Mohan Pal (2011). ***New biocatalysts for asymmetric reduction of heteroaryl ketones and 1,2-Diketones and baeyer villiger oxidation of bicycle[3.2.0] hept-2-en-6-one.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
Supervisor: Dr. R.S.Jolly **Acc. No. : Th-251**

SUMMARY OF THE THESIS

Popularity of biotransformation route compared to chemical route has remained rather low, in spite of the economic and environmental benefits of the bio-route. Lack of the availability of stable strains or enzymes from the different enzyme classes ready for use in a screening for a new product has been a 'burning issue', which has prevented widespread use of the biotransformation route in process chemistry. Although the advantages of the use of enzymes in organic synthesis have been fully illustrated, not much attention has been paid to the systematic exploitation of microorganisms producing new enzymes. The isolation of microorganisms is very important to initiate the study of the microbial transformation of chemicals and exploit new enzymes, because there is wide biological diversity. The recent success in microbial transformation has been based on the screening for microbial enzymes catalyzing new reactions, or by screening known enzymes for an unknown activity with synthetic substrates.

India's landscape encompasses niches, such as deep forests, oceans, mangroves, glaciers, deserts, fresh water lakes, lake containing high salt concentration, etc. Each niche has its own unique microbial diversity. We have access to microbial strains isolated from these diverse niches. This thesis describes the results of our efforts at exploiting the rich microbial diversity of India for the isolation of new enzyme activities and their application in the synthesis of enantiomerically enriched compounds. The thesis has been divided into two independent parts, Part 1 deals with biocatalytic asymmetric reduction of heteroaryl ketones and 1,2-diketones and Part 2 with Baeyer-Villiger oxidation of (±)-*cis*-bicyclo[3.2.0]hept-2-en-6-one.

In summary, this thesis describes isolation of three novel biocatalysts, *Penicillium sp.*, *Alternaria alternata* and *Talaromyces flavus* for asymmetric reduction of heteroaryl ketones and Baeyer-Villiger oxidation of (±)-*cis*-bicyclo[3.2.0]hept-2-en-6-one by *Alternaria alternata*, (i) in addition, *Talaromyces flavus* emerged as a versatile biocatalyst for asymmetric reduction of 1,2-diketones, (ii) thesis also describes purification of dehydrogenase from *Talaromyces flavus* and a Baeyer-Villiger oxidase from *Alternaria alternata* and (iii) using these biocatalysts a number of chiral molecules have been prepared, mostly in high e.e. and high yield. Figure 1 gives a description of the library of chiral molecules, preparation of which has been described in this thesis.

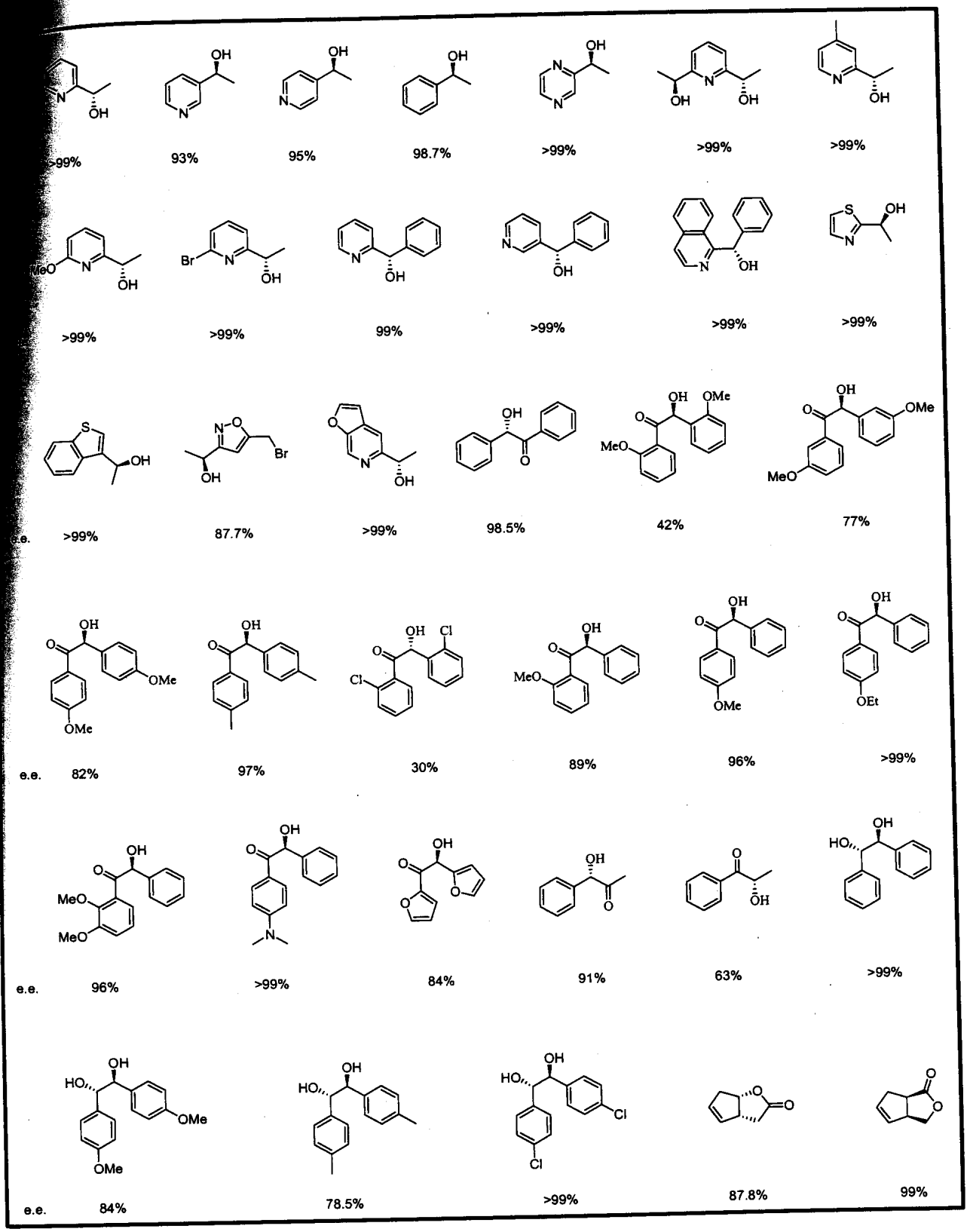


Figure 1: Library of chiral molecules, preparation of which has been described in this thesis

Section 1: Chemical and Biological Asymmetric Reduction of Ketones: A Review

A brief review on the synthesis of enantiomerically pure alcohols, using selected examples from literature has been provided in this section. Both chemical and biological methods of asymmetric ketone reduction to prepare enantiomerically pure alcohols have been discussed. Amongst the chemical methods, metal hydride reduction by improved oxazaborolidenes complexes, catalytic hydrogenations by ruthenium, iridium and iron complexes and hydrogen transfer reactions by ruthenium and iron complexes has been given special attention. Amongst the biological methods, different categories of dehydrogenases, their source and applications of dehydrogenases in synthesis of natural products/pharmaceutically relevant compounds have been discussed.

Part 1, Section 2: New Biocatalysts for Asymmetric Reduction of Heteroaryl Ketones

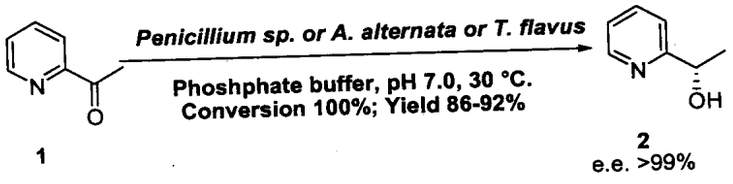
Optically pure chiral 1-heteroaryl-1-alkanols are buildings blocks for various bioactive molecules. Examples: Furo[2,3-c] pyridine thiopyrimidine ether is a clinical candidate for the treatment of HIV infection and AIDS,[1] 2-(2-*tert*-butylamino-1-hydroxyethyl)-benzofuran) acts as β-blocker,[2, 3] (*S*)-phenyl(pyridin-2-yl)methanol has analgetic property.[4] Similarly, several pharmacologically active alkaloids such as akuamidine and heteroyohimidine can be synthesized from 1-heteroaryl-1-alkanols.[5] Several commercial applications of optically pure chiral 1-heteroaryl-1-alkanols have been reported. These include, antihistamine and anticholinergic agent (*S*)-carbinoxamine (Clistin, Palgic),[6] (*S*)-duloxetine used for the treatment of psychiatric and metabolic disorders[7] and optically pure antimalarial drug (*1R*,2*S*)-Mefloquine.[8] Optically pure 1-heteroaryl-1-alkanols also act as chiral building blocks in the synthesis of optically active crown ethers[9] and chiral ligands, which have diverse applications.[10] Other than building blocks, optically pure 1-heteroaryl-1-alkanols are versatile reagents in asymmetric catalysis. Their use in catalytic applications include enantioselective addition of diethylzinc to aldehydes[11, 12] and epoxidation of olefins.[13]

Synthesis of heteroaryl alcohols is of immense industrial interest and biocatalytic ketone reduction is an attractive alternative to drastic chemical methods, not only in terms of environmental concerns but also in economics of the product formation. But inspite of the great synthetic potential that dehydrogenases offer, their utilization as a versatile biocatalyst has remained largely unexploited. The scarcity of suitable and well-characterized ketone reducing dehydrogenases is a barrier to their application. The objective of present work was to isolate and characterize new biocatalysts for enantioselective reduction of heteroaryl ketones with diverse applications. In this section, we report (i) isolation of new biocatalysts

metric reduction of heteroaryl ketone, (ii) purification of a dehydrogenase from a strain (iii) substrate specificity profile of the selected biocatalysts and (iv) ion of the biocatalyst in preparation of intermediates of bioactive compounds and fine als.

(*S*)-1-(Pyridin-2-yl)ethanol **(2)** is common intermediate for synthesis of various ive molecules and can in turn be prepared by enantioselective reduction of 2-pyridine **(1)**. A library of uncharacterized bacteria and fungi collected from a variety of s in India was available with us. We screened 468 bacterial and fungal cultures for their y to reduce **1** to corresponding alcohol **2** and isolated three strains, designated as F9, and S-70 produced desired alcohol **2** in >99% enantiomeric excess (Scheme 1). Fungal tes F9, 8197 and S-70 were identified by Dr. B. D. Shenoy and Dr. Ananthapadmanaban, Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh based morphomolecular characters. F9 has been identified as novel species belonging to genus *nicillium*. Similarly, strains 8197 and S-70 have been identified as *Alternaria alternata* and *laromyces flavus,* respectively.

cheme 1

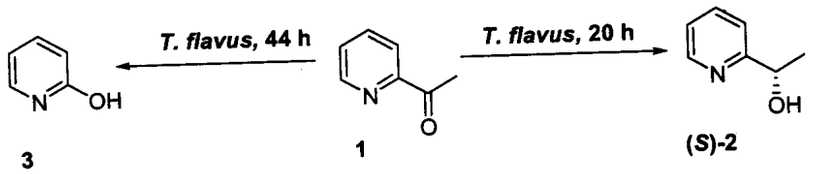


The enzyme responsible for activity has been purified by a 5-step procedure from *Talaromyces flavus*. Approximately 20.2 fold purification was required to obtain electrophoretically homogenous protein, which showed a single band at about ~40 kDa on SDS-PAGE run under reducing conditions. The pure protein reduced 2-acetylpyridine **(1)** to (*S*)-1-(pyridin-2-yl)ethanol **(2)** with e.e. of 96.2%. The molecular weight observed on MALDI was 36.4 kDa, on gel filtration column was 39.8 kDa and that on SDS-PAGE was ~40 kDa. Therefore, the dehydrogenase appears to be a monomer of ~36.4 kDa. The N-terminal sequence, APTNQ _AYDHSV obtained by Edman degradation did not show significant homology with any of reported dehydrogenase in the database.

