effect of Different Formulations

Several media which were suitable for the cultivation of *A.oryzae* that were applied for the screening. Results are tabulated in Table 16.

Throughout the growth media tested Medium C yielded the lowest enantioselectivity (38%) while other media (PDB, MEB, CpDM, GPB) resulted in an enantioselectivity range of 60-75%. Lowest conversion yield was obtained with CpDM. Reaction times were short (in the range of 2.0-3.5h) except medium CpDM where reaction was terminated after 7h.

After this screening Medium D (Med D) was chosen as the medium of bioconversion with the highest yield (91.1%) and ee (80.4%).

Table 16 Effect of media on the bioreduction of benzil to benzoin

Growth media	Growth time(day)	Reaction time(h)	Yield(%)	Ee(%)
PDB	2	3.5	81.0	59.6
MEB	2	3.5	91.4	65.6
CpDM (modified czapex dox broth)	3	7.0	75.8	69.5
GPB	3	4.0	85.6	75.8
Medium C [27]	3	2.0	91.6	38.0
Medium D [27]	**3**	**2.5**	**91.1**	**80.4**

2.4.2.2. Effect of Initial pH of Medium D

Once the growth medium was selected, effect of initial pH was investigated by adjusting initial pH values from 4 to 8. The results are shown in Table 17. Highest enantioselectivity was obtained at neutral pH at 37^{o}C with growing cells. pH profile was not changing over the biotransformation period.

Initial pH was shown to be effective on enantioselectivity. Although adjustment of initial effect did not yield significant differences in the amount of biomass obtained after 3 days growth, it did have an effect on selectivity. The enantioselectivity increased as the initial pH was increased (from pH 4 to pH 7). It suddenly dropped at slightly alkaline initial pH (pH 8). Both pH 6 (83.5%) and pH 7 (86.9%) had higher ee values compared to control (82%), i.e., unadjusted pH. Although conversion yield was lower for pH 7 adjusted medium compared to control, since ee was higher initial pH was chosen as pH 7.

2.4.2.3. Effect of the Physiological State of the Cells and the Buffer pH

Physiological state of the organism was also thought to be another effective parameter on enantioselectivity. In order to test this statement, microorganism was grown for 2.5days in medium D, then wet cells which were filtered off from the supernatant were transferred to Erlenmeyers containing the sterile phosphate buffers with varying pH's (pH 5, 6, 7, 8). As a control experiment *A.oryzae* that was grown again for 2.5 days was kept in the growth medium and substrate was added directly to the growth medium. Results are summarized in Table 18.

Table 17 Effect of initial pH of Medium D on the bioreduction reaction

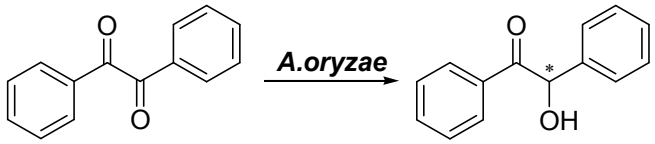
Initial pH	Growth time(day)	Reaction time(h)	Yield(%)	Ee(%)
pH 4	3	6	85.1	67.4
pH 5	3	7	82.0	76.0
pH 6	3	3	85.4	83.5
pH 7	**3**	**1.5**	**83.4**	**86.9**
pH 8	3	4	85	75.0
Uncontrolled pH	3	3	93.0	82.0

pH profiles of the buffers with varying pH's were not changing over the course of biotransformation. At slightly alkaline condition (pH8), although enantioselectivity was not high as acidic conditions (pH 5, pH 6), a satisfactory ee value was obtained minimizing the possibility of partial racemization of (*S*)-benzoin at that pH. This situation can be explained with the short reaction time.

Slightly alkaline condition yielded the longest reaction with 8h. Both employing wet cells at neutral pH (pH 7) and employing growing cells resulted in shorter reaction times, however ee values obtained with these reactions were not as high as pH5.

Wet cells in pH 5 phosphate buffer achieved the desired biotransformation with highest enantioselectivity (94%) and yield (95%). So, this condition was chosen as the optimum condition for this bioconversion. The HPLC spectrum of the reaction achieved by employing wet cells in pH5 phosphate buffer is shown in Fig. 48.

Table 18 Effect of physiological state of the cells and buffer pH on the bioreduction reaction



Physiological state of the cell – pH of the buffer	Reaction time(h)	Yield(%)	Ee(%)
Wet cell- pH 5 phosphate buffer	**3**	**95.0**	**94.4**
Wet cell- pH 6 phosphate buffer	3	90.9	88.5
Wet cell- pH 7 phosphate buffer	1.5	93.1	90.1
Wet cell- pH 8 phosphate buffer	8	97.2	64.2
Medium D (pH 7.2) - CONTROL	2	93.7	85.8

As a result of the optimization studies performed which were discussed earlier; a microorganism achieving the desired bioconversion in moderate selectivity was improved to achieve the same conversion with high stereoselectivity.

2.4.3. Enantioselective Bioreduction of Benzils to the Benzoins

After designing the optimum biotransformation process for *A.oryzae* 5048, symmetrical benzil derivatives were reduced to optically active benzoins with this microorganism on this optimized system. The results are summarized in Table 19.

A.oryzae was able to reduce a good range of benzil derivatives substituted with electron-donating as well as electron-withdrawing properties (Table 19). Absolute configuration of the stereocenters formed were assigned to be (*S*) by comparing optical rotation (alpha) values with the literature. In the case of *ortho*- derivatives are challenging substrates because of steric hindrance. These substrates substituted at *ortho*- position were reduced with high efficiency.

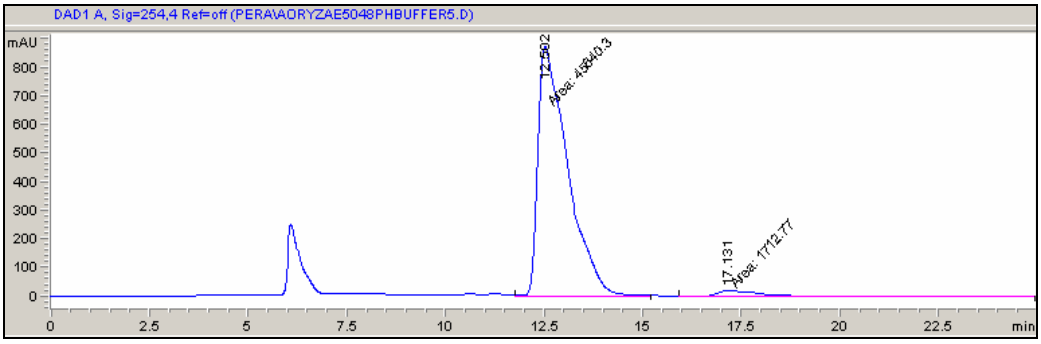


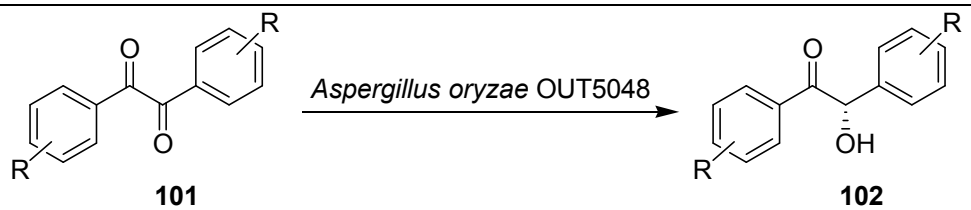
Figure 48 HPLC spectrum of the reaction catalyzed with *A.oryzae* 5048 wet cells in pH 5 phosphate buffer after 3h

Aspergillus oryzae achieved the bioconversion of all substrates at yields 81-99% (except 15% for 2-hydroxy-1,2-bis(4-methoxyphenyl)ethanone, (entry 4)) while ee's were in between 23-94%. This microorganism showed high enantioselectivity with methoxy-substituted benzil derivatives (47-91% ee) while it showed low to moderate enantioselectivity (23-65% ee) with flor-substituted benzil derivatives. Best result among flor-substituted compounds was obtained with 1,2-bis(4-florophenyl)ethane-1,2-dione), (entry 7). *Ortho*-position was favored in the case of methoxy-substituted benzil derivatives [1,2-

bis(2-methoxyphenyl)ethane-1,2-dione], (entry 2). HPLC spectrum for this compound is given in Fig. 49. Benzoins substituted at *ortho* position are problematic and several chemical methods lack syntheses of those. So, whole-cell bioreductions presented herein propose another advantage in this aspect.

In the reduction of benzil, benzoin formed was not further reduced to hydrobenzoin by *A.oryzae*. Reduction to hydrobenzoin was avoided by short reaction time in *Aspergillus oryzae* mediated bioconversion, with prolonged reaction times hydrobenzoin was formed in the reaction medium. In the benzil derivatives with long reduction times trace amount of hydrobenzoin was formed: 1,2-bis(4-methoxyphenyl)ethane-1,2-dione.

Table 19 Microbial bioreduction of benzil derivatives to (*S*)-2-hydroxy-1,2-diphenyl ethanones



101 **102**

Entry	(*S*)-Benzoin (R)	Reaction time(h)	Ee(%)	Conversion(%)
1	H	3	94	95
2	*o*-MeO	72	91	94
3	*m*-MeO	3	84	87
4	*p*-MeO	72	47	15
5	*o*-F	4	23	95
6	*m*-F	2	36	99
7	*p*-F	8	65	81

Enantioselectivity for the benzil derivatives was tried to be improved with the use of additives, as in the case of *A.flavus*. As *Aspergillus oryzae* mediated bioreduction is a fast reaction, it is thought that hydrogen shortage might have occurred in the reaction medium. Therefore, two most popular alcohol derivatives; ethanol and isopropyl alcohol were tried out as additives for benzoin derivatives with low ee (entry 5, 6, 7), however positive effect of these chemicals on enantioselectivity was not observed. Hydrobenzoin formation was not common, so additives acting as enzyme inhibitors were not examined.

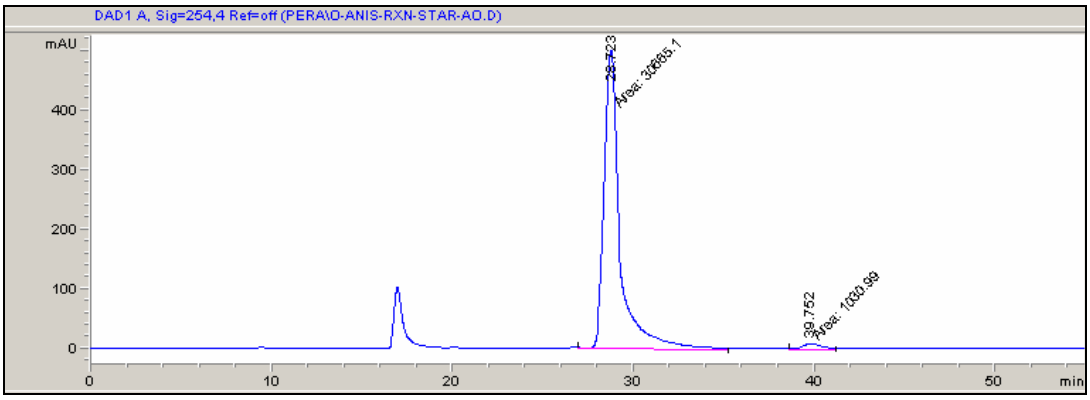


Figure 49 HPLC spectrum of *A.oryzae* mediated reduction of 1,2-bis(2-methoxyphenyl)ethane-1,2-dione

CHAPTER 3

EXPERIMENTAL

3.1. Materials and Equipments

^{1}H-NMR and ^{13}C-NMR spectra were recorded with Brucker-Spectrospin DPX-400, Ultra Shield, High Performance Digital FT-NMR spectrometer using tetramethylsilane (TMS) as internal standard and deuterochloroform ($CDCl_3$) as solvent.

Mass spectra were recorded with Thermo Quest GC-MS in which Phenomenex Zebron capillary GC column (60m length, 0.25mm ID, 0.25μm film thickness) was utilized.

Protein purification was performed with AktaPrime FPLC system.

Flash column chromatography was performed by using Merck Silica Gel 60 (particle size: 40-63μm, 230-400 mesh ASTM).

Solvents used in the extractions were removed by using rotary evaporator.

The chemicals used in the study were purchased from Sigma, Aldrich, Fluka, and Acros Organics.

Microorganisms used were either generous gifts of Osaka University Department of Biotechnology (OUT) or purchased from 4 TUBITAK Marmara Research Center (MRC culture collection).

Lipases were either obtained commercially in lipase basic kit and lipase extension kits from *BioChemika* (Sigma) or generous gifts of Amano PS.

The E. coli BL21 (DE3) PLysS strain purchased from Invitrogen® was used as host to produce the recombinant BAL_{HIS} was. Purification of the hexa-histidine tagged enzyme was performed with Ni^{2+}-NTA affinity column (Invitrogen®).

Optical rotations were measured with a Sucromat polarimeter. Enantiomeric excesses of all the products were determined by Agilent 1100 series HPLC device using appropriate optically active columns. Enzyme production was performed in New Brunswick BioFlo110 Fermentor equipped with pH and temperature probes as well as stirring rate controls.

3.2. General Procedures

3.2.1. BAL Mediated Reactions

3.2.1.1. Production and Purification of BAL

E. coli BL21(DE3)pLysS carrying pUC19-BAL_{HIS} construct was used for BAL (EC. 4.1.2.38) production. Cultures were maintained on LB agar slants that contained (kg/m): yeast extract, 5.0; tryptone, 10.0; NaCl, 10.0; ampicillin, 0.1; agar. Cells from the newly prepared slants were inoculated into the preculture LB medium where it was grown for 8 hours at 37°C, then transferred to the production medium which was also LB medium with an inoculation ratio of 1/10 (i.e.; when 165mL was inoculated a total volume of 1650mL reached). Production was performed in 2L bioreactor. Two hours after the inoculation of the microorganism the production of the enzyme was induced with the addition of isopropyl-β-D-thiogalactopyranosid (IPTG). After the induction microorganism was grown for another 6 hours, then cells were harvested by centrifugation. Cell pellets were taken into a Petri dish where they were left for lyophilization.

For the free enzyme preparation, a crude extract was directly applied to an Ni^{2+}-NTA affinity column and lyophilized for 48 hours.

One unit (U) of BAL activity is defined as the amount of enzyme that catalyses the cleavage of 1µmol benzoin (1.5mmol/L) into benzaldehyde in potassium phosphate buffer (20mmol/L, pH=7) containing $MgSO_4$ (2.5mmol/L), ThDP (0.15mmol/L) and DMSO (20%, v/v) at 30°C.

BAL activity was determined with reverse phase HPLC analysis with Nucleodur C_{18} column at a flow rate of 1mL/min at 254 nm. For this, samples that were taken from the reaction medium were mixed with DMSO (to stop the reaction then diluted with the mobile phase (45% acetonitrile, 0.5% acetic acid, and 54.5% water).

3.2.1.2. General Procedure for the Synthesis of 2-hydroxy-1- arylethan-1-ones from Aromatic Aldehydes

Representative Example: 2-hydroxy-1-(4-hydroxyphenyl)-ethan-1-one [48]

4-Hydroxybenzaldehyde (122mg, 1mmol) was dissolved in a mixture of DMSO (10mL) and potassium phosphate buffer (40mL, 50mM, pH 7, containing $MgSO_4$ (2.5mM) and ThDP (0.15mM)). To this mixture formaldehyde solution (8 mmol, 0.64mL 37% solution) was added. After the addition of BAL (40 U), the reaction was allowed to stand at 37°C. Every 24 h, 30–40 U of BAL and 8mmol of formaldehyde solution were added. After 4 days (checked by TLC), the reaction mixture was filtered and extracted with dichloromethane (3x50mL). After drying the collected organic phase over $MgSO_4$, removal of the solvent under reduced pressure gave the crude product, which was then purified by flash column chromatography (EtOAc:hexane 1:3) to give 135mg (89%) of the desired compound 2-hydroxy-1-(4-hydroxyphenyl)ethan-1-one. Mp: 165–167 °C [49].

^{1}H NMR ($CDCl_3/CCl_4$): δ ppm 3.50 (s, 1H, OH), 4.68 (s, 2H), 4.94 (s, 1H, OH), 6.82 (d, *J*=8.1 Hz, 2H), 7.80 (d, *J*= 8.1 Hz, 2H), Fig. 56.

Spectral data for the other derivatives were as follows;

2-Hydroxy-1-phenylethanone [50]: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 3.37 (s, 1H), 4.76 (s, 2H), 7.41 (t, *J*=7.7, 2H), 7.53 (t, *J*=7.4, 1H), 7.83 (d, *J*=7.6, 2H), Fig. 50.

2-Hydroxy-1*p*-tolylethanone [51]: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 2.35 (s, 3H), 3.49 (br s, 1H, OH), 4.77 (s, 2H), 7.22 (d, *J*=8.1 Hz, 2H), 7.74 (d,

J=8.1 Hz, 2H), Fig. 52; ^{13}C-NMR (100 MHz, $CDCI_3/CCl_4$): δ ppm 22.7, 65.3, 127.8, 129.7, 130.9, 145.2, 198.0, Fig. 53.

2-Hydroxy-1-(4-methoxyphenyl)ethanone [51]: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 3.50 (s, 1H), 3.81 (s, 3H), 4.74 (s, 2H), 6.89 (d, *J*=2.4 Hz, 2H), 7.82 (d, *J*=2.2 Hz, 2H), Fig. 51.

2-Hydroxy-1-(3-methoxyphenyl)ethanone [51]: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 3.36 (s, 1H, OH), 3.79 (s, 3H), 4.74 (s, 2H, CH_2), 7.07 (m, 1H), 7.29 (m, 1H), 7.36 (m, 2H), Fig. 54; ^{13}C-NMR (100 MHz, $CDCI_3/CCl_4$): δ ppm 54.8, 65.0, 111.5, 119.5, 120.1, 129.2, 132.8, 159.6, 197.4, Fig. 55.

