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REVIEW

An Overview on the Enhancement of Enantioselectivity and Stability of Microbial Epoxide Hydrolases

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Abstract Epoxide hydrolases (EHs; 3.3.2.x) catalyze the enantioselective ring opening of racemic epoxides to the corresponding enantiopure vicinal diols and remaining equivalent unreacted epoxides. These epoxides and diols are used for the synthesis of chiral drug intermediates. With an upsurge in the methods for identification of novel microbial EHs, a lot of EHs have been discovered and utilized for kinetic resolution of racemic epoxides. However, there is still a constraint on the account of limited EHs being successfully applied on the preparative scale for industrial biotransformations. This limitation has to be overcome before application of identified functional EHs on large scale. Many strategies such as optimizing reaction media, immobilizing EHs and laboratory-scale directed evolution of EHs have been adopted for enhancing the industrial potential of EHs. In this review, these approaches have been highlighted which can serve as a pathway for the enrichment of already identified EHs for their application on an industrial scale in future studies.

Keywords Epoxide hydrolase · Organic solvents · Ionic liquids · Deep eutectic solvents · Immobilization and directed evolution

Abbreviations

EHs	Epoxide hydrolase
ILs	Ionic liquids
DESs	Deep eutectic solvents
DMSO	Dimethylsulfoxide
HBDs	Hydrogen bond donors
EDA	Ethylenediamine
IDA	Iminodiacetic acid
CPG	Calcium pectate gelled
SA	Sodium alginate
CS	Cellulose sulfate
PMCG	Poly (methylene-*co*-guanidine)
Fmoc-FF	Fluorenylmethoxycarbonyl diphenylalanine
PEI	Polymer, polyethyleneimine
CLEAS	Cross-linked enzyme aggregates
epPCR	Error-prone polymerase chain reaction
PCR	Polymerase chain reaction
ISM	Iterative saturation mutagenesis
CAST	Combinatorial Active Site Saturation Test
B-FIT	B-Factor Iterative Test
ASRA	Adaptive substituent reordering algorithm
FRESCO	Framework for Rapid Enzyme Stabilization by Computational Libraries

Introduction

Chirality is a new emerging subject for academic research as well as for pharmaceutical development. Chirality has substantial effects on the physiological activity of biomolecules as (*R*)- and (*S*)-enantiomer of the racemic drug show different activities in a biological system. According to the strong regulations of US FDA for the manufacture of enantiopure drugs, the pharmaceutical companies are being directed to develop single enantiomeric drugs. Chiral drugs show continuous growth worldwide, and thus, the majority of the topmost selling drugs are chiral.

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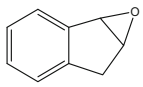
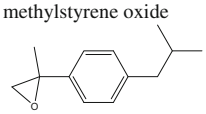
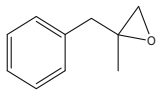
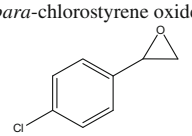
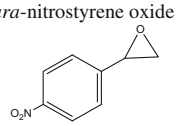
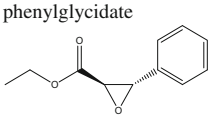
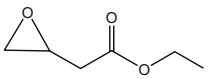
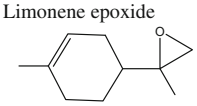
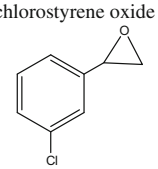
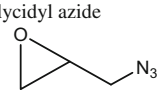
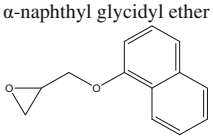
Table 1 Production of chiral drug intermediates using microbial EHs

S. No.	Racemic epoxides	Source of microbial EHs	Chiral drug intermediates	Drugs synthesized	Application of drugs	References
1.	Indene oxide 	*Diplodia gossipina* ATCC 16391	1(*S*),2(*R*) indene oxide	Indinavir (Crixivan)	HIV protease inhibitor MK639	[20]
2.	4-isobutyl-α-methylstyrene oxide 	*Aspergillus niger* LCP521	2-(*p*-Isobutyl phenyl)-1,2-propanediol	(*S*)-Ibuprofen	Ibuprofen is a NSAID (Non-steroidal Inflammatory Drug) commonly used for the relief of symptoms of arthritis, fever and as an analgesic	[21]
3.	2-benzyl-2-methyl oxirane 	*Nocardia* EH1	2,2-dimethyl-3-phenylpropan-1-ol	(*R*)-(-)-Mevalonolactone	Key intermediate in a broad spectrum of cellular biological processes and their regulation	[22]
4.	*para*-chlorostyrene oxide 	*Solanum tuberosum* L* and *Aspergillus niger* LCP521	(*R*)-2-(4-chlorophenyl)propane-1,2-diol	(*R*)-Eliprodil	Neuroprotective agent and is an NMDA (N-methyl-D-aspartate receptor) antagonist	[23]
5.	*para*-nitrostyrene oxide 	*Aspergillus niger* LCP521	(*R*)-1-(4 nitrophenyl)ethane-1,2-diol	(*R*)-Nifenalol	β-adrenergic blocker with antianginal and antiarrhythmic properties	[24]
6.	*trans*-ethyl-3-phenylglycidate 	*Pseudomonas* sp. BZS21	(2*R*, 3*S*)-ethyl-3-phenylglycidate	Diltiazem	Calcium channel blocker	[25]
7.	Ethyl-3,4-epoxybutyrate 	*Acinetobacter baumannii*	(*R*)- ethyl-3,4-epoxybutyrate	(*R*)-γ-amino-β-hydroxybutyric acid and (*R*)-carnitine (vitamin BT)	Neuromediator with antiepileptic and antihypertensive activity Dietary supplement and antiobesity agent	[26]
8.	Limonene epoxide 	*Aspergillus niger* LCP521	2-((*S*)-4-methyl cyclo hex-3-enyl)-propane-1,2-diol	(4*S*,8*S*)-α-bisabolol	Used for the preparation of skin creams, lotions and ointments having anti-inflammatory, bactericidal and antimycotic properties	[27]
9.	3-chlorostyrene oxide 	*Sphingomonas* sp. HXN-200	(*S*)-3-chlorostyrene oxide	BMS-536924	IGF-1R insulin receptor kinase inhibitor helps in reduction of tumour cell proliferation	[28]
10.	Glycidyl azide 	*Aspergillus niger* ZJUTZQ208	(*R*)-glycidyl azide	Linezolid	Antibiotic used for the treatment of infections caused by Gram-positive bacteria	[29]
11.	α-naphthyl glycidyl ether 	Mutant *Bacillus megaterium* ECU1001	(*S*)-naphthyl glycidyl ether	(*S*)-propranolol	β-adrenergic receptor blocking drug	[30]

* *Solanum tuberosum* L. is a plant EH. Epoxide hydrolysis catalyzed by using both plant and fungal EHs in a one-pot sequential bi-enzymatic

With the green wave of biocatalysis, more and more chiral biocatalysts are being discovered as they catalyze a reaction in a regio-, diastereo-, and enantioselective manner under mild reaction conditions in an economic and environmental-friendly manner giving high enantiopurity, yield, specificity, catalytic efficiency, and activity [1–3]. Enzymes are available in so much diversity that they exhibit broad substrate spectra for the generation of enantiopure drug intermediates at ambient temperature and cause much less pollution as compared to chemo-catalytic processes [4]. The biocatalytic scope and potential of enzymes have been expanded by the advancement of new techniques such as 16SrDNA sequencing [5], metagenome screening [6], genome mining [7, 8], etc. for the screening and selection of enzymes.

Chiral epoxides and diols are gaining importance as they are increasingly being used as valuable intermediates in organic synthesis for the production of optically active pharmaceuticals. Epoxides are present commonly in both simple and complex biologically active molecules and can easily undergo stereoselective ring-opening reactions with a varied range of nucleophiles [9]. Therefore, in biocatalytic reactions, epoxides are kinetically resolved by epoxide hydrolases (EHs; E.C.3.3.2.x) producing enantioriched diols and unreacted remaining epoxides from racemic substrates [10]. Enantioselective and enantioconvergent EHs have several advantages as they are ubiquitously present in the environment, can be easily cloned from various microorganisms and produced in abundant amount as recombinant proteins; they are stable proteins and do not require any cofactors for their activities and have broad substrate specificity. Due to these characteristics, EHs are most commonly used commercially important biocatalysts

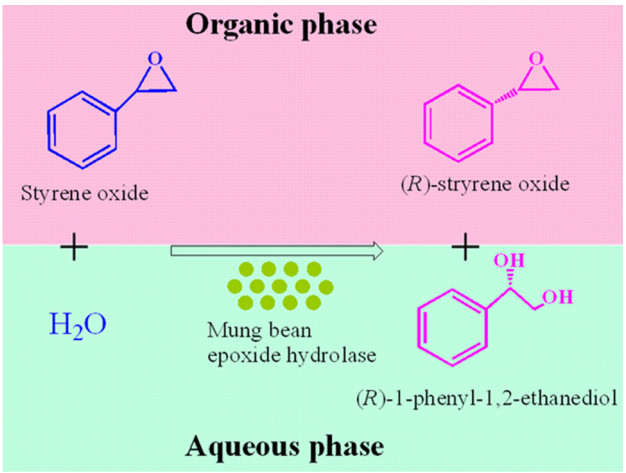


Fig. 1 EH catalyzed the hydrolysis of epoxide in a biphasic system. Mung bean EH catalyzed the hydrolysis of styrene oxide in a biphasic system where remaining unreacted epoxide gets accumulated in the organic phase while formed diol gets collected in the aqueous phase from where they can be easily separated from each other and used for further analysis (adopted from Wenjing et al. [39])

[11]. Microbial EHs have been used on large industrial-scale production for the synthesis of chiral intermediates which are subsequently used in the production of chiral drugs by downstream processing (Table 1). There have also been many patents being granted on the use of microbial EHs for their role in the synthesis of chiral epoxides and diols [12–19].

