Introduction

The interest in chiral tricoordinated sulfur compounds in general and sulfoxides in particular have shifted from purely academic, directed toward the study of their stereochemical behavior to a well established applied synthetic interest. This shift is due to the discovery that chiral sulfoxides are efficient chiral auxiliaries that are able to bring about important asymmetric transformations. In addition to application of chiral sulfoxides as chiral auxiliaries in asymmetric synthesis, a number of biologically significant molecules have in their structure a stereogenic sulfinyl sulfur atom and therefore exist as a pair of enantiomers which may exhibit differential stereochemically dependent metabolism and enzyme inhibition. A number of sulfoxides have found applications in the pharmaceutical industry. Asymmetric oxidation of sulfides to sulfoxides is a subject of current interest. The present thesis describes the result of our effort at characterization of new biocatalysts for the asymmetric oxidation of sulfides. The thesis has been divided into seven sections for convenience.

Section 1: Synthesis of Enantiomerically Pure Sulfoxides: Review of Literature

A brief review on the synthesis of enantiomerically pure sulfoxides, using selected examples from the literature has been provided in this section. Both chemical and biological methods for their synthesis have been discussed. Amongst the chemical methods, titanium and vanadium catalyzed asymmetric oxidation of sulfides has been given special attention. In addition Andersen method, based on nucleophilic substitution on diastereomerically pure (*S*,*S*)-menthyl-*p*-toluene sulfinate and asymmetric sulfoxidation using neighboring group participation have been briefly discussed. Reference has also been made to use of N-sulfonyloxaziridines in the asymmetric synthesis of chiral sulfoxides. Amongst the biological methods, use of whole cells as well as isolated enzymes as biocatalyst has been discussed.

Section 2: Biocatalytic Oxidation of β-Lactams to (*R*)-Sulfoxides

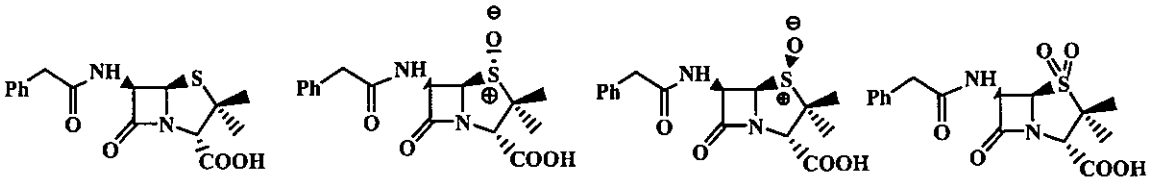
This section describes the development of a method for asymmetric oxidation of β-lactams to (*R*)-sulfoxides in 100% d.e. No over oxidation to sulfone occurred under the reaction conditions.

Penem is a well-known pharmacophore for antibiotics and is widely used in clinic. Recent findings have shown that penem derivatives inhibit mammalian serine and cystine proteases, in addition to bacterial transpeptidase. Since, these proteases have been implicated as important targets for the development of inhibitors as potential therapeutic agents, a number of penem derivatives have been synthesized and evaluated for this activity. In addition, penem derivatives have also been evaluated for the β-lactamase inhibition activity. The activity of penems correlates strongly with the oxidation state of the sulfur moiety in the penem ring. Therefore, a systematic study of the biological activity of these molecules requires simultaneous evaluation of the activity of a penem along with its sulfoxide and sulfone. Whereas (*S*)-sulfoxide and sulfone are easily assessable by direct oxidation of the parent penem by a variety of reagents, preparation of corresponding (*R*)-sulfoxide, generally requires an independent, multistep synthetic route. As a result, (*R*)-sulfoxides are usually not included in the studies aimed at the evaluation of the biological activity of the penem derivatives.

The preferential formation of (*S*)-sulfoxide during oxidation of penicillins has been attributed to the directing effect of the carboxyamido group, most likely through the hydrogen bonding of the peroxide reagent with the amide proton prior to the delivery of reactive oxygen to sulfur atom of the sulfide. We envisaged that the directing influence of amide group is likely to be absent in enzyme catalyzed reactions, since the preliminary reaction of the peroxide occurs at the metal centre (Fe^{III}). The delivery of reactive oxygen will then be dictated by the orientation of the substrate within the binding cavity of enzyme or it will occur from sterically less hindered side.

Biocatalyzed Oxidation of Penicillin G (1) to (*R*)-Penicillin G Sulfoxide (2)

As a first step, selection of a strain, capable of stereoselective oxidation of penicillin G to corresponding (*R*)-sulfoxides was undertaken. Selection of a strain required screening of a library of microorganisms. But a successful rapid screening method requires availability of a standard sample of the product, (*R*)-penicillin G sulfoxide **(2)** in this case. But we were surprised to note that, so far, no method is available in literature for preparation of **2**. Therefore, we devised a 3-stage strategy for the screening work.



1 2 3 4

In the first stage, microorganisms were selected for their ability to oxidize butyl phenyl sulfide. The pure cultures of microorganisms were grown by standard procedures as described in the experimental section (Section 6). The cells were isolated by centrifugation, washed with phosphate buffer pH 7.0 and resuspended in the same buffer at a concentration of 0.5 g/mL and incubated with 2 mg of butyl phenyl sulfide at 200 rpm in an orbital shaker at 37 °C for bacteria, 30 °C for fungi for 12 h. The supernatant was separated by centrifugation and extracted with 2 mL of ethyl acetate. The formation of the butyl phenyl sulfoxide was analyzed by TLC, using standard samples of sulfoxide and sulfone, prepared by oxidation of butyl phenyl sulfide with *m*-chloroperbenzoic acid and oxone®, respectively. The formation of the sulfoxide was further confirmed by ^{1}H NMR.