2-Hydroxy-1-(2-methoxyphenyl)ethanone [51]: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 3.82 (s, 1H, OH), 3.94 (s, 3H), 4.77 (d, *J*=4.8 Hz, 2H), 7.01 (d, *J*=8.8 Hz, 1H), 7.06 (dd, *J*=7.2, 7.2 Hz, 2H), 7.55 (ddd, *J*=7.6 Hz, 2.0 Hz, 2.0 Hz, 1H), 8.05 (dd, *J*=7.6, 1.6 Hz, 1H).

1-(4-Chlorophenyl)-2-hydroxyethanone [52]: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 3.2 (br s, 1H, OH), 4.8 (s, 2H, CH_2), 7.88 (d, *J*=8.8, 2H), 7.51 (d, *J*=8.6 Hz, 2H)

3.2.1.3. Acyloin Reactions of Functionalized Acetaldehyde

3.2.1.3.1. Self-Condensation of Benzyloxyacetaldehyde

1mmol benzyloxyacetaldehyde was dissolved in 50mM potassium phosphate buffer (40mL, pH 7 containing 2.5mM $MgSO_4$ and 0.15mM TPP) containing either 20% DMSO (10mL) or 20% toluene (10mL) as cosolvent. The reaction was started by addition of (40 U) BAL and carried at 37 °C for 24 hours before a further 20 U of BAL was added and monitored via TLC. Reaction was worked-up after 72 hours by extraction with 50mL ethyl acetate (EtOAc) three times. The combined organic layers were washed with brine and dried over $MgSO_4$. Solvent was evaporated with rotary evaporator under vacuum. The product (1,4-bis(benzyloxy)-3-hydroxybutan-2-one) was purified with column chromatography, obtained with 75% yield, 73% ee when toluene was employed as co-solvent. The product was characterized via with ^{1}H NMR and ^{13}C NMR spectroscopy;

^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 3.35 (s, 1H, OH), 3.72 (ddd, *J*=3.7, 10.1, 76.1 Hz, 2H), 4.17 (q, *J*=17.2 Hz, 2H), 4.58-4.30 (m, 5H), 7.37-7.09 (m, 10H), Fig. 57; ^{13}C-NMR (100 MHz, $CDCI_3/CCl_4$): δ ppm 70.86, 73.21, 73.53, 73.63, 75.07, 127.80, 127.93, 128.12, 128.45, 128.54, 132.75, 134.12, 204.90, Fig. 58.

HPLC analysis was done with Chiralpak OA column, UV detection at 254 nm, eluent: *n*-hexane/2-propanol = 80:20, flow 0.8mL/min, 20 °C; R_t's were 14min. and 15 min. respectively, HPLC spectrum of the racemic reference is presented in Fig. 59.

3.2.1.3.2. Cross-Condensation of Benzyloxyacetaldehyde with Acetaldehyde

1mmol benzyloxyacetaldeyhde and 5mmol acetaldehyde were dissolved in 50mM potassium phosphate buffer (40mL, pH 7.0 containing 2.5mM $MgSO_4$ and 0.15mM TPP) containing 20% DMSO (10mL) as cosolvent and BAL (40 U) was added to start the reaction at 37°C. Additional BAL (20 U) was introduced on the daily basis to the reaction medium till the 5th day. Reaction was stopped after 120 hours. Reaction was monitored with TLC. Reaction medium was extracted two times with 100mL EtOAc. The two products could not be separated with column chromatography. These were characterized via GC-MS analysis.

Mass (R_t: 19.09 min.): m/z (relative abundances) 194(M^+)(2); 164(3); 149(5); 133(5); 121(10); 107(63); 91(100); 78(30); 64(45); 50(24); 42(63);
Mass (R_t: 19.32 min.): m/z (relative abundances) 194(M^+)(2); 164(2); 150(3); 133(2); 122(4); 107(63); 91(100); 78(20); 64(38); 50(19); 44(57), Fig. 61.

3.2.1.3.3. Self-Condensation of Dimethoxyacetaldehyde

Firstly, 1mmol dimethoxyacetaldehyde was dissolved in 50mM potassium phosphate buffer (40mL, pH 7 containing 2.5mM $MgSO_4$ and 0.15mM TPP) with 20% DMSO (10mL) as cosolvent. The reaction was started by addition of BAL (40 U) and reaction was monitored via TLC. Additional BAL (20 U) was introduced on the daily basis until the 5th day where reaction was stopped. The

product was characterized via NMR spectroscopy and GC-MS analysis. The specific rotation value for the product is measured as $[\alpha]_D^{20}$: +30 (c 0.5, $CHCl_3$).

^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 3.15 (d, 1H), 3.31 (s, 3H), 3.34 (s, 3H), 3.37 (s, 3H), 3.40 (s, 3H), 4.38 (d, 1H), 4.50 (s, 1H), 4.82 (s, 1H), Fig. 62.

Mass (R_t: 15.80 min.): m/z (relative abundances) 207(M^+)(1) ; 145(4); 117(6); 89(2); 75(100); 59(8); 47(61), Fig. 63.

3.2.2. *Aspergillus flavus* Mediated Whole Cell Multi Reactions

3.2.2.1. Bioreduction of Benzil to Benzoin via Whole Cells

3.2.2.1.1. Selection of the Medium for Bioreduction

For selection of the medium, 100mL of different medium formulations employed for the cultivation of the *A.flavus* were prepared in 250mL erlens as follows:

- Potato dextrose broth (PDB) was prepared by dissolving 8g potato extract and 20g glucose per 1L distilled water.
- Glucose peptone broth (GPB) was formed by preparation of a solution containing 3% glucose and 1% peptone.
- Medium C was prepared according to the literature [41]; [C] = 20g/L glucose, 20g/L yeast extract, 5g/L peptone, 1g/L KH_2PO_4, 2g/L K_2HPO_4, 2g/L $NaNO_3$, 0.5g/L KCl, 0.5g/L $MgSO_4 \cdot 7H_2O$, 0.02g/L $FeSO_4 \cdot 7H_2O$.
- Malt extract broth (MEB)(Oxoid commercially available).
- MGYP was prepared by preparing a solution containing 0.3% malt extract, 1% glucose, 0.3% yeast extract, 0.5% peptone broth.

All the mediums were sterilized at 121°C for 15 min. prior to use.

A. flavus 200120 was grown in potato dextrose agar (PDA) petri dishes. The streaked plates were incubated at 37°C for 3–4 days for spore production and then stored at 4°C until utilized. The surface of the petri plate containing spores was rubbed with a sterile inoculation loop and then transferred to a 250mL sterile Erlenmeyer containing a 100mL different growth medium, in which the organism was grown in a rotary shaker at 37°C for 48h. Reaction was started

with the addition of benzil (21.0mg, 0.1mmol) dissolved in 1mL DMSO. Reaction was monitored with TLC and concluded when benzil was no longer detected in the growth medium. After the reaction was completed, microorganism was filtered off the supernatant, washed with distilled water and the combined aqueous phases were extracted three times with 50mL ethyl acetate (3x50mL). The organic extract was dried over $MgSO_4$, filtered and concentrated and benzoin was purified by flash column chromatography (eluent: 1:3 ethyl acetate/hexane) and it was isolated as a colorless solid.

Enantiomeric excess was determined by the use of HPLC equipped with Chiralpak OD column, UV detection at 254 nm, eluent: n-hexane/2-propanol = 90:10, flow 1.0mL/min, 20°C; R_t (*S*)=13.0 min; R_t (*R*) =19.0 min. HPLC spectra of both *rac*-benzoin and (*R*)-benzoin is given in Fig. 64 and Fig. 65, respectively.

3.2.2.1.2. Investigating the Effect of Substrate Concentration

Same procedure was applied as the Section 3.2.2.1.1, however this time substrate solution was prepared with different concentrations were introduced into the growth medium; PDB. Substrates with varying moles (0.05mmol, 0.1mmol, 0.2mmol, 0.25mmol & 0.5mmol) were dissolved in 1mL and introduced to the growth medium. HPLC analyses were performed as described in Section 3.2.2.1.1.

3.2.2.1.3. Investigating the Effect of the Physiological State of the Cells and the Buffer pH on Enantioselectivity

A. flavus 200120 was grown in potato dextrose agar (PDA) petri dishes. The streaked plates were incubated at 37°C for 3–4 days for spore production and then stored at 4°C until utilized. The surface of the petri plate containing spores was rubbed with a sterile inoculation loop and then transferred to a 250mL sterile Erlenmeyer containing a 100mL growth medium, in which the organism was grown in a rotary shaker at 37°C for 48h. After 2 days resting cells filtered from the growth medium were transferred to 250mL sterile Erlenmeyer either containing 100mL 50mM potassium phosphate buffers with varying pH's (pH 5, pH 6, pH 7, pH 8), or 100mL sterile distilled water then benzil (21.0mg, 0.1 mmol) dissolved in 1mL DMSO was added and the reaction was started. pH

adjustments were done with 1N NaOH and 1N HCl. Conversion was monitored via TLC. After the reaction was completed, microorganism was filtered off the supernatant, washed with distilled water and the combined aqueous phases were extracted three times with 50mL ethyl acetate (3×50mL). The organic extract was dried over $MgSO_4$, filtered and concentrated and benzoin was purified by flash column chromatography. Ee value was determined as described in Section 3.2.2.1.1.

3.2.2.1.4. Investigating the Effect of Additives on Enantioselectivity of the Wet Cell Catalyzed Reduction Reaction of Benzil

A. flavus 200120 was grown in potato dextrose agar (PDA) petri dishes. The streaked plates were incubated at 37°C for 3–4 days for spore production and then stored at 4°C until utilized. The surface of the petri plate containing spores was rubbed with a sterile inoculation loop and then transferred to a 250mL sterile Erlenmeyer containing a 100mL growth medium, in which the organism was grown in a rotary shaker at 37°C for 48h. After 2 days resting cells filtered from the growth medium were transferred to 250mL sterile Erlenmeyer either containing 100mL sterile distilled water then additives were added. Dimethyl sulphate (DMS) was added in a ratio of 1:4 (DMS: substrate). Isopropyl methyl ketone (IPMK) and isobutyl methyl ketone (IBMK) were added as 500μL for 100mL sterile distilled water containing wet cells. 1000μL methanol, ethanol and isopropanol were added for 100mL. For control no additive was introduced to the sterile distilled water with wet cells. After the cells were incubated with additives for 30 mins, benzil (21.0mg, 0.1mmol) dissolved in 1mL DMSO was added and the reaction was started. Conversion was monitored by TLC. After the reaction was completed, microorganism was filtered off the supernatant, washed with distilled water and the combined aqueous phases were extracted three times with 50mL ethyl acetate (3×50mL). The organic extract was dried over $MgSO_4$, filtered and concentrated and benzoin was purified by flash column chromatography. Ee value was determined as described in Section 3.2.2.1.1.

3.2.2.1.5. Bioreduction of Different Benzil Derivatives with *A.flavus*

3.2.2.1.5.1. Synthesis of Symmetrical Benzoin (2-hydroxy-1,2-diphenyl-ethanone) Derivatives

Two methods have been employed for the syntheses of symmetrical benzoins.

Method A: 23.6mmol aromatic aldehyde was dissolved in a mixture of ethanol (15mL) and water (15mL). Reaction was started with the addition of potassium cyanide (5.2mmol). The reaction mixture was refluxed. Conversion was monitored with TLC. After reaction was completed, the reaction mixture was cooled and kept at 4^{o}C to crystallize. After crystallization occurred, the upper liquid fraction was removed and the water remained in the solid part was removed through extraction with ethyl acetate (200mL) three times (100mL water was added to dissolve impurities). Combined organic layers were dried over $MgSO_4$, filtered and solvent was evaporated with rotary evaporator under vacuum. The products were purified from the unconverted aromatic aldehyde via column chromatography with the appropriate EtOAc:Hexane system. Products were characterized with ^{1}H NMR and ^{13}C NMR. The data obtained were in good accordance with the literature values.

Method B: 10mmol aromatic aldehyde was dissolved in 3mL DMF (dimethyl formamide) then potassium cyanide (1.5mmol) was added to this solution. The reaction mixture was heated to 60^{o}C-70^{o}C. Conversion was monitored with TLC. After reaction was completed, the reaction mixture was cooled. To the reaction medium 150mL water was added. Resultant reaction mixture was extracted three times with 75mL ethyl acetate (EtOAc). Combined organic phases were dried over $MgSO_4$, filtered and solvent was evaporated with rotary evaporator under vacuum. The products were purified from the unconverted aromatic aldehyde via column chromatography with the appropriate EtOAc:Hexane system. Products were characterized with ^{1}H NMR and ^{13}C NMR. The data obtained were in good accordance with the literature values.

Spectral data for the derivatives synthesized to be used as standards were as follows;

Rac-2-Hydroxy-1,2-diphenylethan-1-one (Benzoin, **35**): ^{1}H-NMR (400 MHz, $CDCl_3$/ CCl_4): δ ppm 4.58 (d, *J*=6.1 Hz, 1H), 5.97 (d, *J*=6.1 Hz, 1H), 7.29-7.52 (m, 8H), 7.92 (d, *J*=7.9 Hz, 2H) [20b].

Rac-2-Hydroxy-1,2-bis(2-methoxyphenyl)ethan-1-one: ^{1}H-NMR (400 MHz, $CDCl_3$/ CCl_4): δ ppm 3.69 (s, 3H), 3.71 (s, 3H), 4.29 (d, *J*=5.1 Hz, 1H), 5.92 (d, *J*=5.1 Hz, 1H), 6.63-7.69 (m, 8H) [20b].

Rac-1,2-Bis(2-florophenyl)-2-hydroxyethan-1-one: ^{1}H-NMR (400 MHz, $CDCl_3$/CCl_4): δ ppm 4.33 (d, *J*=5.6 Hz, 1H), 5.91 (d, *J*=5.6 Hz, 1H), 6.98-7.30 (m, 6H), 7.55- 7.67 (m, 1H), 7.80-7.91 (m, 1H) [20b].

Rac-1,2-Bis(2-chlorophenyl)-2-hydroxyethan-1-one: ^{1}H-NMR (400 MHz, $CDCl_3$/ CCl_4): δ ppm 4.35 (d, *J*=5.8 Hz, 1H), 6.32 (d, *J*=5.8 Hz, 1H), 7.19-7.47 (m, 8H) [20b].

Rac-2-Hydroxy-1,2-bis(3-methoxyphenyl)ethan-1-one: ^{1}H-NMR (400 MHz, $CDCl_3$/ CCl_4): δ ppm 3.77 (s, 3H), 3.81(s, 3H), 4.54 (d, *J*=6.1 Hz, 1H), 5.90 (d, *J*=6.1 Hz, 1H), 6.82 (d, *J*=8.4 Hz, 1H), 6.86 (t, *J*=2.4 Hz, 1H), 6.94 (d, *J*=6.9 Hz, 1H), 7.07 (d, *J*=8.4 Hz, 1H), 7.22-7.34 (m, 2H), 7.46-7.51 (m, 2H) [20b].

Rac-2-hydroxy-1,2-di*m*-tolylethanone: ^{1}H-NMR (400 MHz, $CDCl_3$/ CCl_4): δ ppm 2.24 (s, 3H), 2.29 (s, 3H), 4.41 (d, *J*=6.1, 1H), 5.80 (*d*, J=5.8, 1H), 6.99 (d, *J*=7.5, 1H), 7.04 (d, *J*=6.4, 2H), 7.15-7.10 (m, 1H), 7.22-7.17 (m, 2H), 7.25 (d, *J*=7.7, 1H), 7.61 (d, *J*=7.7, 1H), 7.68 (s, 1H), Fig. 68.

Rac-1,2-Bis(4-florophenyl)-2-hydroxyethan-1-one: ^{1}H-NMR (400 MHz, $CDCl_3$/CCl_4): 4.12 (d, *J*=5.4 Hz, 1H); 5.86 (d, *J*=5.4 Hz, 1H), 7.09 (m, 2H), 7.12 (m, 2H), 7.25 (m, 2H), 7.84 (m, 2H) [20b].

3.2.2.1.5.2. Synthesis of Symmetrical Benzil Derivatives

20mg copper(II) acetate, 1g (0.0125mmol) ammonium nitrate, 10mmol benzoin derivative and 7mL of an 80% (v/v) aqueous acetic acid solution were put in a 50mL flask fitted with a reflux condenser. The reaction medium was heated with occasional shaking. Nitrogen evolved in the system. Reaction was

monitored with TLC. When reaction was completed, it was cooled and the solution was seeded with a crystal of benzil derivative and allowed to stand for 1 hour. The mixture was filtered at the pump and the solid was dried. If the product was not pure, further purification with column chromatography was performed. The products obtained were characterized with ^{1}H NMR and ^{13}C NMR. The data were in good accordance with the literature values.

Spectral data for the derivatives synthesized were as follows;

Benzil **(45)** [53]: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 7.40 (t, *J*=7.5 Hz, 4H), 7.54 (t, *J*=7.5, 2H), 7.87 (d, *J*=16.8 Hz, 4H).

1,2-Bis(2-methoxyphenyl)ethane-1,2-dione **(71)**: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 6.87 (d, *J*=4.2, 2H), 7.04 (t, *J*=7.6, 2H), 7.45–7.50 (m, 2H), 7.98–8.01 (m, 2H) [53].

1,2-Bis(2-florophenyl)ethane-1,2-dione **(72)**: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 7.06 (t, *J*=9.1 Hz, 2H), 7.27 (t, *J*=7.6 Hz, 2H), 7.53–7.60 (m, 2H), 7.96–8.01 (m, 2H) [53].

1,2-Bis(2-chlorophenyl)ethane-1,2-dione **(73)**: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 7.31-7.35 (m, 2H), 7.46–7.48 (m, 2H), 7.71–7.78 (m, 2H) [53].

1,2-Bis(3-methoxyphenyl)ethane-1,2-dione **(74)**: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 7.09–7.11 (m, 3H), 7.28–7.48 (m, 7H) [53].