EHs have been commercialized from *Aspergillus niger* and *Rhodococcus rhodochrous* as lyophilized powder, both from Sigma-Aldrich [1]; however, still the availability of commercially available EHs is limited [31]. Many novel identified EHs have been established on laboratory scale, but to use them on the large industrial scale, there is a need for the development of viable and stable EHs with high enantiopurity. There has been an increase in the innovative methods being discovered to enhance the enantioselectivity and stability of already identified novel EHs. With enhancement in the enantioselectivity and reusability of EHs, they can be abundantly exploited on an industrial scale for the production of enantiopure drug intermediates.

In this review, various strategies for enhancing the enantiopurity and stability of wild-type and recombinant microbial EHs have been discussed which can be easily produced in an abundant amount in heterologous hosts. Along with the use of organic solvents, detergents, immobilization, and directed evolution methods, some light has also been thrown on the recent use of new organic solvents, ionic liquids, and deep eutectic solvents to enhance the enantioselectivity of EH-catalyzed reactions.

Engineering Microbial EHs for Commercial Use

A rich source of microbial EHs has been made available by various approaches of EH mining. Successful attempts have been made to use these identified EHs in industries for the manufacture of chiral intermediates to be used in the development of drugs [11]. Even though a lot of functional EHs have been identified, still there is a requirement to improve the enantiomeric properties of the available EHs having less stability or enantioselectivity to obtain highly enantioriched epoxides or diols by using various approaches as detailed ahead.

Reaction Media Optimization

Organic Solvents

Earlier, enzymatic catalysis was carried out only in aqueous buffers [32]. In the case of EH-catalyzed reactions as epoxides are nonpolar, hydrophobic substrates and they are chemically unstable, i.e., get auto-hydrolyzed and have low water solubility and hence could not be performed in aqueous media alone. To enhance the efficiency of EH-catalyzed reactions, they are carried out in the presence of

organic solvents in the reaction medium. Organic solvents provide greater stability and also alter the selectivity of an EH in an affirmative way to enhance its activity [33]. These water-miscible and water-immiscible organic solvents were selected on the basis of their log*P* value (logarithm of the partition coefficient of a given solvent in the mixture of 1-octanol and water). log*P* value is used to predict the compatibility of the organic solvent with the enzyme. According to the log*P* value, organic solvents with $\log P < 2$ are not biocompatible, while those having log*P* value between 2 and 4 show moderate compatibility and solvents with $\log P > 4$ are most compatible with enzymes [34]. Initially, the effect of water-miscible/hydrophilic solvents (monophasic) like dimethylsulfoxide (DMSO) ($\log P = -1.3$), dimethylformamide ($\log P = -1$) were analyzed in EH-catalyzed reactions. They showed enhanced activity, especially in *A. niger* catalyzed EH reactions. Hydrolysis of *para*-nitrostyrene oxide using *A. niger* EH was carried out successfully in the presence of 0.2% (*v*/*v*) water-miscible DMSO as a cosolvent which increases its solubility and enhances the reaction rate by avoiding mass-transfer limitations [35]. However, with some substrates and EHs, water-miscible solvents showed inhibitory activity [36]. Even though most of the water-miscible solvents have log*P* value < 2, they show an affirmative effect in EH-catalyzed reactions [7, 37]. Therefore, it can be concluded that log*P* value does not serve as a definite criteria to choose organic solvents [38].

Zaks and Klibanov stated that EHs show greater activity in the presence of hydrophobic/water-immiscible solvents (biphasic) than hydrophilic/water-miscible solvents as EHs require few water molecule clusters for their activity which are completely stripped off by hydrophilic solvents resulting in lowering of their catalytic activity [40]. To improve the resolution of a biocatalyst, its immediate surroundings are modified by adding hydrophobic solvents where epoxide and product diol gets partitioned into two-liquid phases: organic and aqueous phases, respectively. Organic phase serves as an internal substrate reservoir and protects the epoxide from spontaneous hydrolysis. In biphasic reaction, epoxide and diol can be easily separated from each other as diol is present in the aqueous phase, while epoxide is present in the organic phase [36] (Fig. 1). In the hydrolytic reaction of substituted chlorostyrene oxides by EH from *Sphingomonas* sp. HXN-200 using hydrophobic cosolvents, *n*-hexane or *n*-octane or *n*-dodecane with log*P* values 3.5, 4.5 and 6.6, respectively, gave almost similar and high *E*-values as compared to the aqueous system only [28]. Thus, the effect of an additive on enzyme properties cannot be pre-determined and has to be optimized by medium engineering [33]. Various studies were carried out to determine the effects of various water-miscible and water-immiscible cosolvents like *n*-dodecane, heptane, DMSO, glycerol, dioxane, cyclohexane on refining EH stability, activity and enantioselectivity [37, 41–46]. Mostly, cosolvents in the range of 5–10% (*v*/*v*) are sufficient for the improvement in the EH activity at above or below this range, though they have some inhibitory effect on EHs and lower their activity due to enzyme's conformational changes [29, 47]. However, sometimes addition of cosolvents lead to inactivation of enzyme like in case of *Agrobacterium radiobacter* EH in octane/water biphasic system which is due to molecular toxicity (due to contact between organic solvent and EH), interfacial inactivation (due to contact between aqueous/organic solvent and EH) and mass-transfer limitations [9, 48, 49]. The effects of the biphasic system on EH activity and selectivity are yet to be understood completely. As a result, the benefits of the biphasic system with particular cosolvents cannot be generalized and have to be optimized individually [50].

Detergents

Along with organic solvents, non-ionic detergents like Triton X-100, Tween-20 and Tween-80 also help in enhancing hydrolysis and enantioselectivity of EHs. With the addition of 2% (*v*/*v*) Tween-20, the enantioconvergent hydrolysis of styrene oxide by two EHs from *Caulobacter crescentus* and *Mugil cephalus* produced *(R)*-phenyl-1, 2-ethanediol with an enantiopurity of 89%. The mechanism by which addition of detergents increases the enantiopurity and enantioselectivity of racemic epoxides in EHs is still not clear and seems to be reliant on the substrate structure [51]. However, these details have been studied in depth in purified lipases [52]. In lipases, non-ionic detergents (uncharged and having hydrophilic headgroups) are mild which do not interact widely with protein surface and help to enhance lipase activity unlike ionic detergents like sodium dodecyl sulfate which bind protein surface non-specifically and lead to its unfolding and thus lowering their activity [53]. In *Aspergillus brasiliensis*, hydrolysis of styrene oxide exhibits increased activity in the presence of 0.1% (*v*/*v*) Tween-20 and water: isooctane biphasic system [37]. In a peculiar case like in *Bacillus megaterium,* it showed high enantioselectivity toward *(R)*-glycidyl phenyl ether with 5% (*v*/*v*) DMSO as a cosolvent, but when it was replaced by 0.5% (w/v) Tween-80 (an emulsifier), it showed 1.8-fold enhancement in EH activity and its *E*-value 41.2 in the presence of DMSO was raised to 69.3 in the presence of Tween-80 [54]. Hence, the reaction medium has to be optimized with different additives. Triton X-100 and Tween-80 led to the stability of EHs when added above their critical micellar concentration [55].