In the second stage, the selected microorganisms were tested for their ability to convert penicillin G to penicillin G sulfoxide. 4 g of washed cells were incubated with 50 mg penicillin G **(1)** in phosphate buffer at 20 mM substrate concentration for 12 h. The biocatalyst was removed by centrifugation, the supernatant acidified with 3N sulfuric acid and extracted with ethyl acetate. The organic layer was separated and analyzed by TLC. Two strains of bacteria designated as B4W and BIMT 9004 gave a product which corresponded with (*S*)-penicillin G sulfoxide on TLC. However, HPLC analysis on reverse phase, C-18 column of the biotransformed product showed a different retention time compared to (*S*)-penicillin G sulfoxide. The standard sample of (*S*)-penicillin G sulfoxide **(3)** was prepared by oxidation of penicillin G **(1)** with peracetic acid according to literature procedure.

In the third stage, determination of structure of the product and assignment of configuration was undertaken. Strain B4W was selected for further studies as it gave higher conversion rates. The biotransformation product obtained from B4W was analyzed by IR and ^{1}H NMR. The bands at 3371 cm^{-1} (N-H stretch), 1753 cm^{-1} (acid carbonyl), 1652 cm^{-1} (amide carbonyl), 1495 cm^{-1} (aromatic C=C) in the IR spectrum showed that β-lactam structure has remained intact. A band at 1028 cm^{-1} indicated the presence of sulfoxide in the molecule.

^{1}H NMR was in agreement with the sulfoxide structure. Two methyl protons appeared as singlets at δ 0.92 and 1.58. The methylene and H-3 protons resonated as singlets at δ 3.68 and 3.51 respectively. H-5 protons appeared as doublet (J = 4.5 Hz) at δ 5.17 and

H-6 protons as dd (J = 4.5, 8.9 Hz) at δ 5.08. The NH appeared as doublet (J = 8.9 Hz) at δ 6.58. The aromatic protons resonated at δ 7.27-7.44 as multiplet. The ^{13}C NMR spectral data was also in agreement with the sulfoxide structure. The ^{1}H NMR data however was different from corresponding (*S*)-sulfoxide, **3** and sulfone, **4** (Table 1). A sample of sulfone was prepared by oxidation of penicillin G with oxone®. In literature, the penicillin G sulfone has been prepared by oxidation with *m*-CPBA, but we observed that oxone® gave a much cleaner product as no degradation of β-lactams occurred under the reaction conditions.

In ^{1}H NMR of the biocatalyzed product, one of the methyl groups in the product at δ 1.58 appeared downfield compared to penicillin G due to syn-axial effect. The syn-axial is also present for one of the geminal methyl group for (*S*)-sulfoxide and sulfone. The effect was also observed for H-3 and H-6 protons of (*S*)-sulfoxide and sulfone since both protons lie in similar geometrical relation to the sulfur atom. However, H-3 and H-6 protons for the (*R*)-sulfoxide appeared upfield as compared to penicillin G, in all probability due to shielding caused by trans-oriented lone pair on sulfur. H-5 proton in (*R*)-sulfoxide is expected to appear downfield compared to penicillin G because it is located in the deshielding zone of sulfoxide bond. However, the upfield shift has been observed earlier also and has been attributed to the presence of lone pair on sulfur atom. The amide proton appeared at slightly higher field compared to penicillin G, indicating that it is not hydrogen bonded with sulfoxide oxygen; the (*S*)-sulfoxide and sulfone amide appeared downfield due to feasibility of the formation of H-bond with sulfoxide oxygen. Put together all these results suggest that biotransformation product is penicillin G sulfoxide and has (*R*)-configuration.

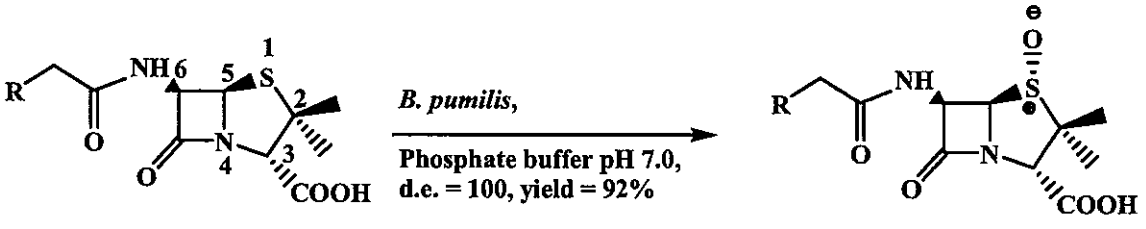
Finally, the structure and (*R*)-configuration was confirmed by conversion of the biotransformed product to its methyl ester (MeI/K_2CO_3/DMF) and comparison of the ^{1}H NMR spectral data with literature value.

