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Prospecting for efficient enantioselective epoxide hydrolases

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Epoxide hydrolases (EHs) from microbial sources have recently been recognized as a versatile biocatalytic tool for the synthesis of enantiomerically pure epoxides and vicinal diols. Keeping in mind the potential of these compounds in pharmaceutical, agrochemical and flavour industries, a range of epoxide substrates have been analyzed using epoxide hydrolase as the catalyst. Enzymatic catalysis is often characterized by exquisite selectivity coupled with limited substrate scope. Hence, research efforts have been on to engineer known EHs for better enantioselectivity and to find novel enantioselective EHs with a wide substrate scope from culturables, non-culturables or from the genome database. Some of the results obtained are promising in terms of the practical utility of these enzymes in the asymmetric hydrolysis of epoxides.

Keywords: Enantioselective; epoxide hydrolase; vicinal diol

Introduction

Enantiopure epoxides and vicinal diols are valuable intermediates in the organic synthesis of chiral pharmaceutical compounds, drugs and agrochemicals[1]. Enantiopure epoxides can be produced chemically by several procedures, like indirect synthesis, stoichiometric resolution using an external chiral agent[2-5] and by the method of hydrolytic kinetic resolution (HKR) of racemic epoxides. HKR, which is a widely accepted method, is an enantioselective process wherein, one of the enantiomers of a racemic epoxide is selectively hydrolyzed to the corresponding product–a vicinal diol, while the opposite enantiomer is left behind unhydrolyzed. Chemically, HKR of racemic epoxides can be done by using chiral (Salen) CoIII complex as the catalyst[6], but this method has been found to be efficient only for terminal epoxides and not for the internal epoxides. In fact, all the chemical methods developed till date have their own limitations in terms of low substrate/catalyst ratios, efficiency and most importantly enantioselectivity.

Biocatalytically, enantiopure epoxides can be produced by monooxygenase-catalyzed stereo-selective epoxidation of alkenes[7]; by halohydrin dehalogenase-catalyzed reversible conversion of vicinal haloalcohols (halohydrins) to epoxides and halide ions[8] (Fig. 1a) and by kinetic resolution of racemic epoxides employing epoxide hydrolases (EHs)[1] (Fig. 1b). The applications of both monooxygenases and halohydrin dehalogenases have remained rather limited due to various reasons. Monooxygenases are the enzymes of the microbial decomposition pathway, in which epoxides are generated merely as intermediates and hence cannot be accumulated. Also, epoxides act as toxins and hence lead to the product inhibition of these enzymes. Halohydrin dehalogenases have been reported from only a few microbes, out of which, the best studied enzyme is HheC (from *Agrobacterium radiobacter* AD1), whose crystal structure and catalytic mechanism have also been elucidated[9]. Since halide release has been found to be a rate limiting step in the conversion of halohydrins to epoxides, attempts were made to modify the halide binding site of HheC by site-directed mutagenesis in order to improve the rate of halide release but to some success only[10]. Rather, HheC has been proven to be an efficient biocatalyst for the formation of enantioenriched β-azido alcohols and β-hydroxy nitriles via enantioselective ring opening of racemic epoxides using azide, cyanide and nitrite ions as the nucleophiles (Fig. 1a)[11-13].

However, both enantiopure epoxides and vicinal diols can be obtained with high enantiomeric excess using epoxide hydrolases (EH; EC 3.3.2), as these enzymes catalyze the enantioselective hydrolysis of

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the racemic epoxide compounds (either available commercially or synthesized chemically in the lab). EHs have a ubiquitous distribution in nature, being found in mammals, plants, insects, bacteria, fungi and yeast. In mammals, EH has been identified to play a role in the detoxification of xenobiotics in liver and also in the regulation of physiological signalling[14]. The mammalian soluble EH (sEH) has been found to be responsible for the degradation of natural inflammation inhibitors (epoxyeicosatrienoic acids; EETs) to the corresponding diols, thus making EH a potential therapeutic target for the screening of anti-inflammatory and cardiovascular drugs[15]. Since it is difficult to obtain larger quantities of mammalian EHs for their application in large-scale industrial production of epoxides, various microbial cultures were screened for having EH activity. Microbial EHs, however, can be obtained in unlimited amounts, especially after their overexpression in the appropriate hosts, due to the possibility of getting their larger biomass by fermentation. Among microbial sources also, due to the low enantioselectivity, only a few organisms could have been exploited for the resolution of racemic epoxides.

For an efficient kinetic resolution of a racemic epoxide, high EH enantioselectivity is a prerequisite. If the enzyme is highly enantioselective, the optical purity of the remaining unhydrolyzed epoxide and the formed diol will be quite high. In the EH catalyzed reactions, the enantiomeric composition of the substrate and/or product is further determined by the regioselectivity of the EH enzyme. Most of the EH enzymes have been found to be completely regioselective i.e. nucleophilic attack is always at the unsubstituted carbon atom of the epoxide ring. As shown in Fig.1b, opening of the epoxide ring at the EH active site can proceed with either inversion or retention of configuration at the carbon atom undergoing nucleophilic attack. In case of an enzyme which is not completely regioselective, this nucleophilic opening of epoxide can occur at either of the two carbon atoms of the epoxide ring. The substitutional pattern of the two carbon atoms of the epoxide ring (if different) also determines the enantiopreference of the enzyme. As a result, both the formed diol and the remaining non-hydrolyzed epoxide can have different enantiomeric composition (ee). Hence, there is a need to calculate ee, both for the substrate (ee_s) and the product (ee_p), independently.

EH enzymes not only have an advantage of no-cofactor requirement but also have been shown to have a broad range of substrate specificities towards both simple and complex epoxides, like aliphatic/aromatic monosubstituted epoxides, di- or tri-substituted epoxides, cyclic meso-epoxides, etc. Because of the potential interest of biochemists in the substrate resolutions catalyzed by the interesting EH enzymes, several reviews have been published on aliphatic/aromatic monosubstituted epoxides[16-21]. Lee and Shuler[19] had reviewed some enzyme evolution studies done on aliphatic and aromatic mono-substituted epoxides in 2007.

The current review specifically focuses on the microbial EHs and their substrate selectivity

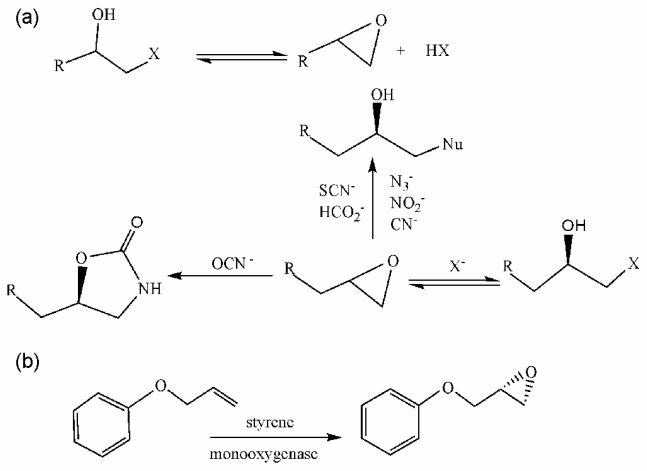


Fig. 1a—Enzymatic methods for the biocatalytic synthesis of epoxides, besides via EHs. (A) Epoxide ring closing and ring opening reactions catalysed by halohydrin dehalogenase; (B) Formation of (R)-phenylglycidyl ether from 3-phenoxypropener by monooxygenase.. Nu represents: N_3, NO_2, CN, SCN, OCHO and X represents: Cl, Br, I.