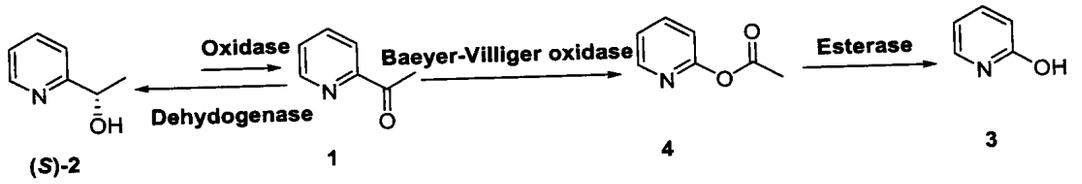
ration of either (*R*)- or (*S*)-2 using whole cells of *Talaromyces flavus*

During the course of enantioselective reduction of **1** by *Talaromyces flavus* it was d that when the reaction was carried out for 20 h, only formation of (*S*)-1-(pyridin-2-hanol **(2)** was observed in >99% e.e., but when the reaction was carried out for another h, **1** was completely converted to 2-pyridinol **(3)** in 44 h (Table 1, Scheme 2). The nation of **3** may be explained by invoking the presence of a Baeyer-Villiger oxidase in the le cells of *Talaromyces flavus* (Scheme 3).

eme 2



heme 3



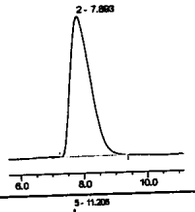
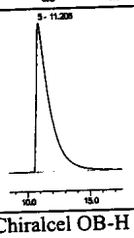
Next we studied the fate of *rac*-**2** on reaction with whole cells of *Talaromyces flavus*. he course of reaction was followed by HPLC and NMR. The results are summarized in able 2. Initially e.e. of (*R*)-**2** increased with increasing formation of 2-pyridinol (**3**). At 50% onversion to **3**, e.e. of (*R*)-**2** reached 98% (Entry 4, Table 2). These results indicate much aster oxidation of (*S*)-enantiomer in comparison to (*R*)-enantiomer of **2**. However, when the eaction was continued further, complete conversion of *rac*-**2** to **3** occurred in 168 h (Entry 5, able 2). Scheme 4 provides a plausible explanation for formation of (*S*)-**2**, (*R*)-**2** and **3**.

At initial stages of reaction, only reduction of **1** was observed, i.e. no Baeyer-Villiger product **4** could be seen. The reason for this observation is not clear. One explanation could be that substrate **1** at higher concentration is inhibitor of BVO enzyme. This path becomes important only after trace concentration of **1** are produced by oxidation of **2**.

It is important to note that both enantiomers of **2** are accessible using same biocatalyst i.e. whole cells of *Talaromyces flavus*. (*S*)-**2** was obtained in >99% e.e. by *Talaromyces*

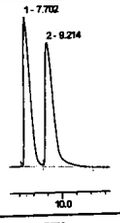
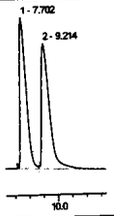
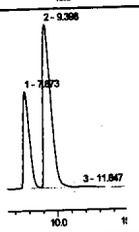
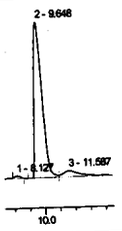
us catalyzed reduction of **1**, whereas (*R*)-**2** was obtained in 98% e.e. by selective dation of *rac*-**2**.

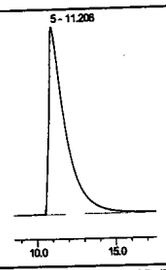
Table 1: *Talaromyces flavus* catalyzed reduction of 2-acetylpyridine **(1)**[a-c]

Entry	Reaction Time (h)	Chiral HPLC	% Conversion to 3	% e.e. of 2 (config.)
1	20		0	>99 (*S*)
2	44		100	--

[a]e.e. was determined by chiral HPLC on Chiralcel OB-H (Diacel, Japan) column. [b]% conversion was determined by ^{1}H NMR. [c]Absolute configuration was assigned based on comparison of sign of optical rotation with literature.

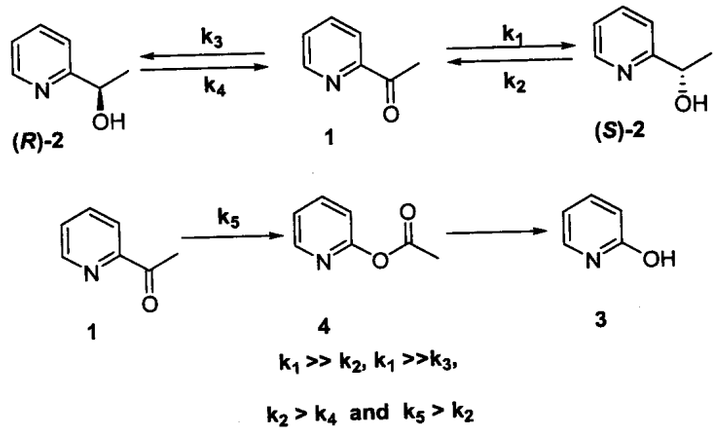
Table 2: *Talaromyces flavus* catalyzed conversion of *rac*-1-(pyridin-2-yl)ethanol **(2)** to 2-pyridinol **(3)** [a-c]

Entry	Reaction Time (h)	Chiral HPLC	% conversion to 3	% e.e. of left alcohol 2 (config.)
1	0		0	0
2	20		0	0
3	48		12.2	36.5 (*R*)
4	96		50	98 (*R*)

5	168		100%	---

[a]e.e. was determined by chiral HPLC on Chiralcel OB-H (Diacel, Japan) column. [b]% conversion was determined by ^{1}H NMR.
[c]Absolute configuration was assigned based on comparison of sign of optical rotation with literature.

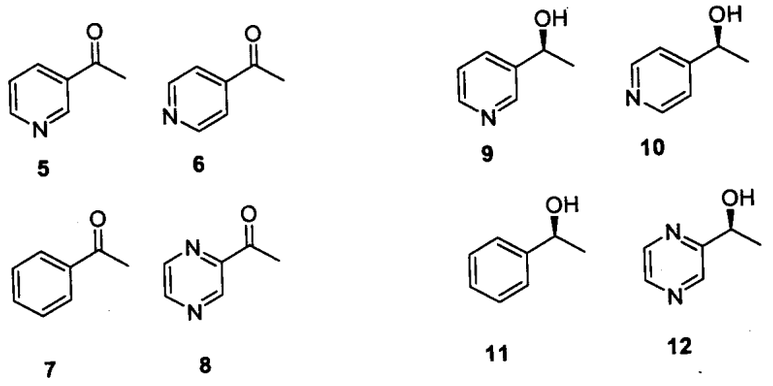
Scheme 4



***Penicillium sp., Alternaria alternata* and *Talaromyces flavus* catalyzed reduction of positional isomers of 2-acetylpyridine: Accessing role of nitrogen atom in catalysis and enantioselectivity**

Having successfully achieved asymmetric reduction of 2-acetylpyridine (**1**) in >99% e.e., the next goal was to study reduction of its positional isomers, viz., 3-acetylpyridine (**5**) and 4-acetylpyridine (**6**). All three strains reduced 3-acetylpyridine (**5**) to (*S*)-1-(pyridin-3-yl)ethanol (**9**) with 86-88% yield and 100% conversion. E.e. of the **9** obtained was in the range of 91-93% with these strains. Similarly 4-acetylpyridine (**6**) was reduced to (*S*)-1-(pyridin-4-yl)ethanol (**10**) by all the three strains with 100% conversion and ~95% e.e. We noticed a small drop in e.e. as the acetyl group moved away from N-atom of pyridine ring. To access the role of nitrogen atom, we studied the reduction of acetylbenzene (**7**), which lacked presence of nitrogen atom in aryl ring. All the three strains produced (*S*)-1-phenylethanol (**11**) in good yield but required much longer time for complete conversion. *Talaromyces flavus* gave very low e.e. of 25%, whereas *Penicillium sp.* gave moderate e.e. of 87.5%. Only *Alternaria alternata* gave good e.e. of 98.7%. With all the three strains, the rate of conversion of **7** was much lower compared to pyridine analogs. For example with *Penicillium sp.*, **1** required 4 h for 100% conversion, whereas **7** required 24 h. Also, the e.e. of the product **11** was poor with all strains except *A. alternata*. Thus, presence of N-atom in the ring appears to

nce the e.e. and rate of reaction. This was further confirmed from the results obtained n the reduction of acetylpyrazine (**8**). All three strains reduced **8** with high conversion rate high e.e. of >99%.

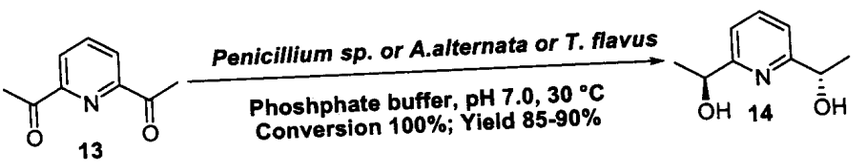


The role of nitrogen atom appears to be opposite to that observed in metal catalyzed symmetric reduction.[14, 15] The ability of nitrogen atom to co-ordinate with metal centre of chemo catalyst typically resulted in decreased enantioselectivity. Although, the reason for improved enantioselectivity due to nitrogen atom has not been investigated in detail, it may be speculated that co-ordination of nitrogen atom in biocatalyzed reactions through H-bond or otherwise may freeze the substrate within the enzyme cavity in proper orientation, which allows enantioface selective delivery of hydride from the bound cofactor. Presumably, proper orientation of substrate with respect to hydride source within the cavity can also explain enhanced rate of reaction for acetylpyridine as compared to acetylbenzene. Moreover, an increment in rate for 2-acetylpyridine is further possible because co-ordination of nitrogen is expected to inhibit stabilizing p-π interactions between nitrogen and carbonyl of acetyl group, thereby making carbonyl more reactive towards hydride reagents.

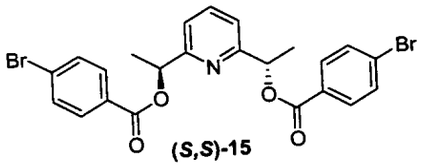
***Penicillium sp., Alternaria alternata* and *Talaromyces flavus* catalyzed reduction of 2,6-diacetylpyridine (13)**

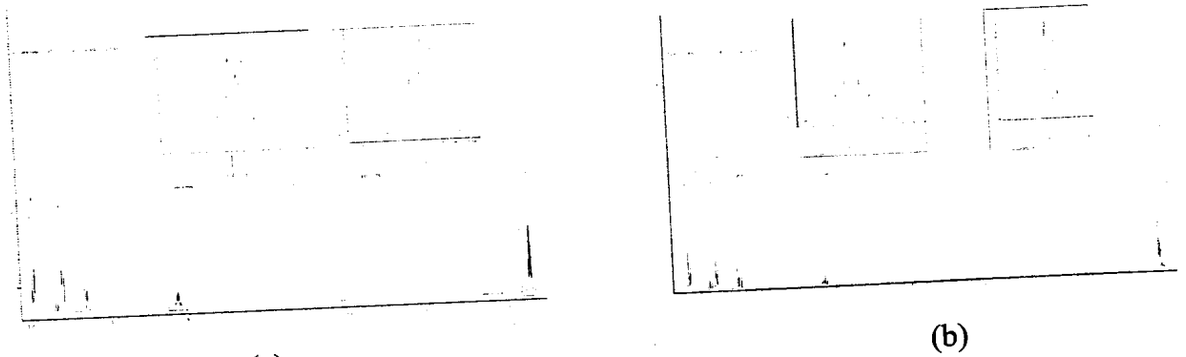
Enantiopure (*S,S*)-2,6-bis(1-hydroxy-ethyl)pyridines **(14)** are useful chiral building blocks in the synthesis of optically active crown ethers. *Penicillium sp., Alternaria alternata* and *Talaromyces flavus* were tested for enantio- and diastereoselective reduction of 2,6-diacetylpyridine **(13)**. All the three strains were able to reduce both carbonyl groups of **13** efficiently with 100% conversion and 85-90% yield to 2,6-bis(1-hydroxyethyl)pyridine **(14)** (Scheme 5).

eme 5



In order to determine the diastereomeric excess of **14**, it was converted to its *p*-omobenzoate ester (**15**). Similarly, *p*-bromobenzoate ester of diastereomeric mixture of **14**, epared by borohydride reduction of **13** was also synthesized for comparison. In ^{1}H NMR of astereomeric mixture **15**, the resonance for methyl and methine protons corresponding to o diastereomers clearly appeared as well separated peaks giving the expected *dl:meso* ratio f 1:1 (Figure 2a). The methine protons appeared as set of two q (J = 6.5 Hz) at δ 6.15 and 6.16. Similarly, methyl protons appeared as set of two d (J = 6.5 Hz) at δ 1.71 and 1.72. However ester of biocatalyzed product **15** showed only one q at δ 6.15 (J = 6.5 Hz) for methine protons and one doublet (J = 6.5 Hz) at δ 1.71 for methyl group (Figure 2b). Thus, based on NMR, the d.e. for biocatalyzed product was estimated to be 100%. E.e. of biocatalyzed diol **14** was determined by HPLC analysis using Chiralcel OD-H column. Based on HPLC results, *dl:meso* ratio for *Penicillium sp., Alternaria alternata* and *Talaromyces flavus* was found to be 99:1, 99:1 and 93:7 respectively. The e.e. of (*S,S*)-**14** was >99% with all the three strains.