Table 1. Comparison of ^{1}H NMR Spectral Data of Penicillin G with Ring Sulfur Moiety in Different oxidation States ($CDCl_3$+DMSO)

	Penicillin G, 1	(*R*)-oxide, 2	(*S*)-oxide, 3	Sulfone, 4
CH_{3B}	1.41 (s), 1.44 (s)	0.92 (s) 1.58 (s)	1.13 (s) 1.57 (s)	1.21 (s) 1.66 (s)
CH_{2B}	3.53 (s)	3.68 (s)	3.44 (s)	3.63 (s)
H-3	4.23 (s)	3.51 (s)	4.42 (s)	4.59 (s)
H-5	5.40 (d, J = 4.1 Hz)	5.17 (d, J = 4.5 Hz)	4.90 (d, J = 4.5 Hz)	4.99 (d, J = 4.5 Hz)
H-6	5.53 (dd, J = 4.1, 8.6 Hz)	5.08 (dd, J = 4.5, 8.9 Hz)	5.85 (dd, J = 4.5, 9.96 Hz)	5.96 (dd, J= 4.5, 9.9 Hz)
NH	6.68 (d, J = 8.6 Hz)	6.58 (d, J = 8.9 Hz)	8.77 (d, J = 9.96 Hz)	8.56 (bs)
Ar	7.17-7.25 (m)	7.27-7. 44 (m)	7.14-7.18 (m)	7.21-7.39 (m)

Thus, B4W catalyzed oxidation of penicillin G **(1)** results in the formation of (*R*)-sulfoxide, **2** as the exclusive product (Scheme 1). No (*S*)-sulfoxide or sulfone could be detected in the reaction mixture.

Scheme 1



1, R = Ph,
5, R = OPh

2, R = Ph
6, R = OPh

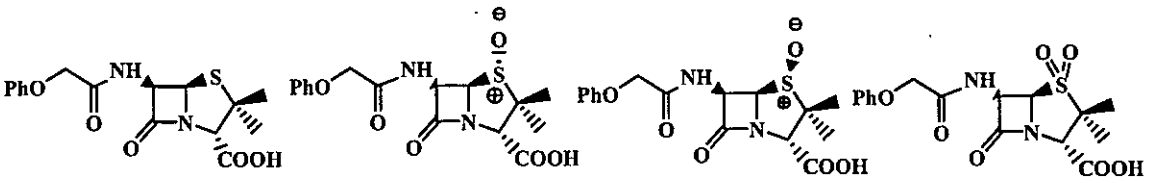
Taxonomy of the Strain

The strain B4W was identified as *Bacillus pumilis* based on the biochemical characterization and 16 s *r*DNA.

***Bacillus pumilis* Catalyzed Oxidation of Penicillin V (5) to (*R*)-Penicillin V Sulfoxide (6) (Scheme 1)**

Incubation of penicillin V **(5)** with *Bacillus pumilis* for 12 h in phosphate buffer, pH 7.0 gave a product, which co-migrated with a standard sample of (*S*)-penicillin V sulfoxide

(7) prepared by oxidation of 5 with peracetic acid. ^{1}H NMR spectral data of the product was in agreement with sulfoxide structure.



5 6 7 8

IR showed that the bands at 3402 cm^{-1} (N-H stretch), 1784 cm^{-1} (lactam carbonyl), 1730 cm^{-1} (acid carbonyl), 1685 cm^{-1} (amide carbonyl) and 1495 cm^{-1} (aromatic C=C) in the IR spectrum showed that β-lactam structure has remained intact. A band at 1064 cm^{-1} indicated the presence of sulfoxide in the molecule. The ^{1}H NMR data however was different from corresponding (*S*)-sulfoxide (7) prepared by oxidation with peracetic acid (Table 2).

In ^{1}H NMR, one of the geminal methyl groups in the biocatalyzed product at δ 1.53 appeared downfield due to syn-axial effect. The syn-axial effect is also observed for H-3 and H-6 protons of (*S*)-sulfoxide. However, H-3 and H-6 protons for (*R*)-sulfoxide resonated upfield compared to pen V, possibly due to shielding caused by trans oriented lone pair on sulfur. The upfield shift of H-5 may be attributed to shielding caused by the trans-oriented lone pair on sulfur atom. Put together all these results suggest that biotransformation product is penicillin V sulfoxide and has (*R*)-configuration. The ^{13}C NMR spectral data was also in agreement with the sulfoxide structure.

Table 2. Comparison of ^{1}H NMR Spectral Data of Penicillin V with Ring Sulfur Moiety in Different Oxidation States ($CDCl_3$+DMSO)

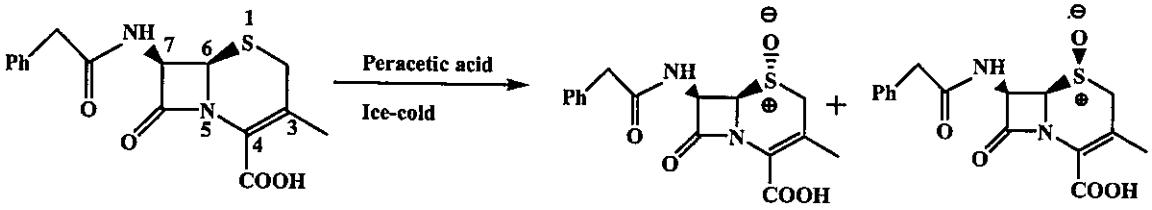
	Penicillin V, 5	(*R*)-oxide, 6	(*S*)-oxide, 7
CH_{3B}	1.48 (s), 1.51 (s)	1.10 (s) 1.53 (s)	1.07 (s) 1.50 (s)
H-3	4.30 (s)	3.53 (s)	4.36 (s)
CH_{2B}	4.48 (s)	4.60 (s)	4.31 (s)
H-5	5.47 (d, J = 4.1 Hz)	5.04 (d, J = 3.8 Hz)	4.90 (d, J = 4.8 Hz)
H-6	5.60 (dd, J = 4.1, 9.3 Hz)	4.85 (dd, J = 3.8, 9.0 Hz)	5.80 (dd, J = 4.8, 10.6 Hz)
Ar	6.81-6.92 (m), 7.19-7.21 (m)	6.90-6.93 (m), 7.24-7.29 (m)	6.68-6.79 (m), 7.03-7.09 (m)
NH	7.24 (d, J = 9.3 Hz)	8.02 (d, J = 9.0 Hz)	8.07 (d, J= 10.6 Hz)

Finally, the structure and (*R*)-configuration was confirmed by conversion of the biotransformed product to its methyl ester (MeI/DMF/K_2CO_3) and comparison of the ^1H NMR spectral data with literature value.