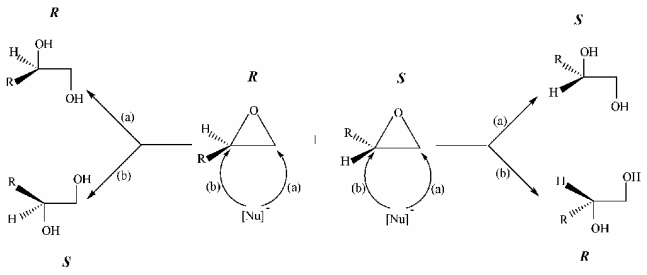


Fig. 1b—Stereochemical pathway of the catalytic mechanism of EH. There will be a formation of either *R*-diol and/or *S*-diol. The enzyme can have enantiopreference of *R*-epoxide or *S*-epoxide, but the ee will be scrambled if the enzyme is not regioselective (i.e. Attack is possible both at (a) the unsubstituted and (b) the substituted carbon atom of the epoxide ring. $[Nu]^-$: Nucleophile.

relationship with the disubstituted epoxides, mesoepoxides and the epoxides having lipophilic ether/ester functionality. This survey of literature describes the significant improvements that have been achieved upon the enantioselectivity of the promising, though limited EHs, found for these special types of substrates on the basis of literature from 2002 to 2010.

What are EHs?

EHs are ubiquitous enzymes responsible for the detoxification of genotoxic compounds and are involved in the synthesis of chemical messengers and secondary metabolites[22]. These enzymes specifically cleave the compounds having an oxirane/epoxide moiety to form the corresponding diols. Majority of the known EHs, with an exception of limonene-1,2-epoxide hydrolase[23], belong to the α/β hydrolase fold superfamily, consisting of a core domain and a cap domain[24]. The core domain, also known as α/β hydrolase fold domain, is composed of a central β-sheet packed with alternating α-helices on both sides (Fig. 2a). The variable cap domain having 5-6 α-helices is positioned on top of the core domain. The substrate binding pocket is located on the NC-loop connecting the core domain and the cap domain. The catalytic triad residues—Asp-His-Asp/Glu i.e. nucleophile-His-acid are located on the loops excursing from the β-sheet in the core domain. The catalytic nucleophile is invariably the Asp residue which is absolutely conserved and attacks the oxirane ring thus forming a covalent-ester-intermediate (Fig. 3). Subsequently, the other two residues of the triad, a histidine and an acid, through proton abstraction, build a charge relay system which activates a water molecule followed by the hydrolysis of an ester intermediate thus leading to the formation of diol product (Fig. 3). In the cap domain, there are two tyrosine residues, which are involved in hydrogen bonding with the oxygen atom of the oxirane ring, thus positioning the substrate properly for the nucleophilic attack by the Asp residue. The α/β hydrolase fold is found in other hydrolases as well like lipases, acetylcholinesterases, carboxypeptidases, haloperoxidases and haloalkane dehalogenases. All these enzymes differ in the type of reactions that they catalyze but all have a similar catalytic triad—nucleophile-His-acid. Though a significant variation has been found in the sequence of these enzymes, all of them have a highly conserved catalytic machinery[25].

One of the characteristics of the EH enzymes is that the sequence similarity is mainly found in some parts of the N-terminal region and around the catalytic triad residues[24]. In fact, the amino acid sequences of the two well-characterized EHs, from *A. radiobacter* AD1 (pdb entry: 1EHY) and *Aspergillus niger* (pdb entry: 1Q07) are only <10% identical, though both enzymes have virtually superimposable active sites and the characteristic α/β hydrolase fold[24]. A sequence homology of only < 20% was found among the 10 putative bacterial EHs identified from the genomic database, which have been shown to possess EH activity after overexpression in *Escherichia coli*[26]. A multisequence alignment of 95 EHs and their phylogenetic analysis led to the division of EH proteins into two superfamilies–the microsomal EH (membrane-bound protein) and cytosolic EH (soluble

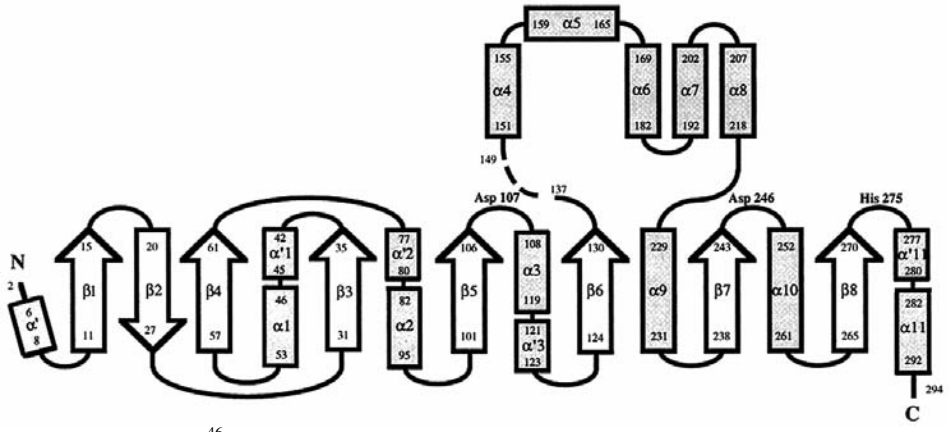


Fig. 2a—EH from *A. radiobacter* AD1[46]. Secondary structure topology diagram and location of the catalytic triad residues, Asp107, Asp246, and His275. Cap domain consists of upper helices α4 to α8, while the rest of the structure forms the core domain. The dashed line represents the missing loop 138-148. Short 3_{10} helices are located at the N terminus (α'), between β3 and α1 (α'1), between β4 and α2 (α'2), and between α3 and β6 (α'3).

proteins) superfamily. Bacterial EHs, which are found in both the superfamilies show a large diversity in their amino acid sequence[24]. It has been anticipated that this broad diversity of bacterial EH sequences is due to their diverse ecological habitats and therefore their exposure to a broad spectrum of substrates.

Despite this low sequence identity, two motifs of the core domain have been found to be highly conserved (Fig. 2b) -the HGXP motif (where X = aromatic residue) and GXS_mXS/T motif (where X = any amino acid and S_m = small amino acid). The function of the GXS_mXS/T motif is unclear while HGXP motif has been proposed to be involved in the stabilization of the oxyanion that is formed on the side chain carbonyl oxygen of the nucleophilic Asp during the hydrolysis of the ester intermediate (Fig. 3).

Substrates of EHs

Glycidyl Ethers

Epoxide compounds have broad applications in areas such as anticancer agents, β-blockers,

