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Review

Epoxide hydrolases from yeasts and other sources: versatile tools in biocatalysis [1]

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Abstract

Major characteristics, substrate specificities and enantioselectivities of epoxide hydrolases from various sources are described. Epoxide hydrolase activity in yeasts is discussed in more detail and is compared with activities in other microorganisms. Constitutively produced bacterial epoxide hydrolases are highly enantioselective in the hydrolysis of 2,2- and 2,3-disubstituted epoxides. A novel bacterial limonene-1,2-epoxide hydrolase, induced by growth on monoterpenes, showed high activities and selectivities in the hydrolysis of several substituted alicyclic epoxides. Constitutively produced epoxide hydrolases are found in eukaryotic microorganisms. Enzymes from filamentous fungi are useful biocatalysts in the resolution of aryl- and substituted alicyclic epoxides. Yeast epoxide hydrolase activity has been demonstrated for the enantioselective hydrolysis of various aryl-, alicyclic- and aliphatic epoxides by a strain of *Rhodotorula glutinis*. The yeast enzyme, moreover, is capable of asymmetric hydrolysis of *meso* epoxides and performs highly enantioselective resolution of unbranched aliphatic 1,2-epoxides. Screening for other yeast epoxide hydrolases shows that high enantioselectivity is restricted to a few basidiomycetes genera only. Resolution of very high substrate concentrations is possible by using selected basidiomycetes yeast strains. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Yeast epoxide hydrolase; *Rhodotorula glutinis*; Substrate specificity; Enantioselectivity; Screening; Epoxide hydrolase characteristics

1. Introduction

1.1. General

Epoxide hydrolases (EC 3.3.2.3) are enzymes that catalyses the *trans* addition of water to epoxides resulting in the formation of the corresponding vicinal diols. These enzymes have been found in all types of living organisms, including mammals, insects, plants and microorganisms, and thus are ubiquitous in Nature. Epoxide hydrolases from higher organisms are induced by a variety of synthetic compounds, toxicants and phytochemicals. They may thus be generally regarded as part of the detoxifying enzyme systems (animals) and defence mechanisms (plants) of these organisms. Epoxide hydrolases from microbial sources usually are present constitutively and their physiological role is still not understood. Some microbial epoxide hydrolases are inducible and are involved in the metabolism of some specific compounds.

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During the past decades, mammalian epoxide hydrolases in particular have been well characterized. Less extensively studied are epoxide hydrolases from insects and plants. It has been observed that the enzymes from these organisms resembles the mammalian types. They also can be distinguished in microsomal and soluble epoxide hydrolases. Studies on epoxide hydrolases from higher organisms in most cases have been set up to investigate their physiological role. Also the molecular mechanism and stereochemical features of these enzymes have been studied in detail. The majority of mechanistic studies also refer to the mammalian enzymes, partly due to the broad range of substrates accepted by these enzymes.

1.2. Biocatalytical potential

From stereochemical investigations, it has been concluded that epoxide hydrolases are of interest in the kinetic resolution of racemic epoxides. Epoxide hydrolases are attractive biocatalysts since they are cofactor-independent and seem to react with a homologous range of epoxides. Moreover, most enzyme preparations are relatively stable. In general, the maximal feasible reaction yield by kinetic resolution is only 50%, related to the racemic substrate. Hydrolytic kinetic resolution of epoxides, however, can result in enantiopure diols as well. For the diols, a maximum reaction yield of 100% can be obtained in specific cases.

Enantiopure epoxides and their corresponding enantiopure vicinal diols are important chiral building blocks in the preparation of more complex enantiopure bioactive compounds or as end products with biological activity. Therefore, a great interest exists in the development of methods for the synthesis of enantiopure epoxides and diols. Various chemical and biological production methods for synthesis of enantiopure epoxides have been reviewed [1–3].

In view of the interest to use biocatalysts for the production of enantiopure compounds, several studies to investigate the potential of microbial epoxide hydrolases have been set up recently. Mammalian enzymes have excellent enantioselectivities for various substrates but microbial enzymes can be applied on an industrial scale. Most microorganisms can be easily cultivated and can produce more enzyme than can be obtained from mammalian cells. A comprehensive review on the biocatalytical potential of known epoxide hydrolases from various sources has recently been published by Archer [4].

In the present paper, a summary of the major characteristics of various epoxide hydrolases will be given and a description of the recently discovered yeast epoxide hydrolase activities in *Rhodotorula glutinis* (*R. glutinis*) and related species will be presented in more detail.

2. Enzymatic mechanism

The commonly accepted epoxide hydrolase mechanism has been elucidated by studies on the two major mammalian epoxide hydrolases, microsomal (mEH) and soluble epoxide hydrolase (sEH), respectively. It is now generally assumed that mEH and sEH are structurally and mechanistically related to each other and also to bacterial haloalkane dehalogenase (HAD) and to bacterial haloacetate dehalogenase (HAcD) [5,6]. Mammalian mEH and sEH, and the bacterial HAD and HAcD, are all members of the same mechanistic class of α/β hydrolase fold enzymes [5,7]. These enzymes are related to each other by their three-dimensional structure and by their two-step catalytic mechanism that involves an alkyl-enzyme ester intermediate [5,7].

Based on findings for HAD, a two-step catalytic mechanism has been postulated for mEH and sEH [5,8]. Single turnover experiments, performed in $H_2^{18}O$, resulted in the lack of ^{18}O in the diol product, indicated that the oxygen incorporated was supplied by the enzyme. From these results, it has been proposed that the enzymatic activity is initiated by an S_N2 type, back side nucleophilic attack of the catalytic

amino acid, leading to the formation of an α-hydroxyester-enzyme intermediate. Subsequently, this acyl-enzyme intermediate is hydrolysed by addition of an activated water to the carbonyl carbon of the ester bond, resulting in regeneration of the active enzyme and release of the diol product. Definite proof for the covalent binding of the epoxide hydrolase to its substrate has recently been reported for mEH and sEH [9]. In this study, visualization of the formed covalent intermediate was by SDS-gel electrophoresis after incubating of either mEH or sEH with radioactive *cis*-9,10-epoxystearic acid. The general accepted mechanism for mEH and sEH is shown in Fig. 1.

From sequence alignments with members of the α/β hydrolase fold enzymes and by site-directed mutagenesis of epoxide hydrolase cDNA, it is proposed that the nucleophilic amino acid is aspartic acid, Asp-226 for mEH and Asp-333 for sEH, respectively. In parallel, the combinations His-431/Asp-352 (mEH), respectively His-523/Asp-495 (sEH), have been determined to perform the activation of the water molecule which hydrolyses the acyl-enzyme intermediate [5,8,10,11].