Ionic Liquids (ILs)

Due to inactivation of EHs by organic solvents, an other class of organic solvents called ILs of comparable polarity has been explored. ILs are organic salts in which the ions (cations and anions) are poorly coordinated, due to which they exist as liquid below 100 °C or at room temperature and are also called as room temperature ionic liquids (RTILs). In them, one ion has delocalized charge and the other component is organic due to which stable crystal lattice is not formed (Fig. 2a, b). ILs do not evaporate as they contain charged cations and anions held together by strong coulombic interactions (hydrogen bonds) which are not easy to break [56]. ILs are truly called "designer solvents" as an appropriate combination of cation and anion can be used to change their structure and solvent properties according to the specific reaction system [57]. ILs serve as cosolvents in dissolving nonpolar substrates in water [58]. The interest in them is increasing rapidly due to their distinctive properties like negligible vapor pressure, high thermal and electrochemical stability, high polarity (due to ionic nature of its components), high solvation power, non-

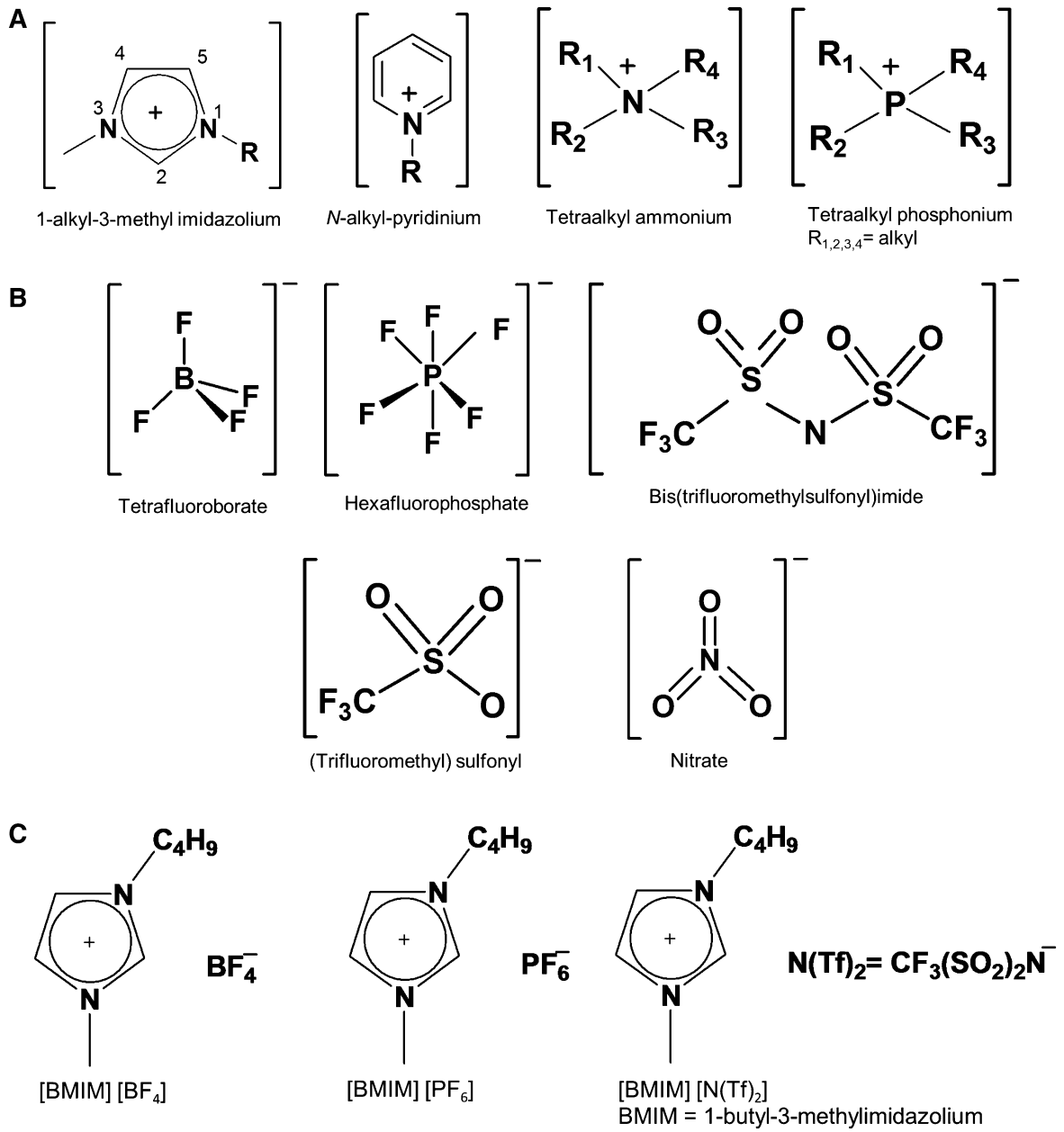


Fig. 2 Structure of ionic liquids. **a** Common cations and **b** anions used in formation of ILs (adopted from Seddon et al. [76]; Xu et al. [68]). **c** Imidazolium-based ILs (adopted from Chiappe et al. [64])

flammable, recyclable, miscibility with water or organic solvents and greater enzyme stability which increases their potential to be used as solvents in enzymatic reactions. Yet, having these properties they cannot be completely labeled as "green" as they have some toxicity and biodegradability issues due to their variable ionic composition [59]. They can be used as cosolvents with both whole cells and free enzymes [60]. Most common ILs used in biocatalysis are imidazolium-based ILs such as BMIM.BF_4 (BMIM = 1-butyl-3-methylimidazolium and BF_4^- = tetrafluoroborate) (Fig. 2c) [61]. Though enzymes are not stable in all ILs, they are stable in some ILs containing BF_4^-, PF_6^- (hexafluorophosphate) and $N(Tf)_2^-$ bis(trifluoromethylsulfonyl)imide anions (Fig. 2c) as BF_4^- spreads its negative charge over four fluorine atoms, PF_6^- over six and $N(Tf)_2^-$ over five atoms. As if the anion or cation is strong, it will disrupt the internal hydrogen bonding of the enzyme and will cause its denaturation. In order to make the anion or cation less strong, charge is distributed over number of atoms, causing its delocalization and this is called lowering of the hydrogen bond basicity, and thus, the weak charge will not be able to disrupt the enzyme and less will be the hindrance with the internal hydrogen bonds of an enzyme. The ILs containing Cl^-, NO_3^-, $CF_3SO_3^-$ (trifluoromethylsulfonate), CF_3COO^- (trifluoroacetate) or CH_3COO^- (acetate) anions denature enzyme due to strong hydrogen bonds between Cl^-, NO_3^-, acetate anions and amino acid side chain of an enzyme (less delocalization of charge), thus promoting unfolding and leading to irreversible aggregation and precipitation of the protein [58]. It was also found that ILs containing chaotropic cations (large-sized and low-charged, weakly hydrated ions which are structure breakers, i.e., decrease the protein stability in solution) and kosmotropic anions (small-sized and highly charged, strongly hydrated ions which are structure makers, i.e., increase the protein stability in solution) stabilize enzymes [62].

There are three generations of ILs. The first generation is mainly composed of cations like dialkylimidazolium and alkylpyridinium derivatives (Fig. 2a) and anions like chloroaluminate and other metal halides which are toxic, non-biodegradable and oxygen sensitive [63]. Due to these limitations, research advanced toward the second generation of ILs composed of anions like halides (Cl^-, Br^-, I^-) or BF_4^-, PF_6^- and cations such as dialkylimidazolium, alkylpyridinium, ammonium and phosphonium (Fig. 2). These are water- and air-resistant and have lower melting points, viscosity, inert, mostly hydrophobic, i.e., immiscible with water and most studied. Their main disadvantage is their high cost because of the purification which is required to remove fluorinated components from the final product [58]. Still, these second-generation ILs have been used in a great number of biocatalytic reactions. The third generation of ILs is called deep eutectic solvents (DESs) that are discussed ahead. ILs can be divided into hydrophilic (water-miscible) and hydrophobic (water-immiscible) ILs. They are generally immiscible with nonpolar organic solvents like hexane; however, some ILs are miscible with polar organic solvents like dichloromethane and tetrahydrofuran. Due to their immiscibility with nonpolar organic solvents and with water, they can be used in biphasic systems for product separation and solvent recovery [60, 61].

There are many reports on the use of ILs in enzymatic reactions. In the case of purified EHs aqueous reactions, use of ILs: BMIM.BF_4, BMIM.PF_6 and BMIM.$N(Tf)_2$ (Fig. 2c) as a reaction medium for the first time has been reported by using cress soluble EH for the hydrolysis of *trans*-β-methylstyrene oxide, which gives corresponding optically active (1*S*,2*R*)-*erythro*-1-phenylpropane-1,2-diol through a stereoconvergent process [64]. Hydrolysis of racemic and meso-epoxides using sEHs from cress and mouse and mEH from a rat in several ILs established the optimized amount of water to be 10% used in these EH reactions [57]. Still, there are very few reports about the use of ILs in EHs. For the first time, the use of hydrophobic IL (C_4MIM.PF_6)/buffer biphasic system (C_4MIM.PF_6 = 1-butyl-3-methylimidazolium hexafluorophosphate) and, in another study, the addition of various hydrophilic ILs as a cosolvent in phosphate buffer/n-hexane biphasic system have improved the performance of mung bean EHs for asymmetric hydrolysis of styrene oxide with product ee 95–97% [65, 66]. Also, the effects of organic solvents and ILs were analyzed using whole cells of microbial EH, *Rhodotorula glutinis* in the hydrolysis of 2-epoxyhexane in hydrophobic solvent or IL/buffer biphasic system. In the presence of mixed hydrophobic solvents 1-hepatanol and dodecane/buffer biphasic system, $E > 100$ was obtained while in (HMIM.BF_4) (1-hexyl-3-methylimidazoliumtetrafluoroborate) IL/buffer biphasic system, E value of only 41 was obtained. This may be attributed to the denaturation of EH that existed in the cell membrane due to an excess of solvent molecules present along the cell membrane raising the hydrophobicity with an increase in alkyl chain length [67]. Although ILs have been used successfully in catalyzing enzymatic reactions with purified EH enzymes in buffered aqueous solutions, the use of whole cells compared to the purified enzyme is interesting as it does not involve purification and isolation of an enzyme. There is only one example as discussed above, where whole cells have been used in buffered EH reaction with ILs, but it leads to decreased enantioselectivity. As there are not enough studies on the use of ILs in whole cell catalysis and mechanisms underlying, this field needs to be explored. However, a recent review by Xu et al. highlighted the toxic effects of ILs on microbial cells and biodegradability of