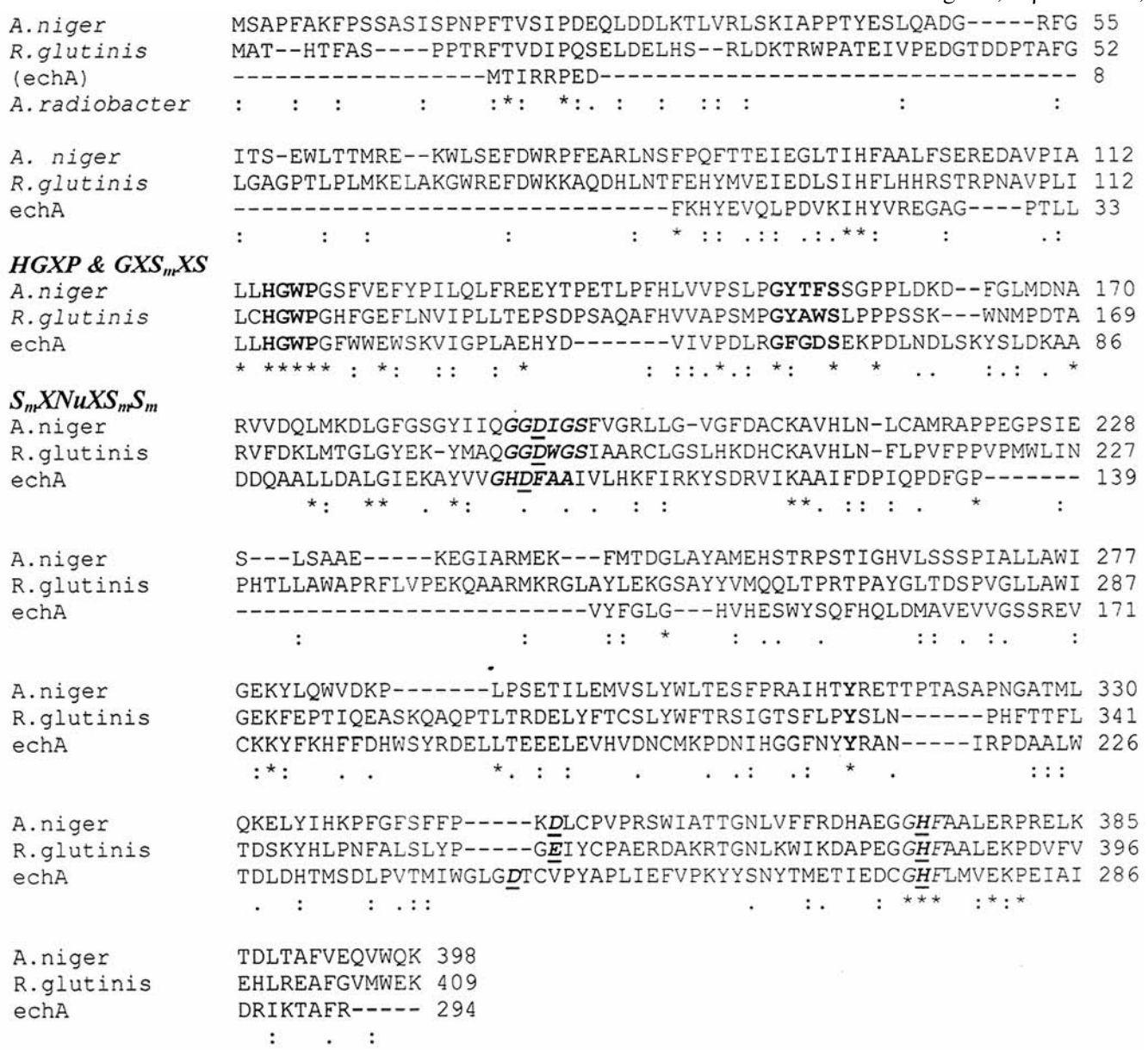


Fig. 2b—Clustal W (1.83) multiple sequence alignment of a fungal, yeast and bacterial EH indicating the conserved regions. The region of oxy-anion is indicated (*HGXP*), while $S_mXNuXS_mS_m$ indicates the genetic motif found in α/β-hydrolase fold enzymes (where S_m = small amino acid, *X* = Any amino acid, *Nu* = Nucleophile). The predicted catalytic triad residues are in bold and underlined with *D/E* being acidic and *H* being the basic member of the catalytic triad. The conserved *Y* (Tyr) residue plays a key role in forming hydrogen bonding with the epoxide ring.

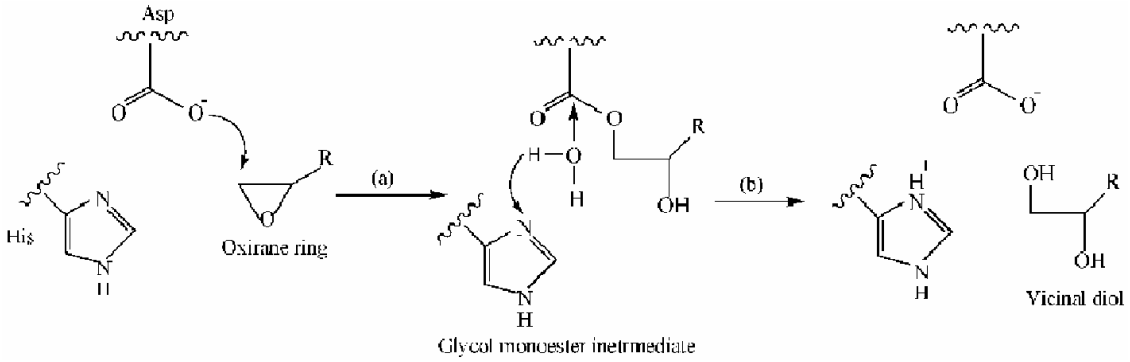


Fig. 3—General mechanism of EHs (a) The epoxide ring is attacked nucleophilically by the carboxylate group of aspartate residue to form a glycol monoester-enzyme intermediate (b) OH^- ion, derived from a water molecule (activated by histidine residue that acts as a proton acceptor) hydrolyzes the intermediate, thus forming vicinal diol and liberating the enzyme. R is any substituent on the substrate.

β agonists, antivirals, antifungals and antibacterials. Since, epoxides are highly reactive electrophiles due to the strain inherent in the three-membered ring and the electro negativity of the oxygen; they react readily with various O~, N-, S- and C-nucleophiles, acids, bases, reducing and oxidizing agents, leading to the formation of bifunctional molecules[27]. Epoxides with the general formula as given in Fig. 4a are commonly known as glycidyl ethers. (*S*)-Aryl glycidyl ethers and their related compounds are potentially useful intermediates for the synthesis of chiral amino alcohols and β-adrenergic receptor blocking agents. To take an example, (*S*)-phenyl glycidyl ether (PGE; Fig. 4a, where R_1 = H and R_2 = Ph) is easily converted into the final β-aminoalcohol products by stereocontrolled nucleophilic addition of amines to the enantiopure epoxide[27]. Vicinal diols, derived from glycidyl ethers, are also key building blocks for the synthesis of pharmaceuticals; e.g. (*S*)-aryl oxy diols are the intermediates for the synthesis of an expectorant, guaifenesin; for a muscle relaxant, mephenesin and an antifungal agent, such as clorphene[28]. Glycidyl ethers and glycidyl esters, having an epoxide in the glycidyl position (Figs 4a & b) and their derivatives are used in the coating compositions as mould prevention agents[29].

Attempts have been made to kinetically resolve these racemic compounds by employing enantioselective EHs from various microbial sources. Since these lipophilic oxiranes have a poor solubility in an aqueous buffer system, causing an apparent low substrate concentration, a very few compounds of this type have been investigated regarding their kinetic resolution by EHs. A yeast, *Trichosporon loubierii*, was found to preferentially hydrolyze the (*S*)-enantiomer of PGE[30] and its analogs, thus yielding

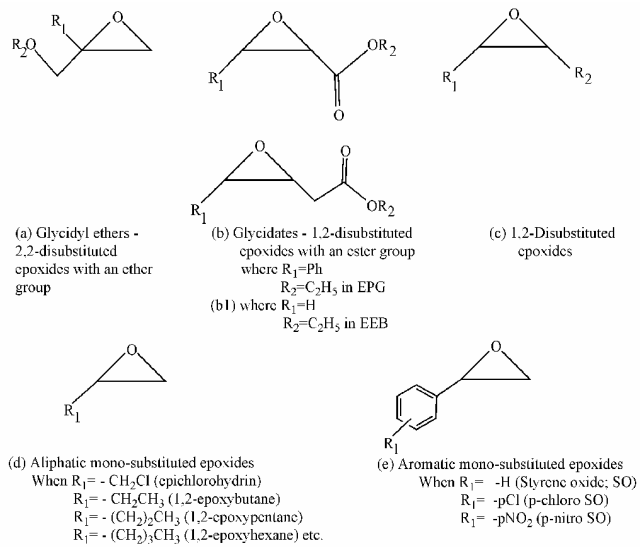


Fig. 4—Structures of the epoxide substrates.