3. Epoxide hydrolases from other sources

3.1. Mammalian epoxide hydrolases

The two major mammalian epoxide hydrolases, microsomal epoxide hydrolase (mEH) and soluble epoxide hydrolase (sEH), are present in the liver of animals. The subcellular distribution and initial characterisation of mammalian epoxide hydrolases has been reviewed by Wixtrom and Hammock [12]. Mammalian sEH and mEH were named after their predominant fractionation to the $100{,}000 \times g$ supernatant and microsomal pellet, respectively. In spite of their relationship, mEH and sEH have different physical properties. Both enzymes also have broad, partially overlapping substrate specificities, but their individual substrate preferences are still quite distinct [9]. In general, it is assumed that mono- and *cis*-disubstituted epoxides bearing a lipophilic substituent are good substrates for mEH [14,15]. For sEH also tri- and tetra-substituted epoxides and in particular, several *trans*-disubstituted epoxides are found to be excellent substrates [13,16].

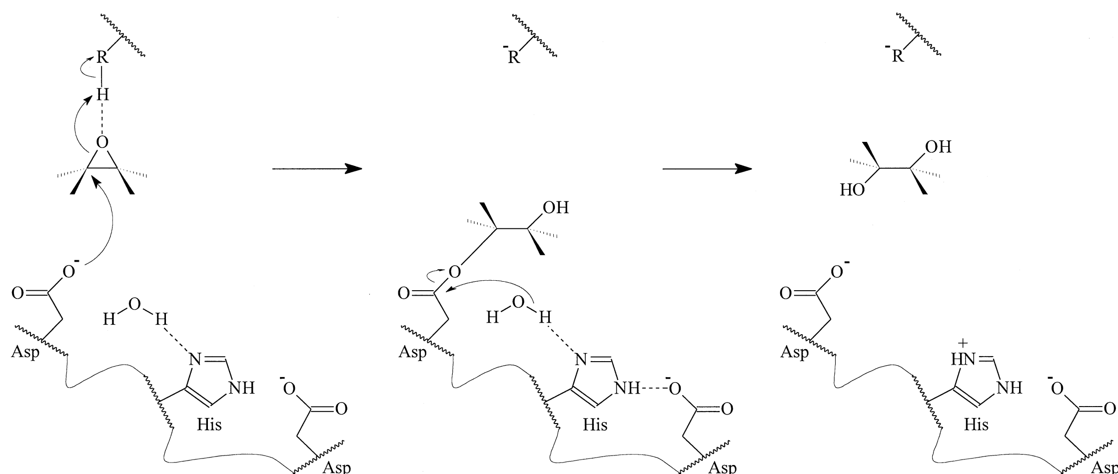

Fig. 1. Proposed catalytic mechanism for mEH and sEH (adapted from [9,10]). In this two-step mechanism the epoxide is opened by a back side nucleophilic attack of an aspartic acid residue which leads to the formation of an α-hydroxyester-enzyme intermediate. Subsequently, the acyl-enzyme intermediate is hydrolyzed by a water molecule, activated by a histidine in cooperation with another aspartic acid, to release the diol product and native enzyme.

3.1.1. Mammalian mEH

The physiological role of mammalian mEH is the hydrolysis of epoxides derived from xenobiotics, including polycyclic aromatic hydrocarbons [17]. In this way, the enzyme is involved in the detoxification processes in higher organisms by conversions of lipophilic substances into more water-soluble, readily excretable compounds. Mammalian mEH is located in the endoplasmatic reticulum and its molecular mass is 52 kDa [18]. Biochemical properties, distribution and regulation of mEH has been reviewed by Seidegard and DePierre [19].

Stereochemical features of mEH have been extensively studied by Bellucci et al. [20–27] and Chiappe et al. [28]. mEH catalysed hydrolysis of several β-alkyl substituted styrene oxide derivatives takes place at the (S) configured carbon atoms, resulting in *inversion* of configuration. The diols formed are enantiopure *threo*-(R,R) diols from the *cis* epoxides and *erythro*-(S,R) diol from the tested *trans* epoxide [23,26]. An increasing size of the chain length in the case of *cis*-β-alkyl substituted styrene oxides was found to cause a decrease in both activity as well as enantioselectivity of the mEH. Similar substrate enantioselectivities for mono-, *cis*- and *trans*-disubstituted substrates are observed in the mEH catalysed hydrolysis of short-chain aliphatic 1,2- and 2,3-epoxides [29,30].

From many reports, showing high substrate enantioselectivities for various racemic substrates and as well high product enantioselectivities for *meso* substrates, it has been concluded that mammalian mEH is a useful biocatalyst for the production of enantiopure epoxides and vicinal diols.

3.1.2. Mammalian sEH

Although mammalian sEH is able to hydrolyse various epoxides derived from xenobiotic compounds, its primary biological function might be the metabolic transformation of epoxides formed from endogenous substrates, especially from fatty acids. This idea is based on the findings that (i) epoxides from fatty acids are the best known substrates for sEH [16,31] and (ii) that sEH is induced by several triglyceride- and cholesterol-lowering hypolipidemic drugs [32–34]. In a study on the regulation of mammalian sEH, it was observed that sEH was concomitantly induced with the peroxisomal β-oxidation by peroxisome proliferators like clofibrate, tiadenol and fenofibrate [32]. mEH however, was found not to be significantly induced by these compounds. On the other hand, there was no induction of sEH by phenobarbital or *trans*-stilbene oxide, both compounds being effective inducers for mEH and other xenobiotic-metabolizing enzymes [35,36], indicating a distinct regulation of sEH and mEH.

In literature, most attention to mammalian epoxide hydrolases has been given to mEH and less to sEH. Reports dealing with substrate enantioselectivities of sEH are particularly scarce. sEH is located in both the cytosol and peroxisomal matrix of liver cells [8]. The native sEH exists in the form of a homodimeric protein, with a subunit molecular mass of about 62 kDa [37], and it has been suggested that the amino-terminal region functions to allow dimerization of the enzyme [38].

Comparative studies on the substrate and product enantioselectivity of sEH and mEH have been done in the case of the hydrolysis of methyl-substituted methylcyclohexene epoxides [25] and also for several alicyclic *meso* epoxides and *cis*-stilbene oxide [22]. From both studies, the authors concluded that in all cases enantioselectivities were lower for sEH than for mEH. Furthermore, no high enantioselectivities for other substrates (or products) have been reported for sEH. Consequently, it can be concluded that mammalian sEH will only have a subsidiary role in biocatalysis.