ILs. Different ILs have a variable amount of toxicity on cells depending on their variable structures. ILs get accumulated in microbial cells and affect the permeability of the membranes. But these studies cannot be generalized, and additional work is required to study the toxic effects of ILs on cell membranes. The detailed mechanism of the effect of ILs on whole cell-based biotransformation are unknown, and further studies are required to determine the interaction of ILs with the cell membranes and influence of reactions within the cell at the enzyme and gene control levels [68].

In bacterial EHs, the use of ILs has not been explored. Therefore, ILs need to be analyzed in enzymatic reactions catalyzed by purified enzymes or whole cells of bacterial EHs to enhance their selectivity and stability. It was reported that even some ILs can denature enzymes. So, to increase the activity and stability of enzymes in those enzyme-inactivating ILs, different enzyme stabilizing approaches have to be adopted as described by Zhao [69]. Cross-linked enzyme aggregates (CLEAS) of mung bean EH efficiently catalyzed the asymmetric hydrolysis of styrene oxide to *(R)*-1-phenyl-1,2-ethanediol in hydrophobic IL ($C_4MIM.PF_6$)/buffer biphasic system giving product ee 95.8% and *E*-value of 151, which can be recycled and reused [70]. However, the defined role of ILs in enzyme catalysis and their stability is still not fully understood [60] even though many reviews have been written on the effect of ILs on enzyme structure and activity [56, 61, 69, 71]. It was found that enzymes are more stable in hydrophobic ILs than hydrophilic ILs as hydrophobic ILs do not eliminate the essential water and enzymes remain in suspended rather than dissolved form in them [71]. Reviews by Naushad et al. and Patel et al. have thrown some light on the effects of ILs and their physicochemical properties; hydrogen bond capacity and hydrophobicity, effect of cations and anions, effect of alkyl chain length in the cations, on stability and activity of proteins which helps in further understanding the role of ILs in protein stabilization. These studies revealed that ILs which are hydrophobic in nature, less viscous, have kosmotropic (order-making) anion and chaotropic (order-breaking) cation augment the activity and stability of enzymes. However, these statements cannot be generalized for all ILs due to different results obtained with them [72, 73]. Hence, further advancements in the understanding of ILs will offer more insights into the mechanism of ILs stabilizing enzymes and how to create their suitable combinations for superior enantioselectivity.

Deep Eutectic Solvents (DESs)

As explained above, due to economic reasons there is a need for advanced ILs called third-generation ILs which are cheaper, stable and biodegradable [59]. They are considered as a viable replacement for organic solvents and second-generation ILs [74]. This requirement is fulfilled by DESs which are a new class of ionic solvents. DESs are mixtures of ammonium salts such as choline chloride or choline acetate and uncharged hydrogen bond donors (HBDs) such as urea, ethylene glycol or glycerol (Fig. 3). Due to the uncharged component, they are not entirely ionic and form a hydrogen bond to the anion of the salts. Due to hydrogen bonding, there is charge delocalization which is responsible for the decrease in the melting point of the mixtures. The molar ratio of the individual components is very important, and at a particular eutectic ratio, 1–4 molecules of hydrogen donor bind per molecule of salt and form a liquid mixture at room temperature [75]. They are more hydrophilic than second-generation ILs and are water-miscible. They can replace polar organic solvents like DMSO, acetone or methanol in enzymatic reactions which can denature enzymes [58]. They are like conventional ILs with the difference that their components are cheaper and they do not require purification after synthesis. ILs are formed by mixing two salts to form two new salts; one is the IL and other is often sodium chloride which must be removed. The removal of salt is complex but necessary as even traces of salt can inhibit enzyme catalysis [76]. Preparation of DESs requires only stirring the components with gentle warming and does not result in any salt formation; therefore, no purification required. Hence, they are least expensive with their cost similar to that of organic solvents [58]. Examples of DESs used in several biocatalytic reactions are ChCl:Gly (choline chloride: glycerol) or ChCl:ET (ET:ethanediol) [59] (Fig. 3).

There are only two examples where DESs are used as cosolvents in aqueous solutions of EH-catalyzed reactions. Regioselectivity of (1*R*, 2*R*)-2-*trans*-methylstyrene oxide was enhanced in the presence of ChCl:Gly or ChCl:ET DESs in aqueous solution when hydrolyzed by purified potato EH by 2.33-fold compared to phosphate buffer

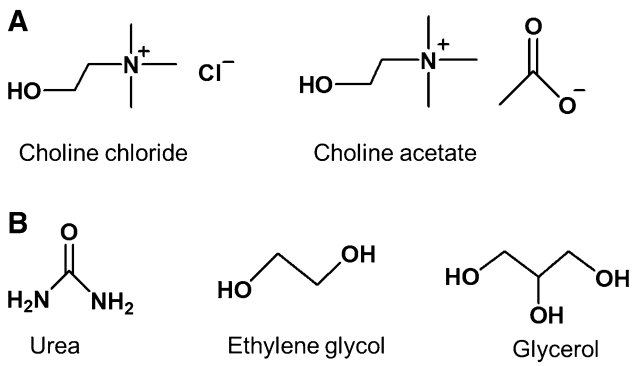


Fig. 3 Structures of commonly used DESs. **a** Cholinium salts and **b** hydrogen bond donors used in the formation of DESs (adopted from Huang et al. [77])

systems only. Above-mentioned DESs can also dissolve 1.5-fold higher epoxide concentration compared to phosphate buffers [78]. DES (ChCl:Gly/buffer mixture) was also used in hydrolysis of styrene oxide by purified recombinant bacterial EH *A. radiobacter* and it led to a 20-fold increase in conversion, which was only 4.6% in the buffer and increased to 92% in 25 vol% ChCl:Gly [79] but with no effect on enantioselectivity. These results showed that advanced DESs can be exploited as new green solvents to enhance the performance of EHs. Their role with whole cells-catalyzed EH biotransformations have been unexplored, which can be viewed as a potential study to apply DESs in EH catalyzed biotransformations. In order to take complete advantage of DESs, it is imperative to understand their role in enzyme stabilization and activity. Huang et al. studied the effects of diverse DESs solvents in lipase-catalyzed reactions and concluded that chemical nature of DESs affects the solvent properties like polarity, viscosity and surface tension. It is established that composition of different salts and HBDs along with the molar ratio between the two play an important role in enhancing enzyme performance [77]. ChCl:Gly (choline chloride: glycerol)-based DESs in a molar ratio (1:2) turns out to be a good choice for lipases due to their favorable properties such as lower viscosity and hygroscopicity, high biodegradability and compatibility with lipases [77]. Also, ChCl:Gly-based DESs showed promising results in EH-catalyzed reactions as described above [78, 79]. However, general guidelines cannot be established as which DES and what molar ratio of salt and HBD can be used for the optimal enzymatic performance.

To understand the structural dynamics of the enzymes in DESs, fundamentals at the molecular level have to be understood. Monhemi research group showed for the first time the molecular dynamics of DESs in lipase-catalyzed reaction. They showed that choline chloride:urea (trade name: Reline) having 9 M urea DESs stabilizes the lipase when urea forms hydrogen bonds with choline and chloride ions as urea molecules have a low diffusion coefficient and cannot reach the protein domains. Further, choline, urea and chloride ions form hydrogen bonds with surface residues of the enzyme which lead to greater enzyme stability instead of denaturing it. In comparison, if only 8 M urea alone in water was used in enzymatic reaction, then there is complete denaturation of the lipase by disrupting intra-chain hydrogen bonds in a direct denaturation mechanism. Hence, these studies proved the stabilizing effects of DESs on enzyme conformation and activity [80].