(*R*)-PGE and (*S*)-diol, with good selectivity (Table 1). The EH enantioselectivity in the yeast lyophilized cells depended on the structure of the aryl group, as the bulky naphthyl group in comparison to phenyl group resulted in lower activity of EH. Also, the enantioselectivity was found to be more with the o-methyl group than with p-methyl group. However, the bacterial soil isolate, *Bacillus megaterium* ECU 1001 preferably hydrolyzed (*R*)-enantiomer of PGE[31], yielding (*S*)-PGE and (*R*)-diol with high enantioselectivity (E = 47.8) (Table 1). Using 5% (v/v) DMSO as the cosolvent for the sparingly water-soluble epoxide PGE (60 m*M*), and the lyophilized bacterial cells (30 g/L), (*S*)-PGE could be obtained with an ee of 99.5% and a yield of 25.6%. Thus, the availability of microbial whole cells having EH activity in the form of lyophilized powder has further

Table 1—Enzymatic hydrolysis of glycidyl ethers

Isolate	R_1	R_2	ee_s (%)	ee_p (%)	Enantio preference	E	Reference
T. loubierii ECU1040	H	-Ph	>99	60	*S*	20	30
	H	2-CH_3-Ph-	>99	72	*S*	41	″
	H	3-CH_3Ph-	95	71	*S*	21	″
	H	4-CH_3-Ph-	88	72	*S*	17	″
	H	Naphthyl	97	59	*S*	15	″
B. megaterium ECU1001	H	-Ph	-	-	*R*	47.8	31
	H	-Ph		100	*R*	94.0	34
B. megaterium ZJUZQ001	H	2-CH_3-Ph-			*R*	73	32
		4-CH_3-Ph-			*S*	21	
B. alcalophilus MTCC10234	H	-Ph derivatives	>99	Up to 89 >97	*R*	Up to 67	33
Rhodococcus sp. R312	CH_3	-CH_2-Ph	>97		*S*	>200	37
R. mucilaginosa DSM 70404	CH_3	-CH_2-Ph	62	13	*R*	8.6	″
C. laurentii M001	H	-CH_2-CH=CH_2	50	38	n.d.	3	38
R. fascians M 022	H	-CH_2-Ph	45	41	*R*	3-4	″
R. mucilaginosa M 002	H	-CH_2-Ph	77	53	*S*	7-8	″
WT-*A. niger* M 200	H	-CH-(CH_3)$_3$	>98	82	*S*	28-32	″
Recombinant-*A. niger* M 200	H	-CH-(CH_3)$_3$	-	-	*S*	60	55
Mutant-*A. niger* M 200	H	-CH-(CH_3)$_3$	-	-	*S*	100	″
WT-*A. niger* LCP 521	H	-Ph	-	56	*S*	4.6	41
Recombinant-*A. niger* LCP 521	H	-Ph	-	74	*S*	10.8	″
Mutant-*A. niger* LCP 521	H	-Ph	94	95	*S*	115	51
WT-*A. radiobacter* AD1 (EchA)	H	4-NO_2-Ph-	-	-	*S*	3.4	45
Mutant S4 -*A. radiobacter*	H	4-NO_2-Ph-	-	-	*S*	32	″
F108A mutant (EchA)	H	4-NO_2-Ph-	-	-	*S*	15	50
F108C mutant (EchA)	H	4-NO_2-Ph-	-	-	*S*	9.1	″
B. subtilis	H	4-NO_2-Ph-	-	-	*R*	22	26
	H	-Ph	-	-	*R*	-	″
D. radiodurans R1	H	4-NO_2-Ph-	-	-	*R*	1.9	″
	H	-Ph	-	-	*R*	1.4	″
N. punctiforme 1	H	4-NO_2-Ph-	-	-	*R*	16	″
	H	-Ph	-	-	*R*	3.7	″

Ph=Phenyl group; WT=Wild-type; n.d.=Not detected

encouraged the organic chemists to use EH as their synthetic tool to obtain enantiopure epoxides and diols. Lyophilized cell powder of another *B. megaterium* isolate ZJUZQ001[32] was used to enantioselectively resolve (*o*,*m*,*p*)-methyl phenyl ethers. Again, different methyl substitution patterns on the phenyl ring were found to play an important role in determining the enantioselectivity of this isolate. Here also, *o*-methyl group substitution resulted in higher enantioselectivity (E= 73) than with *p*-methyl group (E = 21). Moreover, (*o* and *m*)-methyl phenyl ethers yielded (*S*)-epoxide, while the p-methyl PGE resulted in (*R*)-epoxide. Growing cells of *Bacillus alcalophilus* MTCC10234, having EH activity, also showed a preference for (*R*)-methyl-substituted phenyl glycidyl ether derivatives and (*R*)-chloro-derivatives as compared to the nitro-derivatives[33] (Table 1). However, in this case, *m*-methyl PGE was found to be the best substrate with an enantiomeric ratio E=67, leading to enantiopure (*S*)-epoxide with 100% ee and 37% yield in 24 h.

The feasibility of employing EH enzymes in chiral resolutions of racemic epoxides was even strengthened by the stability of these enzymes in organic solvents. Rather, scientists were able to practically improve the enantioselectivity of EH enzymes using water-immiscible organic solvents or two-liquid phase systems. For the resolution of PGE, the EH enantioselectivity of *B. megaterium* ECU 1001 was improved by using a biphasic system[34], 1:5 (v/v) isooctane and potassium phosphate buffer (50 m*M*, *p*H = 8.0). A yield of 44.5% was achieved with 100% ee for 600 m*M* PGE in an isooctane phase with 1560 mg of whole cells in 100 mL buffer. The

enantiomeric ratio was also found to have increased significantly (E = 94.0) with the use of this water-immiscible organic phase as a reservoir for the less-soluble and chemically unstable PGE. On using 5% (v/v) DMSO as the cosolvent with the partially purified EH[35], an enantiomeric ratio of 41.2 was obtained with the hydrolysis of (*R*)-epoxide being 14.4-times faster than the (*S*)-epoxide. The enantiomeric ratio further rose to 69.3, on adding 0.5% w/v Tween-80 as an emulsifier to disperse the water-insoluble substrate PGE. In this emulsion system, the apparent enzyme activity significantly increased by about 1.8-fold with the hydrolysis of (*R*)-epoxide being 25.6-times faster than that of the (*S*)-epoxide. Another bacterial soil isolate, *Bacillus* sp. Z018, isolated from petroleum-contaminated bioremediation site, also showed an enantiopreference for (*R*)-PGE with an enantiomeric ratio of 23.7[36].

The kinetic resolution of (±)-2-methylglycidyl benzyl ether (Table 1) was carried out by employing the whole cells of bacteria, *Rhodococcus* sp. and yeast, *Rhodotorula mucilaginosa* DSM 70404[37]. The three *Rhodococcus* sp. bacterial isolates showed (*S*)-enantiopreference with enantioselectivity up to 71, while the yeast had an enantiopreference for (*R*)-glycidyl ether, though with low enantioselectivity (E = 8.6). An enhancement in the enantioselectivity (E > 200) of the *Rhodococcus* sp. R312 was again achieved by optimization of the various reaction conditions like temperature, substrate conc., catalyst loading, and the use of biphasic system of 1:5 (v/v) isooctane/Tris buffer (50 m*M*, *p*H=8.0). Although the enantiopreference was opposite (*S* and *R*) in these 2 bacterial and a yeast strains respectively, but the regioselectivity was found to be the same as all the three strains exerted a retention of configuration by directing the nucleophilic attack onto the less hindered unsubstituted carbon atom of the epoxide ring only.