Bacillus pumilis Catalyzed Oxidation of Cephalosporin G (9) to (*R*)-Cephalosporin G Sulfoxide (10)

Chemical oxidation of cephalosporin G **(9)** with peracetic acid was studied first. ^1H NMR of the product revealed it to be a mixture of two compounds in 1:1 ratio which was not separated (Scheme 2). The NMR data values assigned to (*R*)-isomer, **10** and (*S*)-isomer, **11** are shown in Table 3. The assignment of configuration is tentative, based on the chemical shift values. Methylene protons in the side-chain amido group for *S*-isomer resonated approximately 0.6 to 0.8 ppm downfield compared to *R*-isomer and parent compound, as expected because of syn effect of sulfoxide oxygen. Similarly, H-7 of (*S*)-isomer appeared about 0.15 ppm downfield compared to (*R*)-isomer. Amido group of (*S*)-isomer appeared 0.5 ppm downfield in all probability due to H-bonding with sulfoxide.

Scheme 2



9 10 11

R/*S* = 1:1

Table 3. Comparison of ^{1}H NMR Spectral Data of Cephalosporin G with Ring Sulfur Moiety in Different Oxidation States

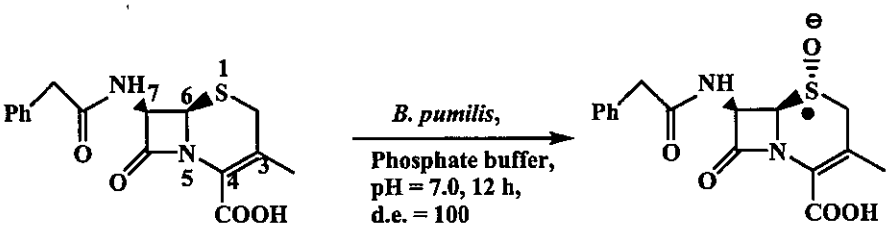
	Ceph G (9)	Biocatalyzed product (*R*)-sulfoxide (10)	Chemically oxidized products[#]	
			(*R*)-sulfoxide (10)	(*S*)-sulfoxide (11)
CH_{3B}	2.12 (s)	1.97 (s)	1.99 (s)	2.11 (s)
H-2	3.18, 3.44 (d, *J* = 17.6 Hz)	3.41, 3.56 (d, *J* = 17.8 Hz)	3.41-3.76 (m)	3.41-3.76 (m)
CH_{2B}	3.31, 3.63 (d, *J* = 14.8 Hz)	3.47, 3.53 (d, *J* = 15 Hz)		4.03. 4.22 (d, *J* = 15 Hz)
H-6	4.93 (d, *J* = 4.5 Hz)	4.72 (d, *J* = 4.5 Hz)	4.76 (d, *J* = 4.5 Hz)	5.3 (d, *J* = 4.2 Hz)
H-7	5.69 (dd, *J* = 4.5, 8.3 Hz)	5.65 (dd, *J* =4.5, 8.7 Hz)	5.69 (dd, *J* = 4.5, 8.7 Hz)	5.85 (dd, *J* = 4.2, 8.5 Hz)
Ar	7.23.7.48 (m)	7.25-7.49 (m)	7.21-7.49 (m)	7.21-7.49 (m)
NH	8.25 (d, *J* = 8.3 Hz)	8.15 (d, *J* = 8.7 Hz)	8.40 (d, *J* = 8.7 Hz)	8.90 (d, *J* = 8.2 Hz)

[#](*R*) and (*S*)-isomers in 1:1 ratio. Mixture was not separated. Assignment of relative configurations are tentative.

Biocatalyzed oxidation of **9** with *Bacillus pumilis* MTCC B6033 under the reaction conditions described above for pen G, gave only one product (Scheme 3). Its ^{1}H NMR spectral data was consistent with sulfoxide structure (Table 3).

The ^{1}H NMR of biocatalyzed product was similar to one of the isomers obtained by peracetic acid oxidation of **9**, which has been tentatively assigned (*R*)-configuration.

Scheme 3



9 **10**

In conclusion, a method (first and only method, so far) for asymmetric oxidation of β-lactams leading to the exclusive formation of (*R*)-sulfoxides, has been developed, which does not require any protection-deprotection step. The method involves the use of a

bacterium as catalyst which has been identified as *Bacillus pumilis* based on morphological and biochemical tests and 16 s *r*DNA sequence analysis.

Section 3: Characterization of the Enzyme of *Bacillus Pumilis* Responsible for Stereoselective Conversion of β-Lactams to *R*-sulfoxides as Catalase-peroxidase (KatG)

This section describes the purification and characterization of the enzyme responsible for oxidation of β-lactams to (*R*)-sulfoxides.

Typically, the enzymes that catalyze oxidation of sulfides to sulfoxides can be categorized into two classes, monoxygenases and peroxidases. The cell free extract, prepared by sonication of the cells of *Bacillus pumilis* was assayed for monooxygenase activity in phosphate buffer, pH 7.0 using penicillin G as substrate and NADPH as co-factor. No change in absorbance at λ_{340} was observed, which rules out the oxygenase as the enzyme responsible for oxidation of penicillin G. Next, the enzyme was assayed for peroxidase activity. The assay mixture contained, in a total volume of 1 mL, 50 mM phosphate buffer pH 6.8, 0.025% (w/w) H_2O_2, ABTS and limiting amounts of enzyme. The consumption of reduced ABTS was followed at 436 nm. The change in absorbance was observed and the specific activity calculated was 31.6 $\mu mol^{-1}min^{-1}mg$ of protein.