3.2. Insect epoxide hydrolases

Insect epoxide hydrolases are either part of the enzymatic detoxification processes, like the mammalian enzymes, or are involved in the regulation of specific pheromones and hor-

mones. Epoxide hydrolase activity in pheromone regulation has been described for the hydrolysis of the sex attractant disparlure (7,8-epoxy-2-methyloctadecane) by tissue homogenates of the gypsy moth *Lymantria dispar* [39]. The role of the epoxide hydrolase is assumed to convert stimulatory pheromones to nonstimulatory products, thus preventing sensory adaptation. Both the (7*R*,8*S*) and (7*S*,8*R*) enantiomers, and as well as two *meso* analogues, are found to be hydrolysed with *inversion* of configuration at the (*S*) configured carbon atom, yielding exclusively the corresponding *threo*-(*R*,*R*) diols [39,40].

Enzymes which are much less substrate specific are the microsomal and soluble epoxide hydrolases from mite species like *Rhizoglyphus robini* [41]. Enzymes from this organism are involved in the insect detoxification system since they are induced by compounds like phenobarbital and *trans*-stilbene oxide and since they accept various epoxide substrates. With styrene oxide as a substrate, most of the epoxide hydrolase activity was found to be located in the microsomal fraction.

Large scale production of insect enzymes is still fairly difficult which strongly hampers biocatalytical applications of insect epoxide hydrolases.

3.3. Plant epoxide hydrolases

Plant epoxide hydrolases are specific for the hydrolysis of *cis* fatty acid epoxides, resulting in *threo* diols [42]. In this way, they are of physiological relevance because these epoxides and their corresponding diols play a key role in the plant defence mechanisms against fungal and bacterial pathogens. These compounds are involved in the biosynthesis of cutine, a polymer being a barrier against pathogen invasion which covers all aerial parts of plants [43]. In addition, fatty acid epoxides are observed to have anti-fungal properties themselves as well [44,45].

The fatty acid epoxide hydrolase from soybean has been studied in more detail by Blee and Schuber [42,46,47]. The authors observed the presence of both a microsomal (minor fraction) and a soluble (major fraction) fatty acid epoxide hydrolase and purified the soluble form from soybean seedlings [42]. The soluble enzyme was characterized as a dimeric protein with a subunit molecular mass of 32 kDa and showed a very high activity for *cis*-9,10-epoxystearic acid (35 μmol/min, mg protein) whereas the *trans* isomer was only hydrolysed with 45% of this activity. Stereochemical features of the enzyme have been studied for the hydrolysis of *cis*-9,10-epoxystearic acid and the *cis*-9,10- and *cis*-12,13-regioisomers of linoleic acid monoepoxides, respectively [46,47]. In the hydrolysis of *cis*-9,10-epoxystearic acid, interestingly, the epoxide hydrolase showed a preference for the (9*R*,10*S*) enantiomer and eventually hydrolysed both enantiomers to the (9*R*,10*R*) corresponding diol, both being the enantiomeric forms with physiological relevance in higher plants.

Plant epoxide hydrolases in principle are useful for the synthesis of enantiopure epoxyfatty acids and dihydroxyfatty acids because of their stereochemical features and their relatively high activities. And, in addition, to obtain large amounts of these plant enzymes will be considerably less difficult than from animal sources. However, their biocatalytical potential should be further determined by investigating the range of substrates accepted.

3.4. Bacterial epoxide hydrolases

Bacterial epoxide hydrolases can be divided in (i) constitutively produced enzymes and (ii) enzymes involved in the metabolism of specific epoxides. The constitutively produced bacterial epoxide hydrolases have been studied by Faber et al. They have screened a large number of bacterial strains for the resolution of 2-mono-, 2,2- and 2,3-disubstituted epoxides [48–51].

Among the various bacterial strains tested, strains from the genera *Rhodococcus*, *Mycobacterium* and *Nocardia* were selected for further studies. Best results were obtained in the highly enantioselective (*E*-values up to 200) hydrolysis of 2-methyl-1,2-epoxyheptane by *Rhodococcus ruber* DSM 43338, *Mycobacterium paraffinicum* NCIMB 10420 and the *Nocardia* strains TB1, H8 and EH1. The latter two *Nocardia* strains were subsequently used in a study on the preparation of enantiopure (*S*) diols up to 98% yield via an enantioconvergent two-step hydrolysis of 2,2-disubstituted epoxides [52]. In this process, a nearly 100% reaction yield was obtained by acid catalyzed chemical hydrolysis of the residual (*R*)-epoxide after biocatalytical resolution of the racemic epoxide by lyophilized *Nocardia* cells. From these studies, it can be concluded that constitutively produced bacterial epoxide hydrolases particularly exhibit high enantioselectivities in the hydrolysis of 2-methyl-2-alkyl- and 2-methyl-2-(aryl)alkyl-epoxides. However, low enantioselectivities were observed in the hydrolysis of unbranched terminal 1,2-epoxides and a number of tested *meso*-substrates were found not be hydrolysed by the selected bacterial strains.

Bacterial epoxide hydrolases which are involved in the metabolism of specific epoxides have some different features in comparison with the constitutively produced enzymes. Metabolism-related enzymes generally have a relatively narrow substrate specificity which is often limited to compounds which are structurally related to the growth substrate of the host organism. Specific activities for growth substrate related compounds are usually relatively high. A typical example of an application of this type of epoxide hydrolase is in the preparation of (1*S*,2*S*)-1-methyl-cyclohexane-1,2-diol via chemo-enzymatic resolution and deracemization of 1-methyl-1,2-epoxycyclohexane by cells of *Corynebacterium* C12 [53]. The involved epoxide hydrolase was induced by growth on 1,2-epoxycyclohexane, the corresponding diol and the 1-methyl diol derivative [54].

A more detailed study was performed on the epoxide hydrolase from *Agrobacterium radiobacter* AD1. The enzyme was purified to homogeneity from epichlorohydrin-grown cells and characterized as a monomeric protein with a molecular mass of 34 kDa [55]. Subsequently, the epoxide hydrolase gene was cloned, expressed in *Escherichia coli* (*E. coli*) and further characterized as a member of the α/β hydrolase fold enzymes [56]. The scope of epoxide hydrolysis has been studied in more detail and from this it has been reported that the recombinant enzyme produced in *E. coli* shows moderate to relatively high enantioselectivities in the hydrolysis of styrene oxide and several styrene oxide derivatives [57].