For an epoxide, allyl glycidyl ether, (Table 1) the slim molecular structure of the allylic residue resulted in poor chiral recognition by the EH microbial isolates discovered by Kotik *et al*[38]. No culture with high enough enantio-selectivity was found, out of the 270 microbial isolates screened. A yeast strain, *Cryptococcus laurentii* M 001, however, showed activity with this substrate but with a poor enantioselective performance. When benzyl group was tested as a substituent of the glycidyl ether (Table 1), EHs of *R. mucilaginosa* M 002 and that of bacterium, *Rhodococcus fascians* M 022 showed opposite enantiopreference. The yeast reacted predominantly with the (*S*)-enantiomer and the bacteria preferring the (*R*)-enantiomer. With an even more substituted ether, tert-butyl glycidyl ether, (Table 1), an enantioselective EH activity was found in a fungal isolate, *A. niger* M 200. The fungal EH enzyme, purified partially, was found to be totally regioselective towards both the (*R*)- and (*S*)-enantiomers of this substrate (i.e. attack is always at the non-substituted carbon atom) though, (*S*)-tert-butyl glycidyl ether is hydrolyzed preferentially by this enzyme to (S)-tert-butoxy-1,2-propanediol.

Enantioselectivity Enhancement by Directed Evolution

Over the years, directed evolution has emerged as a powerful tool to improve enzymatic properties and to tailor them for the specific industrial applications. This molecular engineering technique has been successfully explored by the scientists to improve the enantioselectivity of epoxide hydrolases. EH from the filamentous fungus, *A. niger* strain LCP 521, has been studied in great detail and the recombinant enzyme, produced by an overproducing *A. niger* strain is already commercially available[39,40]. This enzyme was shown to catalyze the kinetic resolution of PGE with quite low enantioselectivity (E = 4.6). To enhance its enantioselectivity, the enzyme was subjected to directed evolution[41], which was improved two-fold (10.8) after only one round of error prone PCR (ep PCR) (Table 1). The improved mutant enzyme contained three amino acid substitutions (Ala217Val, Lys332Glu and Ala390Glu), two of which were far from the enzyme's active site. The mutant also showed EH activity towards other compounds like epichlorohydrin, 4,5-epoxycyclohexene, styrene oxide (SO), nitrostyrene oxides (*p*-nitro SO) (Figs 4d & e) and pyridyloxiranes[18,42-44].

With PGE, the EH of the bacterial species, *A. radiobacter* AD1 (WT-EchA), exhibited an enantioselectivity of 11[45], which is already a little higher than that of the improved *A. niger* mutant. The *A. radiobacter* EH has been well characterized; both structurally[46] and kinetically[47] and various active site mutants have been generated by site-directed mutagenesis to enhance its enantioselectivity[44,48,49]. However, van Loo *et al*[45] successfully improved its enantioselectivity via directed evolution. epPCR

technique was used to create a library of EchA variants. A prescreening of the mutant library was done by an agar plate assay and the subsequent screening of the active clones thus found, was done by spectrophotometric progress curve analysis using p-nitrophenyl glycidyl ether (pNPGE) as the substrate. Clones, which showed increased enantioselectivity were subjected to DNA shuffling and out of this shuffled library, eight improved clones were identified with enantioselectivity 5.5-13-fold greater than that of the wild-type EchA. The substrate, p-NPGE, chosen for screening acted as a mimic of an aliphatic epoxide substrate with the flexible side group such as epoxyalkanes or epihalohydrins (Fig. 4d). On further analyzing the substrate specificity of the clones, the highest improvements in the enantioselectivity were indeed found with epichlorohydrin and 1,2-epoxyhexane. The improved clones were found to have mutations focused around the enzyme's active site and the affected residues were mainly involved in the epoxide ring opening e.g. the mutant S4 had F108I, Y215H, Y205H and E 217V mutations (Table 1). The rationale of these mutational changes can be understood in the light of EH's mechanism of reaction. The reaction mechanism, elucidated from the crystal structure of EchA[46], revealed that D107 is the catalytic nucleophile in the wild-type EchA (Fig. 3), while the proton donor residues, Y215 and Y152 facilitate the epoxide ring opening (Fig. 2a). As a result of the nucleophilic attack of D107, the alkyl-enzyme ester intermediate was formed, which was subsequently hydrolyzed by a water molecule (in turn activated by H275 that acts as a proton acceptor). So, in case of the mutant S4 also, it was noted that the residue F108, next to the active site nucleophile D107, influences the enzyme selectivity or substrate binding. The role of F108 residue of WT-EchA in its enantioselectivity has recently been investigated via site-directed mutagenesis[50]. Here also, pNPGE was used to screen mutants with enhanced enantioselectivity using spectrophotometric progress curve analysis. On screening 200 mutants from this library, five mutants viz. F108A, F108C, F108Q, F108T and F108W with 3- to 7-fold improved enantioselectivity over WT-EchA, were obtained. It was noted that 3- to 9-fold reduction in K_M for the preferred (S)-enantiomer was largely responsible for the improvement of enantioselectivity of the mutants F108I, F108Q and F108T.

Most directed evolution techniques like epPCR and DNA shuffling target the whole gene to generate random mutant libraries of large sizes. Reetz *et al*[51], however, used a method called combinatorial active site saturation test (CAST) to evolve substrate acceptance and/or enantioselectivity of WT-EH of *A. niger* LCP521. This technique generated focused mutant libraries of relatively smaller size that resulted from amino acid randomization at defined positions only. The substrate-binding site of the WT-EH of *A. niger* has been revealed as a narrow hydrophobic tunnel from its X-ray crystal structure[52] (http://www.pdb.ccdc.cam.ac.uk/pdb). The 3D structure of the enzyme was modeled with PGE as the substrate in the binding pocket[51], and six amino acids whose side chains reside next to the binding pocket, were identified. So first of all, six CAST libraries were produced separately by randomization at those six sites, 193/195/196 (A), 215/217/219 (B), 329/330 (C), 349/350 (D), 317/318 (E) and 244/245/249 (F). Then, the gene of an improved mutant originating from one site (here B) was used as a template to perform randomization at the other site (C and D), the process being repeated until the desired degree of catalyst improvement was achieved. The best mutant LW202 having E=115, was found to have a total of nine mutational changes i.e. L215F, A217N, R219S, L249Y, T317W, T318V, M329P, L330Y and C350V. Thus, the enzyme variant LW202 had evolved stepwise in an evolutionary pathway which consisted of five iterative CAST cycles i.e. B→ C→ D→ F→ E. By applying a deconvolution strategy[53] to the five sets of mutations and measuring the enantioselectivity factors of the EH variants, $\Delta G^{\neq}$ values were calculated, which were plotted to construct a fitness landscape of the experimental evolutionary process. With this strategy, out of 5! = 120 evolutionary pathways leading from WT to the best mutant LW202, 55 (~50%) pathways were found to be energetically favourable. Thus, iterative saturation mutagenesis (ISM) has been shown to be a promising approach to more efficient directed evolution. The underlying factors responsible for the enantioselectivity enhancement in the mutant LW202 were analyzed by molecular modeling, MD calculations and X-ray structural studies[54]. The His-tagged enzyme variant LW202 was affinity purified and its kinetic properties were also determined both with (*R*)- and (*S*)-PGE. The evolved enzyme had a pronounced preference for (*S*)-PGE and was found to have much lower k_{cat}/K_M for (*R*)-PGE than that for the preferred (*S*)-PGE. It has been proposed that the ideal

positioning of the preferred (*S*)-substrate in the binding pocket is the underlying reason for this selectivity, which does not happen with the disfavoured (*R*)-enantiomer. With the help of the ligand-free X-ray crystal structure of LW202 resolved at 1.5 Å[54], the binding pockets of WT[52] and that of LW202 mutant were compared. Significant geometrical differences were found between the binding pockets of the two, especially around sites B and E. On manually docking the two enantiomers of the substrate PGE, separately into the binding pockets of the WT and the mutant LW202, it was found that there were severe steric clashes between the (*R*)-substrate and the mutated residues, especially at sites B and E of the mutant, which leads to the non-productive binding of this enantiomer.