1,2-Di*m*-tolylethane-1,2-dione **(75)**: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 2.36 (s, 3H), 7.32 (t, *J*=7.5, 1H), 7.39 (d, *J*=7.5, 1H), 7.69 (d, *J*=8.7, 2H), Fig. 56; ^{13}C-NMR (100 MHz, $CDCI_3/CCl_4$): δ ppm 21.21, 127.52, 129.45, 135.74, 136.14, 142.47, 192.48, Fig. 67 [53].

1,2-Bis(4-florophenyl)ethane-1,2-dione **(76)**: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 7.12 (t, *J*=8.6, 4H), 7.40–7.46 (m, 2H), 7.93–7.97 (m, 4H) [53].

3.2.2.1.5.3. Bioreduction of the Synthesized Benzils

Same procedure was applied as Section 3.2.2.1.4. Wet cells were transferred to Erlenmeyer containing 100mL sterile distilled water and 1mL IPA as additive. Corresponding benzil derivatives were also added at the same order (0.1mmol was dissolved in 1mL DMSO). Work-ups were performed as in Section 3.2.2.1.4. HPLC conditions for the derivatives were as follows:

1,2-Bis(2-methoxyphenyl)ethane-1,2-dione **(71)**: Chiralpak AD-H column, UV detection at 254nm, eluent: *n*-hexane/2-propanol = 90:10, flow 1.0mL/min, 20°C; R_t (*S*) = 30.5 min; R_t (*R*) = 42.2 min for benzoin derivative and R_t = 19 min for benzil derivative, HPLC spectra of 1,2-bis(2-methoxyphenyl)ethane-1,2-dione and *rac*-2-hydroxy-1,2-bis(2-methoxyphenyl)ethanone are presented in Fig. 69 & Fig. 70, respectively.

1,2-Bis(2-florophenyl)ethane-1,2-dione **(72)**: Chiralpak IA column, UV detection at 254nm, eluent: *n*-hexane/2-propanol = 90:10, flow 1.0mL/min, 20°C; R_t (*S*) = 12.0 min; R_t (*R*) = 14.0 min for benzoin derivative and R_t = 6.7 min for benzil derivative.

1,2-Bis(2-chlorophenyl)ethane-1,2-dione **(73)**: Chiralpak IA column, UV detection at 254nm, eluent: *n*-hexane/2-propanol = 90:10, flow 1.0mL/min, 20°C; R_t (*S*) = 12.4 min; R_t (*R*) = 13.8 min for benzoin derivative and R_t = 6.7 min for benzil derivative.

1,2-Bis(3-methoxyphenyl)ethane-1,2-dione **(74)**: Chiralpak AD-H column, UV detection at 254nm, eluent: *n*-hexane/2- propanol = 90:10, flow 1.0mL/min, 20°C; R_t (*R*) = 31.0 min; R_t (*S*) = 40.6 min for benzoin derivative and R_t = 8.9 min for benzil derivative.

1,2-Bis(4-florophenyl)ethane-1,2-dione **(76)**: Chiralpak IA column, UV detection at 254nm, eluent: *n*-hexane/2-propanol = 90:10, flow 1.0mL/min, 20°C; R_t (*S*) = 17.6 min; R_t (*R*) = 16.0 min for benzoin derivative and R_t = 6.4 min for benzil derivative. HPLC spectra of the references for 1,2-bis(4-florophenyl)ethane-1,2-dione and 1,2-bis(4-florophenyl)-2-hydroxyethanone are given Fig. 71 and Fig. 72, respectively.

3.2.2.2. Hydrolysis of Benzoyl Benzoin Coupled with Deracemization

3.2.2.2.1. Synthesis of Protected Benzoins (Benzoyl Benzoins)

To a solution of 5mmol diketone and 5mmol of aromatic aldehyde benzaldehyde in 3mL DMF was added 0.2 equiv. of KCN. The reaction was monitored by TLC. After completion of reaction, the mixture was extracted with excess amount of water (150mL) and ethyl acetate (75mL) three times to get rid of the DMF. Combined organic phases were dried over $MgSO_4$, filtered and solvent was evaporated with rotary evaporator followed by high vacuum furnished the desired product. Product was purified with column chromatography with 1:7 EtOAc:hexane. The product was characterized with ^{1}H NMR and ^{13}C NMR.

The spectral data for the synthesized derivatives were as follows:

2-Oxo-1,2-diphenylethyl benzoate (benzoyl benzoin) **(77)**: ^{1}H-NMR (400 MHz, $CDCl_3$/ CCl_4): δ ppm 7.00 (s, 1H), 7.41-7.25 (m, 7H), 7.53-7.42 (m, 4H), 7.96-7.88 (m, 2H), 8.05 (dd, *J*=1.3, 8.3, 2H), Fig. 73; ^{13}C-NMR (100 MHz, $CDCI_3$/CCl_4): δ ppm 188.18, 163.90, 139.63, 137.91, 133.71, 133.60, 130.85, 130.41, 129.63, 129.48, 129.23, 129.05, 129.00, 128.73, 78.21, Fig. 74 [43].

1-(Naphthalen-2-yl)-2-(naphthalen-6-yl)-2-oxoethyl 1-naphthoate: ^{1}H-NMR (400 MHz, $CDCl_3$/ CCl_4): δ ppm 7.3–7.43 (6H, m), 7.61–7.79 (9H, m), 7.94–7.96 (1H, m), 7.56–8.05 (2H, m), 8.50 (1H, s), 8.60 (1H, s); ^{13}C-NMR (100 MHz, $CDCI_3$/CCl_4): 78.5, 124.8, 125.9, 126.1, 126.8, 126.9, 127.0, 127.2, 128.11, 128.17, 128.5, 128.6, 128.7, 128.9, 129.2, 129.5, 129.9, 130.1, 131.17, 131.91, 132.1, 132.6, 132.8, 132.9, 133.8, 134.0, 136.0, 136.1,166.3, 193.6, [43].

2-Oxo-2-phenyl-1-o-tolylethyl benzoate **(85)**: ^{1}H-NMR (400 MHz, $CDCl_3$/ CCl_4): δ ppm 2.57 (3H, s, Me), 7.19–7.23 (1H, m), 7.27–7.29 (2H, m), 7.38–7.47 (5H, m), 7.52–7.59 (2H), 7.90 (2H, d, *J*=7.4 Hz), 8.13 (2H, d, *J*=7.3 Hz); ^{13}C-NMR (100 MHz, $CDCI_3$/CCl_4): δ ppm 19.9, 75.8, 127.1, 128.7, 129.0, 129.8, 129.9, 130.4, 131.6, 132.8, 133.5, 133.6, 135.5, 137.5, 166.1, 194.1, [43].

1-(2-Florophenyl)-2-oxo-2-phenylethyl benzoate **(89)**: ^{1}H-NMR (400 MHz, $CDCl_3$/ CCl_4): δ ppm 7.03–7.11 (2H, m), 7.25–7.31 (1H, m), 7.34–7.38 (5H,

m), 7.44–7.54 (3H, m), 7.93 (2H, m), 8.03 (2H,m); ^{13}C-NMR (100 MHz, $CDCI_3/CCl_4$): δ ppm 70.7, 116.4 (d, *J*=22 Hz), 121.8 (d, *J*=13 Hz), 125.2 (d, *J*=2.7 Hz), 128.7, 128.9, 129.12, 129.7, 130.4, 130.5 (d, *J*=1.9 Hz), 131.7 (d, *J*=8.2 Hz), 133.6, 134.0, 134.7, 160.5 (d, *J*=250 Hz), 165.9, 192.8, [43].

3.2.2.2.2. Lipase Mediated Hydrolysis of Benzoyl Benzoin

5mg benzoyl benzoin was dissolved in 300mL DMSO in a 1.5mL eppendorf tube and then 700mL phosphate buffer with different pHs were added. Reactions were started with the addition of lipase (5 U) and monitored with TLC. Reactions were stopped at 50% conversion for all lipases except for PLL where dynamic kinetic resolution occurred. Reaction mediums were extracted with 1mL chloroform. The organic phase was analyzed with HPLC using Chiralpak OD column, UV detection at 254nm, eluent: n-hexane/2-propanol = 90:10, flow 1.0mL/min, 20°C; R_t (*S*) = 13.0 min; R_t (*R*) = 19.0 min for benzoin and R_t's were 9.0 min, 11.0 min for benzoyl benzoin.

3.2.2.2.3. *Aspergillus flavus* Mediated Hydrolysis of Benzoyl Benzoin

Microorganism was grown in 400mL PDB for 2 days then 0.4mmol (126mg) dissolved in 4mL DMSO was added to the growth medium. Reaction was monitored via TLC. After the reaction was complete, the fungal biomass was filtered off and the mixture was extracted three times with 200mL of EtOAc. The combined organic layers were washed with brine and dried over $MgSO_4$. The crude product was purified by flash column chromatography. Reaction medium was analyzed with HPLC as described in Section 3.2.2.2.2.

3.2.2.2.4. *Aspergillus flavus* Mediated Hydrolysis of Benzoyl Benzoin Derivatives

Same procedure was applied as in Section 3.2.2.2.3. However this time products were not analyzed with HPLC, products were characterized with ^{1}H NMR.

Spectral data for the products [1-(2-florophenyl)-2-hydroxy-2-phenylethanone and 2-(2-florophenyl)-2-hydroxy-1-phenylethanone] obtained as a result of

hydrolysis of 1-(2-florophenyl)-2-oxo-2-phenylethyl benzoate by *A.flavus* was as follows;

^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 4.39 (d, *J*=6.1, 1H), 4.42 (d, *J*=6.0, 1H), 5.83 (d, *J*=5.9, 1H), 5.88 (d, *J*=5.8, 1H), 6.99 (t, *J*=8.5, 2H), 7.05 (t, *J*=8.5, 2H), 7.35-7.26 (m, 6H), 7.39 (t, *J*=7.7, 2H), 7.52 (t, *J*=7.4, 2H), 7.87 (d, *J*=7.7, 2H), 7.93 (dd, *J*=5.5,8.6, 2H).

3.2.2.3. *Aspergillus flavus* Mediated Deracemization of Benzoin Coupled with Reduction

3.2.2.3.1. Investigating the Effect of Potato Extract Concentration on the Deracemization and Reduction of Benzoin

Same procedure was applied as the Section 3.2.2.2.3. This time PDB mediums were prepared by dissolving potato extract with different concentrations (4g/L, 8g/L, 12g/L, 16g/L) and 20g/L glucose in distilled water. Then substrate solution (0.4mmol was dissolved in 4mL DMSO) was added. Work-up procedure was same also.

Reaction medium was analyzed with HPLC Chiralpak AD-H column, UV detection at 254nm, eluent: *n*-hexane/2-propanol = 90:10, flow 0.7mL/min, 20 °C; R_t (*R*-benzoin)=29.0min;R_t(*S*-benzoin)=39.0min; R_t (*meso*-hydro-benzoin)=25.5min; R_t (*S,S*-hydrobenzoin)=22.0 min; R_t (*R,R*-hydro-benzoin)=23.2 min.

Purified hydrobenzoins were analyzed with HPLC Chiralpak OJ column, UV detection at 254nm, eluent: *n*-hexane/2-propanol = 90:10, flow 1.0mL/min, 20°C; R_t (*meso*-hydrobenzoin)=23.3 min; R_t (*S,S*-hydrobenzoin)=17.6 min; R_t (*R,R*-hydrobenzoin)=19.8 min.

3.2.2.3.2. Investigating the Effect of Sugar Concentration on the Deracemization and Reduction of Benzoin

Same procedure was applied as the Section 3.2.2.2.3 except growth mediums were prepared with varying sugar concentrations (10g/L, 20g/L, 30g/L, 40g/L). HPLC analyses were done as described in Section 3.2.2.3.1.

3.2.2.3.3. Deracemization of Different Benzoin Derivatives with *A.flavus*

Microorganism was grown for 2 days in PDB medium (8g/L potato extract & 20g/L glucose). To 400mL growth medium 0.4mmol benzoin derivative dissolved in 4mL (1%) DMSO was inoculated. The reaction was carried out until the observation of the hydrobenzoin formation; work-up was done as in Section 3.2.2.2.3.

For the syntheses of new benzoin derivatives that were used for deracemization were synthesized as described in Section 3.2.2.1.5.1. Spectral data for these derivatives are given below:

2-Hydroxy-1,2-di*o*-tolyethanone **(98)**: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 2.27 (s, 3H), 2.31 (s, 3H), 4.31 (d, *J*=6 Hz, 1H), 5.89 (d, *J*=5.6 Hz, 1H), 6.99-7.06 (m, 5H), 7.09 (d, *J*=8.0 Hz, 1H), 7.22 (t, *J*=8.0 Hz, 1H), 7.28 (d, *J*=8.0 Hz, 1H), [20b].

2-Hydroxy-1,2-di*p*-tolyethanone **(99)**: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 2.30 (s, 3H), 2.36 (s, 3H), 4.52 (d, *J*=5.8 Hz, 1H), 5.88 (d, *J*=5.8 Hz, 1H), 7.16 (d, *J*=8.1 Hz, 2H), 7.18-7.22 (m, 4H), 7.83 (d, *J*=8.1 Hz, 2H), [20b].

2-Hydroxy-1,2-bis(4-methoxyphenyl)ethanone **(100)**: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 3.76 (s, 3H), 3.85 (s, 3H), 4.46 (d, *J*=5.7 Hz, 1H), 5.84 (d, *J*=5.7 Hz, 1H), 6.82 (d, *J*=8.6 Hz, 2H), 7.16 (d, *J*=8.6 Hz, 2H), 7.24 (d, *J*=8.6 Hz, 2H), 7.85 (d, *J*=8.6 Hz, 2H), [20b].

HPLC analyses were done as described below:

2-Hydroxy-1,2-dio-tolyethanone: Chiralpak OD column, UV detection at 254 nm, eluent: n-hexane/2- propanol = 90:10, flow 1.0mL/min, 20 °C; R_t (*R*) = 13.8 min; R_t (*S*)=11.8 min.

2-Hydroxy-1,2-dip-tolyethanone: Chiralpak OD column, UV detection at 254 nm, eluent: n-hexane/2- propanol = 90:10, flow 1.0mL/min, 20°C; R_t (*R*) = 11.2 min; R_t (*S*)=9.4 min.

2-Hydroxy-1,2-bis(3-methoxyphenyl)ethanone: HPLC analysis conditions for this compound was given in Section 3.2.2.1.5.3.

2-Hydroxy-1,2-bis(4-methoxyphenyl)ethanone: Chiralpak IA column, UV detection at 254nm, eluent: n-hexane/2- propanol = 90:10, flow 1.0mL/min, 20 °C; R_t (*R*) = 36.0 min; R_t (*S*)=41.0 min.

Only with 2-hydroxy-1,2-bis(4-methoxyphenyl)ethanone a detectable hydro-benzoin formation (10%) was observed which was separated with column chromatography and characterized with ^{1}H-NMR and ^{13}C-NMR. Since ^{1}H-NMR spectrum contained a mixture of *meso*- and D,L- two data were given:

^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 2.83 (s, 2H), 3.68 (d, *J*=3.6, 6H), 4.47 (s, 1H), 6.64 (d, *J*=8.6, 4H), 6.90 (d, *J*=8.6, 4H); ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 2.83 (s, 2H), 3.71 (d, *J*=3.6, 6H), 4.60 (s, 1H), 6.72 (d, *J*=8.6, 4H), 7.05 (d, *J*=8.6, 4H), Fig. 76; ^{13}C-NMR (100 MHz, $CDCI_3/CCl_4$): δ ppm 158.78, 132.05, 128.08, 113.95, 78.75, 54.30, Fig. 77.

3.2.2.3.4. Effect of the Physiological State of the Cells and the Buffer pH

Same procedure was applied for the growth of microorganism as in Section 3.2.2.2.3. After 2 days, cells were filtered from the growth medium and taken into Erlenmeyers containing 400mL 50mM phosphate buffer at different pH's (pH 5.1, pH 6, pH 7, pH 8) and then 0.4mmol benzoin dissolved in 4mL DMSO was added. As control experiments, cells were filtered from the growth medium washed and then transferred to the new growth medium (400mL PDB) denoted as Control (1) and wet cells were transferred to the sterile (400mL) distilled water denoted as Control (2). Afterwards benzoin (0.4mmol in 4mL DMSO) was inoculated to the each control experiment. Reactions were monitored via TLC. Work-up was performed as described in Section 3.2.2.2.3. Analyses were done with HPLC as described in Section 3.2.2.3.1.

3.2.3. *Aspergillus oryzae* 5048 Mediated Reduction of Benzil Derivatives

3.2.3.1. Reduction of Benzil with Different *Aspergillus Species*

Seventeen different Aspergillus species [13 from Osaka University Department of Biotechnology (OUT) and 4 from TUBITAK Marmara Research Center (MRC culture collection)] were grown in potato dextrose agar (PDA) petri dishes. The streaked plates were incubated at 37°C for 3–4 days for spore production and then stored at 4°C until utilized. The surface of the petri plate containing spores was rubbed with a sterile inoculation loop and then transferred to a 250mL sterile Erlenmeyer containing a 100mL growth medium (PDB) prepared with dissolving 8g/L potato extract and 20g/L glucose in 1L distilled water. Reactions were monitored with TLC. Work-up procedure and HPLC analysis were done in as in Section 3.2.2.1.1.