An interestingly novel bacterial epoxide hydrolase has very recently been studied by van der Werf et al. [58]. They have purified and characterized a limonene-1,2-epoxide hydrolase from *Rhodococcus erythropolis* (*Rh. erythropolis*) DCL14. It was found that this epoxide hydrolase was induced during growth on monoterpenes, with highest enzyme levels by growth on the monoterpene (+)-isomers. In cells of *Rh. erythropolis* DCL14, which was routinely grown on (+)-limonene, all the epoxide hydrolase activity was located in the cytosolic fraction. The enzyme was purified to homogeneity and was subsequently characterized as a monomeric protein of 17 kDa. The enzyme thus has an exceptional low molecular weight for epoxide hydrolases. Comparison of the N-terminal amino acid sequence of this enzyme resulted in no substantial homology with any other protein present in the databases. Experiments with selective inhibitors of α/β hydrolase fold epoxide hydrolases and a leukotriene A_4 hydrolase inhibitor showed no inhibition of the limonene-1,2-epoxide hydrolase [59,60]. And, in addition, there neither was inhibition detected with imidazole-modifying compounds, suggesting that, unlike in α/β hydrolase fold epoxide hydrolases, a catalytic His was not involved in the mechanism of this enzyme. From these results, it has been suggested that this limonene-1,2-epoxide

hydrolase possibly belongs to a separate class of epoxide hydrolases. The substrate specificity was found to be narrow and thus is in agreement with what has been reported for other metabolism-related epoxide hydrolases. Accepted substrates were limonene-1,2-epoxide, 1,2-epoxycyclohexane, 1-methyl-1,2-epoxycyclohexane and indene oxide. The stereochemistry of the hydrolysis of these four substrates was studied in detail and it was found that from (+)- and (−)-limonene-1,2-epoxide, the (1*S*,2*R*)-isomers were only hydrolysed after complete hydrolysis of the (1*R*,2*S*)-isomers. The substrate enantioselectivity in the hydrolysis of 1-methyl-1,2-epoxycyclohexane was observed to be the opposite of that reported for the hydrolysis of this compound by *Corynebacterium* C12 [53]. Remarkably, the formed diols were in both cases in the (1*S*,2*S*) configurations indicating the involvement of different catalytic mechanisms.

Bacterial epoxide hydrolases are easily accessible tools in organic synthesis because large scale production of these biocatalysts is relatively easy. However, each of these enzymes also has its limitations. The constitutively produced bacterial epoxide hydrolases are useful in the resolution of di-substituted epoxides only. In addition, the reported specific activities are in most cases not very high. On the contrary, high specific activities are typical for the inducible enzymes, which regrettably only act on a very limited range of substrates. It is, however, to be expected that in the near future some of these limiting features will be improved by genetic engineering.

3.5. Fungal epoxide hydrolases

Studies on epoxide hydrolases from filamentous fungi were originally set up to investigate the biocatalytical potential of these enzymes. In fact, the real breakthrough in the interest for using microbial epoxide hydrolases in organic synthesis was by a report of the group of Furstoss. In this paper, they described the hydrolysis of styrene oxide by cells of two fungal strains via two distinct mechanisms [61]. Hydrolysis of racemic styrene oxide by *Aspergillus niger* (*A. niger*) LCP 521 proceeded with *retention* of configuration at the chiral center, resulting in the (*S*)-residual epoxide and the (*R*)-diol. With cells of *Beauvaria sulfurescens* (*B. sulfurescens*) ATCC 7159 hydrolysis of styrene oxide resulted in the (*R*)-residual epoxide while the (*S*)-epoxide was converted with *inversion* of configuration to the (*R*)-diol as well. In subsequent studies, the substrate specificities and mechanistic differences of these two fungi were investigated in more detail. The observed enantiocomplementarity of these fungi was confirmed in a study on the hydrolysis of several *para*-substituted styrene oxides [62]. The residual epoxides were always in the (*S*)-configuration in the case of *A. niger* whereas (*R*)-epoxides were obtained by use of *B. sulfurescens*. Another comparative study on the substrate specificities of the two fungi showed that only non-subtituted derivatives were accepted by *A. niger*, whereas a number of differently subtituted styrene oxide derivatives were also substrates for *B. sulfurescens* [63]. Concerning the catalytic mechanism of *B. sulfurescens* it was concluded that the attack by the enzyme takes place at the more hindered carbon atom of (*S*) configuration and subsequently proceeded with *inversion* of configuration of this chiral center. Hydrolysis of substituted styrene oxides has also been studied by others with another *Beauvaria* strain, *B. densa* CMC3240 [64]. Reaction rates of the epoxide hydrolysis by *B. densa* CMC3240 were found to be greatly affected by the substituent position, and decreased in the order *para* > *meta* > *ortho* for both chlorinated and methylated series of substituted styrene oxides. The authors also suggested that the epoxide hydrolase of *B. densa*, and also of *B. sulfurescens*, most likely was located in the membrane fraction of these fungi. The cellular localization of the epoxide hydrolase from *A. niger* had previously been determined to be associated with a soluble protein [65]. The dif-

ference in cellular localization might be the origin of the different characteristics of regioselectivity and substrate specificity of the epoxide hydrolases from *Aspergillus* and *Beauvaria* spp. [64]. The applicability of fungal epoxide hydrolases has also been investigated towards the hydrolysis of different substituted aliphatic epoxides [66]. For this study, a number of new fungi was selected as well from a screening using 1,2-epoxyhexane and *trans*-2,3-epoxyhexane as substrates. Hydrolysis of aliphatic epoxides however, proceeded with both low enantioselectivities and activities.

Hydrolysis of indene oxide has been studied with different fungal strains. The interest for the production of enantiopure indene oxide is caused by the possibility to use (1*S*,2*R*)-indene oxide as a precursor to the side chain of the HIV protease inhibitor MK 639 [67]. Preparative-scale resolution of indene oxide yielding the commercially valuable (1*S*,2*R*)-residual epoxide was successful by use of cells of *Diplodia gossipina* [67]. Resolution of indene oxide by *B. sulfurescens* yielded the opposite (1*R*,2*S*)-epoxide enantiomer [63].

Fungal epoxide hydrolases are promising biocatalysts which are constitutively present in fungal cells when grown on simple carbon sources. Production of these biocatalysts is therefore possible in large amounts as well. They have a relatively broad substrate specificity. High enantioselectivities are particularly found for the hydrolysis of aryl- and substituted alicyclic substrates but not for aliphatic epoxides. The difference in the enantioselectivity and regioselectivity as observed for the enzymes from *Aspergillus* and *Beauvaria* spp. is a very interesting phenomenon of the epoxide hydrolases from filamentous fungi.