While performing the assays for peroxidase activity, frothing and effervescence in the reaction mixture were observed upon addition of H_2O_2, indicating that a catalase activity might be present. Catalase activity was confirmed on following the decrease in absorbance at λ_{240} nm corresponding to the consumption of hydrogen peroxide. The specific activity for the cell free extract for the catalase was 33.3 $\mu mol^{-1}min^{-1}mg$ of protein. In literature, catalases and peroxidase are known for catabolism of hydrogen peroxide. The former is characterized by electron pair transitions in which H_2O_2 is decomposed to O_2 and H_2O, whereas the latter is characterized by single electron transfers resulting in the oxidation of various organic compounds by H_2O_2. A novel type of hydroperoxidase, so-called catalase-peroxidase (KatG) has been recently proposed, which exhibited both catalatic and peroxidatic activities. This class of enzymes is distinct from typical catalases and peroxidases, with respect to a number of physical, chemical and structural features.

In order to find out the nature of enzyme/s responsible for *Bacillus pumilis* catalatic and peroxidatic activities, activity stain (zymography) of the protein separated by PAGE run under non-denaturing conditions was performed. The gel was sliced into two pieces. For the catalase activity, one piece of the gel was incubated in 3% H_2O_2 solution for 15 min. The solution was poured off and gel was immersed in 1:1 mixture of 2% potassium ferricyanide

and 2% $FeCl_3$. Yellow bands of catalase activity appeared on a dark background almost immediately. For peroxidase staining, the other half of the sliced gel after over-night incubation with ABTS solution was treated with H_2O_2. Green bands appeared against a light-green background, confirming the presence of peroxidase. The mobility of the bands for both the activities in the gel was same. The results suggested that a dual activity catalase-peroxidase may be the enzyme responsible for the oxidation of penicillins.

Although peroxidases have been widely used in organic synthesis (reviewed in section 1), no synthetic application for catalase-peroxidase has been reported, except that of halogenation of dimedone. However, the application of catalase-peroxidase in bioremediation of textile dye effluent has been described. The stereoselective oxidation of β-lactams to (*R*)-sulfoxides; to the best of our knowledge is the first attempt at the exploitation of this class of enzyme in organic synthesis.

For the purification of the enzyme, the cell lysate was cooled to 4 °C and solid ammonium sulfate was added to 20% saturation. The pellet was removed and the supernatant was brought to 90% saturation with solid ammonium sulfate while maintaining the temperature at 4 °C. The precipitated proteins were collected by centrifugation, and the pellet was dissolved in phosphate buffer (50 mM, pH 7.0). The solution was applied to a phenyl sepharose high performance fast flow column, pre-equilibrated with 1.0 M Na_2SO_4 in phosphate buffer. The bound proteins were eluted with linear gradient of 1.0-0 M Na_2SO_4 in phosphate buffer (10 mM, pH 7.0). The active fractions were pooled and loaded onto a Q-sepharose column pre-equilibrated with phosphate buffer (10 mM, pH 7.0). The bound proteins were eluted with a linear gradient of 0-1.0 M NaCl in the same buffer. The active fractions were pooled and concentrated in an ultrafiltration cell using a 30 kDa membrane. Desalting of the protein was done with dilutions with distilled water and finally concentrating to a volume of 1 mL. Finally, the gel filtration chromatography was performed with a Sephacryl S-200 column previously equilibrated with phosphate buffer (50 mM, pH 7.0) containing 0.15 M NaCl. The column was run with the same buffer and active fractions were collected. The protein after gel-filtration was found to be electrophoretically homogenous. Approximately 25-fold purification was achieved in about 45% yield in 5-step purification procedure. The peroxidatic and catalatic activities always co-eluted in same fractions which indicated that both activities are present in the same protein.

The purified enzyme from *Bacillus pumilis* showed a molecular weight of 62.4 kDa on SDS-PAGE run under reducing conditions. The molecular weight of the native protein as determined by MALDI-TOF was 62.14 kDa. The molecular mass of the protein from gel-filtration data was calculated to be ~70.5 kDa. These results suggest that the catalase-

peroxidase from *Bacillus pumilis* exists as a monomer in the native form. Typical catalase-peroxidases reported in literature are either homo-dimers or homo-tetramers. In general, the identical subunits of catalase-peroxidases were 78-85 kDa in size, as has been demonstrated for the subunits in *E. coli*, *Streptomyces* sp. IMSNU-1, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Rhodobacter capsulatus* and *Bacillus stearothermophilus*.

The catalase-peroxidase of *Bacillus pumilis* exhibited a narrow pH range of 6.5 to 8.0 for catalase activity. The maximum activity was observed at pH 7.2. The enzyme showed temperature optima for catalase activity at 37 °C. The activity decreased drastically at temperatures above 45 °C. These properties are similar to the typical catalase-peroxidases reported in literature.

The catalase and peroxidase activity of the purified enzyme was 818 and 743 μmolmin^{-1}mg^{-1} of the protein, respectively. The apparent V_{max} and K_m of the catalase-peroxidase were 4.61 μmolmin^{-1}mg^{-1} and 3.82 mM respectively. The catalase-peroxidase of B*acillus pumilis* has K_m and catalytic efficiency as defined by k_{cat}/K_m similar to that of other bacterial catalase-peroxidases.