3.2.3.2. Optimization of Fermentation Conditions

3.2.3.2.1. Investigating the Effect of Different Formulations on Enantioselectivity of *A.oryzae* Mediated Reduction of Benzil to Benzoin

Aspergillus oryzae 5048 was grown in potato dextrose agar (PDA) petri dishes. The streaked plates were incubated at 37°C for 3–4 days for spore production and then stored at 4°C until utilized. The surface of the petri plate containing spores was rubbed with a sterile inoculation loop and then transferred to a 250mL sterile Erlenmeyer containing 100mL different growth mediums prepared as described below:

Potato dextrose broth (PDB) was prepared by dissolving 8g potato extract and 20g glucose in 1L distilled water. Medium B, medium C, medium D were prepared according to the literature [41]. [B] = 30g/L glucose (20g/L in reference), 10g/L peptone, [C] = 20g/L glucose, 20g/L yeast extract, 5g/L peptone, 1g/L KH_2PO_4, 2g/L K_2HPO_4, 2g/L $NaNO_3$, 0.5g/L KCl, 0.5g/L $MgSO_4 \cdot 7H_2O$, 0.02 g/L $FeSO_4 \cdot 7H_2O$; [D] = 4g/L yeast extract, 15g/L starch, 1 g/L K_2HPO_4, 0.5g/L $MgSO_4 \cdot 7H_2O$. Czapek Dox medium (modified) and malt extract broth (MEB) were purchased from Oxoid. Modified Czapek Dox medium: 30g/L sucrose, 2g/L $NaNO_3$, 0.5g/L KCl, 0.5g/L magnesium glycerophosphate

($C_3H_7MgO_6P$), 0.01g/L $FeSO_4$, 0.35g/L K_2SO_4. Malt extract broth: 17g/L malt extract, 3g/L mycological peptone.

Work-up procedure and HPLC analysis performed were same as in Section 3.2.2.1.1.

3.2.3.2.2. Investigating the Effect of Initial pH of Medium D on Enantioselectivity of *A.oryzae* Mediated Reduction of Benzil to Benzoin

Aspergillus oryzae 5048 was grown in potato dextrose agar (PDA) petri dishes. The streaked plates were incubated at 37°C for 3–4 days for spore production and then stored at 4°C until utilized. The surface of the petri plate containing spores was rubbed with a sterile inoculation loop and then transferred to a 250mL sterile Erlenmeyer containing 100mL Medium D (Med. D) in which the organism was grown in a rotary shaker at 37°C for 60h. Initial pH of medium D's were adjusted to pH4, pH5, pH6, pH7, pH8 and unadjusted (control) pH with 1N NaOH and 1N HCl. Prepared mediums were sterilized at 121°C and 15 min. Again work-up procedure and HPLC analysis were as described in Section 3.2.2.1.1.

3.2.3.2.3. Investigating the Effect of the Physiological State of the Cells and the Buffer pH on Enantioselectivity of *A.oryzae* Mediated Reduction of Benzil to Benzoin

Microorganism was prepared as described before (Section 3.2.3.2.2) and transferred to 5 Med. D whose pH was adjusted to pH 7, each of them were 100mL, after microorganism was grown for 60h in a rotary shaker at 37°C. After 2.5 days resting cells filtered from the growth medium were transferred to 250mL sterile Erlenmeyer containing 100mL 50mM buffers with different pH's (pH 5, pH 6, pH 7, pH 8) and benzil (0.1mmol) dissolved in 1mL DMSO was added to each buffer containing wet cells and the reactions was started. pH adjustments were done with 1N NaOH and 1N HCl. For control experiment benzil (0.1mmol) dissolved in 1mL DMSO was added to the growth medium (Med D). Conversion was monitored by TLC. After the reaction was completed, microorganism was filtered off the supernatant, washed with distilled water and the combined aqueous phases were extracted three times with 50mL ethyl acetate (3×50mL). The organic extract was dried over $MgSO_4$, filtered and

solvent was evaporated with rotary evaporator under vacuum. Product was purified with column chromatography. HPLC analysis was performed as described in Section 3.2.2.1.1.

3.2.3.3. Enantioselective Bioreduction of Benzils to the Benzoins

Same procedure was applied as Section 3.2.3.2.3. After microorganism was grown for 60h resting cells filtered from the growth medium were transferred to 250mL sterile Erlenmeyer containing 100mL 50mM pH 5 phosphate buffer, and then substrate [benzil, (0.1mmol)] dissolved in 1mL DMSO was added and the reaction was started. pH adjustments were done with 1N NaOH and 1N HCl. Conversion was monitored by TLC. After the reaction was completed, same work-up procedure was applied (Section 3.2.2.1.1) and benzoin was purified by flash column chromatography (eluent: 1:3 ethyl acetate/hexane) and it was isolated as a colorless solid (20.1mg, 95% yield); (94% ee); m.p. 135°C [18a]; $[\alpha]_D$ ^{20}:+108 [a]D +114.9" (c 1.5, acetone) (+114.9°C 1.5, acetone, for >97.7% ee) [54]. HPLC conditions were as in Section 3.2.2.1.1

Enantiomeric excess was determined by the use of HPLC equipped with chiral column using authentic (*R*)- and (*S*)-benzoins, as references. Benzoin was obtained with 94% ee. All racemic benzoins are synthesized according to the literature and as described in Section 3.2.2.1.5.1 and used as reference [3a–d]. 1,2-Bis(3-florophenyl)-2-hydroxyethan-1-one was synthesized as Section 3.2.2.1.5.1 and characterized with ^{1}H-NMR.

1,2-Bis(3-florophenyl)-2-hydroxyethan-1-one: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 4.45 (d, *J*=4.8 Hz, 1H), 5.77 (d, *J*=4.8 Hz, 1H), 6.96-7.32 (m, 2H), 7.40-7.62 (m, 4H), 7.67-7.71 (m, 2H).

1,2-Bis(3-florophenyl)ethane-1,2-dione which was one of the substrates was synthesized as described in Section 3.2.2.1.5.2 and characterized via ^{1}H-NMR.

1,2-Bis(3-florophenyl)ethane-1,2-dione: ^{1}H-NMR (400 MHz, $CDCl_3/CCl_4$): δ ppm 7.27–7.32 (m, 2H), 7.40–7.46 (m, 2H), 7.60–7.67 (m, 4H).

HPLC analyses of other 2-hydroxy-1,2-diphenyl ethanone derivatives:

2-Hydroxy-1,2-diphenyl ethanone: Described in Section 3.2.2.1.1.

HPLC analysis protocols of 2-Hydroxy-1,2-bis(2-methoxyphenyl)ethanone, 2-Hydroxy-1,2-bis(3-methoxy-phenyl)ethanone & 1,2-Bis(2-florophenyl)-2-hydroxyethanone were described in Section 3.2.2.1.5.3.

2-Hydroxy-1,2-bis(4-methoxyphenyl)ethanone: Described in Section 3.2.2.3.3.

1,2-Bis(3-florophenyl)-2-hydroxyethanone: Chiralpak IA column, UV detection at 254 nm, eluent: n-hexane/2-propanol = 90:10, flow 1.0mL/min, 20^{o}C; R_t (*R*) = 12.7 min; R_t (*S*) = 16.5 min.

CHAPTER 4

CONCLUSION

In this study described here, both enzyme and microorganism mediated syntheses of optically active α-hydroxy ketones were achieved.

For the enzymatic conversions benzaldehyde lyase [BAL, (EC 4.1.2.38)] was firstly used in the hydroxymethylation of aromatic aldehydes with formaldehyde via acyloin linkage in high yields. The products were obtained in high yields starting from simple, easily available aromatic aldehydes so this method presents an efficient methodology for the syntheses of hydroxyacetophenones. BAL was also employed in self- and cross- condensation of functionalized acetaldehydes such as benzyloxyacetaldehyde and dimethoxyacetaldehyde which were not employed as donors previously by this enzyme. As the result of self-condensation of benzyloxyacetaldehyde, 1,4-bis(benzyloxy)-3-hydroxybutan-2-one was obtained with 73% ee and 75% yield, while self-condensation of dimethoxyacetaldehyde resulted in 3-hydroxy-1,1,4,4-tetramethoxybutan-2-one **69** which is equivalent to is the protected dialdehyde **70** with pharmaceutical importance at a yield of 63% and $[\alpha]_D^{20}$ value of 30. In the cross-condensation reaction of benzyloxyacetaldehyde with acetaldehyde, cross-condensation products other than the self-condensation products were obtained. These aliphatic aldehydes were acted as donors not acceptors.

For the whole cell mediated reactions, firstly *A.flavus* was used in multi-reactions including reduction, hydrolysis and deracemization. Benzil was reduced to benzoin with 98% yield and 97% ee with the introduction of isopropanol as additive while benzil derivatives were converted to the relevant benzoins up to 96% yield and 88.4% ee. In another reaction by *A.flavus* benzoyl benzoin was tried to be hydrolyzed in an enantioselective manner. However the reaction has proven to proceed by nonselective hydrolysis coupled with deracemization. If the reaction was kept longer than the benzoin was further reduced to the hydrobenzoin. The reaction conditions were optimized to

handle the reaction in a selective manner. By bioprocess engineering the course of the reaction (whether mainly deracemization or reduction would take place) was taken under control.

In another biotransformation study whole cells of *A. oryzae* were used in the reduction of benzils. Symmetrical benzils were converted to (*S*)-benzoin derivatives with the yields up to 99% while ee's up to 94% with this microorganism. The reductions by *A.oryzae* proceed smoothly; hydrobenzoin formation was not observed but if so only in trace amounts.

The processes employed in this study have been shown to be efficient as they can be performed under mild reaction conditions using simple procedures and they provided good to excellent yields and enantiomeric excesses.