4. Yeast epoxide hydrolases

The potential of epoxide hydrolase activity in yeasts has not been studied until recently. It is, however, of interest to screen yeasts for this enzyme activity because these unicellular organisms are (i) more easily to cultivate, especially on a large-scale and (ii) in whole-cell biotransformations can be removed easily from reaction mixtures in a simple centrifugation step.

To screen for epoxide hydrolase activity in yeasts, we have tested a number of different yeast strains for enantioselective hydrolysis of styrene oxide **4**, the substrate which is most widely accepted by known epoxide hydrolases. From this screening, a strain of *R. glutinis* was eventually selected for further studies.

4.1. Epoxide hydrolysis by R. glutinis

Enantioselective epoxide hydrolysis by yeasts has been first demonstrated for the hydrolysis of several aryl, alicyclic and aliphatic epoxides by cells of *R. glutinis* CIMW 147 [68]. The epoxide hydrolase of this yeast was produced constitutively when grown on various simple carbon sources. *R. glutinis* was routinely grown in a chemostat culture on a mineral medium supplemented with glucose as carbon source. The presently known range of accepted epoxides **1** to **20** by *R. glutinis* CIMW 147 is given in Fig. 2.

4.1.1. Scope of epoxide hydrolysis

The substrate specificity and enantioselectivity of the epoxide hydrolase from *R. glutinis* has been investigated. The results from the hydrolysis of epoxide **1** to **13** and from epoxide **14** to **20** by glucose-grown cells of *R. glutinis* are summarized in Tables 1 and 2, respectively.

The hydrolyses of the *meso* epoxides **1** to **3** are of special interest because in these cases enantiopure *trans* diols can be obtained, possibly in 100% theoretical yield. In this context, the results obtained after more than 98% conversion of the epoxide, for 1,2-cyclopentanediol (1*R*,2*R*)-**2a** (> 98% e.e. and 98% yield) and 1,2-cyclohexanediol (1*R*,2*R*)-**3a** (90% e.e. and 93% yield) are rather encouraging. Somewhat unsatisfactory was the yield in the case of *threo* 2,3-butanediol (2*R*,3*R*)-**1a** (90% e.e. and 78%

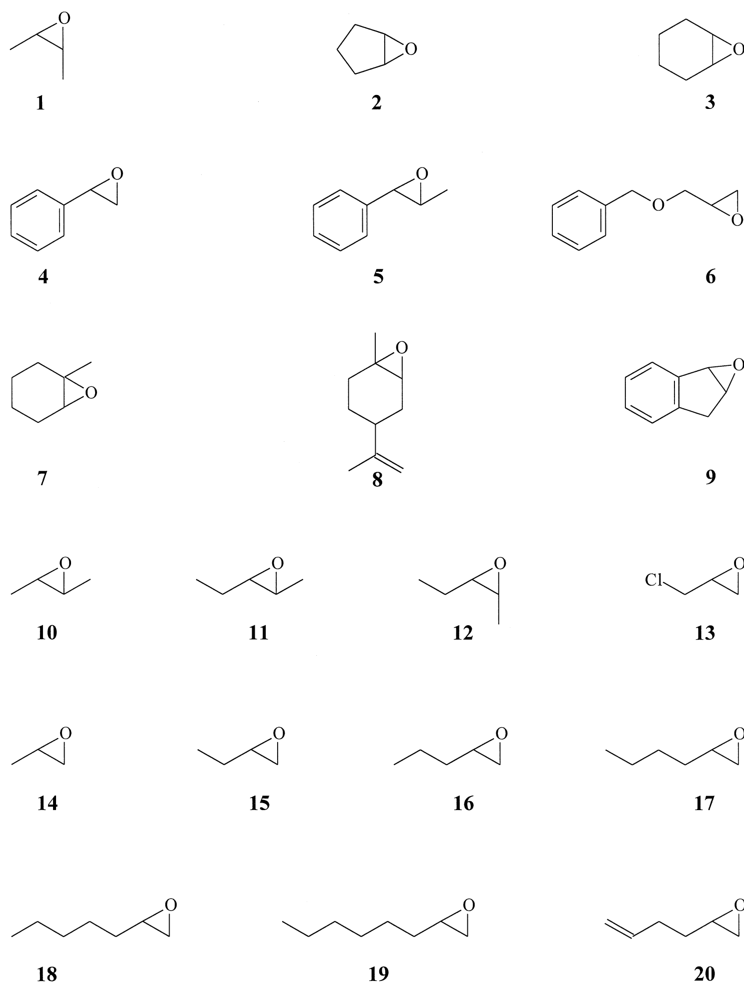

Fig. 2. Scope of epoxide hydrolysis by *R. glutinis*.

yield). The *meso* compound 1,2-epoxycyclooctane was not hydrolysed at all by cells of *R. glutinis*. In general, asymmetric hydrolysis of *meso* epoxides still is rather exceptional for a microbial epoxide hydrolase [4].

In the hydrolysis of racemic epoxide **4** to **20**, the reaction was terminated when the residual epoxide reached an e.e. of more than 98%. The aryl epoxides **4**, **5**, and **6** were good substrates for the epoxide hydrolase of *R. glutinis* with maximum activity for benzyl glycidyl ether **5**. High enantioselectivity was found in the hydrolysis of *trans*-1-phenyl-1,2-epoxypropane **5**. A complete resolution of epoxide (1*R*,2*R*)-**5** (e.e. > 98%) was achieved at a conversion of 55% and resulted in the formation of diol (1*R*,2*S*)-**5a** with an e.e. of 98%, thus being satisfactory results for a resolution process. Hydrolysis of epoxide **5** has been studied as well with mammalian mEH [23] and with the fungus *B. sulfurescens* [63]. Mammalian mEH catalyzed hydrolysis of epoxide **5** resulted in epoxide (1*R*,2*R*)-**5** and diol (1*S*,2*R*)-**5a** both with low enantiomeric purity and low yield. With *B. sulfurescens* better results were obtained for the residual epoxide (1*R*,2*R*)-**5** (e.e. 98%, yield 30%) and diol (1*R*,2*S*)-**5** (e.e. 90%, yield 38%) [63]. From this comparison it can be concluded that the hydrolysis of epoxide **5** by *R. glutinis* and *B. sulfurescens* proceeded with similar