In 2010, Gutierrez et al. studied the effect of DESs on the whole cell-catalyzed reactions in aqueous solutions and stated that hydration of the individual components of the DESs would result in separation of the hydrogen-bonded supramolecular complexes and the DESs would become a simple solution of its individual components and its special features will then disappear. Since whole cells cannot be used in non-aqueous conditions, i.e., in pure ILs or DESs without any buffer, therefore, Gutierrez and co-authors proposed freeze-drying of bacterial whole cells in an aqueous solution of DESs in its pure state, thereby preserving the bacterial integrity, viability, and non-dissolution of DESs [81]. In contradiction to the above-said statement, Huang et al. revealed that DESs solubilized in aqueous solution existed as an intact cation–anion–HBD complex due to the strong ionic interactions between the cation and the anion and HBD [77]. There have been very few reports on the study of toxic effects of DESs on cells. Preliminary studies suggest that DESs individual components (such as ChCl or urea) may be toxic to the cells and this toxicity can be weakened by incorporating these individual components into eutectic mixtures [77, 82]. This varying degree of toxic effects of DESs depends on the structural alteration of the HBDs and the tested organisms to some degree [68, 83]. Henceforth, to make a certain statement regarding the toxicity of DESs on cells requires more extensive investigations. A recent review by Smith et al. [84] discussed the properties and synthesis of DESs detailing their application in metal processing, extraction, organic synthesis, etc. other than biocatalysis. Thus, DESs have a great potential for further research to be undertaken, increasing the types of DESs and expanding their applications [84].

Immobilization

To implement EHs industrially, apart from improving their enantioselectivity there is also need to enhance their reusability so as to improve their commercial value. Therefore, immobilization of EHs is a prerequisite for their use as industrial biocatalysts. Though immobilization renders enzymes reusable but it also produces minor distortion in enzyme structure which may alter its activity (rate of the reaction per milligram of enzyme), selectivity (production of one product among several other products) and specificity (discrimination between substrates) either in a positive or negative manner [85–87]. Thus, different immobilization protocols have to be considered for improving the functional properties of enzymes to that of corresponding soluble enzymes. Mateo et al., and Rodrigues et al., discussed various aspects on how immobilization of an enzyme affects various properties which become favorable or unfavorable for an enzyme catalyzed reaction [85, 86]. Enzymes get inhibited by high concentration of substrates or reaction products which decrease the enzyme activity. Inhibition can be reduced by enzyme immobilization due to partial blocking or distortion of the inhibition site. Also, the rigidification of enzyme by multi-point

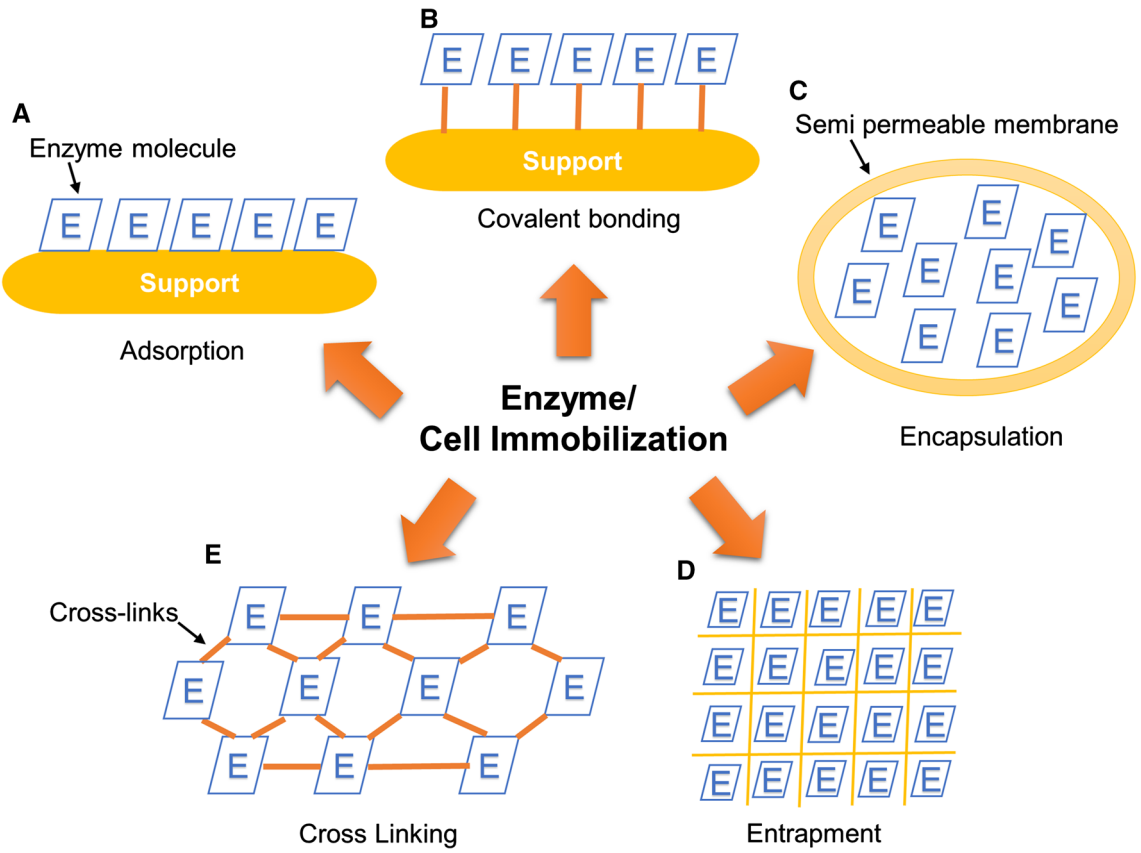


Fig. 4 Different methods used in EHs immobilization. **a** Adsorption, **b** covalent bonding, **c** encapsulation, **d** entrapment, **e** cross-linking

covalent attachment immobilization prevents enzyme conformational changes induced by any detergent or organic cosolvent or temperature change and thus enzyme retains its structure even under drastic conditions compared to the free soluble enzyme. So, under the drastic conditions, the activity of the free enzyme is decreased while that of the immobilized enzyme is stable. Thus, immobilization results in higher activity of enzymes due to a decrease in enzyme inhibition or avoiding alteration of the enzyme structure and not due to the formation of a more active conformation of the enzyme after immobilization [85]. By immobilizing the enzyme on a porous support, it gets fully dispersed which prevent aggregation and other inactivation phenomenon. Porous support also protects enzymes from detergents if they are present in high concentration in the reaction medium and results in increased enzyme activity by protecting the enzymes from inhibition or distortion [88]. Porous immobilization of an enzyme also produces internal pH gradients, diffusional limitations and partition effects of substrate or products which in some instances are positive for enzyme activity by decreasing K_m if substrate concentration is below that is required to saturate the enzyme and the enzyme environment permits partitioning of the substrate toward the enzyme and vice versa [88]. These changes affect enzyme performance. A hyperactivated form of enzyme induced by an effector or reaction medium is stabilized by immobilization and enhances enzyme performance [88]. Improvement in enzyme activity or specificity cannot be achieved by following only one immobilization protocol. It can be achieved by preparing a large library of enzymes by different immobilization methods focusing on different enzyme regions or different enzyme-support interactions or generating different enzyme microenvironments [86]. If an enzyme already has desired properties, they should be unaltered by using mild immobilization protocols. Enzyme activity can also be improved by site-directed mutagenesis and to reuse enzyme it should be stabilized by immobilization [86]. Various research papers have been published on understanding the concepts of enzyme immobilization and the use of best possible immobilization protocols depending on the nature of the enzymes [86–89]. Thus, immobilization is an ever growing tool to develop improved enzymes to be used in industries.

Immobilization of EHs from various organisms has been implemented since a long time to improve their stability and reusability [70, 90–92]. Microbial EHs were immobilized using different approaches according to the suitability and requirement of immobilizing a particular EH as discussed ahead (Fig. 4).

Adsorption

Physical adsorption of the enzyme onto the surface of water-insoluble carriers is the simplest method of enzyme immobilization (Fig. 4a). Examples of adsorbents are ion-exchange matrices, porous carbon, glasses, clay, hydrous metal oxides and polymeric aromatic resins. This method causes little or no conformational change of the enzyme or destruction of its active site center as it involves binding mainly by hydrogen bonds, multiple salt linkages and van der Waal's forces. It is a simple and cheap method as it requires no reagent and involves minimum activation steps. However, if there is a weak binding force between enzyme and a carrier, enzyme will get leaked and this method is little slower than other immobilization methods [87]. The operational stability of recombinant *A. niger* EH used in hydrolysis of racemic *p*-chlorostyrene oxide was increased by adsorption onto Acurrel EP 100 by 142% compared to free EH, which was further increased by two-fold by immobilization on diethylaminoethyl–cellulose, demonstrated for the first time in the presence of neat organic solvent heptane: dioxane in 80:20 (*v*/*v*) [45]. Also, in another study (*R*)-epichlorohydrin was produced from 1,3-dichloro-2-propanol by a two-step biocatalysis in a specially designed reactor using recombinant *E. coli cells* harboring haloalcohol dehalogenase and EH from *A. radiobacter* AD1, immobilized by adsorption onto perlite adsorbent. In this work, free enzymes were not used for immobilization so as to avoid the time & cost involved in the extraction of the enzymes. Also, enzymes are more stable inside the cells, it being their natural environment. By adsorption, cells are in direct contact with the reaction solution containing the substrate which eliminates the mass-transfer limitations. Two-phase reaction system was used to reduce the spontaneous chemical hydrolysis and to prevent the reverse reaction of the formed epichlorohydrin by haloalcohol dehalogenase. The yield of racemic epichlorohydrin formed by conversion of haloalcohol in the first step of the reaction catalyzed by immobilized haloalcohol dehalogenase in presence of 40% (*v*/*v*) cyclohexane was 73% and the yield of (*R*)-epichlorohydrin with ee $\geq$ 99% increased from 19.2 to 26.4% in the second step by optimizations. The reaction can be carried out continuously using immobilized cells for 3–5 times [93].