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APPENDIX

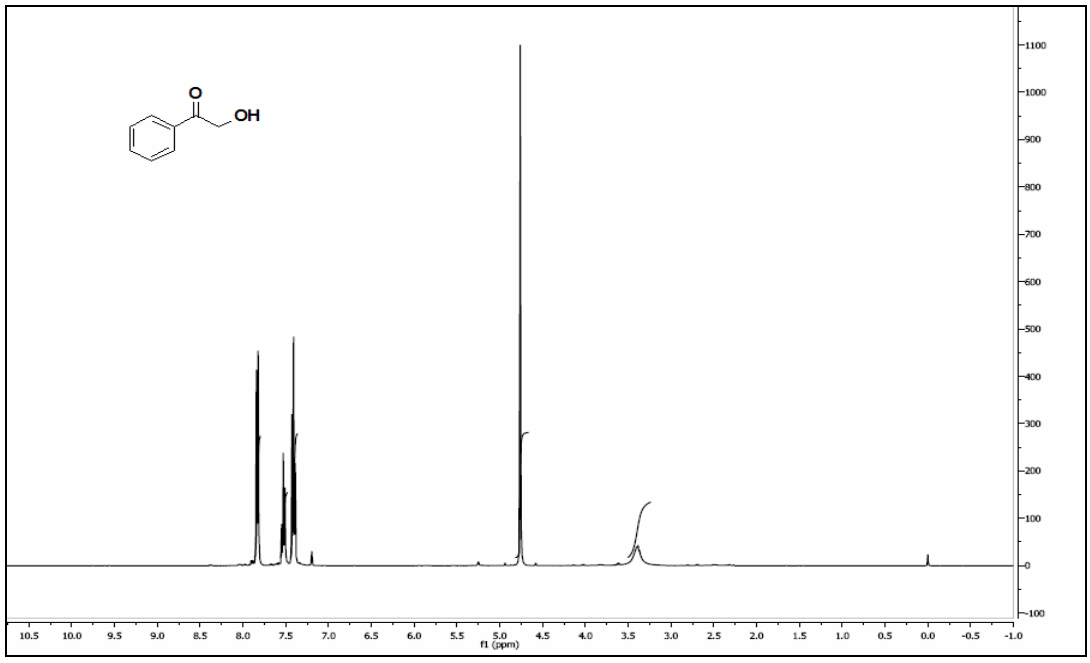

Figure 50 ^{1}H-NMR spectrum of 2-hydroxy-1-phenylethanone

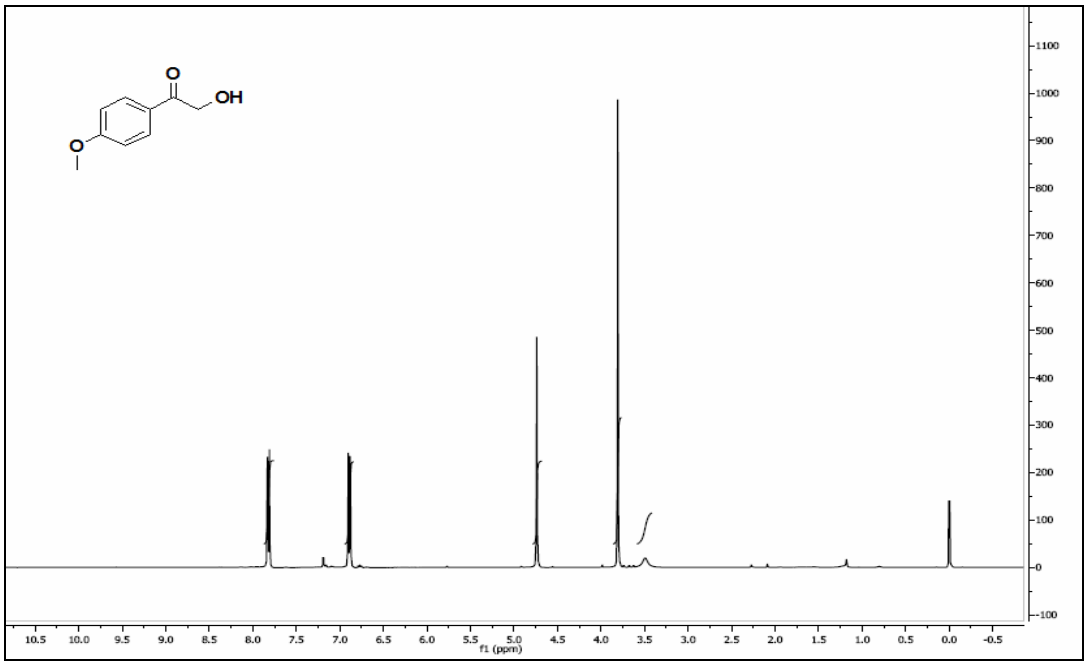

Figure 51 ^{1}H-NMR spectrum 2-hydroxy-1-(4-methoxyphenyl)ethanone

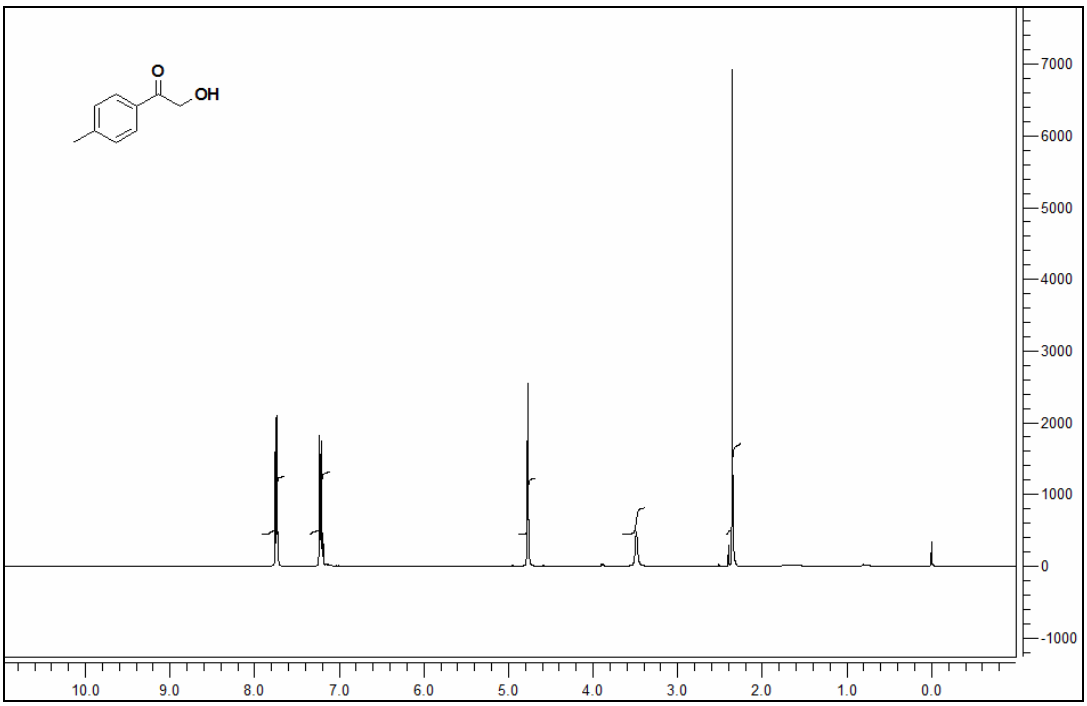

Figure 52 ^{1}H-NMR spectrum of 2-hydroxy-1*p*-tolylethanone

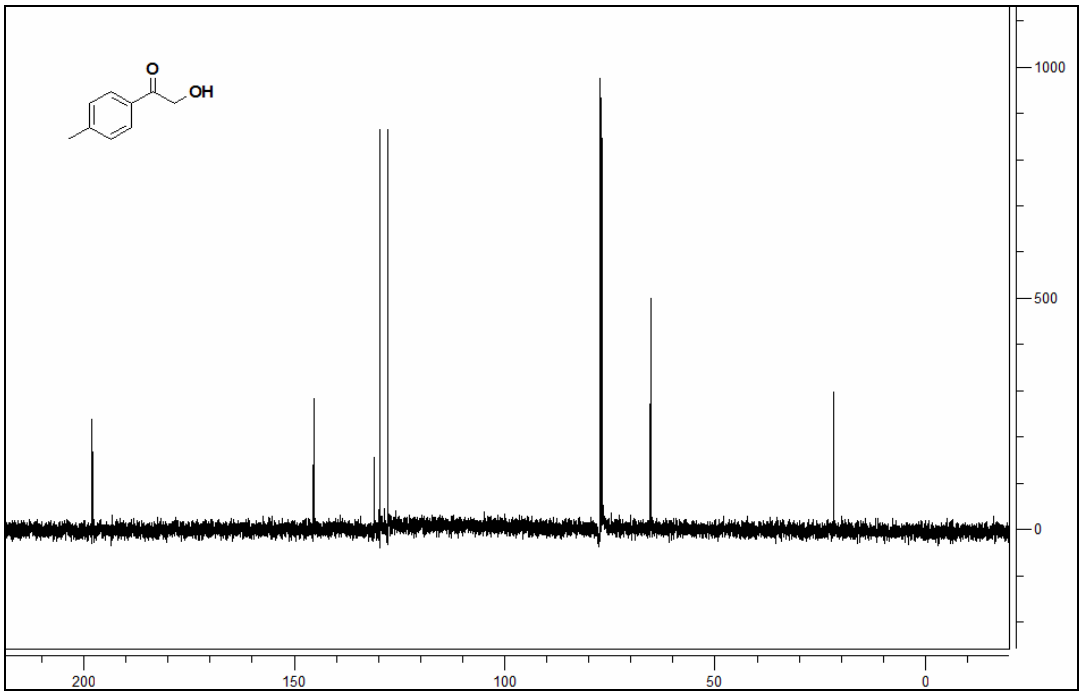

Figure 53 ^{13}C-NMR spectrum of 2-hydroxy-1*p*-tolylethanone

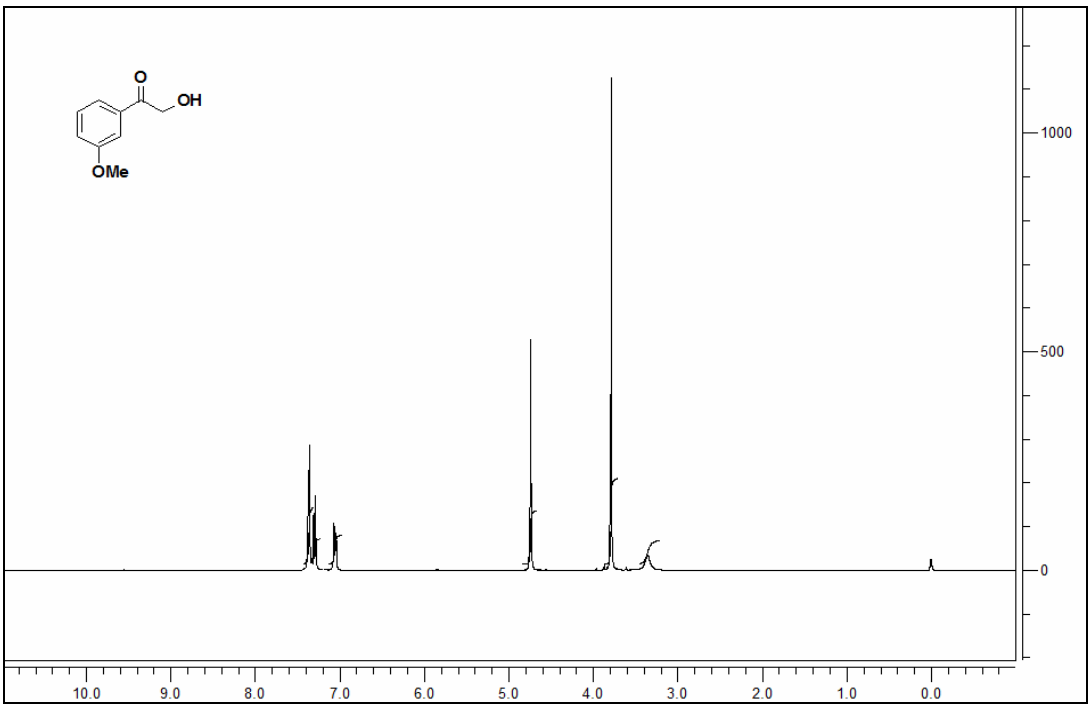

Figure 54 ^{1}H-NMR spectrum of 2-hydroxy-1-(3-methoxyphenyl)ethanone

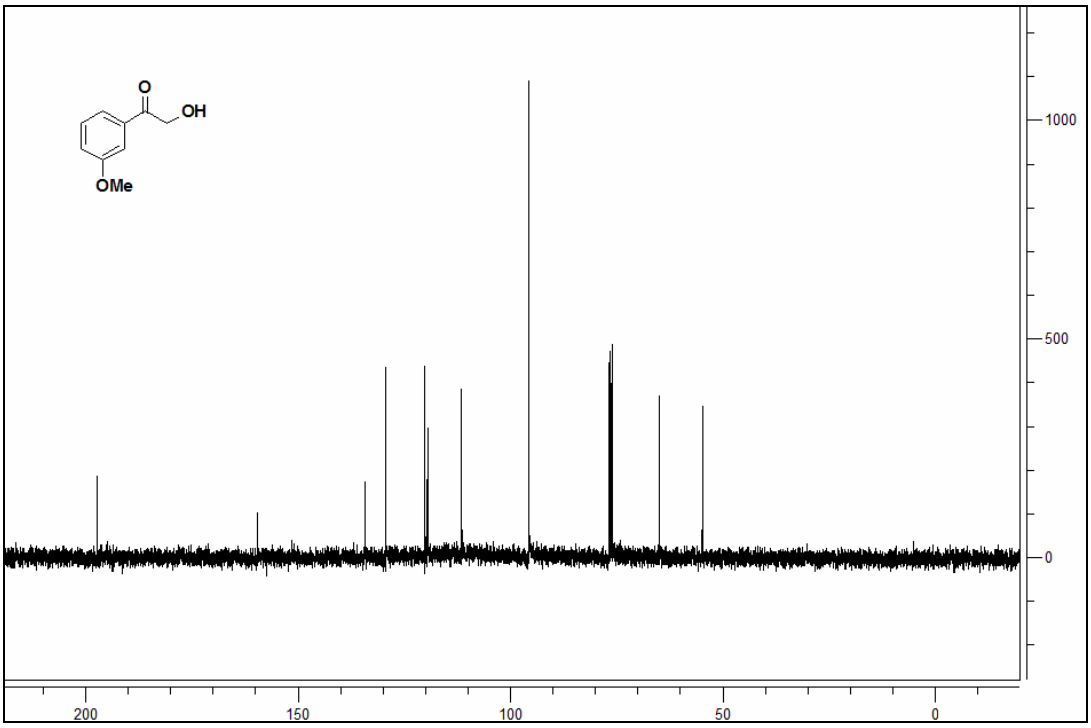

Figure 55 ^{13}C-NMR spectrum of 2-hydroxy-1-(3-methoxyphenyl)ethanone

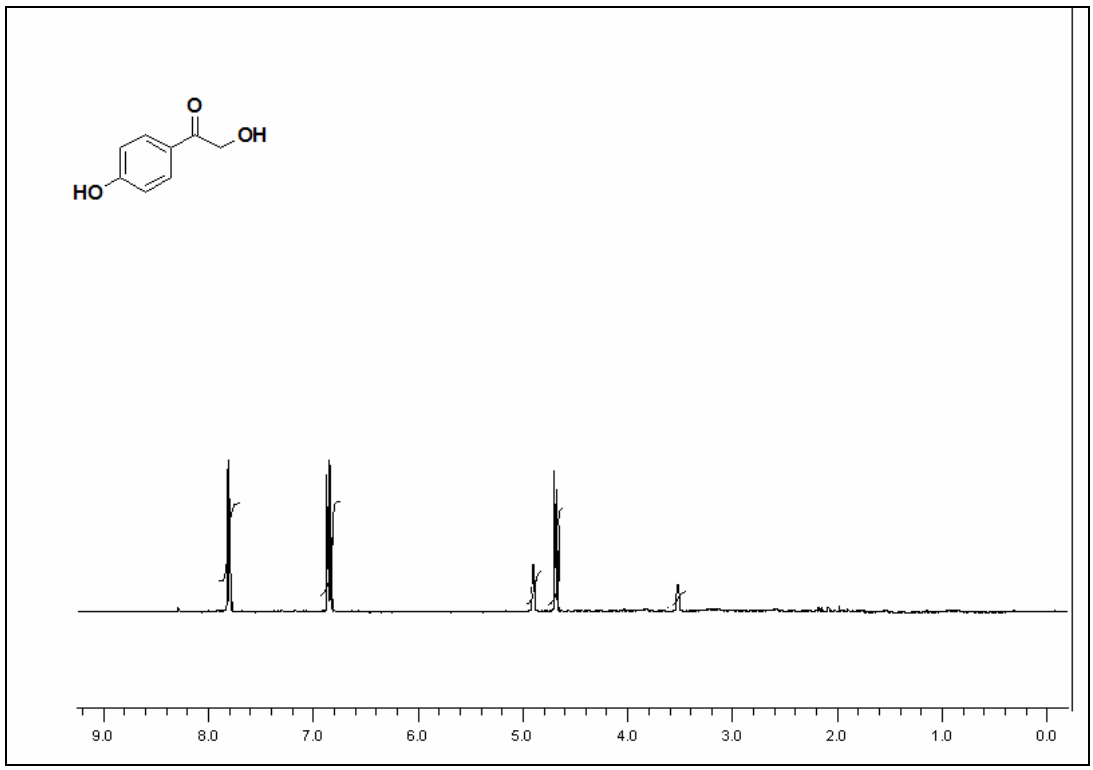

Figure 56 ^{1}H-NMR spectrum of 2-hydroxy-1-(4-hydroxyphenyl)ethanone

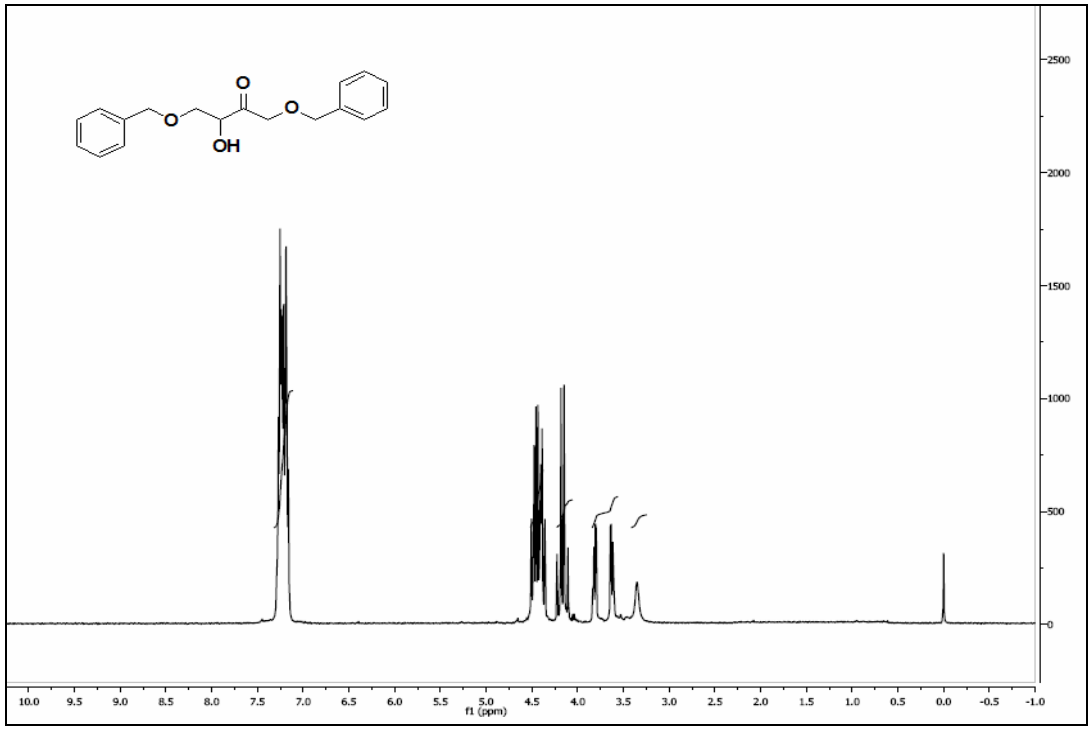

Figure 57 ^{1}H-NMR spectrum of 1,4-bis(benzyloxy)-3-hydroxybutan-2-one

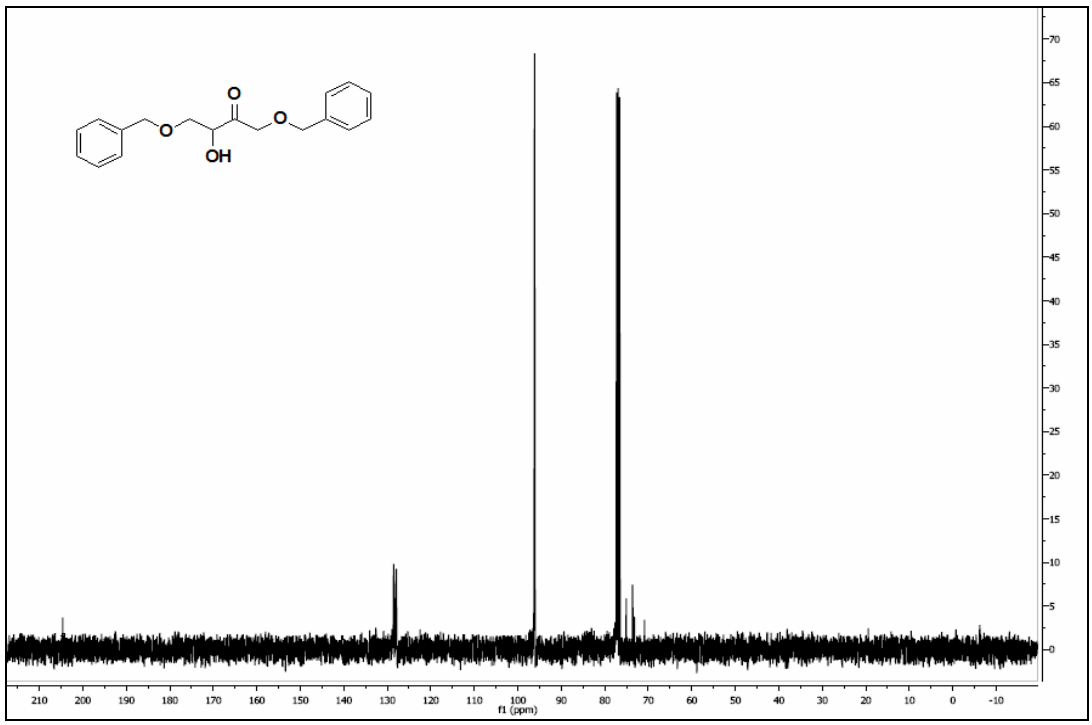

Figure 58 ^{13}C-NMR spectrum of 1,4-bis(benzyloxy)-3-hydroxybutan-2-one

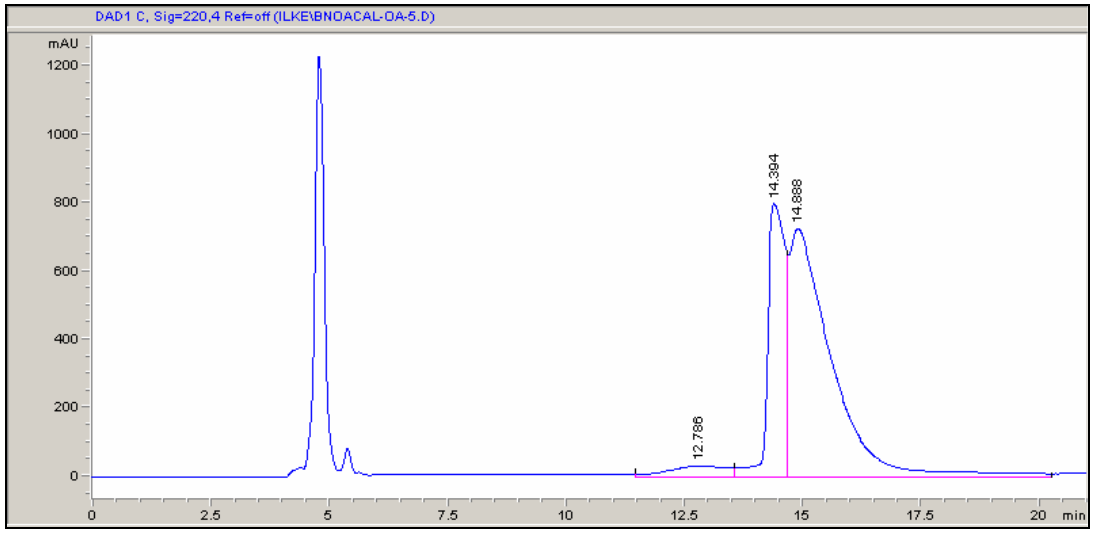

Figure 59 HPLC analysis of *rac*-1,4-bis(benzyloxy)-3-hydroxybutan-2-one

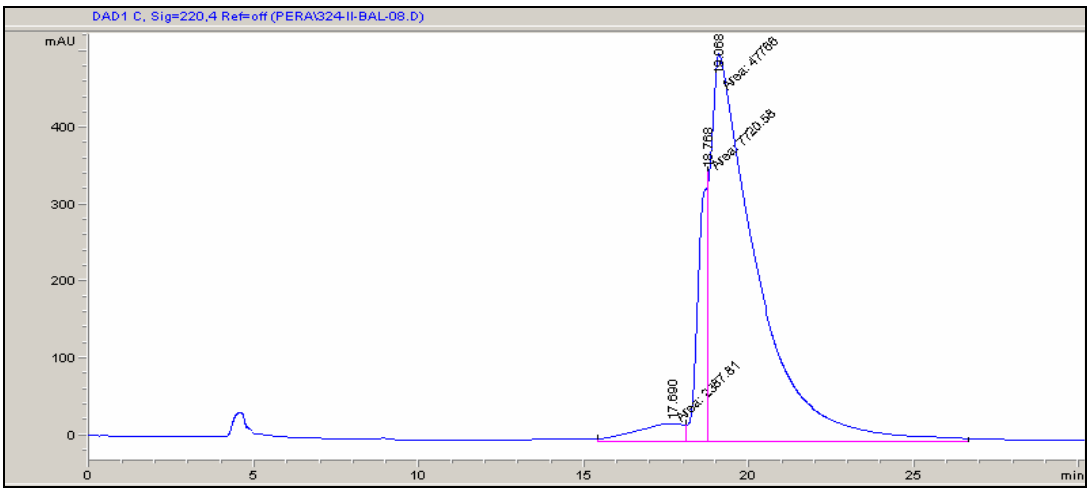

Figure 60 HPLC analysis of BAL catalyzed 1,4-bis(benzyloxy)-3-hydroxybutan-2-one

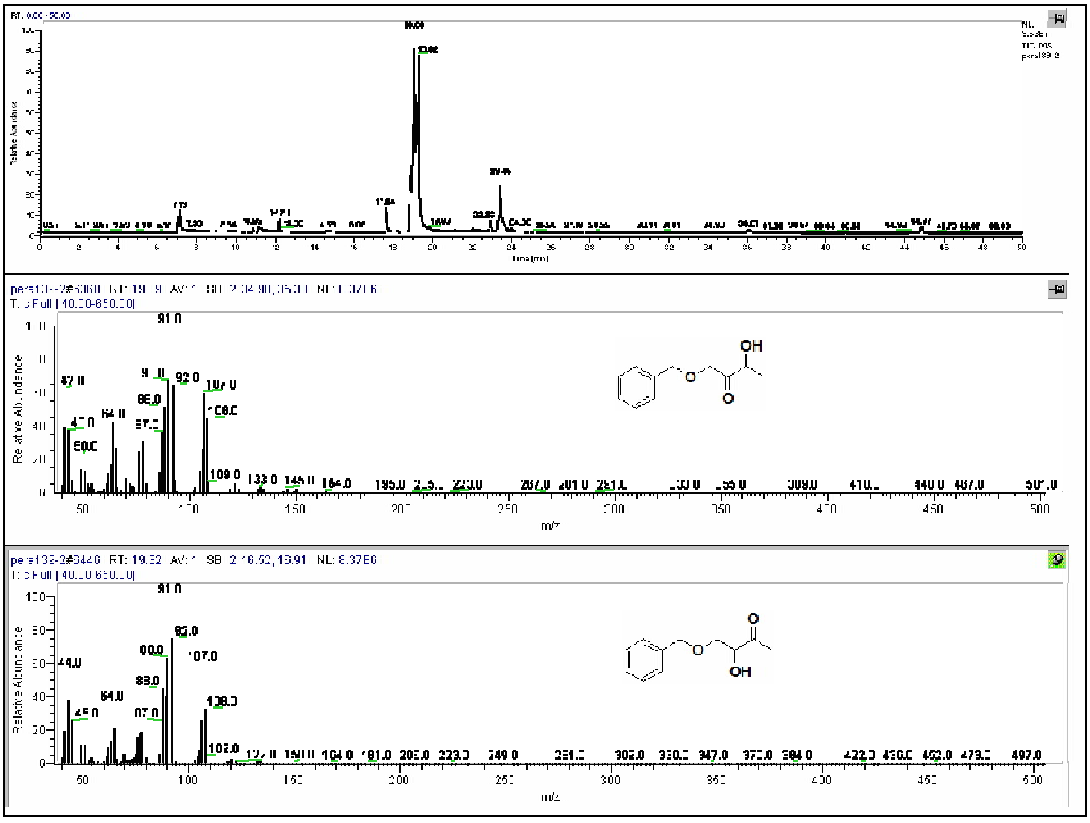

Figure 61 Mass spectra of 4-(benzyloxy)-3-hydroxybutan-2-one and 1-(benzyloxy)-3-hydroxybutan-2-one