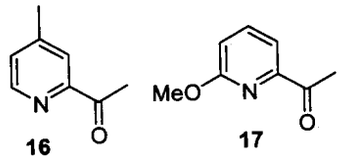


(a) (b)

Figure 2: ^{1}H NMR of *p*-bromobenzoate ester **15** (a) prepared from diastereomeric mixture of 2,6-bis(1-hydroxyethyl)pyridines **(14)** and (b) prepared from biocatalytic product 2,6-bis(1-hydroxyethyl)pyridines **(14)**

***Penicillium sp., Alternaria alternata* and *Talaromyces flavus* catalyzed reduction of substituted 2-acetylpyridines**

All the three strains, *Penicillium sp., Alternaria alternata* and *Talaromyces flavus* were able to reduce 2-acetyl-4-methylpyridine **(16)** to produce (*S*)-1-(4-methylpyridin-2-yl)ethanol **(19)** in 84-86% yield, >99% e.e. Next, we studied the reduction of 2-acetyl-6-methoxypyridine **(17)** with *Penicillium sp., Alternaria alternata* and *Talaromyces flavus.* All the three strains produced (*S*)-1-(6-methoxypyridin-2-yl)ethanol **(20)** in 86-89% yield. *Penicillium sp.* and *Talaromyces flavus* gave >99% e.e., but e.e. obtained with *Alternaria alternata* was 97.4%. Similarly 2-acetyl-6-bromopyridine **(18)** was reduced to (*S*)-1-(6-bromopyridin-2-yl)ethanol **(21)** by all the three strains. *Penicillium sp.,* and *Alternaria alternata* gave e.e. of >99%, whereas *Talaromyces flavus* gave e.e. of only 85.6%.



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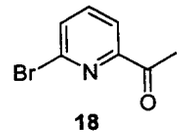

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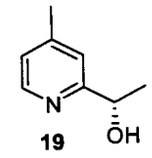

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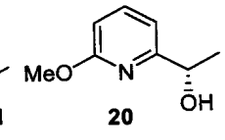

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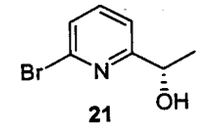

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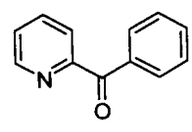
***Penicillium sp., Alternaria alternata* and *Talaromyces flavus* catalyzed reduction of aryl heteroaryl ketones**

The enantioselective hydrogenation of aryl heteroaryl and bis-heteroaryl ketones by biocatalytic means has not been studied much. The known biocatalysts generally produced alcohols with low e.e.[16] Keeping in view that many of compounds which belong to this class have diverse pharmacological properties, we tested our biocatalysts *Penicillium sp., Alternaria alternata* and *Talaromyces flavus* for reduction of diaryl ketones **(22-24)**.



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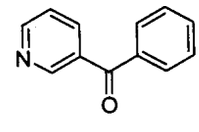

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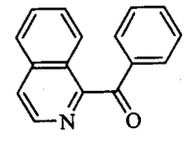

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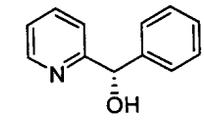

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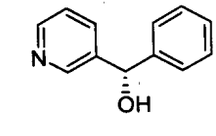

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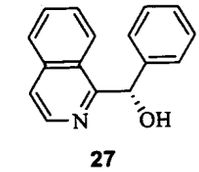

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Initially we studied *Penicillium sp.*, *Alternaria alternata* and *Talaromyces flavus* talyzed reduction of 2-benzoylpyridine **(22)** to corresponding alcohol **25**. (*S*)-henyl(pyridin-2-yl)methanol **(25)** has analgetic properties.[4] All the three strains were able to duce **22** to **25**. *Alternaria alternata* gave best results, producing **25** in 88% yield and 99% e. at 100% conversion. *Talaromyces flavus* gave poor results; not only the conversion rate as poor, alcohol **25** was produced in 21.7% e.e. It is important to note that, so far a naximum of 86% e.e. for **25** has been reported in literature by biocatalytic means using *Rhizopus arrhizus* and *Camellia sinensis* cell culture,[16] whereas *Alternaria alternata* roduced the product in 99% e.e.

Next, we studied the asymmetric reduction of 3-benzoylpyridine **(23)**. Although, all hree strains were able to reduce **23**, only *T. flavus* gave e.e. of >99%. The e.e. with *Penicillium sp.* and *Alternaria alternata* was 9.6% and 36% respectively. Finally, we showed asymmetric reduction of 1-isoquinolinylphenyl ketone **(24)** by whole cells of *Penicillium sp.*, *Alternaria alternata* and *Talaromyces flavus*. With *Penicillium sp.* and *Alternaria alternata*, e.e. of biocatalyzed product **27** was 77.6% and 76% at 60% conversion, respectively but with *Talaromyces flavus* >99% e.e. at 80% conversion was obtained. Thus *Talaromyces flavus* was found to be best biocatalyst for the preparation of (*S*)-phenyl(pyridin-3-yl)methanol **(26)** and (*S*)-isoquinolin-1-yl(phenyl)methanol **(27)** in high e.e. while *Alternaria alternata* was effective in preparation of (*S*)-phenyl(pyridin-2-yl)methanol **(25)** in high e.e. of >99%.

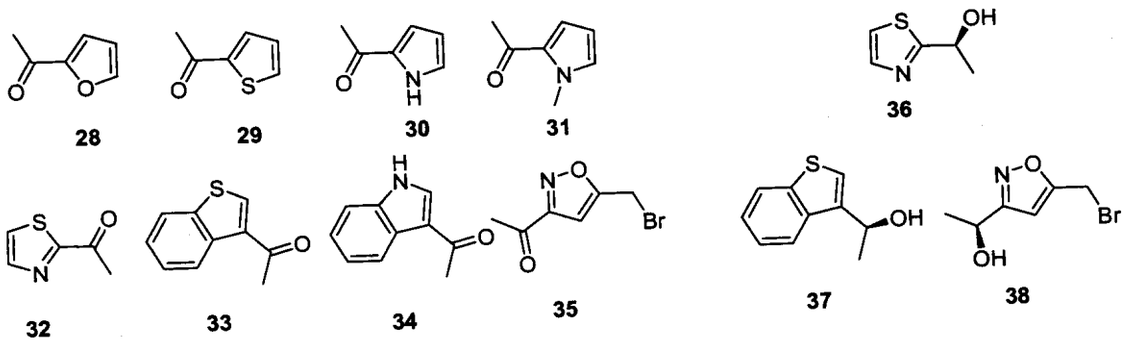
Penicillium sp., Alternaria alternata and _Talaromyces flavus_ catalyzed reduction of five membered heteroaryl ketones

In order to extend the scope of these bicatalysts we studied the reduction of various five membered heterocyclic ketones **(28-35)**. Five membered heterocycles 2-acetylfuran **(28)**, 2-acetylthiophene **(29)**, 2-acetylpyrrole **(30)**, *N*-methyl-2-acetylpyrrole **(31)** and 3-acetylindole **(34)** were not reduced by any of the biocatalyst. All three strains were able to reduce 2-acetyl thiazole **(32)** to desired alcohol **36**. Whereas, 100% conversion could be achieved with *Penicillium sp.* and *Alternaria alternata*, only 20% conversion occurred with *Talaromyces flavus* in 16 h. Only *Alternaria alternata* was able to give **36** in >99% e.e. at 100% conversion. The e.e. obtained with *Penicillium sp.* and *Talaromyces flavus* was 85% and 47% respectively.

All three strains were able to reduce bicylic heteroaryl ketone 3-acetylthianapthene **(33)** to desired alcohol **37**. Whereas 100% conversion could be achieved with *Penicillium sp.*

n 36 h, 50% conversion was observed with *Alternaria alternata* and *Talaromyces flavus in* 48 h. All the three strains produced (*S*)-**37** in >99% e.e.

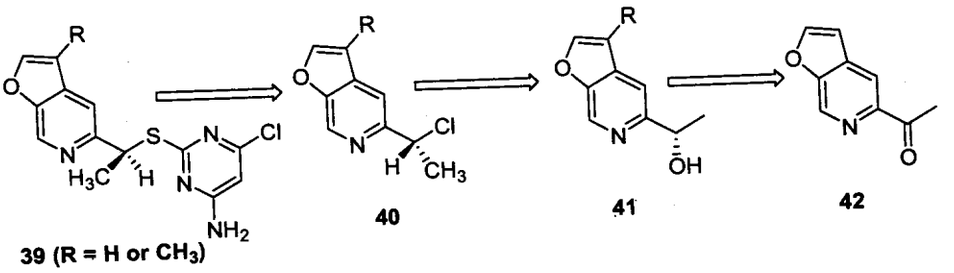
Isoxazole is another category of heterocycles which are important building blocks for a wide variety of compounds such as α, β-unsaturated oximes, β-hydroxy ketones and γ-amino alcohols. We synthesized isoxazole 1-(5-(bromomethyl)isoxazol-3-yl)ethanone **(35)** according to literature method.[17] *P. sp.* and *T. flavus* reduced **35** to (*S*)-1-(5-(bromomethyl)isoxazol-3-yl)ethanol **(38)** with 100% conversion as confirmed by ^{1}H NMR. The e.e. was obtained in the range of 84.6-87.7%. Although starting material completely disappeared on reaction with *A.alternata*, we failed to isolate desired alcohol from the reaction.



Application of *Penicillium sp., Alternaria alternata* and *Talaromyces flavus* in synthesis of (*S*)-5-(1-hydroxyethyl)furo[2,3-c]pyridine (41): A key intermediate for HIV-1 reverse transcriptase inhibitors PNU-142721 and PNU-109886

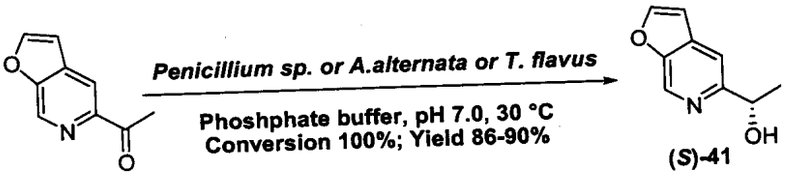
Furo[2,3-c]pyridine thiopyrimidine ethers PNU-142721 or PNU-109886 **(39)** have been described as HIV-1 reverse transcriptase inhibitors. The (*S*)-enantiomer of these compounds was found to be at least 100 times more potent than the corresponding (*R*)-enantiomer against variant virus. Retrosynthetic scheme for **39** shows the requirement of (*S*)-5-(1-hydroxyethyl)furo[2,3-c]pyridine **(41)** as a key intermediate in the synthesis of **39** which can in turn be synthesized by enantioselective reduction of **42** (Scheme 6).[1]

Scheme 6

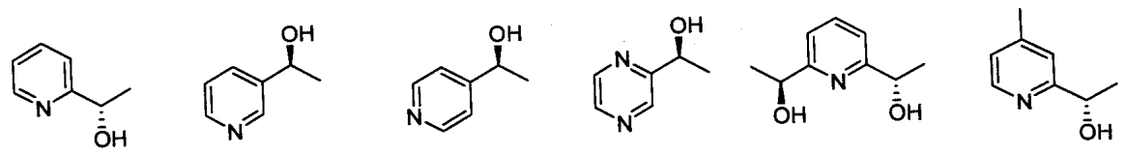


To test our newly discovered biocatalysts *Penicillium sp., Alternaria alternata* and *Talaromyces flavus*, we synthesized **42** according to procedure of Whiska *et al.*[1] All the three biocatalysts were able to reduce **42** to **41** with 100% conversion and 86-90% yield (Scheme 7). All the three strains gave e.e. of >99% and the absolute configuration was assigned as *S* based on optical rotation $[\alpha]_D^{25}$ = -35.1 (c 0.51, $CHCl_3$) [lit. $[\alpha]_D$ = -35.8 (c 0.51, $CHCl_3$) 99.9% e.e. (*S*)].[1] Preparative scale reaction showed that *Penicillium sp.* reduced 10 mM substrate in 4.5 h, *Alternaria alternata* took 14 h and *Talaromyces flavus* took 12 h for 100% conversion.