Table 1
Hydrolysis of aryl, alicyclic and aliphatic epoxides by *R. glutinis*

Epoxide (residual substrate)						Diol product		
	Reaction rate[a]	e.e.	Abs. conf.	Yield	Reaction time (h)		e.e.	Abs. conf.
1	0.52	–	*meso*	< 2%	13.0	**1a**	90%	(2*R*,3*R*)
2	0.29	–	*meso*	< 2%	11.0	**2a**	> 98%	(1*R*,2*R*)
3	2.1	–	*meso*	< 2%	1.4	**3a**	90%	(1*R*,2*R*)
4	6.5	> 98%	(*S*)	18%	0.8	**4a**	48%	(*R*)
5	0.91	> 98%	(1*R*,2*R*)	45%	1.3	**5a**	> 98%	(1*R*,2*S*)
6	12.7	> 98%	(*R*)	14%	0.2	**6a**	33%	(*S*)
7	0.31	> 98%	(1*S*,2*R*)	26%	6.0	**7a**	37%	(1*R*,2*R*)
(−) − **8**	1.2	> 98%	(1*S*,2*R*,4*S*)	48%	1.2	(−) − **8a**	> 98%	(1*R*,2*R*,4*S*)
(+) − **8**	0.64	> 98%	(1*S*,2*R*,4*R*)	28%	3.2	(+) − **8a**	30%	(1*R*,2*R*,4*R*)
9	11.4	> 98%	(1*R*,2*S*)	22%	0.2	**9a**	54%	(1*R*,2*R*)
10	0.07	> 98%	(2*R*,3*R*)	47%	18.3	**10a**	–[b]	*meso*
11	0.21	> 98%	(2*R*,3*R*)	48%	3.4	**11a**	54%	(2*S*,3*R*)
12	1.2	> 98%	(2*R*,3*S*)	48%	0.6	**12a**	> 98%	(2*R*,3*R*)
13	2.0	> 98%	(*R*)	10%	1.0	**13a**	22%	(*S*)

[a] Initial rate of epoxide hydrolysis in nmol/min, mg dw.
[b] > 98% *cis* diol.
Hydrolysis of 10 mM epoxide in 20 ml reaction mixture with glucose-grown cells of *R. glutinis* (dry weights ranging from 1.0 to 1.5 g).

stereochemistry. The enantioselectivity in this reaction was in favour of the yeast enzyme.

Alicyclic epoxides **7** to **9** were all hydrolysed to (1*R*,2*R*) diols with moderate to high enantioselectivities. The residual epoxides from racemic 1-methyl-1,2-cyclohexane **7** and limonene oxide **8** in both cases were the (1*S*,2*R*) enantiomers. Hydrolysis of epoxide **7** by two different bacterial epoxide hydrolases has been studied as well [53,58]. In these two other cases, the reactions yielded (1*S*,2*S*) diols but with resolution of the opposite epoxide enantiomers. Both these findings are thus different from the results obtained by *R. glutinis* and could possibly be caused by the involvement of different mechanisms. The enantioselectivity and activity in the hydrolysis of limonene oxide **8** by *R. glutinis* were strongly influenced by the position of the sidechain at the stereogenic C4 carbon atom. High selectivity and activity was

Table 2
Hydrolysis of unbranched aliphatic 1,2-epoxides by *R. glutinis*

Epoxide (residual substrate)						Diol product			
	Reaction rate[a]	e.e.	Abs. conf.	Yield	Reaction time (h)		e.e.	Abs.	Yield conf.
14	1.2	> 98%	(*S*)	13%	6.0	**14a**	25%	(*R*)	82%
15	2.3	> 98%	(*S*)	21%	3.0	**15a**	29%	(*R*)	78%
16	7.2	> 98%	(*S*)	40%	0.7	**16a**	66%	(*R*)	54%
17	50.8	> 98%	(*S*)	48%	0.4	**17a**	83%	(*R*)	47%
18	106.1	> 98%	(*S*)	44%	0.3	**18a**	73%	(*R*)	52%
19	85.2	> 98%	(*S*)	38%	0.3	**19a**	55%	(*R*)	60%
20	34.6	> 98%	(*S*)	46%	0.4	**20a**	74%	(*R*)	49%

[a] Initial rate of epoxide hydrolysis in nmol/min, mg dw.
Hydrolysis of 20 mM epoxide in 10 ml reaction mixture with glucose-grown cells of *R. glutinis* (dry weights ranging from 0.1 to 0.5 g).

found when the *iso*-propenyl group was in the (4*S*) configuration of (−)-limonene oxide and decreased dramatically in case of (4*R*) configuration of the alkyl substituent. Resolution of indene oxide **9** by *R. glutinis* cells was unsatisfactory, partly caused by the chemical instability of indene oxide **9**. The yield of epoxide (1*R*,2*S*)-**9** as well as the e.e. of diol (1*R*,2*R*)-**9a** was very similar to the results obtained by the hydrolysis of indene oxide **9** by *B. sulfurescens* [63].

Activity for methyl substituted 2,3-epoxides, represented by substrates **10** to **12**, was low in the case of *trans* substituted side chains and higher when the side chains were *cis* situated or extended in chain length. Enantioselectivity for epoxide **10** to **12** was in all cases very high, resulting in residual (2*R*) epoxides with nearly the maximal feasible yields of 50%. Similar high enantioselectivities have been reported for the mammalian mEH catalyzed hydrolyses of epoxide **10** to **12** [30].

Epichlorohydrin **13** was a poor substrate for the epoxide hydrolase of *R. glutinis*. Both activity as well enantioselectivity were relatively low as was in the case of mammalian mEH catalyzed hydrolysis of this epoxide [29].

4.1.2. Resolution of unbranched aliphatic 1,2-epoxides

Unbranched aliphatic 1,2-epoxides, also described as mono substituted terminal aliphatic epoxides, are not bearing the functional substituents which are required for enantioselectivity of epoxide hydrolases from various sources. On the other hand, the absence of reactive side groups is a possible advantage for excluding undesired side-reactions while using crude enzyme preparations or whole cell biocatalysts. Enantioselective hydrolysis of these epoxides is therefore of interest. Substrate specificity and enantioselectivity of the yeast epoxide hydrolase towards unbranched aliphatic epoxides has been investigated very recently [69]. Results from the hydrolysis of epoxide **14** to **20** by glucose-grown cells of *R. glutinis* are summarized in Table 2.

From the results presented, it is evident that the yeast epoxide hydrolase has a preference for substrates with a chain length of six carbon atoms and more. The high enantioselectivity observed for the hydrolysis of epoxide **18** and, in particular, for epoxide **17** is remarkable. The enantiomeric purities of the diols, obtained after complete resolution of the corresponding epoxides, were in all cases low. Enantiopure diols could only be obtained at low conversions. The effect of the substrate chain length on the initial reaction rate and on the enantiomeric ratio E in the resolution of epoxides **14** to **19**, is shown in Fig. 3a and b, respectively. In conclusion, enantioselectivity for this range of epoxides showed an optimum in the hydrolysis of 1,2-epoxyhexane ($E = 84$).