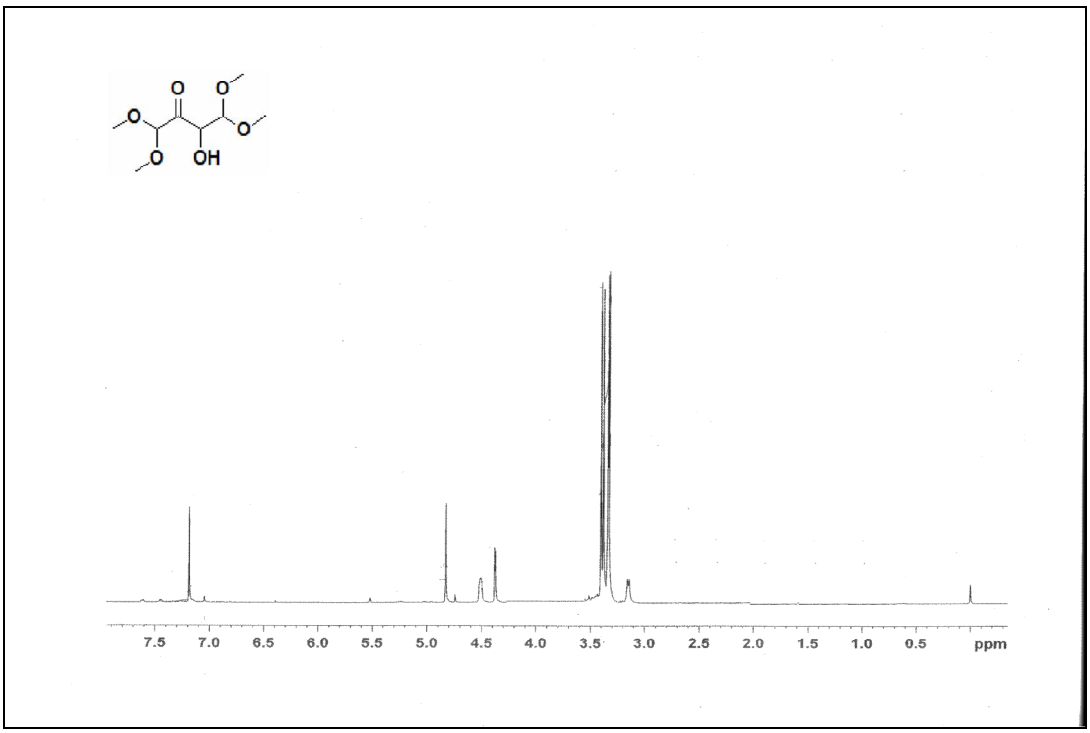

Figure 62 ^{1}H-NMR spectrum of 3-hydroxy-1,1,4,4-tetramethoxybutan-2-one

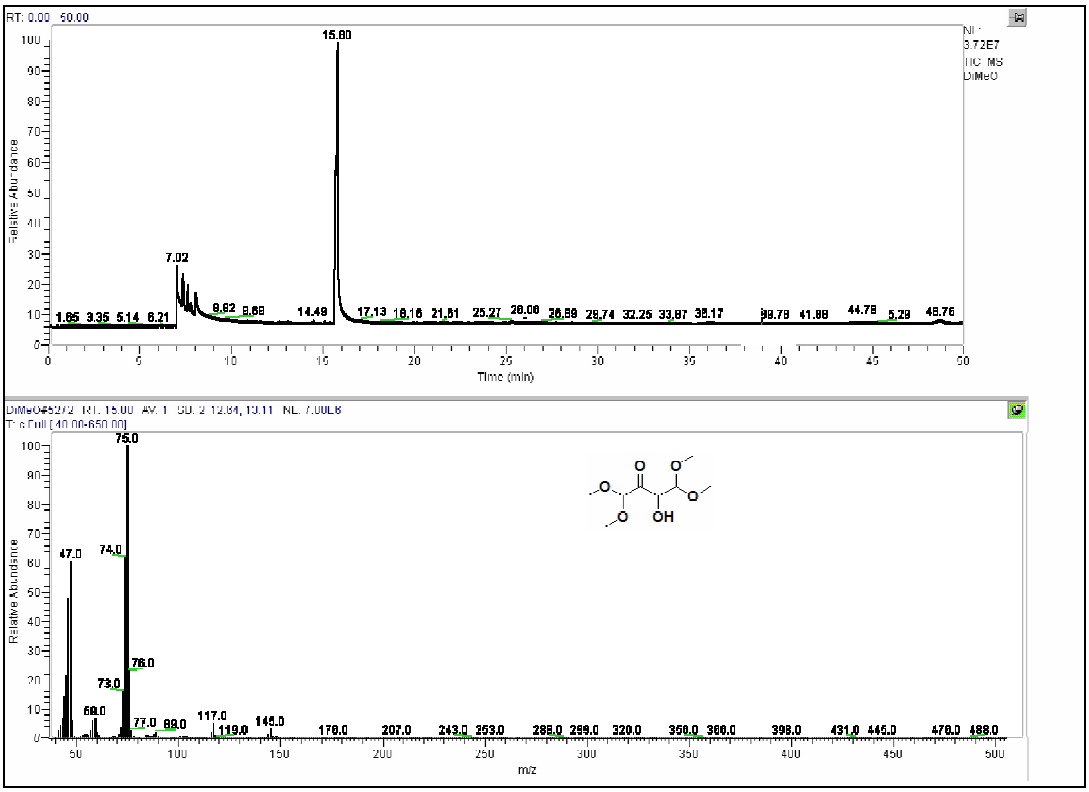

Figure 63 Mass spectrum of 3-hydroxy-1,1,4,4-tetramethoxybutan-2-one

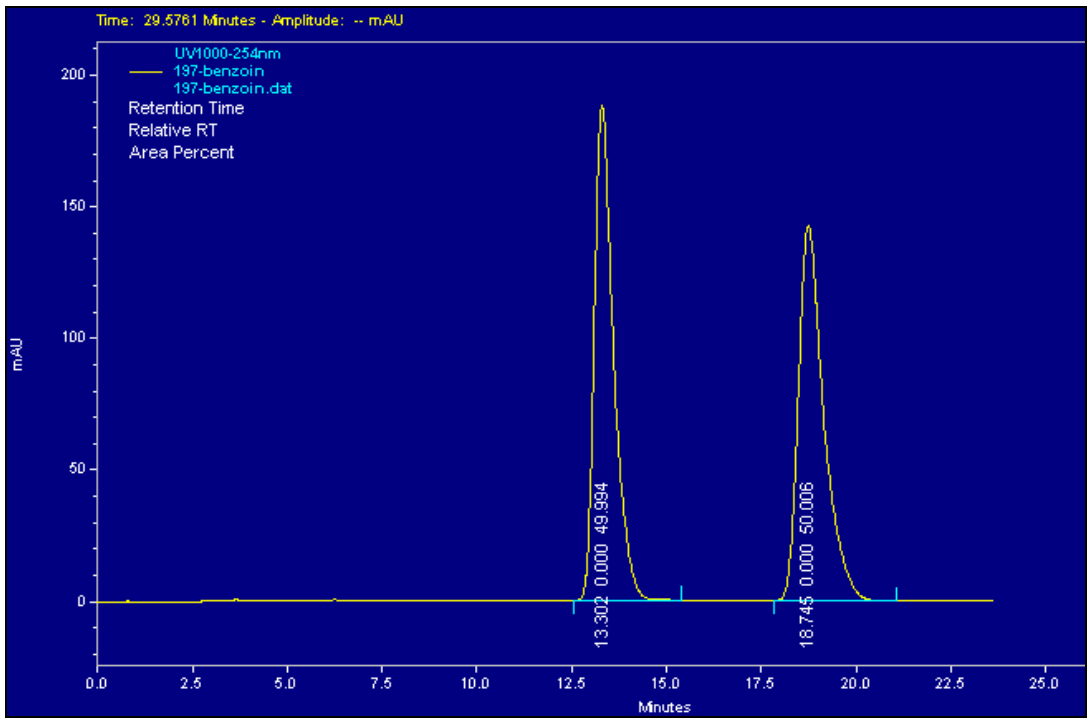

Figure 64 HPLC spectrum of racemic benzoin

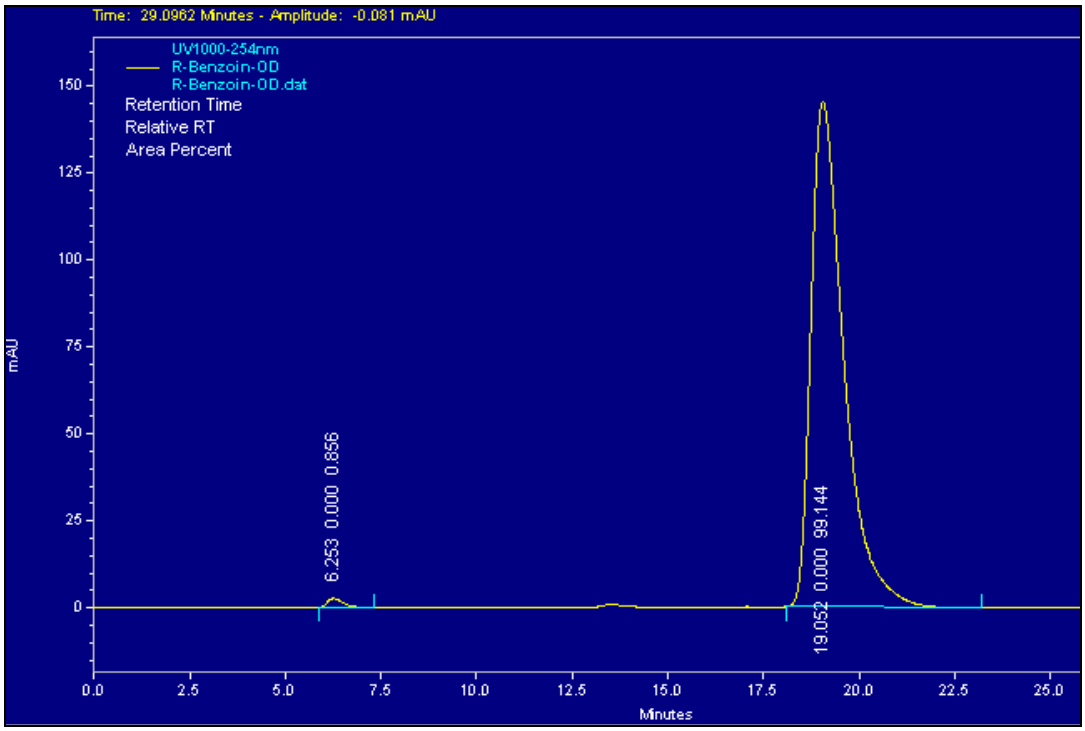

Figure 65 HPLC spectrum of (*R*)-benzoin

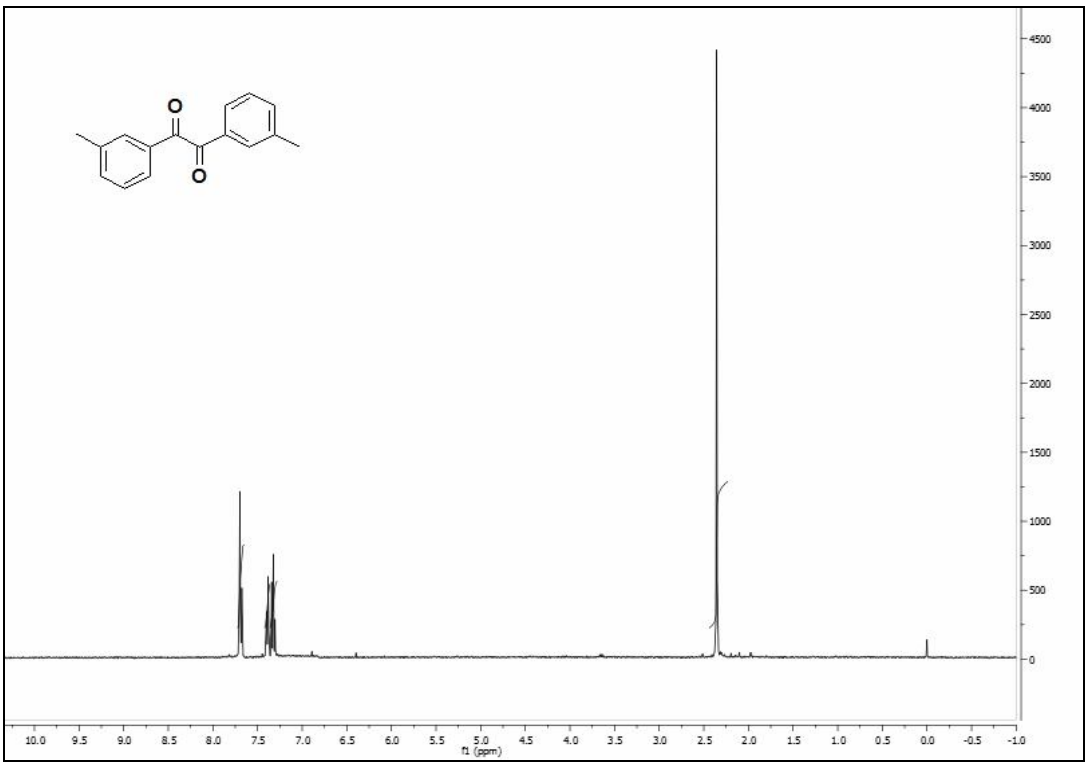

Figure 66 ^{1}H-NMR spectrum of 1,2-dim-tolylethane-1,2-dione

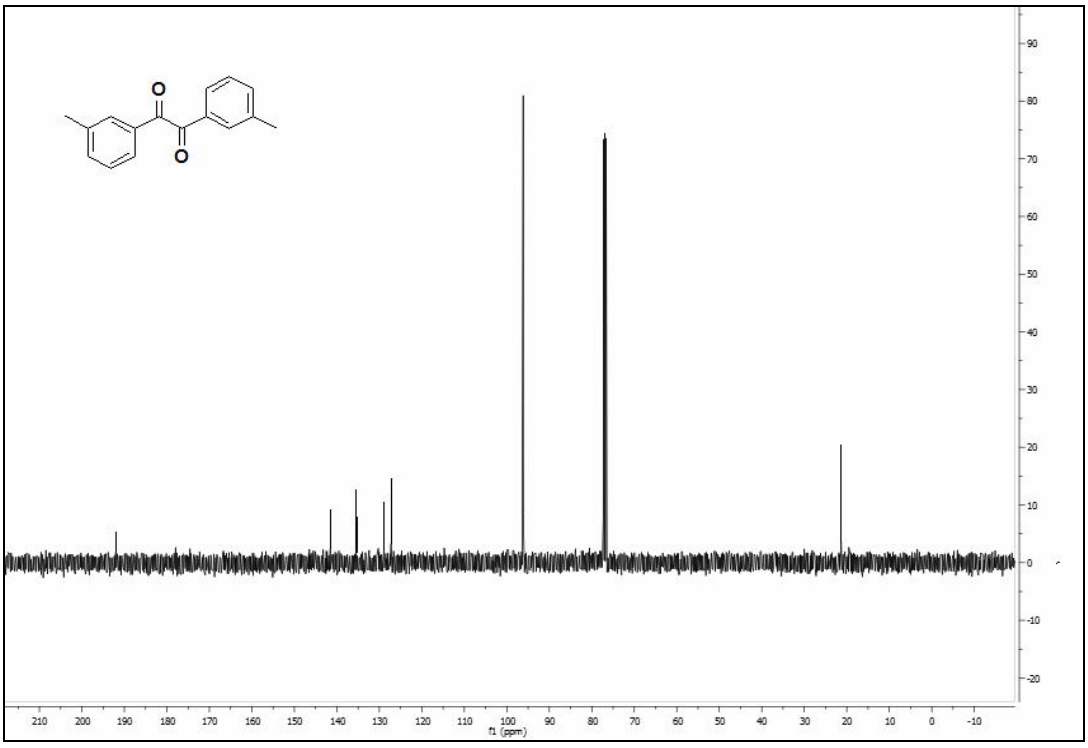

Figure 67 ^{13}C-NMR spectrum of 1,2-di*m*-tolylethane-1,2-dione

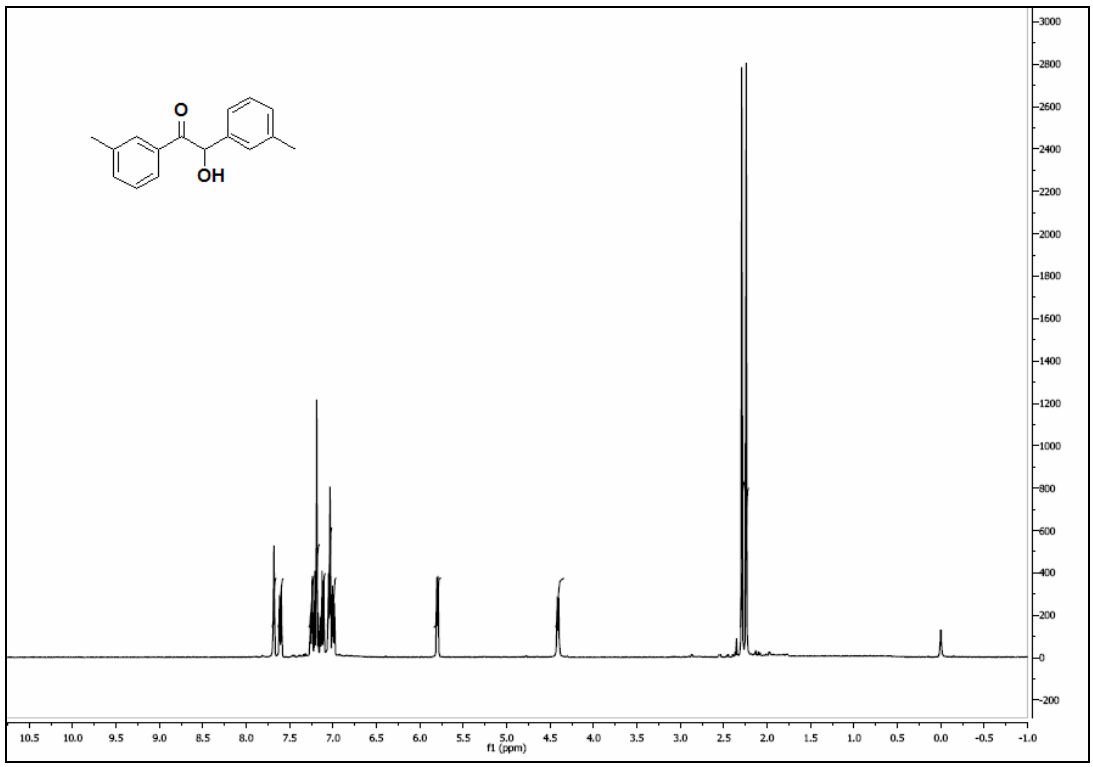

Figure 68 ^{1}H-NMR spectrum of 2-hydroxy-1,2-di*m*-tolylethanone

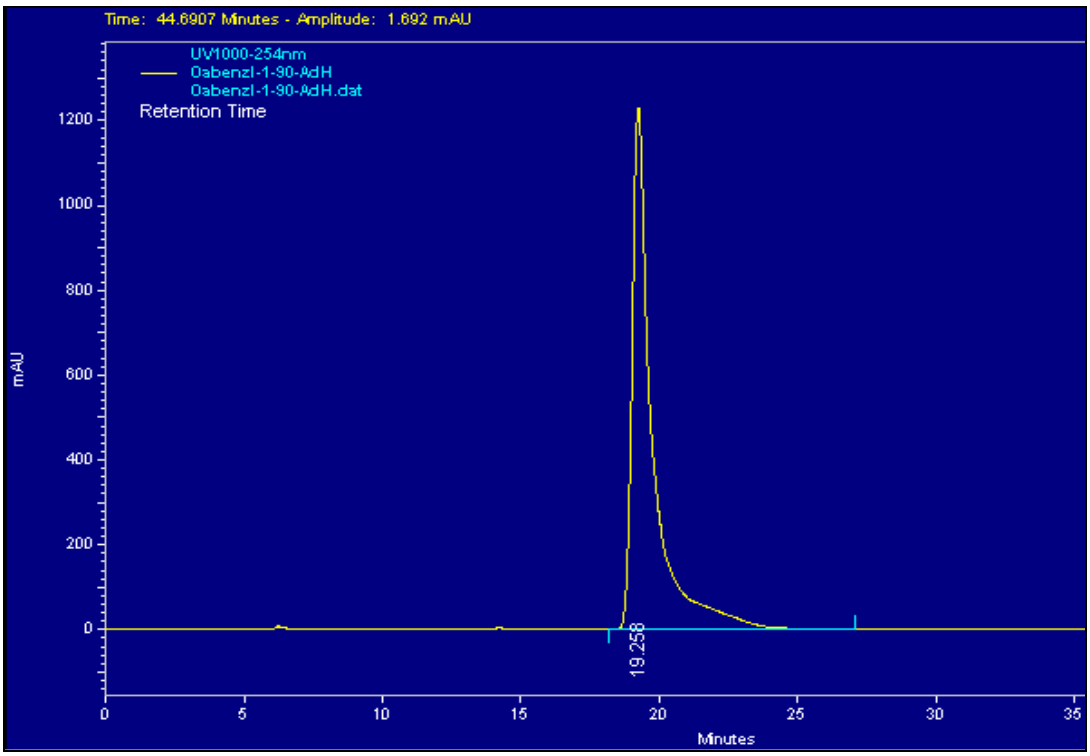

Figure 69 HPLC spectrum of *o*-anis benzyl

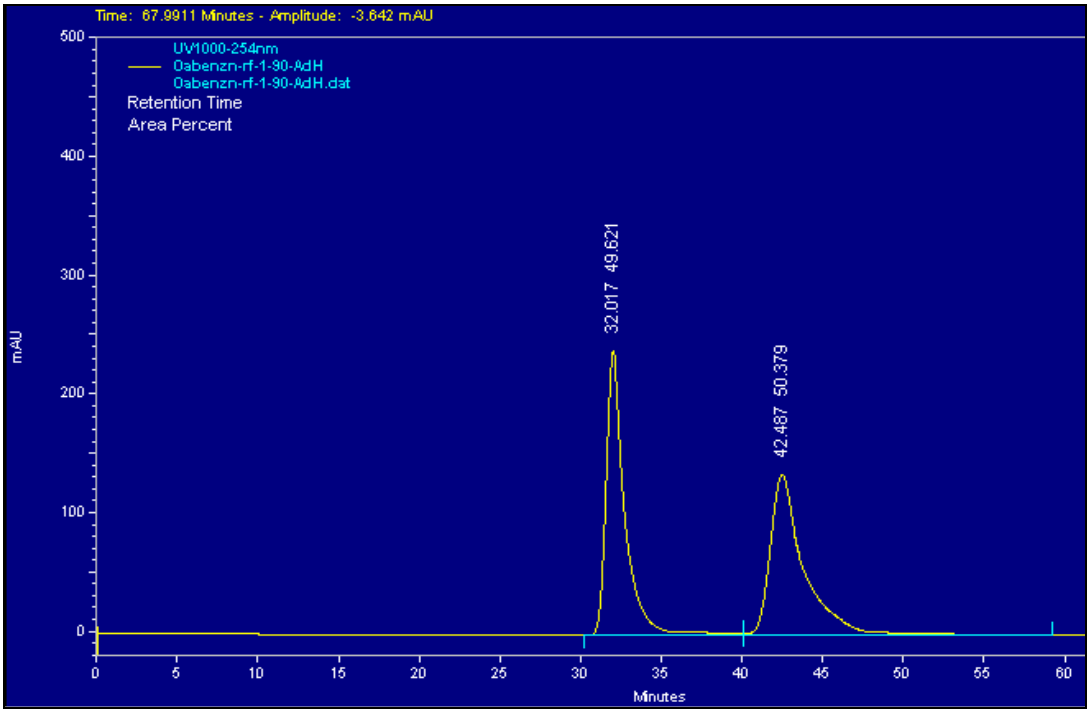

Figure 70 HPLC spectrum of *o*-anisoin

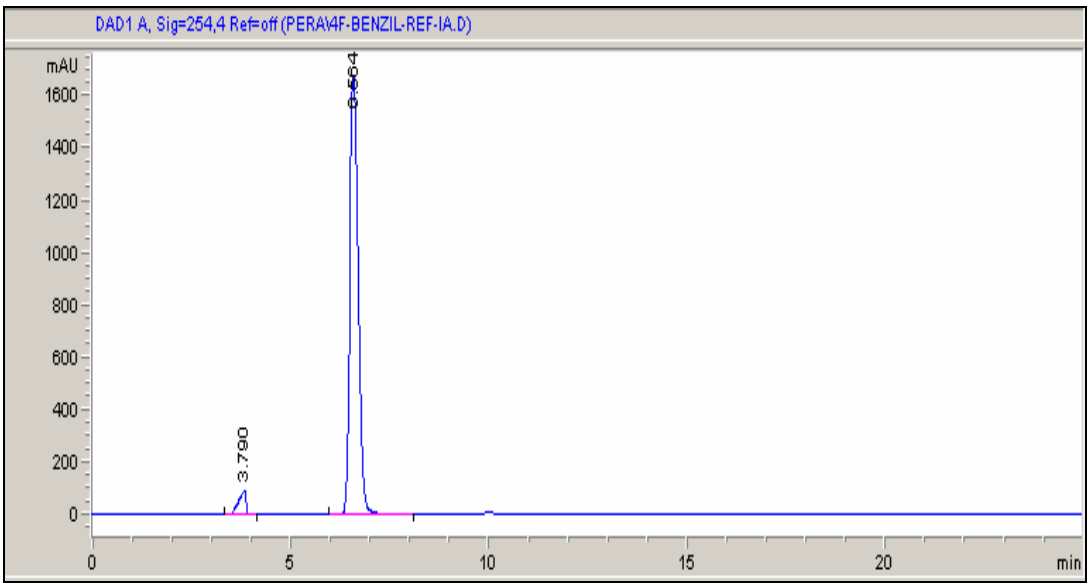

Figure 71 HPLC spectrum of 1,2-Bis(4-florophenyl)ethane-1,2-dione

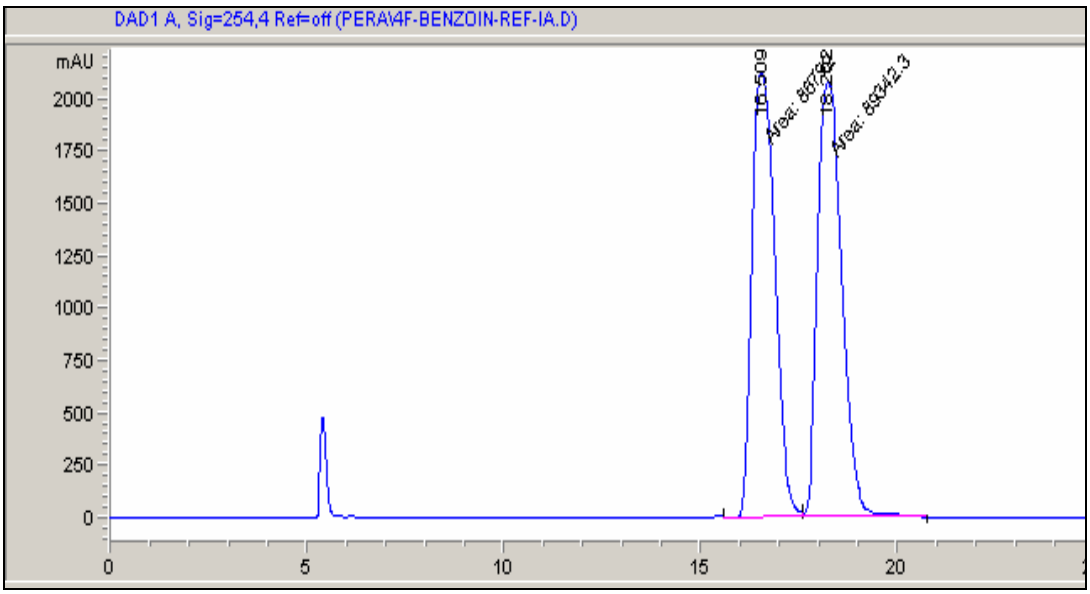

Figure 72 HPLC spectrum of 1,2-Bis(4-florophenyl)-2-hydroxyethan-1-one

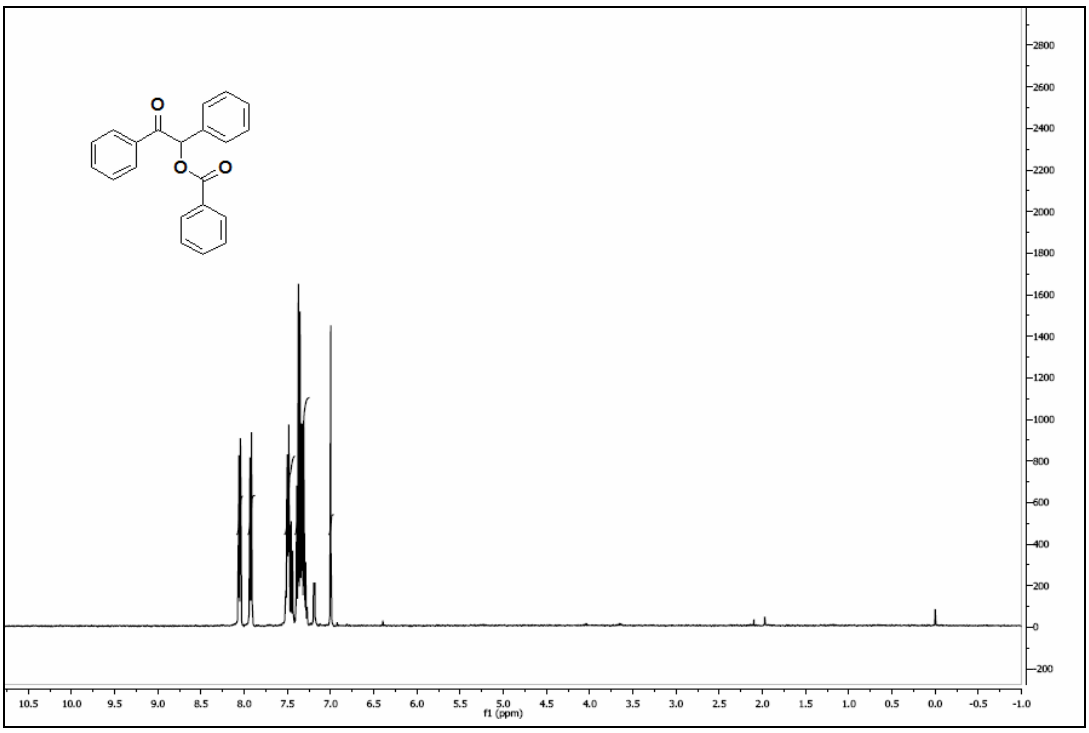

Figure 73 ^{1}H-NMR spectrum of 2-oxo-1,2-diphenylethyl benzoate

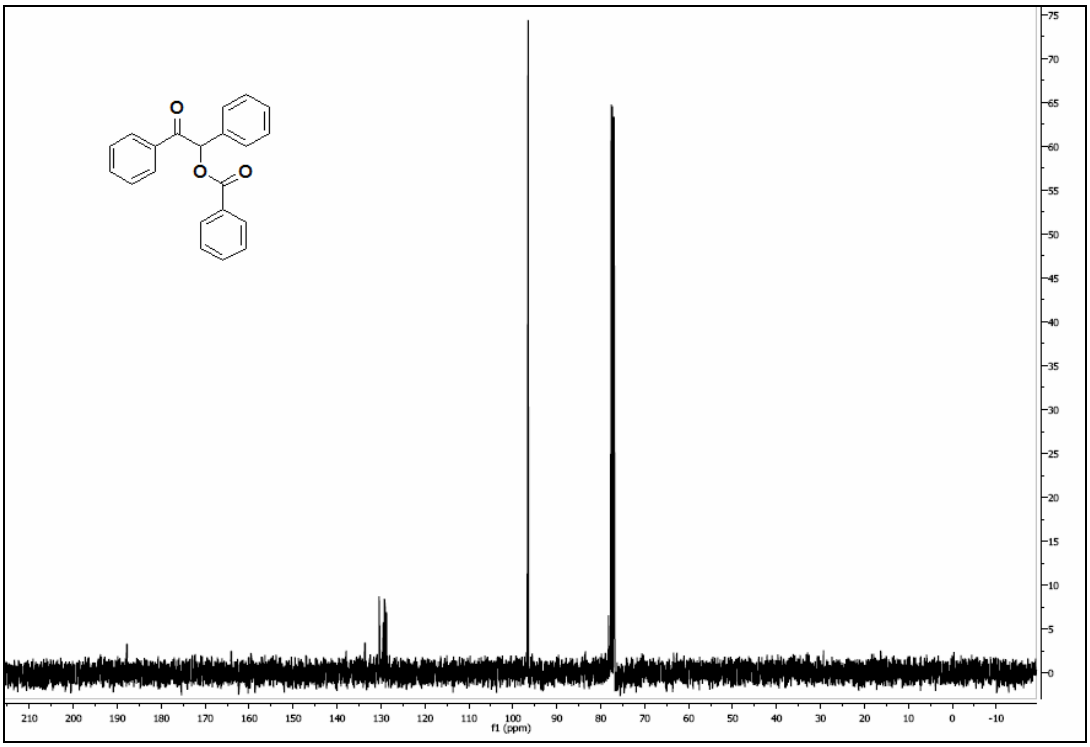

Figure 74 ^{13}C-NMR spectrum of 2-oxo-1,2-diphenylethyl benzoate

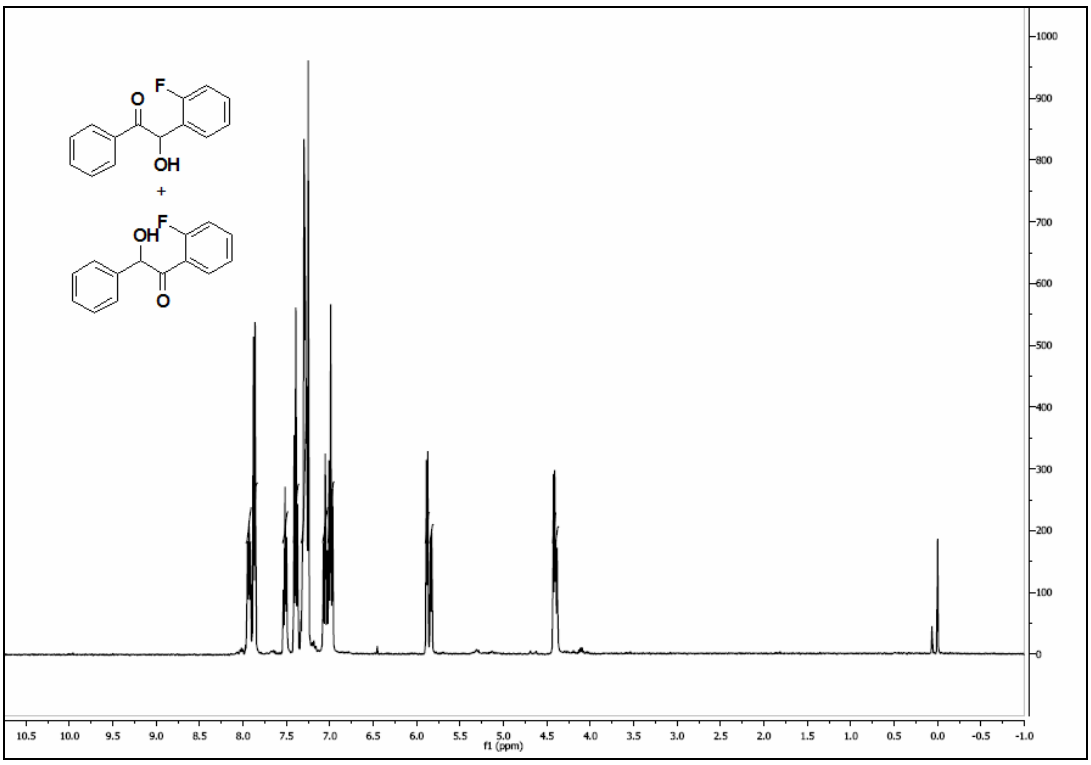