Depending on the pH of the solution and the isoelectric point, the surface of the enzyme bears charges which are exploited for immobilization of the enzyme on ion exchangers as carriers. Ion exchangers can be positively (diethylaminoethyl–cellulose) or negatively (carboxymethyl cellulose) charged depending upon charge on the enzyme and form strong ionic and polar interactions with the enzyme. Ionic immobilization is dependent on the pH and salt concentration during immobilization, and high salt concentration can lead to ion exchange and washing out of the enzymes. Therefore, pore size of the ion exchanger has to be considered as an important parameter while considering other factors [87]. Semi-purified *Nocardia* EH1 was stabilized by immobilization through ionic bonding to diethylaminoethyl–cellulose and showed 225% more activity in the presence of Triton X-100 than free enzyme although with somewhat decrease in enantioselectivity. Whole cells showed $E > 200$ and after ionic binding they showed $E = 174$ which was also comparable. After five cycles, immobilized enzyme retained >50% of its initial activity, while whole cells lost 90% of their activity [94]. Thus by immobilization, significant stabilization was achieved and allowed the enzyme to be reused though after numerous optimizations with different carriers and immobilization techniques.

Covalent Bonding

In covalent bonding, enzyme is tightly bound through amino or carboxyl groups to a carrier and as a result, prevents leaching of an enzyme (Fig. 4b). Formation of a multiple covalent bonds between enzyme and a carrier restricts conformational flexibility and hence leads to protein unfolding and denaturation [87]. Wide variety of carriers with functional groups are available for covalent bonding, thus making it the most commonly used immobilization method. Carriers must be chosen so that they do not affect the active site of the enzyme and hence do not cause any loss of activity. EH from *A. niger* is commercially available and has good activity and enantioselectivity but in order to improve its operational stability and reusability, it has been immobilized by various methods, each trying to make it more industrial friendly [45, 92, 93, 95–99]. In one of the methods, it was covalently immobilized on epoxide matrix obtained by derivatization of silica-gel with epoxy groups. Covalent attachment of an enzyme to an insoluble matrix forms stable linkages which prevents leakage of an enzyme. This immobilized EH retained 90% of its initial activity, showed enhanced stability in the presence of 20% (*v*/*v*) DMSO, with 99% ee_s and 92% ee_p by hydrolyzing *p*-nitrostyrene oxide and can be reused for over nine cycles without any loss of enzyme activity and change in enantioselectivity [98]. Another method in stabilization of recombinant *A. niger* was developed using the second generation of epoxy-activated modified supports (Eupergit® C) having multipoint covalent attachment strategy. Eupergit® C is made by co-polymerization of *N*, *N*-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide and have macroporous beads of 150 µm. Eupergit® is a registered trademark of Röhm GmbH & Co. KG. Eupergit® C is stable within a pH range of 1 and 12

and has a good chemical and mechanical properties. It has high density of oxirane groups on the surface of the beads due to which it binds enzymes at various sites of its structure and shows multi-point covalent attachment. Other form of Eupergit® C is Eupergit® C 250L which is also used as a carrier for immobilization of enzymes and has the same chemical structure as Eupergit® C but has larger pores of 250 μm [100]. Immobilization of *A. niger* was initially carried out on Eupergit® C/Eupergit® C 250L and on their various modified supports formed by linking ethylenediamine (EDA) or iminodiacetic acid (IDA). After various optimizations, Eupergit® C/EDA appeared to be the best support for immobilization of *A. niger* as it lead to 70% retention of initial enzymatic activity and a stability factor of 30. *E*-value was also improved from 25 to 56 and 40 to 100 in case of styrene oxide and *p*-chlorostyrene oxide, respectively. Moreover, enzyme can be reused for over 12 cycles without any loss of activity or change in *E*-value [96]. To explore the potential of Eupergit® C 250L as support for immobilization of *A. niger* EH, it was modified by pre-treatment with EDA and activated by glutaraldehyde and catalyzed hydrolytic kinetic resolution of racemic styrene oxide. Various parameters of free and immobilized EH were compared simultaneously which showed that free EH retained 52 and 33%, while immobilized EH retained 90 and 75% of its maximum activity at 40 and 60°C, respectively. Immobilized EH showed 2.5-fold higher enantioselectivity than free EH and retained 90% of its initial activity after 5 reuses. On a preparative scale, immobilized EH gave 50% yield and 99% ee for both epoxide and diol forms. Thus, various supports of Eupergit® having different functional groups showed diverse immobilization and activity yields which have to be optimized before using for immobilization [99]. Comparison of the covalent immobilization of *A. niger* EH on modified Florisil® and Eupergit® C supports and by adsorption on Lewatit® VP OC 1600 support was performed which indicates that Florisil® is a good support for EH immobilization and immobilized EH preparations are promising and effective catalysts for the preparation of enantiopure epoxides or diols than their free forms [92].

Encapsulation

Encapsulation involves immobilization of cells or enzymes within a spherical particle formed of polymers like sodium alginate in which they are enclosed within a polymeric semi-permeable membrane which permits bidirectional diffusion of low molecular weight substrates or products except for enzymes or cells (Fig. 4c). Due to instability encountered with the use of purified wild-type EH from *Nocardia* sp. EH1, encapsulation with whole cells was performed [55]. Wild-type whole cells of *Nocardia tartaricans* were encapsulated in capsules made of sodium alginate (SA) and cellulose sulfate (CS) as polyanions and the polycation poly (methylene-*co*-guanidine) (PMCG) in the presence of $CaCl_2$ as gelling and NaCl as anti-gelling agents in the polycation solution (SA-CS/PMCG capsule) [101]. Comparison of *N. tartaricans* cells entrapped in calcium pectate gelled (CPG) beads to encapsulated whole cells of *N. tartaricans* in (SA-CS/PMCG capsule) was performed in the biotransformation of disodium *cis*-epoxysuccinate to disodium L-(+)-tartrate. Activity of *cis*-epoxysuccinate hydrolase encapsulated in (SA-CS/PMCG capsule) increased to about 20-fold and that too in less time compared to only three-fold when entrapped in CPG-beads. These results indicated that encapsulation of *N. tartaricans* cells is better than entrapment for production of L-(+)-tartrate. In recent times, an EH from *Sphingomonas* sp. HXN 200 was immobilized by a novel, simple and efficient method of enzyme encapsulation using one step, template free synthesis of organic–inorganic hybrid capsules in aqueous phase [102]. These capsules are made of sodium silicate as inorganic precursor to enhance the stability of capsules and *N*-fluorenylmethoxycarbonyl diphenylalanine (Fmoc-FF) as the organic self-assembling unit with a cationic polymer, polyethyleneimine (PEI) used to instantaneously activate the interfacial self-assembly of Fmoc-FF and polycondensation of silicate, leading to the synthesis of Fmoc-FF/PEI/SiO2 (FPSi) multilayered organic–inorganic capsules. These capsules gave >90% enzyme loading efficiency, <3% enzyme leakage after 48 h incubation and are mechanically stable. The immobilized enzyme catalyzed hydrolysis of cyclohexene oxide to (1*R*, 2*R*)-cyclohexane-1,2-diol with 88% ee and retained 87% productivity even after 20 cycles with a total reaction time of 80 h. Thus, this new immobilization method is proficient, practical and upgraded than other reported encapsulation methods which show enzyme leakage, low loading capacity, low mechanical stability, or low recyclability.