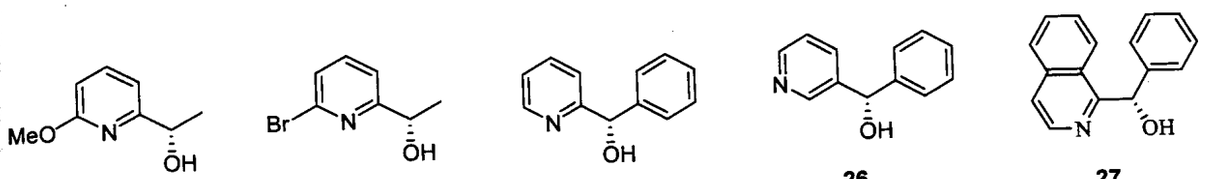
Scheme 7



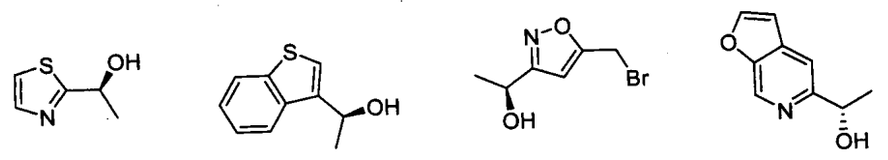
In conclusion, we have (i) described isolation of three new biocatalysts, which reduced 2-acetylpyridine to corresponding (*S*)-alcohol in >99% e.e. and >90% yield, (ii) identified the selected biocatalysts as *Penicillium sp., Alternaria alternata* and *Talaromyces flavus*, (iii) purified protein responsible for activity from *T. flavus*. The protein was found to be a monomer of ~36.4 kDa, (iv) N-terminal sequence obtained from the purified protein by Edman degradation did not show significant similarity with any of the known dehydrogenase in database. The protein, therefore appears to be novel, (v) all three biocatalysts were able to reduce a range of acetylpyridines, aryl heteroaryl ketones and 5-membered heteroaryl ketones, (vi) Using appropriate biocatalyst a library of following chiral heteroaryl alcohols was prepared in good to excellent e.e. and 86-92% yield. All the reactions were done on preparative scale and structure of all the compounds was established by NMR and (vii) (*S*)-**41** obtained in >99% e.e. and 90% yield is a crucial intermediate for HIV-1 reverse transcriptase inhibitors PNU-142721 and PNU-109886.



	2	9	10	12	14	19
e.e.	>99%	93%	95%	>99%	>99%	>99%



	20	21	25	26	27
e.e.	>99%	>99%	99%	>99%	>99%



	36	37	38	41
e.e.	>99%	>99%	87.7%	>99%

Part 1, Section 3: Biocatalytic Asymmetric Reduction of 1,2-Diketones to α-Hydroxyketones or 1,2-Diols

α-Hydroxyketones are key intermediates in the synthesis of compounds of pharmaceutical interest such as antifungal agents, antitumor antibiotics (Olivomycin A, Chromomycin A3, and epothilones),[18] selective inhibitors of amyloid-β-protein (for Alzheimer's disease treatment),[19] farnesyl transferase inhibitors Kurasoin A and B,[20] antidepressant drugs (bupropion and its metabolites),[21] urease inhibitors,[22] etc. In addition, α-hydroxyketones can be easily converted to α-aminoalcohols and 1,2-diols which are precursors of various biologically active molecules as well as chiral ligands and auxillaries in stereoselective organic synthesis. This prompted us to explore our newly discovered biocatalysts *Penicillium sp., Alternaria alternata* and *Talaromyces flavus* for asymmetric reduction of 1,2-diketones.

This section of thesis reports (i) application of *T. flavus* as a versatile biocatalyst for regio- and enantioselective reduction of 1,2-diketones to either α-hydroxyketones or 1,2-diols in high e.e. and high yield and (ii) evidence in support of the involvement of multi-enzyme

stem to explain pH dependent selective formation of either α-hydroxyketones or 1,2-diols *T. flavus* catalyzed asymmetric reduction of 1,2-diketones.

Enantioselective reduction of benzil (43) to benzoin (44) and hydrobenzoin (45) by *Penicillium sp., Alternaria alternata* and *Talaromyces flavus*

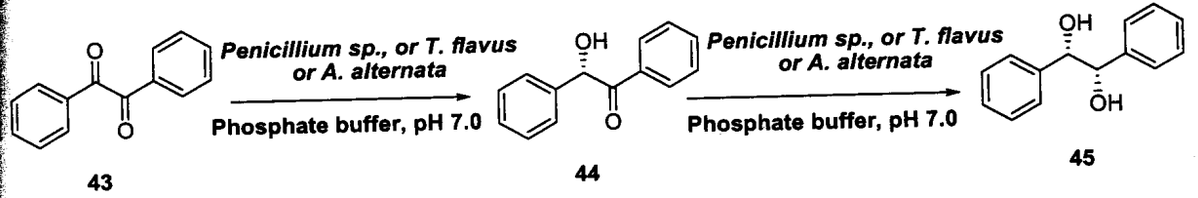
In previous section, we have described three biocatalysts *Penicillium sp., Alternaria alternata* and *Talaromyces flavus* for enantioselective reduction of heteroaryl ketones. We tested all three strains for enantioselective reduction of benzil **(43)**. All the three strains were able to reduce **43** to mixture of benzoin **(44)** and hydrobenzoin **(45)** in varying ratio (Scheme 8; Table 3). The ratio of **44** to **45** was determined by ^{1}H NMR. The single methine proton of pure *rac*-**44** appeared as bs at δ 5.95. **45** prepared by sodium borohydride reduction of **43** showed two bs at δ 4.72 and 4.84 corresponding to two methine protons of *dl* and *meso* pair. The ratio of **44/45** in biocatalyzed product in the reaction mixture was determined from integral values of resonance peaks at δ 5.95, 4.72 and 4.84. The biocatalytic reduction was carried till 100% consumption of **43** using 5 mM substrate concentration.

The e.e. of **44** was determined by chiral HPLC using Chiralcel OD-H column. *T. flavus* gave e.e. of >99% (Entry 3, Table 3), whereas *Penicillium sp.* and *A. alternata* gave e.e. of 95% and 91%, respectively (Entry 3, Table 3). The absolute configuration of *Talaromyces flavus* catalyzed product **44** was assigned as (*S*) based on the comparison of optical rotation data with literature . $[\alpha]_D^{25} = +114.8$ (c 1.5, acetone) [lit. $[\alpha]_D^{19} = +115.0$ (c 1.5, acetone), 99% e.e. (*S*)].[23]

The *dl/meso* ratio of **45** was determined by ^{1}H NMR while e.e. of **45** was determined by chiral HPLC using Chiralcel OJ column. A sample of *dl/meso*-**45** prepared by sodium borohydride reduction of **43** on chiral HPLC using Chiralcel OJ column resolved into three peaks with retention time of 16.2, 18.8 and 22.6 min. The peaks at 16.2 and 18.8 min appeared in almost 1:1 ratio and were assigned to *dl* pair of **45**. The peak eluting at 22.6 min was therefore assigned to *meso*-**45**. The ratio of *dl/meso* in borohydride reaction was found to be 5:95, indicating the predominant formation of *meso* product in the reaction. Similar ratio was also obtained by comparison of integral values of resonance peaks at δ 4.84 and 4.72, corresponding to methine protons of *meso* and *dl* pair, respectively in ^{1}H NMR of the **45** obtained from borohydride reduction of **43**. Thus, *dl/meso* ratio in all cases was calculated from ^{1}H NMR. In contrast to predominant formation of *meso* product in borohydride reduction, the biocatalyzed reduction produced *dl* as the major product. The ratio of *dl/meso*

btained from *Penicillium sp.*, *A.alternata* and *T. flavus* was 65/35, 94/6 and 80/20, espectively (Table 3). The e.e. of *dl* pair of **45** was also determined. *T. flavus* gave e.e. of 9% while the e.e. of 93 and 85% was obtained in case of *Penicillium sp. and A. alternata* espectively. The absolute configuration of *T. flavus* catalyzed **45** was assigned as (1*S*,2*S*) based on comparison of optical rotation data of *T. flavus*. [$[\alpha]_D^{25}$ = -91.0 (c 1.05, ethanol)] [lit. $[\alpha]_D^{25}$ = +91.6 (c 1.05, ethanol), 99.9% e.e. (*R*,*R*)].[24, 25] Thus in *dl* pair the peak eluting at 16.2 min correspond to (1*S*,2*S*) configuration (Figure 3a).

Scheme 8



43 44 45

Table 3. Enantioselective reduction of benzil **(43)** to benzoin **(44)** and hydrobenzoin **(45)** by *Penicillium sp., Alternaria alternata* and *Talaromyces flavus*[a-c]

Entry	Strain	% Conv.	Reaction time (h)	pH	Ratio of 44:45	Benzoin (44)		Hydrobenzoin (45)	
						Absolute config.	% e.e.	*dl/meso*	% e.e. (config.)
1	*Penicillium sp.*	100	24	7.0	1:0.21	*S*	95	65/35	93 (*S*,*S*)
2	*A. alternata*	100	18	7.0	1:1.53	*S*	91	94/6	85 (*S*,*S*)
3	*T. flavus*	100	37	7.0	1:0.94	*S*	>99	80/20	99 (*S*,*S*)

[a]% Conversion, ratio of **44:45** and *dl/meso* ratio was determined by ^{1}H NMR. [b]e.e. of **44** and **45** was determined by chiral HPLC on Chiralcel OD-H and OJ (Diacel, Japan) column, respectively. [c]Absolute configuration was assigned based on comparison of sign of optical rotation with literature.

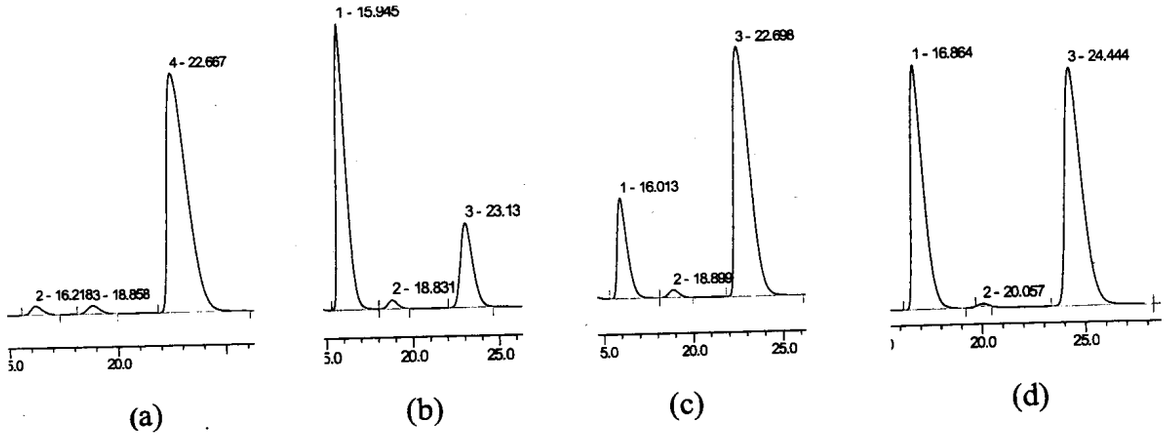


Figure 3: HPLC profiles of **45** obtained from (a) borohydride reduction (b) *Penicillium sp.* (c) *Alternaria alternata* and (d) *Talaromyces flavus* catalyzed reduction

Effect of pH on *Talaromyces flavus* catalyzed asymmetric reduction of benzil (43)

Demir *et al.* have reported very interesting effect of pH on *Rhizopus oryzae* catalyzed reduction of **43**.[26, 27] In view of this, we studied the effect of pH on *T. flavus* catalyzed asymmetric reduction of **43**. The reactions were done at 5 mM substrate concentration at various pH between 4.0 and 9.0 in appropriate buffers. Aliquotes were drawn at 14, 37, 144, and 264 h and formation of **44** and **45** was studied by ^{1}H NMR and chiral HPLC as described above. The results have been summarized in Table 4. It was observed that (i) at pH 4.0-6.0, **44** was formed as major product in high e.e. and (*S*)-configuration; only negligible amount of **45** was produced (ii) the amount of **45** increased with increasing pH. At pH 7.0, almost 1:1 ratio of **44** to **45** was observed at 100% conversion. While e.e. of benzoin **(44)** was >99%, hydrobenzoin was also produced in good *dl/meso* ratio of 80/20 with >99% e.e. (Entry 14, Table 4) and (iii) partial racemization of (*S*)-**44** was observed at all pH when the reaction was continued for longer duration and (iv) at pH 4.0 and 5.0, not only diol was formed in poor ratio, it gave poor *dl/meso* ratio. The diol was formed in poor e.e. and had (*R,R*) configuration, whereas, at pH 6.0-8.0, (*S,S*)-diol was produced in high e.e.