Figure 75 ^{1}H-NMR spectrum of 1-(2-florophenyl)-2-hydroxy-2-phenyl-ethanone and 2-(2-florophenyl)-2-hydroxy-1-phenylethanone

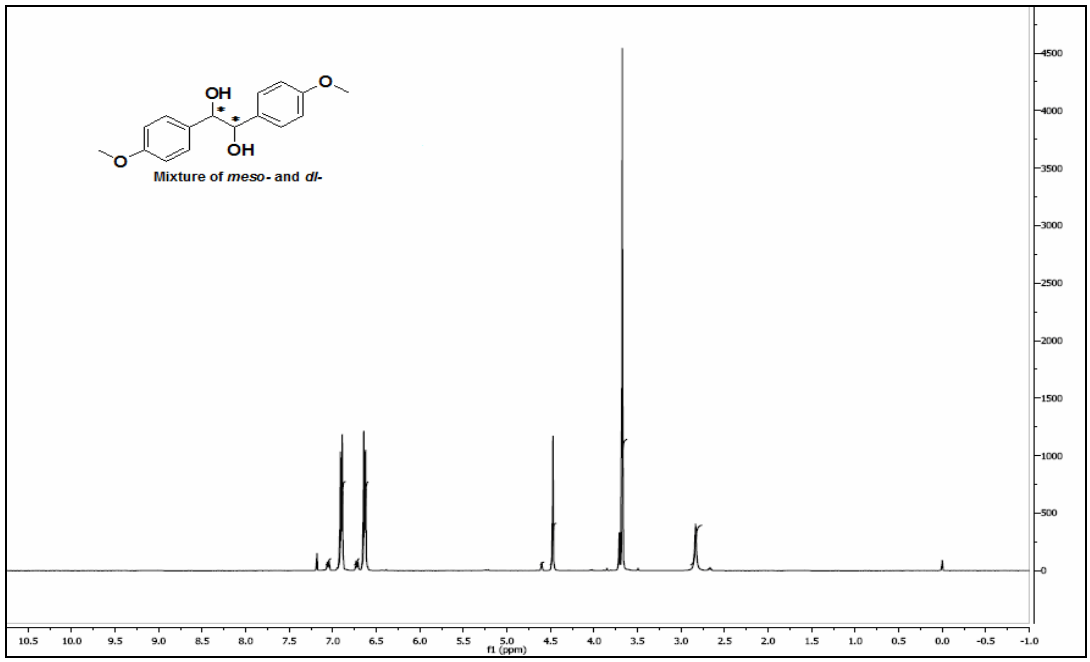

Figure 76 ^{1}H-NMR spectrum of *meso*- and D,L-1,2-bis(4-methoxyphenyl)ethane-1,2-diol

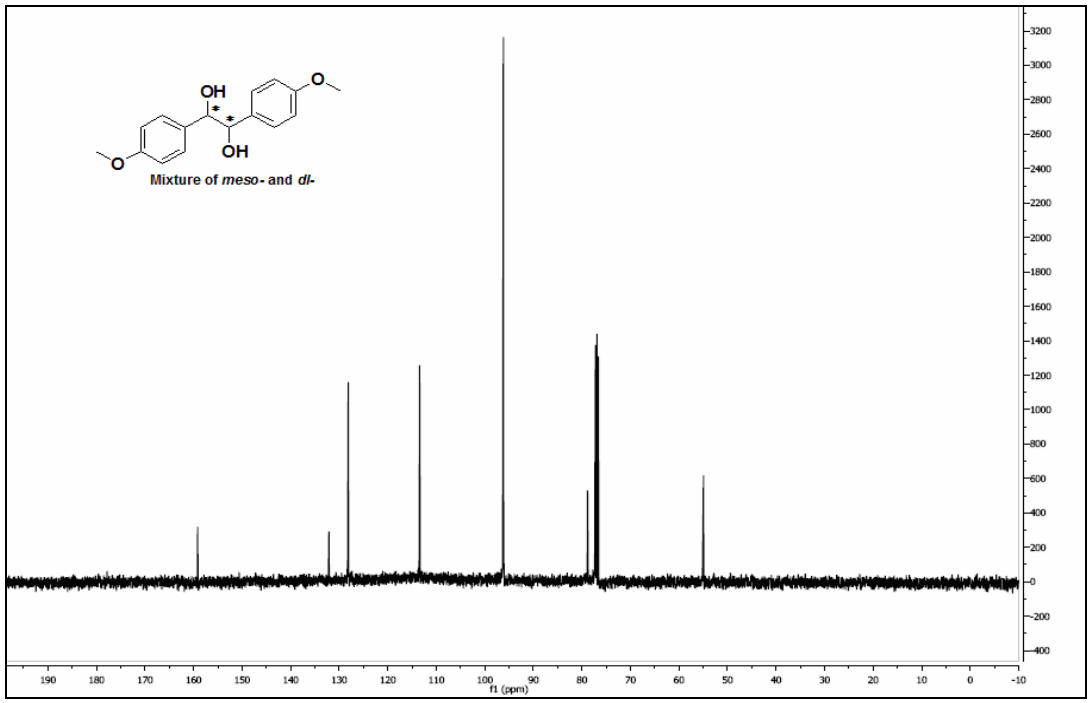

Figure 77 ^{13}C-NMR spectrum of *meso*- and D,L-1,2-bis(4-methoxyphenyl)ethane-1,2-diol

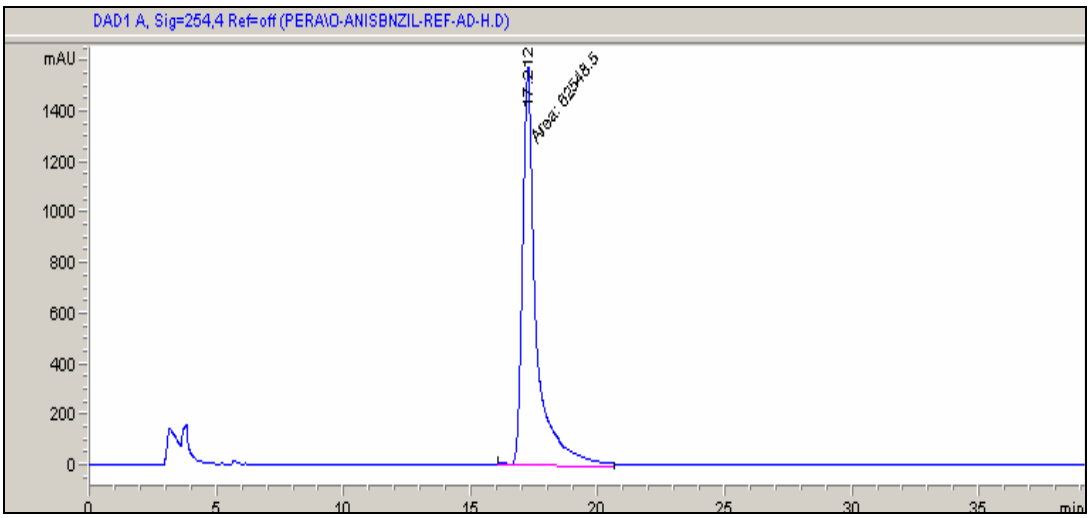

Figure 78 HPLC spectrum of 1,2-Bis(2-methoxyphenyl)ethane-1,2-dione

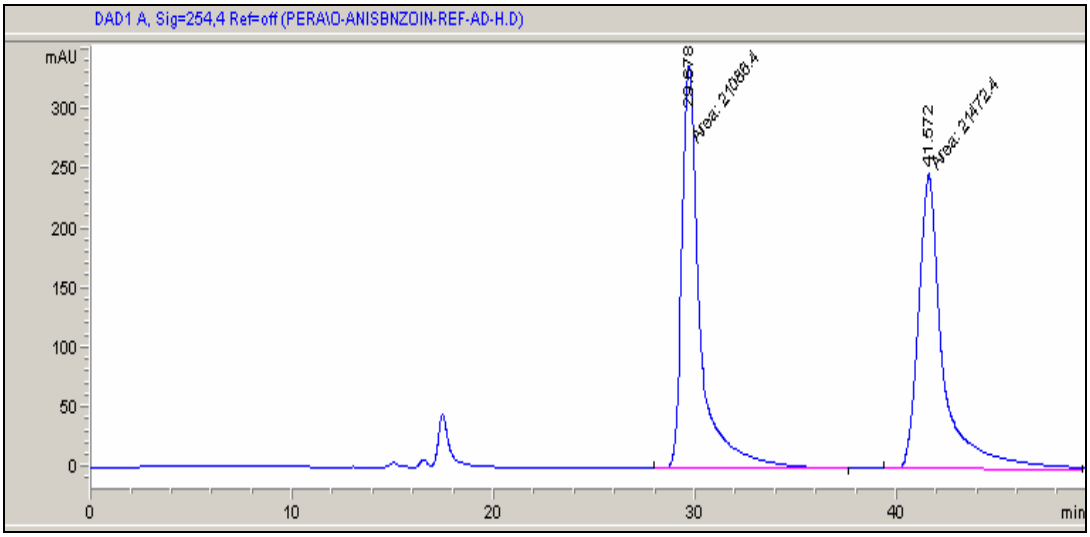

Figure 79 HPLC spectrum of 2-Hydroxy-1,2-bis(2-methoxyphenyl)ethanone

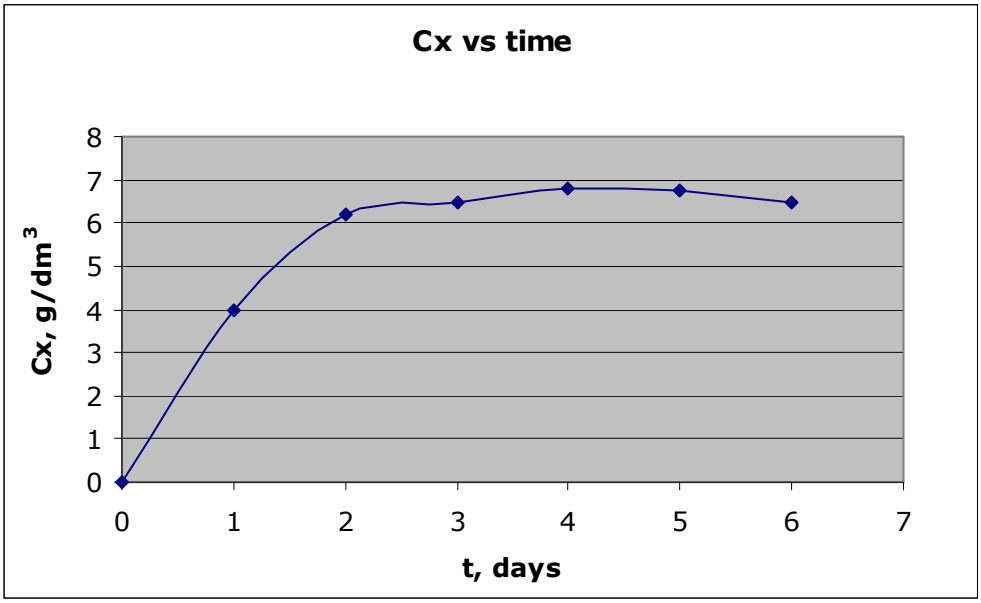

Figure 80 Growth curve of *Aspergillus flavus*

CURRICULUM VITAE

PERSONAL INFORMATION

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EDUCATION

Degree	Institution	Year of Graduation
BS	METU Food Engineering	2002
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WORK EXPERIENCE

Year	Place	Enrollment
2003-2008	METU, Graduate School of Natural and Applied Sciences	Research Assistant
2001 August	Sütaş	Intern Engineering Student
2000 July	Nuhun Ankara	Intern Engineering Student

FOREIGN LANGUAGES

Advanced English, Intermediate Spanish, Basic German

PUBLICATIONS

1. Demir, A. S.; Ayhan, P.; Demirtaş, U.; Erkılıç, U. *Fusarium roseum* and *Aspergillus oryzae*- mediated enantioselective reduction of benzils to benzoins. J. Mol. Catal. B: Enzym., 2008, 55, 164-168.