In addition to immobilization by encapsulation in capsules, nanoparticle encapsulation is also gaining importance as a new method for immobilization of biocatalysts. Nanoparticles are particles sized between 1 and 100 nm and behave as a whole object with respect to their transport and properties. In nanobiocatalysis, enzymes are immobilized by incorporating in nanostructured materials. It is a rapidly growing area as it involves immobilization of enzymes on large surface area of nanostructured materials which improves enzyme loading and uniform size distribution of nanomaterials along with their nanomaterial properties like conductivity and magnetism which led to improved enzyme properties with regard to stability and activity in nanobiocatalytic systems [103]. Use of magnetic nanoparticles is now growing as they can be separated from the reaction solution by applying an external magnet which

can be reused for the next reaction thus significantly reducing the cost. However, covalent bonding between the enzyme and nanoparticle results in loss of activity due to restriction of the active site of the enzyme [103]. Therefore, enzymes were immobilized on nanoparticles so as to retain their biological activity using concept of attachment of histidine-tagged proteins to Ni^{2+} ions by assembling NiO nanoparticles on the surface of magnetic silica which lead to the formation of Fe_3O_4-silica-NiO superstructure. Histidine-tagged EH from *Rhodotorula glutinis* was immobilized using the above approach and lead to >70% retention of enzyme activity after immobilization and >98% enantiopurity of (*S*)-styrene oxide during the first 6 cycles of hydrolysis of styrene oxide after magnetic separation which make it reusable [104]. There are not much reports about the use of magnetic nanoparticles in microbial EHs, but different modifications of magnetic nanoparticles had been utilized in improving stability, enantioselectivity and reusability of EH in marine fish *Mugil cephalus* [105, 106] and plant EH *Solanum tuberosum* [107]. These immobilizations will aid in understanding the interactions between enzymes and nanoscale materials, which led to the utilization of nanobiocatalytic approaches in immobilization of microbial EHs which also leads to their enhanced stability [108].

Entrapment

Entrapment methods are generally used for whole cell immobilization and rarely for pure enzymes as there is a problem of enzyme leakage from the gel. This can be avoided by increasing the degree of cross-linking and thus reducing pore size, but this also has mass-transfer and diffusional limitations. Their advantage is that they are fast, cheap, easy, involve mild conditions, and there is less loss of enzyme activity. In entrapment method, cells are not bound to the matrix and they are physically entrapped in the gel matrices network (Fig. 4d). Cells are protected from the outside surroundings and have a high retention of cell viability. Entrapment is carried out by mixing the biocatalyst in monomer solution followed by polymerization, initiated by a change in temperature or by a chemical reaction. Commonly used polymers for cell entrapment matrices include agar, alginate, κ-carrageenan and polyacrylamide [109]. Cells of *Rhodosporidium toruloides* were immobilized in calcium alginate beads for the enantioselective resolution of racemic-1,2-epoxyoctane. Alginate is cheap, readily available and has the ability to form a gel under mild conditions. It is composed of varying proportions of mannuronic and guluronic acid. The initial activity of immobilized cells was lower than the free counterpart though they were extremely stable and can be reused even after 8 batches [110]. Recently, a *cis*-epoxysuccinate hydrolase has been identified in proteobacteria *Labrys* sp. BK-8 for the production L-(+)-tartaric acid. For industrial applicability, wild-type EH containing cells were immobilized using κ-carrageenan gelation method. Carrageenan is obtained from red sea weed *Rhodophyceae* and consists of sodium, potassium, calcium, and magnesium sulfate esters of galactose and 3, 6-anhydrogalactose units. Immobilized cells showed >99% conversion rate and ee >99.5% with storage ability of >90 days compared to free cells. So, *Labrys* sp. BK-8 is a good stable alternative to earlier isolated EHs for the production of L-(+)-tartaric acid [91].

Cross-Linking

In cross-linking, enzymes are not fixed to a carrier. Here, enzymes act as its own carrier. Enzymes are first aggregated by the addition of precipitants and then cross-linked by a cross-linker such as glutaraldehyde by the formation of covalent bonds leading to three-dimensional cross-linked aggregates (Fig. 4e). This method involves very little desorption. For practicality of this method, a catalyst should be unchanged after the reaction and should be recyclable [87]. Cross-linked enzyme aggregates (CLEAS) technology has a simple method of preparation and high enzyme loading capacity with low loss of activity and avoids inactivity by the solid supports, and CLEAS are not leached in aqueous media [111]. There was no study on the preparation of CLEAS from microbial EHs; until CLEAS were prepared and characterized for the first time in mung bean EHs. Hydrolysis of styrene oxide to (*R*)-1-phenyl-1,2-ethanediol by free mung bean EHs in the presence of organic solvent or ILs lead to their deactivation [39, 65, 66]. To enhance the industrial potential, CLEAS of mung bean EH were prepared which in the presence of *n*-hexane/buffer biphasic system gave 93.5% ee of the product and retained more than 50% of the initial activity after 8 batches of reuse [112]. When the above-said reaction was catalyzed using mung bean EH CLEAS in the biphasic system containing second-generation hydrophobic IL C_4-MIM.PF_6 and buffer, ee_p increased to 95.8% with *E*-value of 151 and reusability was also improved compared to *n*-hexane/buffer biphasic system [70]. Based on these encouraging results, CLEAS immobilization method can also be used to enhance the stability of microbial EHs to improve their industrial performance.

All these successful immobilization techniques with free and whole cells of EHs implied that immobilization greatly enhances the stability and activity of EHs and making them reusable. With every day advancement in immobilization methodology, EHs immobilization can be made more productive, though there is still need for trial and error assay and make libraries of immobilized EHs with different

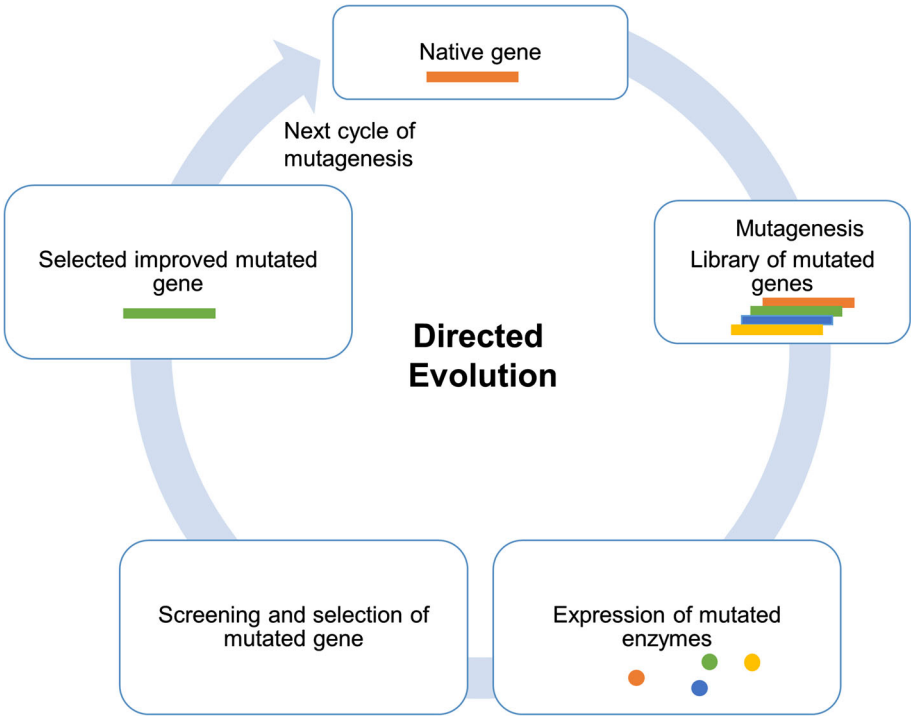


Fig. 5 General steps in the directed evolution. In directed evolution, residues in parent gene are mutated and a library of mutated genes are created. After protein expression and screening, best mutant genes are selected which are again passed through further rounds of mutagenesis till desired results are obtained

immobilization protocols, which can then be screened, since one method cannot yield optimum results. Immobilized EHs should be reused for a number of batches as there is considerable cost involved in their immobilization. While immobilizing the next batch of biocatalyst using the same support, it should be efficiently done without a decrease in any activity and stability parameters. For the successful application of EHs industrially, they should not only be stable, active and reusable but should also be prepared with lower cost using inexpensive immobilization strategies.

Engineering of the Epoxide Hydrolase

Directed Evolution

For the synthesis of a chiral pharmaceutical precursor, highly specific and stereoselective EHs are needed which are being engineered by directed evolution to have improved properties like thermal robustness, regio- and enantioselectivity [113, 114]. Directed evolution involves random gene mutagenesis, expression of mutant enzymes and then screening or selecting the best hit for a given property. The gene of the best mutant variant of the initial mutant library is then used as a template for another round of mutagenesis, screening and expression, and the procedure is repeated again and again till the mutant with the desired improved functional property has been achieved (Fig. 5) [115].

Directed evolution studies on EHs were initially performed using error-prone polymerase chain reaction (epPCR) where there is no need for any structural information of an enzyme and random mutations were introduced in an enzyme during polymerase chain reaction (PCR) by reducing the fidelity of DNA polymerase. New improved enzymatic properties were introduced by repeating the mutagenesis and selection (screening) cycles [116]. Using only one round of epPCR, *E* value of wild-type *A. niger* EH was more than doubled from 4.6 to 10.8 [117]. DNA shuffling is another mutational method where structural information of enzyme is again not a prerequisite but unlike epPCR, it allows recombination of beneficial mutations from diverse multiple genes. In the process of DNA shuffling, DNA sequences of multiple homologous genes are randomly fragmented by DNaseI and fragments of the desired size are purified from agarose gel and then reassembled at a region of high sequence identity by cycles of PCR. Finally, after recombination, PCR amplification of recombined fragments with primers was performed to generate full-length chimeras which are then cloned into an expression vector and screened for desirable function [118]. Wild-type EH from *A. radiobacter* has an *E*-value of 11 which after epPCR and DNA shuffling of active site residues was raised to 20-fold on hydrolysis of various epoxide substrates [119].