The EHs of *A. niger* strains LCP521 and M 200 have 89.4% identity and the catalytic triad, Nucleophile-His-Acid, is also found at the same positions, D192, H374 and D348 (Fig. 2b). Besides these common structural features and motifs, both the enzymes have same enantiopreference and so, M200 EH is expected to have similar structure of the substrate-binding tunnel region as that of LCP521. To improve its EH activity, the amino acids of the substrate-tunnel region of *A. niger* M 200 EH were substituted[55]. It was found that the replacement of A217 by Val led to a six-fold higher activity towards allyl glycidyl ether while the mutant A217L showed enhanced E-value of 100 towards tert-butyl glycidyl ether (Table 1). Earlier attempts on the enantioselectivity enhancement had also pointed towards the residue 217 as one of the important sites for interaction with the epoxide substrate.

Thus, from the above studies it is clear that to meet the growing demand of EHs in synthetic applications, more efficient technologies are being developed to generate mutants of the existing EHs. Though directed evolution has been able to address the low enantioselectivity problem, further improvements on EHs is yet to be done using this molecular technique. Substrate specificity, stability in organic solvents, less sensitivity to product inhibition are the other enzymatic properties of EH that need to be worked on, in future for them to be used commercially in the industrial organic synthesis.

Mining the Genomic Database for Enantiospecific EHs

Another successful bioinformatics approach that has been explored by several groups is the mining of genomic database for putative EH-encoding genes, using EH-specific sequence motifs, and their expression in the surrogate host cells. Though most of the known EHs have been found to have a low sequence identity, yet the presence of some conserved sequence motifs like nucleophillic elbow (Sm-X-Nu-X-Sm-Sm), the G-X-Sm-X-S/T motif, the H-G-X-P oxyanion hole and the Asp-His-Asp catalytic triad have helped to annotate a α/β hydrolase fold gene as an EH (Fig. 2b). By using these conserved EH sequence motifs for the bioinformatics analysis of the vast microbial genome databases available, the other structurally and mechanistically related α/β hydrolase gene sequences such as those of esterases and dehalogenases can easily be separated out. Using this bioinformatics approach in our lab, we have successfully identified, cloned and overexpressed a few novel EH bacterial genes and have also characterized one of them in detail (unpublished results).

Putative microbial EHs from the genomic database of different phylogenetic groups were first identified by van Loo *et al* in 2006[26]. The amino acid sequences of the known EHs were used as queries. They cloned 10 putative EH-encoding genes in *E. coli* and tested them for their biocatalytic potential. All the cloned active EHs were produced as fusion proteins with maltose binding protein, MalE. The cloned EHs from *Bacillus subtilis*, *Deinococcus radiodurans* R1 and *Nostoc punctiforme* were found to be (*R*)-specific towards phenyl glycidyl ether (Table 1) but (*S*)-specific towards an aryl epoxide (SO). The same enantiopreference for aryl glycidyl ethers has been reported in other bacterial species viz. *B. megaterium*[31,32], *B. alcalophilus*[33] and *Rhodococcus fascians* M022[38]. The EH of *A. radiobacter*[56] shows an opposite enantio-preference (*S* and *R*) towards aryl glycidyl ethers and aryl epoxides, respectively (Table 1).

An EH-encoding gene from *Caulobacter crescentus* was also identified by genome data mining[57] and the recombinant protein was found to be (R)-specific towards SO and indene oxide[58]. Three EH-encoding genes (*eeh1*, *eeh2* and *eeh3*) were identified from the genome sequence of a marine bacterium, *E. litoralis* HTCC2594[59]. All the 3 genes were cloned in *E. coli*, expressed as His-tagged proteins and purified. The 3 enzymes were found to have different enantioselectivities towards SO, PGE and other epoxides. *eeh1* was enantioselective for (*R*)-SO and (*S*)-PGE while *eeh3* was enantioselective

for (*S*)-SO but could hydrolyze (*R*)-PGE and (*S*)-PGE at the same rate. *eeh2*, though showed EH activity, was found to have rather no enantiopreference.

The availability of the genome sequences of about 100 marine organisms, determined by Moore Foundation, facilitated the development of bacterial EHases from marine environment. A putative EHase (SEH) having 42% homology with EEH1 of *E. litoralis* HTCC2594 was found in *Sphingophyxis alaskensis*[60]. The thus encoded recombinant EHase was cloned and purified from *E. coli*. Though, the purified SEH displayed EH activity towards various aliphatic and aromatic epoxides, but without any enantioselectivity at all. In *Novosphingomonas aromaticivorans*, the putative EHase (NEH) had 67% homology to EEH1 of *E. litoralis* HTCC2594[61]. The recombinant purified NEH could preferentially hydrolyze (*R*)-SO (from 17 m*M* racemic SO) with 99% ee and (*S*)-SO was obtained with 11.7% yield in 20 min. A putative EH-encoding gene (*REH*) from *Rhodobacterales bacterium* HTCC2654 has been found to have 26% identity to EEH3[62]. The recombinant (*S*)-specific REH protein could yield 38.4% (*R*)-PGE from 29.2 m*M* racemic PGE in 20 min with 99.9% ee.

Besides, having an application in industrial biocatalysis, microbial EHs have also been shown to play a key role in natural product biosynthesis, like that in enediyne antitumour antibiotic C-1027, produced by *Streptomyces globisporus* SB 1010. A putative EH sequence (SgcF), identified by a similar bioinformatics approach, from the C-1027 biosynthetic gene cluster of this actinomycetes[63], was indeed found to encode an EH when expressed in *E. coli*. The EH had complementary enantio- and regiospecific activities towards both (*S*)-SO and (*R*)-SO, which was used as a mimic of the enediyne core epoxide, and hence formed an enantio-enriched (R)-diol exclusively, from both the enantiomers of SO. This characterization study of SgcF helped in defining the function of SgcF as an EH responsible for the formation of (*R*)-vicinal diol, as a key enediyne intermediate in the biosynthesis of the chromoprotein antitumour antibiotic C-1027.

Glycidates

Enantiopure glycidates are important compounds in chiral drug synthesis. In particular, (2R,3S)-3-phenylglycidate is an efficient intermediate for taxol and taxotere side chains[64]; (2R,3S)-3,4-methoxy phenyl) glycidate is a key intermediate for Diltiazem[65] used in the treatment of angina and hypertension.