In summary, pH 5.0 was found to be optimal for production of (*S*)-**44** (Entry 6, Table 4; Scheme 9) and pH 7.0 for (*S,S*)-**45** (Entry 14, Table 4; Scheme 9). In fact, when the substrate concentration was reduced to 2 mM, the reaction at pH 7.0 resulted in exclusive production of **45** in *dl/meso* ratio of 94/6 and >99% e.e. with (*S,S*) configuration (Scheme 9). Thus, depending on pH either (*S*)-**44** or (*S,S*)-**45** can be selectively obtained in high e.e. and high *dl/meso* ratio. In contrast to *Rhizopus oryzae* we did not observe pH dependent reversal of enantioselectivity with *T. flavus*. However, we did observe drop in e.e. of (*S*)-**44** when the reaction was continued for longer period of 144-264 h. (*S*)-**44** did not show any racemization at pH 4.0 or 9.0 in absence of biocatalyst. These results do suggest presence of racemase activity in *T. flavus*.

Scheme 9

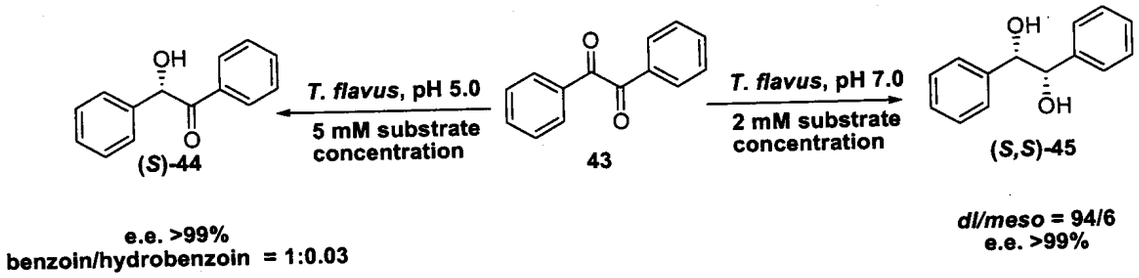


Table 4: Effect of pH on *Talaromyces flavus* catalyzed asymmetric reduction of benzil **(43)**[a-c]

Entry	pH	R.Time (h)	% Conv.	44:45	% e.e. of 44 (config.)	*dl:meso*	% e.e. of 45 (config.)
1	4.0	14	22	1:0	97 (*S*)	n.d.	n.d.
2		**37**	**29**	**1:0**	**97 (*S*)**	**n.d.**	**n.d.**
3		144	90	1:0.025	86 (*S*)	33/67	33.3 (*R,R*)
4		264	100	1:0.08	70 (*S*)	25/75	45.9 (*R,R*)
5	5.0	14	66	1:0	97 (*S*)	n.d.	n.d.
6		**37**	**100**	**1:0.035**	**99 (*S*)**	**43/57**	**91 (*S,S*)**
7		144	100	1:0.94	96 (*S*)	47/53	2.5 (*S,S*)
8		264	100	1:1.3	74 (*S*)	52/48	35 (*R,R*)
9	6.0	14	80	1:0.07	>99 (*S*)	56/44	**>99** (*S,S*)
10		**37**	**100**	**1:0.393**	**>99 (*S*)**	**75/25**	**>99 (*S,S*)**
11		144	100	1:1.2	96 (*S*)	88/12	>99 (*S,S*)
12		264	100	1:1.6	78 (*S*)	88/12	97.2 (*S,S*)
13	7.0	14	50	1:0.392	>99 (*S*)	80/20	>99 (*S,S*)
14		**37**	**100**	**1:0.94**	**>99 (*S*)**	**80/20**	**>99 (*S,S*)**
15		144	100	1:1.76	82 (*S*)	82/18	>99 (*S,S*)
16		264	100	1:2.14	81 (*S*)	84/16	97.6 (*S,S*)
17	8.0	14	65	1:0.174	>99 (*S*)	77/23	>99 (*S,S*)
18		**37**	**100**	**1:0.441**	**>99 (*S*)**	**78/22**	**>99 (*S,S*)**
19		144	100	1:1.07	80 (*S*)	83/17	96.2 (*S,S*)
20		264	100	1:1.26	76 (*S*)	88/12	63.7 (*S,S*)
21	9.0	14 h	65	1:0.25	>99 (*S*)	85/15	>99 (*S,S*)
22		**37**	**98**	**1:0.51**	**>99 (*S*)**	**80/20**	**96 (*S,S*)**
23		144	100	1:0.95	76 (*S*)	80/30	93.7 (*S,S*)
24		264	100	1:1	71 (*S*)	86/14	78.2 (*S,S*)

[a]% Conversion, ratio of **44:45** and *dl/meso* ratio was determined by ^{1}H NMR. [b]e.e. of **44** and **45** was determined by chiral HPLC on Chiralcel OD-H and OJ (Diacel, Japan) column, respectively. [c]Absolute configuration was assigned based on comparison of sign of optical rotation with literature. n.d. = not detected

In order to confirm presence of deracemase activity in *T. flavus*, we studied the reduction of *rac*-**44** with *T. flavus* as catalyst at various pHs in the range of 4.0-9.0 in appropriate buffers. The results are summarized in Table 5. The following observation made from Table 5 may be highlighted (i) whereas no change occurred at pH 4.0, the reaction at pH 5.0 was very sluggish, (ii) at pH 6.0-9.0 and at about 50% conversion, the (*S*)-**44** was recovered in ~30% e.e. and, **45** was obtained in *dl/meso* ratio of approximate 3/7 (Scheme 10). The results suggests that both (*S*) and (*R*)-**44** are substrate for reductase enzyme, but (*R*)-enantiomer reacts faster than (*S*)-enantiomer, which made partial resolution of *rac*-**44**

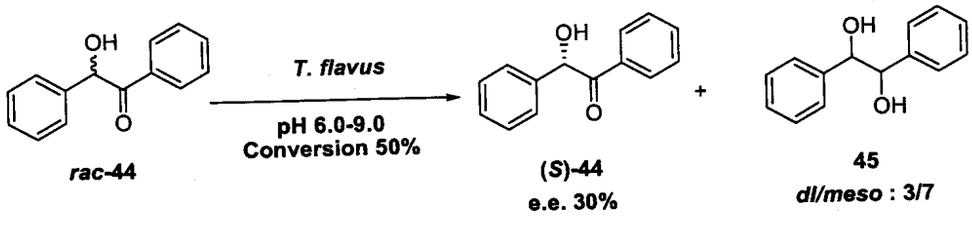
possible at about 50% conversion, (iii) it was interesting to note that when the reaction was continued further at pH 7.0-9.0, complete conversion of **44** to **45** occurred to give *dl/meso* ratio of 3:7 in about 24 h. However, continuation of reaction for another 72 h, the ratio of *dl/meso* changed to 1:1; the enantiomeric excess of *dl* form was 96-98% (Scheme 11). Continuation of the reaction for longer times or addition of fresh cells did not change *dl/meso* ratio. These results may be explained by invoking presence of an oxidase activity, which converted **45** to *rac*-**44**, which was re-reduced to hydrobenzoin. However, attempts to recover *rac*-**44** from this reaction were not successful (Scheme 12).

Table 5: pH dependent reduction of *rac*-benzoin **(44)** by *Talaromyces flavus*[a-c]

Entry	pH	R.Time (h)	% Conv	% e.e. of unreacted 44 (config.)	*dl:meso* of 45	% e.e. of 45 (config.)
1	4	24	0	n.d.	n.d.	n.d.
2		96	0	n.d.	n.d.	n.d.
3	5	24	33	15	26/74	78.5 (*S,S*)
4		96	48	22	30/70	85 (*S,S*)
5	6	12	50	32	30/70	86 (*S,S*)
6		24	100	--	23/77	93.5 (*S,S*)
7		**96**	**100**	--	**50/50**	**97.9 (*S,S*)**
8	7	12	48	30	28/72	84.6 (*S,S*)
9		24	92	--	30/70	95.4 (*S,S*)
10		**96**	**100**	--	**50/50**	**98.5** (*S,S*)
11	8	12	53	29	26/64	85.9 (*S,S*)
12		24	100	--	28/72	93.9 (*S,S*)
13		**96**	**100**	--	**47/53**	**96.7** (*S,S*)
14	9	12	47	31	26/64	83.8 (*S,S*)
15		24	100	--	28/72	94.2 (*S,S*)
16		96	100	--	50/50	95.6 (*S,S*)

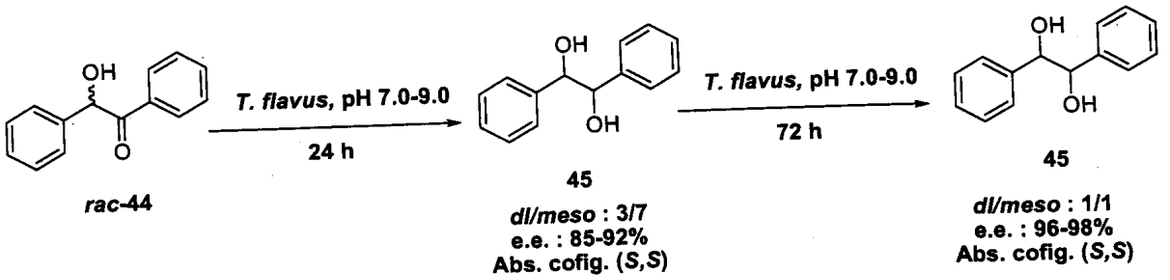
[a]% Conversion and *dl/meso* ratio was determined by ^{1}H NMR. [b]e.e. of **44** and **45** was determined by chiral HPLC on Chiralcel OD-H and OJ (Diacel, Japan) column, respectively. [c]Absolute configuration was assigned based on comparison of sign of optical rotation with literature. n.d. = not determined.

Scheme 10

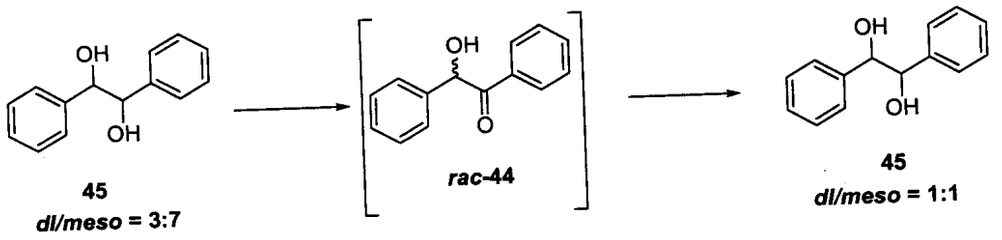


rac-44 → (*S*)-44, e.e. 30% + **45**, *dl/meso* : 3/7

Scheme 11



Scheme 12



Evidence in support of involvement of multi-enzyme system in *T. flavus* catalyzed reduction of benzil (43)

T. flavus catalyzed bioreduction of benzil **(43)** resulted in (i) formation of (*S*)-**44** in >99 e.e. at pH 5.0 and (ii) (*S,S*)-**45** in >99% e.e. along with *meso*-**45** in 94:6 ratio at pH 7.0. In principle, these results may be explained by invoking a single enzyme, which has pH dependent substrate specificity or by invoking two enzyme system, each of which has different pH profile for activity. In addition to above pH dependent activity, conversion of *meso*-**45** to *dl*-**45** was also observed, which in all probably should involve oxidation of an hydroxyl group to keto group to generate *rac*-**44** as intermediate. Put together, these results indicate the presence of multiple enzyme system in *T. flavus*, which is involved in the reduction of **43**. Thus, we fractionated proteins of *T. flavus* in an attempt to isolate various activities as shown in Figure 4. SDS-PAGE, run under reducing conditions for F2 and F3 are shown in Figure 5. F3 exhibited only one band at ~40 kDa in SDS-PAGE (Figure 5a).

All the fractions, including the pure protein were studied for asymmetric reduction of **43**, *rac*-**44** and (*S*)-**44** at pH 5.0 and 7.0. The results are summarized in Table 6 and Table 7. The presence of following enzymes may be inferred from these results.

(*S*)-selective benzil reductase

Fraction F2 converted benzil **(43)** to benzoin **(44)** with (*S*)-selectivity at pH 5.0 as well as pH 7.0 (Entry 5 and 6, Table 6). No trace of hydrobenzoin **(45)** could be detected in

these reactions, which clearly shows that F2 is devoid of any benzoin reductase activity. However, lower e.e. of 91% was obtained with F2 compared to 98.5% obtained with whole cells at pH 5.0. The improved e.e. in whole cells is likely to be due to conversion of (*R*)-**44** to *meso*-**45** by benzoin reductase activity present in whole cells, but absent in Fraction F2.