2. Demir, A. S.; Ayhan, P.; Sopacı, Ş. B. Thiamin pyrophosphate dependent enzyme catalyzed reactions: stereoselective C-C bond formation in water. CLEAN: SOIL, AIR, WATER, 2007, 35(5), 406-412.

3. Çalık, P.; Yılgör, P.; Ayhan, P.; Demir, A. S. Oxygen transfer effects on recombinant benzaldehyde lyase production. Chemical Engineering Science 2004, 59, 5075 – 5083.

4. Demir, A. S.; Hamamcı, H.; Ayhan, P.; Duygu, A. N; İğdir, A. Ç.; Çapanoğlu D. Fungi mediated conversion of benzil to benzoin and hydrobenzoin. Tetrahedron: Asymmetry 2004, 15, 2579–2582.

5. Demir, A. S.; Ayhan, P.; İğdir, A. Ç.; Duygu, A. N. Enzyme catalyzed hydroxy-methylation of aromatic aldehydes with formaldehyde. Synthesis of hydroxyaceto-phenones and (*S*)-benzoins. Tetrahedron 2004, 60, 6509–6512.

PROJECTS

- Yaygınlaştırılmış Ulusal ve Uluslararası Projeler (YUUP) BIOCAT: Kiral ilaç temel maddelerinin üertim ve sentez teknolojilerinin gelistirilmesi (Teknoloji Projesi) – *as Researcher*.

- Simetrik olmayan Kiral Benzoinlerin Biyoteknolojik-Kimyasal Yöntemlerle Üretim Tekniklerinin Geliştirilmesi (TÜBITAK) – *as Researcher*.

- Kamu Projesi (Sağlık Bakanlığı, Hacettepe Üniversitesi): RNA "Splicing" Hatalarının Neden Olduğu Kalıtsal Hastalıklarda İlaç Araştırma-Geliştirme Çalışmaları – *as Researcher*.