The promising result for the resolution of 1,2-epoxyhexane **17** was subsequently confirmed by hydrolysis of its corresponding unsaturated substrate analog 1,2-epoxy-5-hexene **20**. Unsaturated epoxides are interesting building blocks in the stereoselective synthesis of cyclic ethers via bromine assisted epoxide ring expansion [70,71]. The synthesis of *cis*- and *trans*-2,5-dimethyltetrahydrofuran, 2-methyltetrahydropyran and oxepane has been described using epoxide **20** as starting material [71]. Enantioselectivity in the hydrolysis of epoxide **20** by cells of *R. glutinis* was slightly lower ($E = 60$) than it was in the hydrolysis of its saturated substrate analog **17** (Fig. 4).

4.1.3. Stereochemical aspects

From the results from Tables 1 and 2, it is concluded that hydrolysis of terminal epoxides by cells of *R. glutinis* proceeds via *retention* of configuration of the chiral centre, as a result from the attack at the less hindered carbon atom. Enantioselectivity was, without any exception, preferential towards hydrolysis of the (*R*)-epoxide enantiomers leading to the formation of the corresponding (*R*) diols in excess. The apparent divergent results obtained for epoxide **6** and **13**, and diols **6a** and **13a**, are caused by a change in priority leading to a

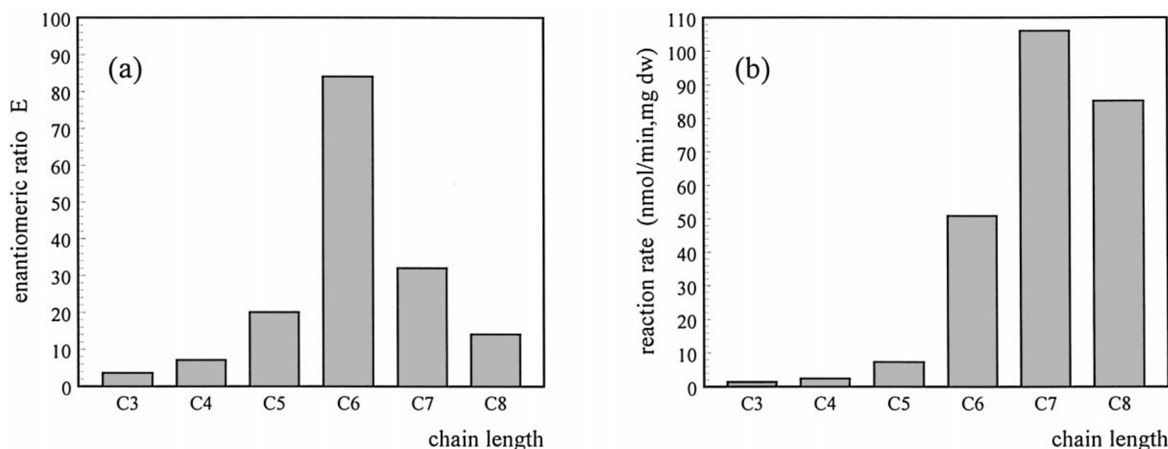

Fig. 3. Effect of the substrate chain length on the enantioselectivity E (a) and reaction rate (b) in the hydrolysis of unbranched aliphatic 1,2-epoxides **14** to **19** by glucose-grown cells of *R. glutinis*.

different stereochemical notation of these compounds although the relative configurations in fact have not changed.

In the hydrolysis of internal epoxides, another general stereochemical pattern can be recognized. All internal epoxides tested are hydrolysed with *inversion* of configuration by the preferential attack at the (S) configured stereogenic centre. With the exception of epoxide **12**, the attack occurred at the more hindered, not methyl-substituent bearing, carbon atom of the tested methyl-substituted internal epoxides. With respect to the *meso* compound 1,2-epoxycyclohexane **3**, the preferential attack was towards the 3,4-anticlockwise conformer of this epoxide, referring to the helicity about the 3,4 C–C bond [4]. Subsequently, the (S) configured stereogenic centre was hydrolysed with *inversion* of configuration to the $(1R,2R)$ diol. The stereochemical patterns of hydrolysis of terminal and methyl-substituted internal epoxides by *R. glutinis* are given in Fig. 5.

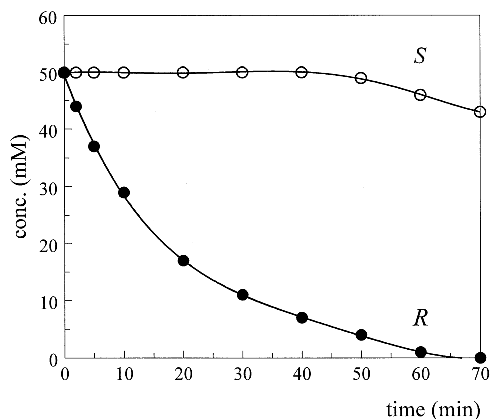

Fig. 4. Resolution of 100 mM 1,2-epoxyhexane by glucose-grown cells of *R. glutinis*. Hydrolysis of the (R)-epoxide (●) followed by subsequent slow hydrolysis of the (S)-epoxide (○).

4.1.4. Cellular enzyme localization

The epoxide hydrolase from *R. glutinis* was found to be located in the membrane fraction, obtained by ultra-centrifugation at $100,000 \times g$ of crude cell free extracts of glucose-grown cells [72]. Solubilisation of the enzyme from this membrane fraction yielded a stable preparation of the epoxide hydrolase which could be stored for several months at $-20°C$ or when lyophilized. The subsequent purification and characterization of the enzyme is in progress and will be reported in due course.