The absorption spectra of native enzyme exhibited maxima at 406, 503 and 635 nm. Addition of 10 mM KCN shifted the soret band to 425 nm. The bands at 500 and 635 nm disappeared but a new band at 548 nm appeared. The absorption maxima of catalase-peroxidase at 406, 503, and 635 nm are similar to those of high spin heme proteins with a histidine as the fifth ligand, but very different from that of cytochrome P450 (391, 500 and 646 nm) with a cysteine as the fifth ligand as well as that of cytochrome b, low spin, hexacoordinate species. The absorption maxima of CN-adduct of catalase-peroxidase are virtually identical to those of the low spin, hexacoordinate CN-adducts of horseradish peroxidase, lignin peroxidase and manganese peroxidase, but distinct from that of the low spin, hexacoordinate CN-adduct of cytochrome P450. These absorption patterns suggest that the fifth ligand to the heme iron of catalase-peroxidase can be a histidine. The treatment of the enzyme with pyridine-NaOH-dithionite, customarily used to determine pyridine hemochrome, gave a typical spectrum of iron protoporphyrin IX, whose absorption bands appeared at 418, 524 and 556 nm. On the basis of the molar absorption coefficient of 33,200 $M^{-1}cm^{-1}$ at 556 nm for the pyridine hemochrome, the heme content was calculated to be 1.05 mol of heme per mol of protein. It has been reported that catalase-peroxidases found in other bacterial systems have low heme content. The enzyme of *E. coli* HP-1 had 2.07 mol of heme per mol of tetrameric protein, that of *H. halobium* had 1.43 mol of heme per tetramer and that of *R. capsulata* had 1-1.2 mol of heme per tetramer. The reduction of enzyme with sodium dithionite caused concentration dependent decrease in soret band. Typical catalases

are not reduced or are only slightly reduced by dithionite but the catalase-peroxidases are readily reduced. The inhibition of the enzyme by KCN, NaN_3, and hydroxylamine also indicated that the enzyme from *B. pumilis* is a typical heme protein.

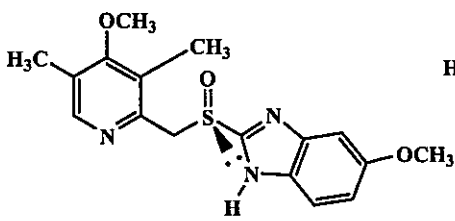
In conclusion, a catalase-peroxidase (KatG) has been purified from *Bacillus pumilis* MTCC B6033 and has been shown to be the enzyme responsible for oxidation of β-lactams to (*R*)-sulfoxides. The protein is a monomer of 62.14 kDa as evident from SDS-PAGE, MALDI-TOF mass analysis and gel-filtration data. It is a heme containing protein showing characteristic heme spectra with soret peak at 406 nm and visible peaks at 503 and 635 nm. The treatment of the enzyme with pyridine-NaOH-dithionite gave spectra typical of iron-protoporphyrin IX.

The KatG of *Bacillus pumilis* is similar to other KatGs of bacterial origin in terms of its spectral properties, narrow pH range and effect of enzyme inhibitors etc., the major properties that distinguish *Bacillus pumilis* KatG from other bacterial KatGs are: (i) the enzyme is a momomer whereas catalase-peroxidase of other bacteria are dimers or tetramers, (ii) it contains one heme per momomer, whereas KatGs of other bacteria have low heme content of about one per dimer or two per tetramer and (iii) 12-residue, N-terminal sequence obtained by Edmann degradation did not show significant similarity with any other KatG.

Section 4: Biocatalytic Synthesis of Proton Pump Inhibitors

This section describes isolation of a new biocatalyst for preparation of (*S*)-omeprazole in >99% e.e.

5-Methoxy-2-{[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl-1H-benzimidazole; Omeprazole, is a highly potent gastric acid secretion inhibitor and was firstly marketed by Astra in 1988 under the name Prilosec®. It has a stereogenic center at the sulfur atom, and exists as two enantiomers, (*S*)-enantiomer **12** and (*R*)-enantiomer **13**. The sales in the first quarter of 2006 were $ 12 billion for Nexium® and $ 3 billion for Prilosec®. (*S*)-enantiomer, Nexium® has been shown to have much better pharmacological properties compared to omeprazole or its (*R*)-enantiomer.



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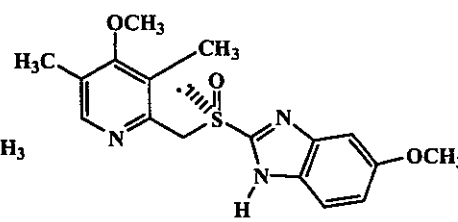

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The objective of the present work was to provide a biocatalyst for the preparation of (*S*)-omeprazole **(12)** in high e.e. Another objective was to test the selected biocatalyst for asymmetric oxidation of lansoprazole and rabeprazole sulfide. The only known literature method for the biocatalytic oxidation of omeprazole sulfide and lansoprazole sulfide failed to give any enantiomeric excess, although the same strain gave e.e. >99% for oxidation of rabeprazole sulfide.

The catalase-peroxidase reported in Section 3 was successful in oxidizing omeprazole sulfide but the enantiomeric excess was very poor. Therefore, we decided to continue screening of microorganisms for the desired activity. During the course of these initial studies, it became apparent that the product omeprazole is not very stable in buffers at pH 7.0. Omeprazole is a pro-drug, which in presence of hydrogen ions rearranges to the active form which, irreversibly binds to the gastric pump, thereby, deactivating it. It was, therefore, decided to perform the screening on model substrate benzyl phenyl sulfide and then test the selected microorganisms for their ability to oxidize omeprazole sulfide. Approximately 300 strains of bacteria and 100 strains of fungi were screened for the oxidase activity.