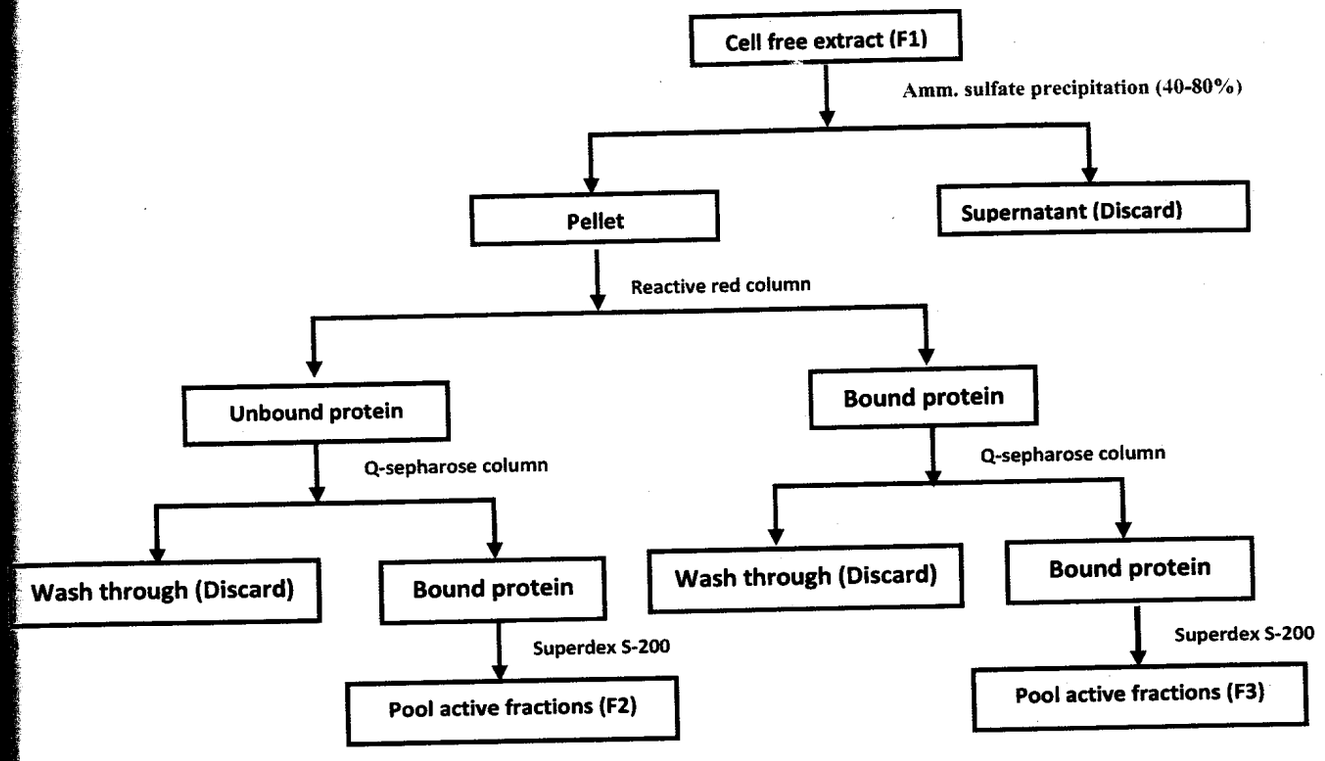
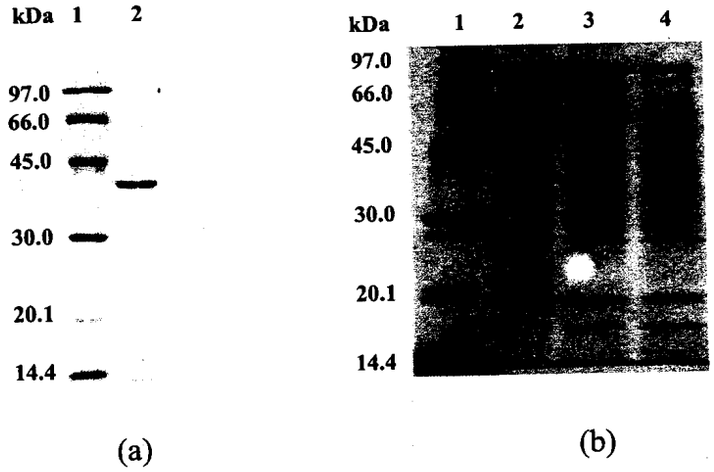


Figure 4: Summary of attempted fractionation of proteins of *T. flavus*. Fraction 1 to 3 were used for asymmetric reduction of benzil **(43)**, *rac*-benzoin **(44)** and (*S*)-benzoin **(44)**



(a) (b)

Figure 5: SDS-PAGE of Fraction 2 and 3 (Figure 6) run under reducing conditions (a) Fraction 3; *Lane 1:* molecular wt. markers; *Lane 2:* purified protein. (b) Fraction 2; *Lane 1;* molecular wt. markers, *Lane 2, 3 and 4* are different fractions from size exclusion chromatography step.

Table 6: Asymmetric reduction of benzil **(43)** and *rac*-benzoin **(44)** with protein Fraction 1 to 3 (Figure 4) obtained from *T. flavus*[a-c]

ntry	Fraction	pH	Benzil (43) as substrate				*rac*-benzoin (44) as substrate			
			% Conv.	e.e. of 44 (config.)[2]	% 45 (*dl/meso*)[2]	% e.e. of 45 (config.)	% Conv.	% e.e. of 44 (config.)	45 (*dl/meso*)	% e.e. of 45 (config.)
1	Whole cells	5.0	100	98.5 (*S*)	~3	--	48	28 (*S*)	26/74	75 (*S,S*)
2		7.0	100	n.d.	100 (94/6)	>99 (*S.S*)	100	n.d.	50/50	97 (*S,S*)
3	F1	5.0	20	90 (*S*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4		7.0	40	86 (*S*)	7 (78/22)	80 (*S,S*)	15	11 (*S*)	16/84	86 (*S,S*)
5	F2	5.0	50	91 (*S*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6		7.0	50	96 (*S*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	F3	5.0	<5	48 (*S*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8		7.0	16	75 (*S*)	20 (100:0)	>99 (*R,R*)	20	36 (S)	99/1	>99 (*R,R*)
9		7.0	25	6 (*R*)	33 (100:0)	>99 (*R,R*)	50	25 (S)	100:0	>99 (*R,R*)

[a]% Conversion and *dl/meso* ratio was determined by ^{1}H NMR. [b]e.e. of **44** and **45** was determined by chiral HPLC on Chiralcel OD-H and OJ (Diacel, Japan) column, respectively. [c]Absolute configuration was assigned based on comparison of sign of optical rotation with literature. n.d. = not determined.

Table 7: Asymmetric reduction of (*S*)-benzoin with various fractions of protein of *T.flavus*[a-c]

Entry	Fraction	pH	*S*-Benzoin (44) (>99% e.e.) as substrate			
			% Conv.	% e.e. of unreacted 44 (config.)	45 (*dl/meso*)	% e.e. of 45 (config.)
1	Whole cells	7.0	80	100 (*S*)	80:20	>99 (*S,S*)
2	F3	7.0	50	32 (*S*)	100 :0	53% (*S, S*)

[a]% Conversion and *dl/meso* ratio was determined by ^{1}H NMR. [b]e.e. of **44** and **45** was determined by chiral HPLC on Chiralcel OD-H and OJ (Diacel, Japan) column, respectively. [c]Absolute configuration was Assigned based on comparison of sign of optical rotation with literature.

(*R,R*)-selective benzoin reductase

The pure protein (F3, Entry 8 and 9, Table 6) showed (*R,R*)-selectivity for reduction of **43** at pH 7.0. F3 produced only (*R,R*)-**45**, no trace of (*S,S*)-**45** or *meso*-**45** could be detected either by NMR or HPLC. However, the conversion rate for this enzyme was very poor compared to whole cell requiring 24 h for 25% conversion compared to whole cells. Also, the (*R,R*)-selectivity is not strict, because when (*S*)-**44** was used as substrate (*S,S*)-**45** was

produced in 53% e.e. (Entry 2, Table 7). This results indicate that pure protein (F3) also has epimerase activity. F3 did not show any appreciable activity at pH 5.0.

(*S*)-benzoin to (*R*)-benzoin epimerase activity

When, the pure protein (F3, Entry 2, Table 7) catalyzed conversion of (*S*)-**44** (>99% e.e.) to **45** was stopped at 50% conversion, the e.e. of remaining (*S*)-**44** was found to be only 32%. This clearly suggested that the pure protein (F3, Entry 2, Table 7) in addition to benzoin reductase activity also has *S* to *R* epimerase activity. However conversion rate for this activity was also very poor.

(*S,S*)-selective benzoin reductase

Whole cell contain a very strong (*S,S*)-selective benzoin reductase activity at pH 7.0, but not at pH 5.0. However this activity could not be recovered from any of the fractions except F1 (Entry 4; Table 6), which showed trace amount of the activity, clearly indicating that enzyme responsible for this activity is unstable outside the cell. This is inspite of the presence of protease inhibitor in buffer at the time of preparation of cell free extract from whole cells. Several attempts to isolate protein corresponding to this activity were unsuccessful.

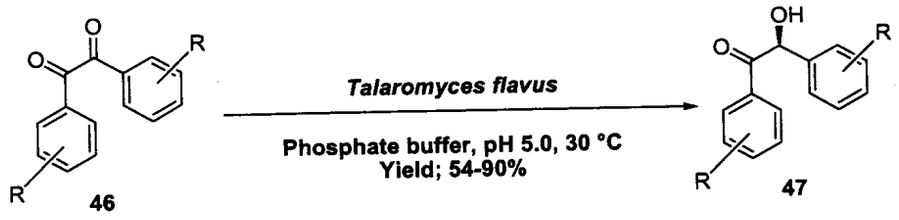
A case for whole cell vs pure enzyme

The use of pure enzyme in carbonyl reduction has advantage that it produces products in high quality and yield, but suffers from the drawback that cofactor and cofactor recycling system has to be added to derive the reaction to completion. The whole cell system provides cofactor recycling within cells, but suffers from disadvantage in terms of product quality and/or yield because of the presence of competing activities within the cells. However, in case of *T. flavus* the conversion rate, product quality and yields obtained were much better with whole cells compared to purified or semi-purified enzyme system. Apparantly, the competing activities are balanced in the favour of desired products in case of *T. flavus* catalyzed asymmetric reduction of benzil **(43)**.

***Talaromyces flavus* catalyzed asymmetric reduction of symmetrical benzil derivatives**

In order to study the scope of *T. flavus* catalyzed reduction of benzil, various symmetrical benzil derivatives **46** were synthesized. In all examples, *T. flavus* reduced **46** to give corresponding benzoin **47** in 54-90% yield (Scheme 13).

Scheme 13



Entry	R	% e.e. (config.)	% Yield	Reaction Time (h)
1	H	98.5 (*S*)	90	30
2	2-OCH_3	42 (*S*)	90	96
3	3- OCH_3	77 (*S*)	85	96
4	4- OCH_3	82 (*S*)	54	96
5	4-Me	97 (*S*)	84	96
6	4-EtO	n.r.	n.r.	n.r.
7	2-Cl	30 (*R*)	62	96

n.r.= no reaction

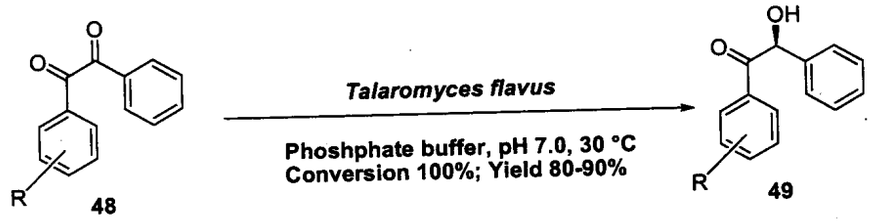
The following observations are worthy of note, (i) the substitution of phenyl ring with +I or –I substituent considerably slowed down the reaction. Whereas, benzil was reduced in 30 h, substituted benzil typically required about 96 h for the reaction to go to completion. No reduction occurred in case of 4-ethoxy substituent, (ii) the substitution of electron donating methoxy group or electron withdrawing chloro group resulted in reduced enantioselectivity. The loss of enantioselectivity was considerably higher in case of chloro substitution compared to methoxy substitution, (iii) the e.e. in case methoxy substitution followed order 4-methoxy > 3-methoxy > 2-methoxy. It indicates that in addition to electronic effects, steric effects in the vicinity of carbonyl group also play a role. Thus, e.e. increased as the methoxy group was moved away from 2-position to 3-position of phenyl group from 42% to 77%. The e.e. was 82% when -OCH_3 group was moved to 4-position, (iv) an interesting observation was the reversal of stereoselectivity in case of 2-chloro substitution. Whereas unsubstituted benzil and methoxy or methyl substituted benzil produced corresponding benzoin predominantly in *S*-configuration, 2-chloro substituted benzil produced corresponding benzoins in *R*-configuration and (v) the substitution of methyl group at 4-position of phenyl group has minimal effect on the enantioselectivity of the biocatalyst.