Enantiopure glycidates can be obtained by the catalytic action of lipases[66], esterases or epoxide hydrolases. However, owing to the practical problems in the lipase/esterase mediated hydrolysis of racemic glycidates, the organic biochemists have tried to resolve these compounds by the catalytic action of EHs. Only a few microbes having EH activity towards glycidates have been isolated from the natural biodiversity. Among actinomycetes, EH activity has been found in *Streptomyces antibioticus*, *S. arenae* and *S. fradiae*[67]. *S. antibioticus* Tu4 cells could hydrolyze ethyl-3-phenyl glycidate (EPG, Fig. 4b) with the remaining substrate (ee_s) of 81% ee after 2.5 h reaction. In another study, the enantioselective EH of *Pseudomonas* sp. could produce (2R,3S) ethyl-3-phenylglycidate with 95% ee and 26% yield in 12 h from 0.2% (w/v) of the racemate[68]. Bacterial EH from the newly isolated (*S*)-specific *Acinetobacter baumannii*[69] showed enantiomeric ratio (E) of 33, with the substrate ethyl-3,4-epoxybutyrate (EEB), resulting in >99% ee and 46% yield of its (*R*)-enantiomer. EEB is also a terminal epoxide (Fig. 4b) having an ethyl ester group like EPG. (*R*)-EEB can directly be converted to (*R*)-γ-amino-β-hydroxybutyric acid, which acts as a neuromediator with antiepileptic and antihypertensive activity as well. (*R*)-EEB is also a useful intermediate for the synthesis of salicylate enamide anticancer agents Lobatamide C and new nitrogen mustards, which are modified carnitine analogs[69].

Disubstituted Epoxides and Meso-epoxides

In case of disubstituted oxiranes, since the steric requirements are similar at both the carbon atoms of the epoxide ring, the ring-opening can occur at both positions, but at different ratios. As a result, E value must be calculated on the basis of the conversion ratio and the enantiomeric excess of the residual epoxide[70]. In most cases, it was found that the hydrolysis proceeded in an enantioconvergent manner i.e. only one diol, (*R*,*R*)- or (*S*,*S*)- was formed as the sole product. This happens when one substrate enantiomer is hydrolyzed with retention of configuration while the other substrate enantiomer is hydrolyzed with inversion of configuration (Fig. 1b). Out of the 10 cloned putative EHs identified from the database of sequenced genomes[26] as described earlier, only a few enzymes showed activity with these sterically more demanding α,β-disubstituted epoxides (Fig. 4c). EH

from *Rhodopseudomonas palustris* was found to be active towards trans-stilbene oxide (Fig. 4c, R_1 = Ph; R_2 = Ph), but not towards cis-stilbene oxide. *N. punctiforme* EH was also found to be active with trans-stilbene oxide (E value-not mentioned)[26].

The regioselective conversion of the (*R*,*S*)-configured cyclo meso-compounds (Table 2) also results in optically enriched vicinal diols due to the inversion of configuration at one of the two stereocenters. In 2003, Chang *et al*[71] reported *Sphingomonas* sp. HXN-200 to be the first known bacterial EH capable of catalyzing the hydrolysis of carbocyclic meso-epoxides like epoxycyclohexane (Table 2). An EH enzyme with this substrate specificity, though found earlier in a yeast[72] and fungal[73] source, had quite low enantioselectivity.

Screening of Novel EHs in Environmental DNA

The discovery for novel EHs is not just limited to their screening from the limited culture collections but rather has even been attempted from the unculturables. The genome of uncultured microbes, known as metagenome, has been recognized as a rich source of novel biocatalysts. In 2004, Zhao *et al* generated DNA expression libraries directly from the environmental samples collected from various global habitats[74]. The metagenomic library was screened for the novel EHs with unique amino acid sequences by activity-based high-throughput assays. All new EHs had a conserved aspartate residue, which is an active-site nucleophile that attacks at the epoxide ring in the first step of catalysis. The bulky Cis-stilbene oxide with varying substituent groups and dipyridyl derivatives were analyzed, for activity with the environmental EH enzyme BD 8877 (Table 3). It was able to form disubstituted (R,R)-1,2-ethane diols with enantioselectivity >80%, though it showed the highest specific activity and enantiomeric excess (ee) with bis(2-pyridyl) epoxide. BD 9126 EH predominantly formed S,S-diols with cis-stilbene oxide and 2-chloro-cis-stilbene oxide.

Hydrolytic regioselective ring-opening of meso-epoxides has thus far only been described for enzymatically by EH enzymes[74], and no chemical catalysis of these conversions has been reported with organometallic (Salen) Co (III) catalysts, though these metal catalysts hydrolyze the terminal epoxides in an enantioselective manner[6]. Epoxide hydrolases can produce optically enriched vicinal (*R*,*R*)-diols or (*S*,*S*)-diols in 100% theoretical yield through regioselective hydrolysis of meso-epoxides. The three F108 EchA site-saturation mutants, as earlier described[50], were improved for their enantioselectivity as compared to the wild-type (EchA). They showed upto>150-fold higher activities towards cis-disubstituted and meso-epoxides. The EH mutant F108C, could convert cis-2, 3-epoxybutane to (2R,3R)-2,3-butanediol with a 7-fold improved activity than WT-EchA and >99% ee (Table 3). The EH mutant F108A was also used for the preparative

Table 2—Enzymatic hydrolysis of cyclic meso-epoxides

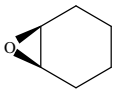
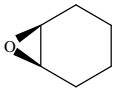
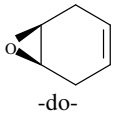
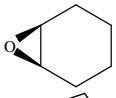
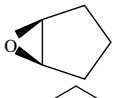
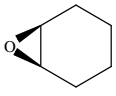
Isolate	Epoxide	Structure	ee_p (%)	Enantiopreference	Reference
Sphingomonas sp. HXN-200	Epoxycyclohexane		87	(*R*,*R*)	71
N. punctiforme 1	Epoxycyclohexane		97	(*S*,*S*)	26
BD 10159	4,5-Epoxycyclo hexene		76	(*S*,*S*)	74
BD 9883	-do-	-do-	91	(*R*,*R*)	″
BD 9883	Epoxycyclohexane		96	(*R*,*R*)	″
BD 10721	Epoxycyclopentane		90	(*R*,*R*)	″
F108A mutant (EchA)	Epoxycyclohexane		>99	(*R*,*R*)	50

Table 3—Enzymatic hydrolysis of disubstituted epoxides

EH	R_1	R_2	ee_p (%)	Enantio-preference (Formed diol)	
eEH BD 8877	-Ph	-Ph	99	*(R,R)*	74*
	2-Cl-Ph-	2-Cl-Ph-	98	*(R,R)*	″
	2-F-Ph-	2-F-Ph-	80	*(R,R)*	″
	3-Cl-Ph-	3-Cl-Ph-	98.5	*(R,R)*	″
	4-Cl-Ph-	4-Cl-Ph-	>99.5	*(R,R)*	″
	2-Pyridyl	2-Pyridyl	99	*(R,R)*	″
	3-Pyridyl	3-Pyridyl	97	*(R,R)*	″
	4-Pyridyl	4-Pyridyl	98	*(R,R)*	″
eEH BD 8876	-Ph	-Ph	99.5	*(R,R)*	″
eEH BD 9300	-Ph	-Ph	96	*(R,R)*	″
eEH BD 9883	-Ph	-Ph	99	*(R,R)*	″
eEH BD 9126	-Ph	-Ph	99	*(S,S)*	″
	2-Cl-Ph-	2-Cl-Ph-	99	*(S,S)*	″
F108C mutant (EchA) eEH Kau2	$-CH_3$	$-CH_3$	>99	*(R,R)*	50
	-Ph	$-CH_3$	99.5	*(R,S)*	76

where eEH-environmental EH.
*References

scale conversion of epoxycyclohexane to cyclohexane diol as it showed >150-fold higher activity than the WT-EchA and could yield 9.8 g of (*R*,*R*)-cyclohexane-1,2-diol with an ee of 98% from 13.5 g of epoxycyclohexane (Table 2).