The strains ASW 112, ASW 115, ASW 119, ASW 138, ASW 142, ASW 145, ASW 235, BIMT 9004, BIMT 9068 and BIMT 9087 gave sulfoxide as the exclusive product. The conversion rate was high with ASW 115, ASW 119, ASW 138, BIMT 9068 and BIMT 9087 amongst the strains, which produced benzyl phenyl sulfoxide as the exclusive product. The strains ASW 118, ASW 132, ASW 134, ASW 139, ASW 140, BIMT 9001, BIMT 9068 and MTCC B6033 gave both sulfoxide and sulfone in the biocatalyzed reaction but only in the strain ASW 140 the formation of sulfone was faster than the sulfoxide. The strains ASW 124, ASW 128, ASW 129, ASW 131, ASW 144, ASW 149, ASW 221, ASW 228, ASW 361 and ASW 362 gave sulfone as the exclusive product but the reaction was faster in strains ASW 129, ASW 131, ASW 228 and ASW 362.

The strains, which gave sulfoxide, were selected and its enantiomeric excess determined by chiral HPLC. The e.e. was found to be in the range of 10 to >99%. The strains showed selectivity of either (*R*) or (*S*)-sulfoxide. The strains ASW 119, ASW 112, MTCC B6033, BIMT 9001 and ASW 235 gave (*R*)-sulfoxide as the major isomer though the e.e. was very less for MTCC B6033 and ASW 235. The strains ASW 115, ASW 118, ASW 132, ASW 134, ASW 138, ASW 139, ASW 140, ASW 142, ASW 145, BIMT 9004, BIMT 9068 and BIMT 9087 gave (*S*)-enantiomer but the e.e. varied from 9.5 for ASW 115 to 100 for BIMT 9004. One of the strains BIMT 9004 produced (*S*)-sulfoxide in 100% e.e.

The strain BIMT 9004 has been identified as *Staphylococcus saprophyticus* based on the morphological and biochemical characteristics. The strain *Staphylococcus saprophyticus* BIMT 9004, which gave 100% e.e. with benzyl phenyl sulfoxide, was tested for its asymmetric oxidation of omeprazole sulfide, but no conversion to omeprazole could be detected by TLC or HPLC. We also tested all the strains which gave benzyl phenyl sulfoxide, but none of them was able to oxidize omeprazole sulfide.

In view of the disappointing results obtained in following an indirect two-step approach, we decided to continue screening of microorganisms, but using omeprazole sulfide as the substrate instead of a model substrate. As mentioned earlier, omeprazole is prone to undergo degradation at pH 7.0 or lower, it was decided to do screening at pH 8.0-8.5. Although it would compromise the optimal reaction conditions for fungi and bacteria, yet the stability of the product omeprazole at pH above 8.0 would increase the probability of finding a suitable strain. Approximately, 400 strains of bacteria and 100 strains of fungi were screened for oxidase activity as described previously. Two strains of fungi designated as FIMT 9401 and FIMT 9434 produced omeprazole. Most of other strains tested were inactive or showed negligible activity. The structure of the product was confirmed by ^{1}H NMR spectral data, which was indistinguishable from the standard data of omeprazole spectra.

The microorganisms FIMT 9401 and FIMT 9434 have been identified as *Aspergillus carbonarious* and *Curvularia lunata*, respectively based on 16s *r*DNA sequence analysis. *Aspergillus carbonarious* produced (*S*)-omeprazole **(12)** in >99% e.e. The enantiomeric excess of omeprazole was determined by HPLC on chiral column using 250x4.6 mm chiralcel OB-H (Daicel, Japan). Elution was done with hexane:iso-propanol 90:10 at a flow rate of 1.0 mLmin^{-1} and the detection was done at 302 nm. The configuration was assigned as *S* based on retention times reported in literature.

Aspergillus carbonarious was able to produce lansoprazole and pantoprazole from the corresponding sulfide. They were assigned (*S*)-configuration based on the –ve sign of optical rotation, but their e.e. could not be determined as these compounds failed to resolve on OB-H and OD-H columns.

Cell-free extract obtained from *Aspergillus carbonarious* failed to oxidize ABTS in presence of H_2O_2, thereby ruling out the presence of a peroxidase. The monooxygenase activity was assayed with 5 mM omeprazole sulfide, 0.1 mM NADPH in 50 mM phosphate buffer pH 7.0 and 50 µL of cell-free extract in a total volume of 1 mL. The consumption of reduced coenzyme was followed at 340 nm using molar absorption coefficient of 6800 M^{-1}cm^{-1} for calculation. The enzyme showed activity of 1327 units per mL of cell-free extract

(1 unit = the amount of enzyme which catalyzed the reduction of 1 μmol of NADPH per min under specified conditions). The separation of membrane and cytoplasmic fractions was performed by ultra-centrifugation at 100,000 *g*. A dark-brown membrane pellet and cytoplasmic fractions were separated. The membrane fraction was washed twice with phosphate buffer pH 7.0. Both the fractions were tested for monooxygenase activity using NADPH as co-factor. Both the fractions were devoid of monooxygenase activity. Reconstitution of cytoplasmic fraction by combining cytoplasmic and membrane fraction regenerated nearly the whole activity. These results suggest that the enzyme responsible for activity is a multi-component monooxygenase.

In conclusion, a fungus *Aspergillus carbonarius*, has been isolated which produced (*S*)-omeprazole **(12)** in >99% e.e. by the oxidation of its precursor sulfide. Approximately, 400 strains of bacteria and 100 strains of fungi were screened to isolate the organism. The enzyme responsible for activity has been identified as a multi-component monooxygenase.