***Talaromyces flavus* catalyzed asymmetric reduction unsymmetrical benzil derivatives**

Various unsymmetrical benzil **(48)** derivatives were synthesized using crossed-benzoin condensation. The cell suspension was incubated with benzil derivatives **(48)** at 2

mM concentration at 30 °C on an orbit shaker at 200 rpm. In all examples, regio- and enantioselective reduction occurred to give corresponding benzoin in 80-90% yield (Scheme 14).

Scheme 14



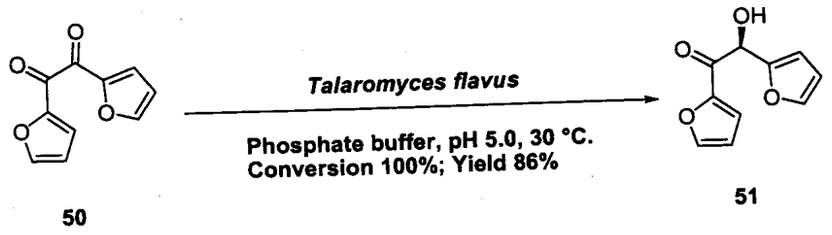
Entry	R	% e.e. (config.)	% Yield	Reaction Time (h)
1	2-OCH_3	89 (*S*)	91	17
2	4- OCH_3	96 (*S*)	88	17
3	4-EtO	>99 (*S*)	84	24
4	2,3-OCH_3	96 (*S*)	80	96
4	4-(*N*)Me_2	>99 (*S*)	85	48

Noteworthy features of *T. flavus* catalyzed asymmetric reduction of unsymmetrical benzils have been described below. In all the examples studied, the carbonyl α to unsubstituted phenyl group was regioselectively reduced, (i) there was no effect of substitution on the enantioselectivity as e.e. of 96-99% was obtained in all examples studied except when methoxy group was present at 2-position of phenyl ring, which gave e.e. of 89%. This is in contrast to symmetrical benzils, where substitution had determintal effect on e.e. of product, (ii) *N*-substituent was well tolerated as dimethylamino group at 4-position of phenyl ring produced the desired product in >99% e.e. and 85% yield, (iii) no hydrobenzoin was produced in any of the examples studied even when the reactions were carried out for longer times (iv) the e.e. and regioselectivity obtained in *T. flavus* catalyzed reaction is higher compared to any report in literature so far. In literature *S. cerevisiae* catalyzed regioselective reduction of these substrates has been reported but e.e. of products was not calculated.[28] With *Xanthomonas oryzae* mixture of both regioisomers were obtained.[29]

***Talaromyces flavus* catalyzed asymmetric reduction α-furil (50) and arylalkyl analog of benzil (52)**

α-Furil **(50)** was synthesized according to the literature procedure.[30, 31] *T. flavus* reduced diketone **50** to (***S***)-**51** in 86% yield and 84% e.e. (Scheme 15).

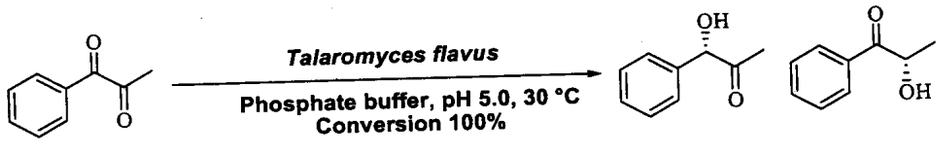
Scheme 15



50 51

Besides the diaryl 1,2-diketones, we also studied the enantio- and regioselective reduction of aralkyl 1,2-diketone with whole cells of *Talaromyces flavus*. Thus, commercially available 1-phenylpropane-1,2-dione **(52)** on biocatalyzed reduction produced a mixture of (*S*)-1-hydroxy-1-phenylpropan-2-one **(53)** and (*S*)-2-hydroxy-1-phenylpropan-1-one **(54)** in a ratio of 2:1 as determined by ^{1}H NMR in 24 h (Scheme 16). The e.e. of mixture was determined by chiral HPLC using Chiralcel OD-H column as described in literature.[32] E.e of **53** was found to be 91% while for **54** was 63%. The configuration of both the products was *S* based on elution order on OD-H column as reported.[32]

Scheme 16



52 53 54

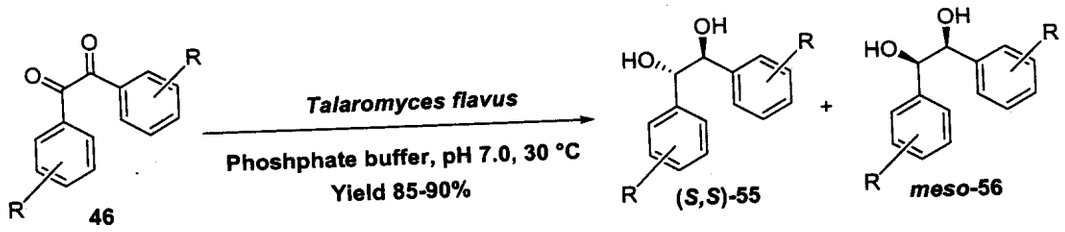
e.e. 91% 63%

53/54 = 2:1

***Talaromyces flavus* catalyzed asymmetric reduction of benzil and its derivatives to corresponding 1,2-diols**

We have already demonstrated that biocatalytic reduction of benzil by *Talaraomyces flavus* at pH 7.0 leads to predominant formation of chiral 1,2-diols. We extended this study to various derivatives of benzil in order to study the effect of substituent on conversion rate and e.e. of the product and also to prepare synthetically important chiral 1,2-diols in high e.e. *Talaromyces flavus* catalyzed reduction of symmetrical benzil derivatives **(46)** produced mixture of corresponding 1,2-diols **(55** and **56)** in 85-90% yield (Scheme 17).

Scheme 17



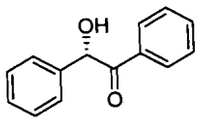
Entry	R	*dl/meso*	% e.e. (config.)	% Yield	Reaction Time (h)
1	H	94/6	>99 (*S,S*)	86	56
2	2-OCH_3	n.r.	n.r.	n.r.	n.r.
3	3-OCH_3	89/11	n.d.	88	48
4	4-OCH_3	38/62	84 (*S,S*)	89	168
5	4-Me	67/33	78.5 (*S,S*)	87	168
6	4-EtO	n.r.	n.r.	n.r.	n.r.
7	2-Cl	10/90	>99 (*S,S*)	85	168
8	3-Cl	70/30	n.d.	88	48
9	4-Cl	67/33	>99%(*S,S*)	86	168

n.r. = no reaction. n.d.= not determined

The following observations are worthy of note, (i) the presence of a substituent at 2 or 4 position of phenyl ring slowed down the reaction, whereas substituent at 3-position had much less effect on the conversion rate. No reaction occurred when a OCH_3 group was present at 2-position of benzil, whereas 2-chloro derivative required 168 h for the reaction to go to completion. 4-Methoxy and 4-methyl derivatives also required about 168 h for the reaction to go to completion. Surprisingly no reaction reaction occurred with 4-ethoxy substituent, (ii) the substitution of +I methoxy group or –I chloro group resulted in reduced *dl/meso* ratio. Whereas benzil gave *dl/meso* ratio of 94/6, 3-methoxy and 4-methoxy derivative gave *dl/meso* ratio of 89/11 and 38/62, respectively. Chloro substituent at 2, 3 and 4-position gave *dl/meso* ratio of 10/90, 70/30 and 67/33, respectively and (iii) the presence of methoxy or methyl substituent at 4-position resulted in decreased e.e. of 78.5-84% while with 2 and 4-chloro substituent e.e. of >99% was obtained.

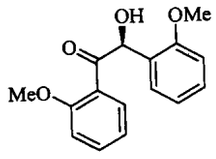
In conclusion, we have described (i) *Talaromyces flavus* as a versatile biocatalyst for asymmetric reduction of 1,2-diketones, (ii) At pH 5.0 *T. flavus* reduced benzil **(43)** to benzoin **(44)** in 98.5% e.e. and 90% yield, (iii) At pH 7.0 *T. flavus* reduced benzil **(43)** to hydrobenzoin **(45)** in *dl/meso* ratio of 94/6, *dl* pair was produced in >99% e.e. and (1*S*,2*S*) configuration, (iv) we have demonstrated that pH dependent selective production of either **44** or **45** in *T.*

flavus catalyzed reduction of **43** is due to presence of different enzymes and not due to single enzyme having pH dependent substrate specificity, as presumed earlier, (v) *T. flavus* was able to reduce symmetrical benzils, unsymmetrical benzils and other 1,2-diketones giving good to excellent e.e. In all the examples studied, exclusive monoreduction to α-hydroxyketone occurred at pH 5.0 and exclusive direduction to 1,2-diols at pH 7.0, (vi) in unsymmetrical benzils, carbonyl α to unsubstituted phenyl ring was preferentially reduced at pH 5.0 and (vii) a library of following α-hydroxyketones and 1,2-diols was prepared using *T. flavus* as biocatalyst.



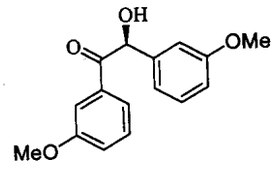
44

e.e. 98.5%



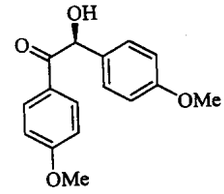
47 a

42%



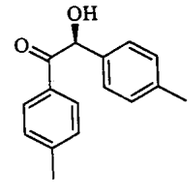
47 b

77%



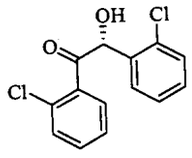
47 c

82%



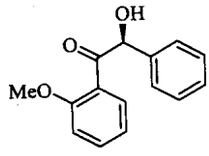
47 d

97%



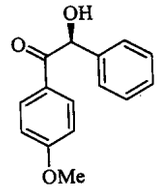
47 f

e.e. 30%



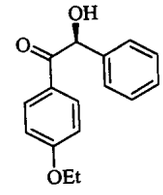
49 a

89%



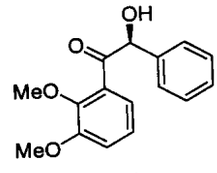
49 b

96%



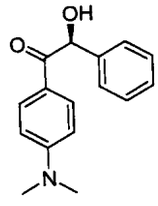
49 c

>99%



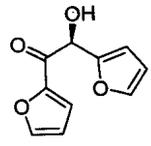
49 d

96%



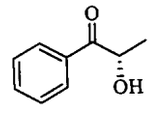
49 e

e.e. >99%



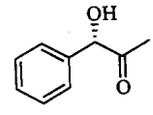
51

84%



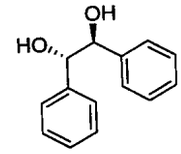
53

91%



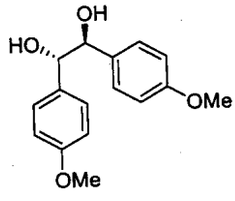
54

63%



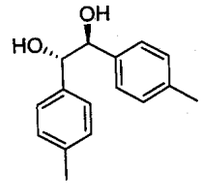
45

>99%



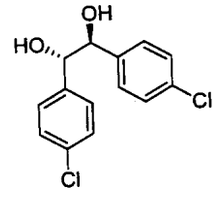
55 c

84%



55 d

78.5%



55 h

>99%

e.e.

Part 2, Section 1: Regiodivergent Baeyer-Villiger Oxidation of (±)-*cis*-Bicyclo[3.2.0]hept-2-en-6-one: An Overview

A brief review on the synthesis of enantiomerically pure lactones by biocatalyzed Baeyer-Villiger oxidation, using selected examples from literature has been provided in this section. An overview of Baeyer-Villiger monooxygenases, their reaction mechanism, structural features, biocatalytic applications has been listed. As a specific example biocatalytic methods for regiodivergent Baeyer-Villiger oxidation of (±)-*cis*-bicyclo[3.2.0]hept-2-en-6-one has been reviewed.