Another approach to identify EH genes in a metagenomic DNA library has recently been developed by Kotik *et al*[75,76]. They have amplified the EH-specific gene fragments directly from the metagenome using degenerate primers targeted to the conserved EH motifs (Fig. 2b) followed by its assemblage by genome-walking. The three recombinant EHs obtained were expressed in *E. coli* and characterized with a range of epoxides. Both the recombinants, Kau2 and Kau8, were found to have a broad substrate spectrum with Kau2 preferring for (*R*)-epoxides (attacking on non-substituted carbon of the epoxide ring) but had a complementary regioselectivity for (*S*)-epoxides, thus ultimately resulting in (R)-diol with a high ee-value. The highest enantioselectivity (E > 200) was achieved for the disubstituted epoxide, trans-1-phenyl-1, 2-epoxypropane (Fig. 4c, R_1= Ph; R_2= CH_3). On a preparative scale, the EH Kau2 was able to biotransform 600 m*M* of this racemic epoxide to (1*R*, 2*R*)-epoxide with an ee of 99.3% and 48% yield in a biphasic system having 8% (v/v) ethanol as the co-solvent. The corresponding (1*R*, 2*S*)-diol had 99.5% ee in 46% yield. The third recombinant enzyme was found to have quite low enantioselectivity towards aliphatic terminal epoxides i.e. 1, 2-epoxyhexane (E=5.9), 1,2-epoxyoctane (1.5) and 1,2-epoxydecane (2.1) (Fig. 4d), while its activity towards other disubstituted epoxides or meso-compounds was not determined.

Enantioconvergent Hydrolysis of Terminal Aliphatic and Aromatic Epoxides

Despite its widespread applications, EH-catalyzed HKR, like all other classic kinetic resolutions, has a major limitation that the theoretical yield of each enantiomer is always restricted to 50%. Enantioconvergent strategy is one of the most efficient methods to overcome the 50% yield limitation. This process employs two EHs, one that attacks the unsubstituted carbon atom of the epoxide ring e.g. (*R*)-enantiomer (Fig. 1) yielding (*R*)-diol (retention of configuration), while the second EH attacks the substituted carbon atom of (*S*)-epoxide, and again producing (*R*)-diol (Inversion of configuration), thus leading to the production of enantiomerically enriched vicinal-diol with a theoretical yield of 100%. Only a few examples of enantioconvergent bioconversions exist. Some groups have attempted the enantioconvergent synthesis of enantiopure diols with a high yield using two EH enzymes having complementary enantio- and regioselectivity, a (*S*)-specific plant EH and the other (*R*)-specific bacterial EH[77]; a (*R*)-specific marine fish EH and the other (*S*)-specific bacterial EH[78]. Such hydrolysis can also be done using a single EH having complementary enantioselectivity and regioselectivity towards epoxides[76,79,80]. The recent progress on EH-mediated enantioconvergent bioconversions to prepare chiral aliphatic and aromatic epoxides and alcohols, has been reviewed by Lee[81].

The (*R*)-specific marine fish EH was engineered by site-directed mutagenesis[82] to improve the catalytic reaction rate of the enzyme towards an aromatic epoxide SO. The active site of fish *Mugil cephalus* EH was modeled using X-ray crystal structure of *A. niger* as the template[52]. On superimposition, certain differences between the active sites of the two EHs

were found, even though the topologies of the two were similar. So, a triple point mutant of *M. cephalus* EH was created by site-directed mutagenesis—mutation F193Y for spatial orientation of the nucleophile (D199), E378D for good charge relay in the active site (as unlike bacterial EHs, fish EH has glutamate instead of aspartate in its charge-relay system) and W200L for removing the electron density overlap between W200 and Y348. The resulting (*R*)-specific EH mutant showed 35-fold faster reaction rate to achieve 98% ee of (*S*)-styrene oxide formed from 10 m*M* racemic styrene oxide.

These studies clearly show that EHs have been engineered successfully to have higher activities and improved enantioselectivities, by applying various molecular and enzyme engineering techniques in order to make them efficient biocatalysts in the asymmetric synthesis of epoxides and diols.

Conclusions and Future Perspectives

The ubiquitous distribution of epoxide hydrolases (EHs) and hence a wide range of substrate specificity and enantioselectivity makes these enzymes an ideal choice for biotechnological applications. As a source of biocatalysts, microorganisms are more suitable than higher organisms like mammals, because it is easier to cultivate large amounts of biomass to obtain larger quantities of enzymes as biocatalysts. The microbial wild-type and recombinant EHs that have been characterized till date have all been found to be soluble in the cytosol (both soluble and microsomal ones) and thus could easily be purified (Table 4). The intracellular nature of these enzymes combined with the advanced molecular biology tools has made the production of EHs on a preparative scale much easier.

From this review of the recent studies done, it is very clear that EHs are promising biocatalysts for the preparation of chiral epoxides and vicinal diols. The

Table 4—Physico-chemical properties of the characterized microbial EHs

Organism	Class	Subunit size	Optimum *p*H/Temp.	$K_{cat\ (S^{-1})}$ (for R/S SO)	Reference
S. carzinostaticus ATCC15944	nd	44.5 kDa	8.0/25°C	137/33 min^{-1}	83
eEH Kau2	nd	nd	7.5/40°C	nd	76
eEH Kau8			7.5/36°C		
S. globisporus SB 1010	nd	44.5 kDa	8.0/28°C	4.3/48	63
eEH pSEEH9	Soluble	~34 kDa	7.5/25°C	nd	75
P. chrysosporium	Soluble	Monomer/42 kDa	9.0/40°C	nd	84
N. aromaticivorans	Microsomal	42 kDa	6.5/45°C	27.28/13	61
S. alaskensis	Microsomal	49 kDa	6.5/35°C	7.42/10	60
A. niger M200 EH	Microsomal	Dimer/ ~43.0 kDa	7.5/28°C	8.5	55
A. niger SQ-6	Microsomal	41 kDa	7.5/37°C	172 for pNSO	85
B. rhodina	nd	Partially purified (28-40 kDa)	nd /30°C	nd	86
E. litoralis HTCC2594 rEEH1	Microsomal	41 kDa	6.5/50°C	66.1/34.1	59
rEEH2	Soluble	33.4 kDa	7.5/55°C	2.17/2.24	〃
rEEH3	Soluble	34.5 kDa	8.0/45°C	1.08/3.34	〃
R. opacus ML-0004	nd	Monomer/28 kDa	7.5/30°C	nd	87
R. mucilaginosa AY627310	Microsomal	1979 bp ORF having 9 introns (44 kDa)	7.5/25°C	nd	88
C. crescentus	nd	37 kDa	8.0/37°C	nd	58
M. tuberculosis R_V2740	Similar to LEH	Dimer	nd	nd	89
Rv3617	nd	35 kDa			90
Rv1938	nd	40 kDa			90
R. paludigenum	High homology with *R. glutinis*	1600 bp ORF having 6 introns (46 kDa)	nd	nd	91
R. erythropolis DCL 14	New class of EH (LEH)	Monomer/16.5 kDa	7.0/50°C	nd	23

eEH- environmental EH
nd- Not determined
LEH- Limonene-1,2-epoxide hydrolase

use of EHs for biotechnological applications requires them to perform under the demanding industrial conditions. This includes acting on substrates for which the currently known arsenal of EHs was not evolutionarily selected. The potential use of EHs in the industrial applications cannot be fully realized until the following limitations of EHs are urgently dealt with: (1) the number of enzymes available is small, (2) the spectrum of substrates for the known EHs is limited, and (3) enantioselectivity is low, which is an utmost important factor to be determined for the practical utility of these recently discovered enzymes. Studies highlighted herein show that though various approaches have been tried to develop EHs with desired enantioselectivity in the last decade. Yet, novel EHs need to be discovered through a continued mining of new EHs from the nature's vast reserves or identifying novel enzymes in metagenome/environmental samples or through bioinformatics based *in-silico* screening of our expanding genome databases. Equally important is to improve the stereoselectivity and activity of the existing and new EHs using protein engineering technologies.

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