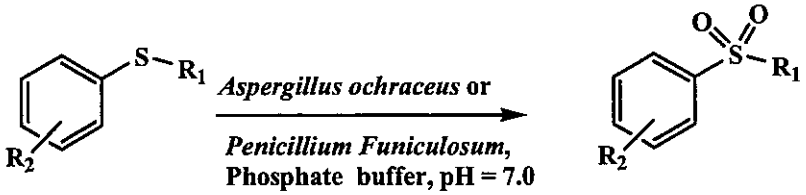
Section 5: Biocatalytic Oxidation of Sulfides to Sulfones

This section describes a method for the biocatalyzed oxidation of sulfides to sulfones. During the screening of microorganisms for the oxidation of sulfides (Section 2 and 4), it was noticed that a number of strains of microorganisms, were able to oxidize butyl phenyl sulfide and benzyl phenyl sulfide, but the desired sulfoxide was either not obtained or obtained only as a minor product. A close observation of the reaction showed that instead, sulfone formation had occurred in these cases. The use of sulfones in organic synthesis has become a classic strategy in the synthesis of many of the most demanding and sophisticated complex molecules. From the methodological point of view, sulfones have been employed in the preparation and functionalization of a wide variety of products by stabilizing α-radicals, α-anions and acting as cationic synthons. This prompted us to explore the formation of sulfones by biocatalytic route.

Approximately 20% of the strains tested (400 bacterial and 200 fungi) showed the formation of sulfone with conversion rate varying from 3 to 100% based on TLC analysis. Two strains of fungi, *Aspergillus ochraceus* MTCC 5245 and *Penicillium Funiculosum*, MTCC 5246 catalyzed oxidation of sulfides, **14-17** to corresponding sulfones **(18-21)** in 90-95% yield (Scheme 4). In all these examples, the product was different from corresponding standard sulfoxide prepared by oxidation with *m*-chloroperbenzoic acid but well corresponded with the standard sample of sulfone prepared by oxidation of the

corresponding sulfides with oxone®. The identity of sulfones in all cases was confirmed by ^{1}H NMR.

Scheme 4

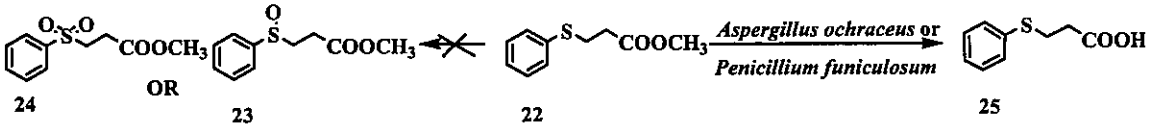


14 R_1= methyl, R_2 = H,
15 R_1= ethyl, R_2 = H,
16 R_1= butyl, R_2= H,
17 R_1= methyl, R_2 = 4-chloro

18-21

Methyl 3-(phenylthio) propionate **22**, prepared by conjugate addition of thiophenol on methyl acrylate on biocatalyzed reaction with *Aspergillus ochraceus* and *Penicillium Funiculosum* failed to give the desired sulfoxide **23** or sulfone **24**, but TLC showed complete consumption of starting material and formation of a high polarity compound. The NMR spectral data of the isolated product showed absence of ester methyl protons, instead a broad singlet was present at δ 11.12. The product was identified as **25** (Scheme 5). No sulfoxide or sulfone of either ester, **22** or acid, **25** could be isolated from the reaction mixture.

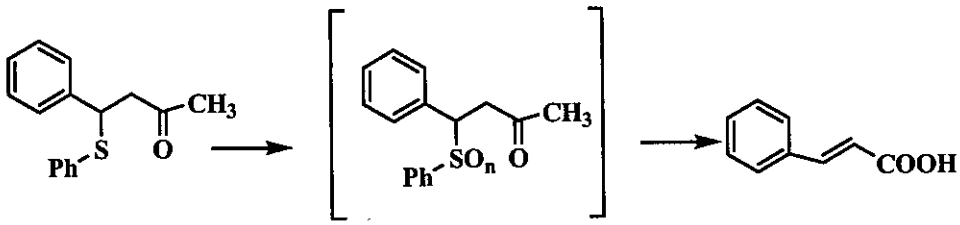
Scheme 5



24 **23** **22** **25**

4-phenyl-4-(phenylsulfonyl)butan-2-one **26** also failed to produce any sulfoxide or sulfone, but instead gave a product, which was identified as trans-cinnamic acid **28** based on ^{1}H NMR spectral data. In all probabilities the trans-cinnamic acid was produced via oxidation of sulfide to either sulfoxide or sulfone **(27)**. But neither sulfoxide nor sulfone could be isolated from the reaction mixture. Oxidation of **26** with H_2O_2 or oxone® also resulted in the formation of **28**, which indicated lability of the sulfoxide/sulfone under the reaction conditions (Scheme 6).

Scheme 6



26 27, n =1 or 2 28

In conclusion, two strains of fungi, *Aspergillus ochraceus* MTCC 5245 and *Penicillium funiculosum* MTCC 5246 have been identified which oxidized aryl alkyl sulfides to corresponding sulfones in high yield. The method is environmentally benign as it requires no organic solvents and can be carried under mild reaction conditions. However, methyl 3-(phenylthio)propionate **(22)** failed to give desired sulfone, instead only the hydrolysis of the ester functionality occurred to give acid, **25**. Oxidation of 4-phenyl-4-(phenylsulfonyl)butan-2-one **(26)** occurred, but sulfoxide/sulfone readily eliminated under the reaction conditions to give trans-cinnamic acid **(28)**.

Section 6 and Section 7

All experimental procedures and methods for section 1 to 5 have been described in Section 6. Section 7 is the bibliographic section.