As epPCR and DNA shuffling generates large libraries which have to be screened, this is considered to be a time-consuming method, so there is a need to adapt these directed evolution practices to become more time-efficient

by using automated robotic techniques and reducing the library size to such an extent so that gas chromatography/high performance liquid chromatography methods should be sufficient to monitor the regio- and enantioselectivities of the EHs [113]. With the increase in the number of available X-ray structures of EHs, target enzymes can be modeled on them to identify specific sites for protein engineering using homology modeling algorithms [120]. Therefore, different protein engineering methods were explored to generate more focussed libraries.

Saturation mutagenesis is one of the methods where targeted mutations of certain amino acids were performed based on the structure of the enzyme's active site. A library of mutants containing all possible mutations in one or more pre-determined target positions in a gene sequence was created and screened for functional improvements [121]. Site saturation mutagenesis of the residue flanking nucleophilic Asp in *A. radiobacter* AD1 EH enhanced the substrate scope and enantioselectivity of EH toward styrene and *meso*-epoxides [122]. *Agromyces mediolanus* ZBJ120203 EH was engineered using saturation mutagenesis. It catalyzed the hydrolysis of racemic epichlorohydrin and using directed evolution, its *E*-value was increased from 12.9 to 90 and enantiopure *(S)*-epichlorohydrin was obtained with >99% ee [123]. In cascade biocatalysis, two mutated enzymes halohydrin dehalogenase and EHs from *Agrobacterium radiobacter* AD1 and *Agromyces mediolanus* ZBJ120203, respectively, were constructed using saturation mutagenesis and catalyzed enantioselective biotransformation of prochiral 1,3-dichloro-2-propanol in a two-step, two enzyme reaction giving (*S*)-epichlorohydrin with >99% ee and 91.2% yield [124].

A more focused approach for library generation is the iterative saturation mutagenesis (ISM) which is based on the Cartesian view methods (mathematical methods of scientist Descartes with emphasis on logical analysis) built on 3D protein structure which is analyzed for the specific regions which influence its catalytic property. Basically, in this method, 4–5 sites are selected which consist of 1–3 amino acids (or more) each. Each of these sites is then randomized by saturation mutagenesis to form different mutant libraries which are screened, and the best mutant is sequenced. Till here, the steps are same as in other methods of directed evolution, forming focused libraries except that the systematic view is taken. While in epPCR and DNA shuffling, the whole gene is subjected to whole anew mutagenesis even though amino acids at only a few positions are significant for specific properties. After the first round of mutagenesis of ISM, the best hit serves as a template and the next round of saturation mutagenesis was undertaken at other sites except for the one which was earlier mutated. In each pathway, each site is considered only once and mutated till the desired results are obtained. ISM maximizes the possibility of obtaining additive/and or cooperative effects of newly introduced mutations [115]. Metagenome-derived wild-type EH Kau2 has been made enantioconvergent using ISM with five amino acid replacements with an ee of 93% of *(R)-para*-chlorophenylethane-1,2-diol [125]. epPCR increased expression rate 50 times while ISM of wild-type EH increased *E*-value to 160, from 4.6 of *A. niger* EH, in the hydrolysis of glycidyl phenyl ether [126].

Further modifications of ISM were developed: Combinatorial Active Site Saturation Test (CAST), for enhancing the substrate scope and enantioselectivity & B-Factor Iterative Test (B-FIT), to increase the thermostability of enzymes [113]. Both CAST and B-FIT follow the same mutational strategy. CASTing is based on the 3D structure of the enzyme, where two or three amino acids lying next to the active site of an enzyme are chosen as loci for saturation mutagenesis forming focused libraries. After initial identification of best mutant, it is used as a template for further rounds of saturation mutagenesis at different sites and is repeated till all the sites have been covered creating higher-quality smarter libraries with high catalyst diversity leading to distant effects [127]. Wild-type EH from *A. niger* was initially subjected to directed evolution by using epPCR which led to *E*-value of only 10.8. Later, it was evolved by using CAST technique by which it showed a great leap in *E*-value to 115. The main concern in directed evolution is to generate higher-quality enzyme libraries. To achieve small high-quality libraries, Reetz et al. applied NNK codon degeneracy (N: adenine/cytosine/guanine/thymine; K: guanine/thymine) involving 32 codons relating to 20 amino acids and NDT codon degeneracy (D: adenine/guanine/thymine; T: thymine) involving 12 codons relating to 12 amino acids in ISM of EH from *A. niger* to improve its enantioselectivity. Out of the two, NDT library proved to be of much higher quality and at the same time more focused than NNK library [113].

To generate focused libraries, different algorithms like adaptive substituent reordering algorithm (ASRA) employed with ISM are also available in addition to codon degeneracy which can be used for designing and assessing the different mutagenic libraries [128]. A smart library of *Bacillus megaterium* ECU1001 EH was created which could produce *(S)*-enantiomer of β-blockers (used in cardiovascular therapies) as they are 10–500 fold more potent than *(R)*-enantiomer in binding to the β-adrenergic receptor [129]. In another study of *Bacillus megaterium* ECU1001 EH, an active tunnel which has *(R)*-enantioselectivity toward epoxides was present for substrate access or product release. It was modified by protein engineering to unblock the hindrance at the site for the easy release of the products. Bulky pharmaco epoxide α-naphthyl glycidyl ether was then efficiently resolved and used as a precursor of β-

blocker *(S)*-propranolol [30]. Recently, enantioselectivity of wild-type LEH was enhanced or inverted significantly using CAST and ISM approach with single amino acid mutagenesis at a large randomization site comprising of 10 residues with drastically reduced library size [130].

Besides enhancing the enantioselectivity of EHs, directed evolution has also been applied to increase the thermostability of EHs. For applied biocatalysis, an enzyme should have long shelf-life and should be stable at varied temperatures. B-FIT approach based on B factors was used to increase the thermal robustness of *A. niger* EH. With several rounds of ISM, the best variant of *A. niger* EH has 21°C increase in T_{50}^{60} value (temperature at which half of the enzyme activity was maintained after heat treatment for 60 min) [131]. B factors are atomic displacement parameters gained from X-ray data. Amino acids showing highest B factors values are chosen for mutagenic studies as they have a high degree of thermal motion and thus flexibility [132]. Another EH, i.e., limonene EH, from *R. erythropolis* DCL14 was made thermostable with an increase in the apparent thermal denaturation temperature of ~35 °C by using the strategy Framework for Rapid Enzyme Stabilization by Computational Libraries (FRESCO) [133].

A number of outstanding reviews were written on the directed evolution of enzymes detailing the advancement of methods in directed evolution, which can be applied to create more advanced enzymes with smart mutant libraries [134–139]. Widersten et al. described the relationship between EHs structure and function in connection to biocatalysis and also highlighting the concepts of directed evolution [140].

Jochens et al. showed that site-directed mutagenesis can also interconvert the enzyme activities within the α/β-hydrolase family [141]. Esterase from *Pseudomonas fluorescens* was converted to EH with few amino acid substitutions at the active site alongwith substitution of a loop. Even though the catalytic activity of the developed EH was too low for commercial purpose, it suggests that the directed evolution has opened the prospect of applying catalysis to non-natural reactions. It revealed that by changing few amino acids in an enzyme, it can show promiscuous behavior to another enzyme [141].

From the past, great advances have been made in the field of directed evolution. With the use of directed evolution techniques, EHs have been continuously made more selective in their properties to be used in the chiral world. With rapid developments in the computational field and availability of multiple algorithms and softwares, smart libraries are being generated, thus reducing the labor work to a great extent and saving both the time and money. Constant developments in the field of directed evolution will offer more powerful and stable biocatalysts for various industrial uses.

Conclusions

In the present scenario, biocatalysis has been increasingly employed as a method of choice for the synthesis of chiral drug intermediates. With the increase in the number of microbial EHs, they are being exploited as unlimited sources of eco-friendly EHs. To make EHs industry friendly and to be used on a large scale with high enantiopurity, stability and reusability, various studies had been undertaken. Reaction media optimization to obtain chiral epoxides or diols was performed by using various hydrophilic and hydrophobic organic solvents, detergents and ionic liquids. Due to the rising concern of green and eco-friendly environment, EH optimizations with new kind of greener ionic solvents (deep eutectic solvents) were also performed though the studies are in their preliminary stages. Various immobilization approaches have increased the reusability of EHs, consequently improving the capital gain of the industry. With a vast pool of protein engineering techniques, immobilized engineered EHs have contributed as promising biocatalysts. These strategies seem to be powerful tools to establish EHs as industrial biocatalysts in the market.

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