Present Work: Results and Discussions

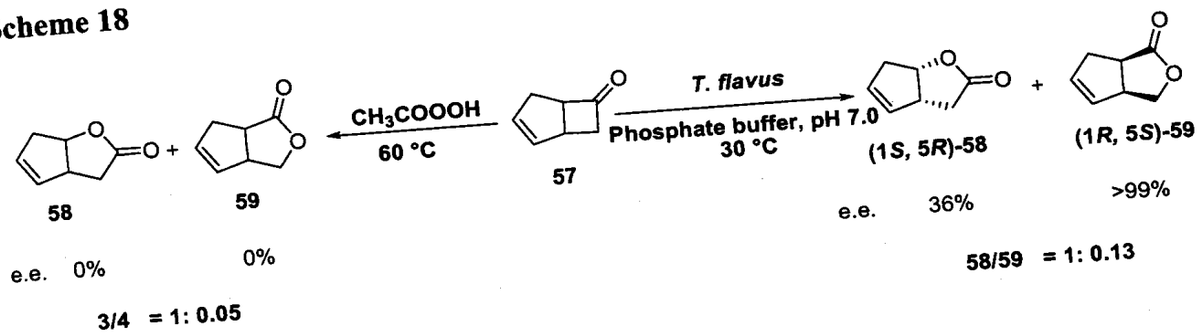
Talaromyces flavus catalyzed BV oxidation of (±)-*cis*-bicyclo[3.2.0]hept-2-en-6-one (57)

Both normal lactone (**58**) and abnormal lactone (**59**) are amenable from (±)-*cis*-bicyclo[3.2.0]hept-2-en-6-one (**57**) by Baeyer-Villiger oxidation, are important intermediates in the synthesis of various biologically important molecules. BV oxidation of **57** with classical peroxidants leads to the predominant formation of normal lactone **58**. Also no enantioselective variant of this reaction has been reported. Biological BV oxidation, in contrast to chemical methods provides **58** and **59** in good yield and high e.e.

While studying asymmetric reduction of 2-acetylpyridine by *Talaromyces flavus*, we observed formation of 2-pyridinol in the reaction mixture under specified conditions, which indicated presence of BV oxidase activity in *Talaromyces flavus*. This gave us an opportunity to test this biocatalyst for BV oxidation of **57**. Thus, **57** was incubated with whole cells of *Talaromyces flavus* in phosphate buffer (50 mM, pH 7.0) at 30 °C. The reaction was monitored by TLC, which revealed complete disappearance of starting material after 18 h. After usual work up, the products were analyzed by ^{1}H NMR. The ^{1}H NMR revealed the product to be a mixture of normal lactone **58** and abnormal lactone **59** (Scheme 18). Assignment of resonances to normal and abnormal lactone was based on comparison with literature.[33] Thus, normal lactone **58** showed resonances at 2.42 (dd, J = 1.7, 17.8, 1H), 2.66-2.71 (m, 3H), 3.45-3.53 (m, 1H), 5.10-5.13 (m, 1H), 5.54-5.58 (m, 1H) and 5.75-5.79 (m, 1H) ppm and the abnormal lactone **59** showed resonances at 2.71-2.76 (m, 3H), 3.11 (dt, J = 2.4, 7.5, 1H), 3.53-3.61 (m, 1H), 4.22 (dd, J = 1.4, 9.2, 1H), 4.41 (dd, J = 7.2, 9.2, 1H), 5.61-5.65 (m, 1H) and 5.82-5.86 (m, 1H) ppm. The resonance at δ 5.12 corresponding to H-1 of normal lactone (**58**) and δ 4.24 correspond to H-4 of abnormal lactone (**59**) appeared in uncomplicated zone of NMR of mixture of these compounds. The ratio of normal/abnormal lactone in mixture was calculated based on comparison of integral values for resonance at δ

5.12 and δ 4.24. Enantiomeric excess of **58** and **59** was determined by using chiral GC using capillary column (B-PM, β-cyclodextrin, permethyl) from Astech with dimensions 30 m×0.25 mm. A standard sample of a mixture of lactone **58** and **59** was prepared by oxidation of **57** with peracetic acid (Scheme 18). The results were disappointing in *T. flavus* catalyzed oxidation as poor e.e. of 36% was observed for normal lactone **58**. Although e.e. of abnormal lactone **59** was found to be >99%, it was obtained in poor ratio of only 13%. This prompted us to screen microbial diversity for new BV oxidase with desired property.

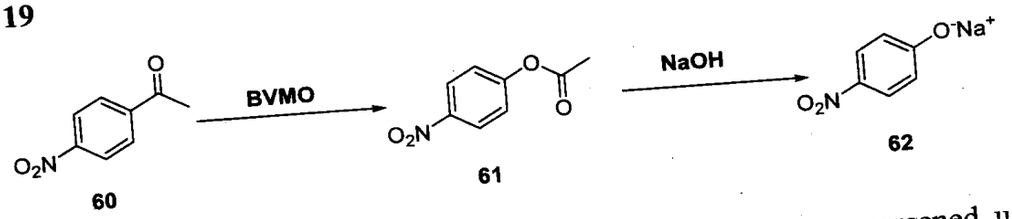
Scheme 18



Screening of microbial diversity of India for Bayer-Villiger oxidation

We developed a colorimetric method for the rapid screening of microorganisms. The principle is shown in Scheme 19. 4-Nitroacetophenone **(60)** was selected as substrate, which on oxidation would produce 4-nitrophenylacetate **(61)**. We observed that compound **61** undergoes rapid hydrolysis under basic conditions to *p*-nitrophenolate ion **(62)**, which can be monitored spectrophotometrically at 410 nm.

Scheme 19



226 strains of fungi, isolated from diverse niches in India were screened using this method. In fact, the yellow colour of *p*-nitrophenolate was visible to naked eye in samples which tested positive. In all, 12 strains were selected. Out of the 12 shortlisted strains, 6 were able to oxidise ketone **57** to a mixture of normal and abnormal lactone (Table 8). The ratio of normal/abnormal lactone was determined based on ^{1}H NMR. Only one strain designated as 8197 gave normal/abnormal ratio of 1:1.18 indicating that stereodivegent BV oxidation of ketone **57** might have occurred (Entry 4, Table 8). The strain 8197 described as *Alternaria*

alternata is same strain, which has been described for asymmetric reduction of heteroaryl ketones. It is very interesting to note that with **57** as substrate no reduction of carbonyl occurred as no signals corresponding to the reduced product could be observed either in ^{1}H NMR or GC.

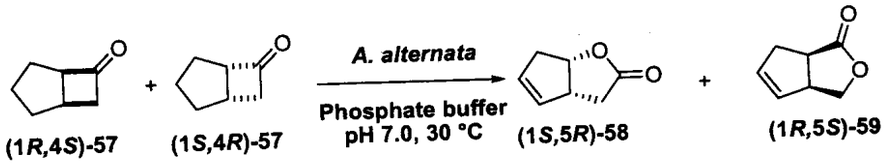
Table 8: Ratio of normal/abnormal lactone obtained in BV oxidation of (±)-*cis*-bicyclo[3.2.0] hept-2-en-6-one **(57)** catalyzed by various fungal strains

Entry	Strain No.	Strain Name	Normal /abnormal lactone ratio[a]
1	8223	*Aspergillus flavus*	1:0.19
2	8204	*Trichoderma harzanium*	1:0.20
3	8188	*Aspergillus flavus*	1:0.19
4	8197	*Alternaria alternata*	1:1.18
5	8237	*Aspergillus flavus*	1:0.20
6	2556	*Aspergillus fumigatus*	1:0.19

[a]Determined by ^{1}H NMR method

The e.e. of the products obtained from *Alternaria alternata* catalyzed reaction was determined as described previously (Page 257). Based on GC profiles e.e. of normal lactone **58** and abnormal lactone **59** in *Alternaria alternata* catalyzed reaction have been determined as 87.8% and 98.9% respectively. Similarly the e.e. for product obtained from other strains was also determined and found to be in the range of 35-38% for normal lactone and ~ 99% for abnormal lactone. The absolute configuration was assigned based on comparison of GC retention times with literature values.[34] Thus, absolute configuration was assigned as (-)-(1*S*,5*R*) for normal lactone **(58)** and (-)-(1*R*,5*S*) for abnormal lactone **(59)** in case of *A. alternata* (Scheme 20). Based on these results strain 8197, identified as *Alternaria alternata* was selected for stereodivergent BV oxidation of **57**.

Scheme 20



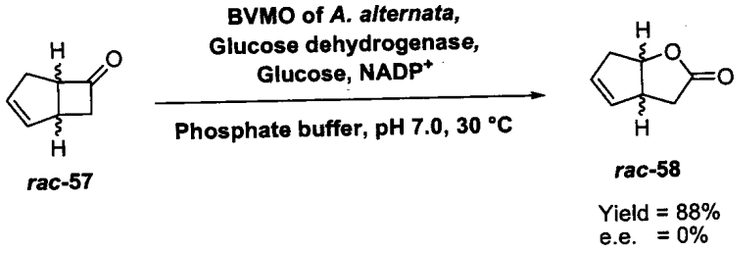
The enzyme responsible for activity has been purified by a 4-step procedure from *Alternaria alternata*. Approximately 43-fold purification was required to obtain electrophoretically homogenous protein, which showed a single band at about ~42 kDa on SDS-PAGE run under reducing conditions. The native molecular weight observed for

Baeyer-Villiger monooxygenase was calculated 42.6 kDa. Thus, Baeyer-Villiger monooxygenase appears to be a monomer.

BV oxidation of (±)-*cis*-bicyclo[3.2.0]hept-2-en-6-one (57) with pure BV oxidase of *Alternaria alternata*

Finally, the biotransformation of (±)-*cis*-bicyclo[3.2.0]hept-2-en-6-one **(57)** was attempted using purified enzyme. The reaction was done using 30 µg of pure enzyme, 784 nmol of $NADP^+$, 14 units of glucose dehydrogenases, 0.5 mmol glucose, 5 mg (±)-*cis*-bicyclo[3.2.0]hept-2-en-6-one **(57)** in phosphate buffer (50 mM, pH 7.0) (final volume 2 ml) and contents were incubated at 30 °C for 10 h.

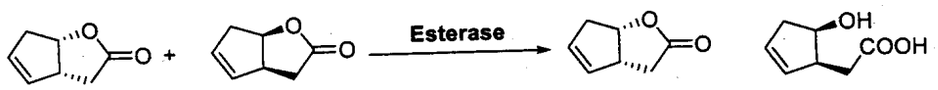
Scheme 21



We were surprised to find that the pure BV oxidase produced only normal lactone **58** in high yield but 0% e.e. (Scheme 21). Addition of cofactor FAD/$NADP^+$ or FMN/NAD^+ to the reaction mixture did not alter the outcome of the reaction. We also tested other fractions, i.e. cell-free extract, ammonium sulphate precipitate, reactive red elute and wash through obtained during purification of BVO using flavin cofactor FAD or FMN in the presence of $NADP^+$ or NAD^+. But we failed to isolate any other BVO activity, inspite of several attempts. All the fractions produced only racemic lactone **58**, no trace of abnormal lactone **59** could be detected. These results indicate that another BVO, which was present in the whole cells has been lost during the purification procedure. A total activity of only 5.04 units was obtained in cell-free extract from ~ 450 g cells, which also support loss of BVO activity.

The pure BVO produced *rac*-**58**, whereas whole cells produced (1*S*,5*R*)-**58** in 87.8% e.e. A possibility exists that an esterase exists in the whole cells, which stereospecifically hydrolyzed (1*R*,5*S*)-**58** to corresponding acid **63**, leading to resolution of lactone **58** (Scheme 22). But inspite of several attempts, we failed to isolate even a trace of **63** from the reaction mixture, thereby ruling out this possibility.

Scheme 22



(1*S*,5*R*)-58 (1*R*,5*S*)-58 (1*S*,5*R*)-58 (1*R*,5*S*)-63

Conclusions

In conclusion we have described (i) isolation of a new biocatalyst for Baeyer-Villiger oxidation of (±)-*cis*-bicyclo[3.2.0]hept-2-en-6-one **(57)**, (ii) the new biocatalyst has been identified as *Alternaria alternata,* (iii) *Alternaria alternata* catalyzed BV oxidation of **57** produced normal lactone **(58)** and abnormal lactone **(59)** in the ratio of 1:1.18. The e.e. of normal lactone **(58)** and abnormal lactone **(59)** was 87.8% and 98.9%, respectively, (iv) a Baeyer-Villiger oxidase has been purified from *Alternaria alternata* in 0.9% yield, (v) the purified protein oxidized lactone **57** to normal lactone **(58)** in high yield but 0% e.e. Apparently, the major Baeyer-Villiger oxidase activity of *A. alternata* was lost during the purification procedure, indicating its instability in cell free environment.