4.2. Distribution of epoxide hydrolases in other yeast species

To investigate the distribution of epoxide hydrolase activity within the yeast domain, an

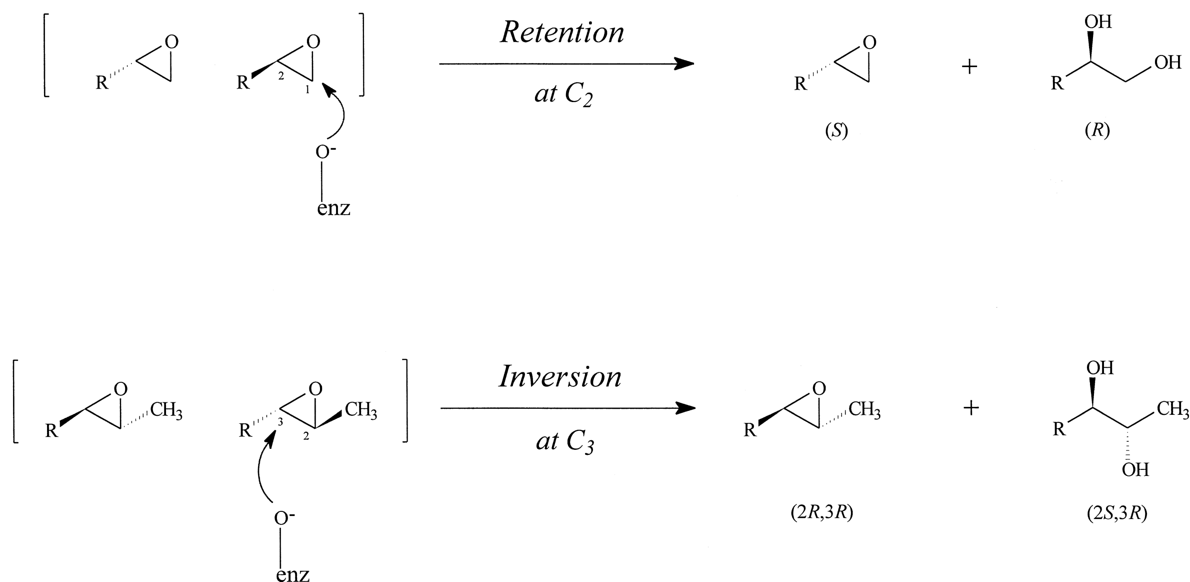

Fig. 5. Stereochemistry of epoxide hydrolysis by *R. glutinis*. *Retention* of configuration by the preferential attack at the (*R*) configured stereogenic centre of terminal epoxides. *Inversion* of configuration by the preferential attack at the (*S*) configured stereogenic centre of methyl-substituted internal epoxides.

extensive screening of yeast strains from 25 different genera has been set up [73]. The yeasts were tested for the enantioselective hydrolysis of 1,2-epoxyoctane **19**. Hydrolysis of 1,2-epoxyoctane by *R. glutinis* CIMW 147 proceeded with a high reaction rate but, however, with a relatively low enantioselectivity towards this substrate [69].

From the results in Table 3, it can be concluded that, although epoxide hydrolase activity is fairly widespread, enantioselectivity for the hydrolysis of 1,2-epoxyoctane was observed in very few yeasts only. In these cases, (*R*)-1,2-epoxyoctane was preferentially hydrolysed via *retention* of configuration to (*R*)-1,2-octanediol. High enantioselectivity was found to be restricted to only a few basidiomycetes genera, which include *Trichosporon*, *Rhodosporidium* and *Rhodotorula*. Two strains, *R. araucariae* CBS 6031 and *Rhodosporidium toruloides* CBS 349, showing both high activity and enantioselectivity ($E > 100$) were selected for further studies. Highly enantioselective hydrolysis of 1,2-epoxyoctane has not been reported for epoxide hydrolases from other sources.

Preparative scale hydrolysis of 1,2-epoxyoctane **19** was tested with the two selected *Rhodotorula* and *Rhodosporidium* strains, respectively [73]. To this, the epoxide concentration was raised to 1000 mg epoxide in a total volume of 20 ml. Such a substrate concentration, calculated to be theoretically 500 mM, exceeds the maximum solubility of this compound. Under these conditions, substantial amounts of epoxide will be separated from the aqueous phase during the reaction. Chemical hydrolysis will be minimized and larger quantities of substrate can thus be resolved. This high epoxide concentration had no significant adverse effect on the tested biocatalysts since there was only about 14% and 16%, respectively, decrease in reaction rate, compared with the rates for 20 mM epoxide. Similar results were obtained in the preparative scale hydrolysis [69] of 100 mM and 500 mM 1,2-epoxyhexane **17** by *R. glutinis* CIMW 147 (Fig. 4).

It is demonstrated that high yield resolution of unbranched aliphatic 1,2-epoxides is restricted to basidiomycetes yeasts. Further studies can give more information to which extend

Table 3
Distribution of epoxide hydrolase activities for 1,2-epoxyoctane in yeasts

Genera	Number of yeasts strains		
	Screened	With activity	With $E > 40$
Bullera	1	1	
Candida	29	11	
Cryptococcus	3		
Debaryomyces	5	1	
Galactomyces	1		
Geotrichum	13	2	
Kloeckera	1		
Kluyveromyces	1		
Lipomyces	15	2	
Myxozyma	5	1	
Pachysolen	2		
Phaffia	2		
Pichia	37	8	
Rhodosporidium	5	4	2
Rhodotorula	11	5	4
Sporabdomyces	1		
Sporidiobolus	1	1	
Sporobolomyces	1		
Sporopachydermia	2		
Trichosporon	8	6	1
Wickerhamia	1		
Williopsis	3		
Wingea	1	1	
Yarrowia	4	3	
Zygozyma	3		
Unclassified	31	8	

substrate specificity and enantioselectivity are strain dependent.

Yeast epoxide hydrolases are versatile biocatalysts with a broad substrate specificity, and even unbranched terminal aliphatic 1,2-epoxides were hydrolysed enantioselectively. The unicellular appearance of yeasts is a considerable advantage in the production of the epoxide hydrolase enzyme and in bioconversions using whole cells.

5. Concluding remarks

The development of methods for the application of epoxide hydrolases is of interest because these enzymes are attractive biocatalysts. They are cofactor-independent and can react in general with a homologous range of epoxides. At the moment, much attention is given to the microbial enzymes because of their relatively easy availability on a large scale. On the other hand, however, techniques for large-scale production of microorganisms containing recombinant enzymes from other sources regains the interest for these other enzymes as well.

For resolution of many interesting epoxides there are now suitable biocatalysts with epoxide hydrolase activity available. The versatility of epoxide hydrolases has recently been extended by the exceptional enantioselective hydrolysis of unbranched aliphatic 1,2-epoxides by *R. glutinis* and related yeast species. And, in addition, kinetic resolutions of very high substrate concentrations by the yeast enzymes greatly facilitates possible applications of the method on a preparative scale. Because of its remarkable broad substrate specificity, it will be of interest to study the membrane bound epoxide hydrolase of *R. glutinis* in more detail. It can be concluded from literature that membrane associated epoxide hydrolases from eukaryotic sources perform better in view of substrate specificity and enantioselectivity than their soluble counterparts. Examples of such membrane associated enzymes are the mammalian mEH and the microbial enzymes from *Beauvaria* spp.

Future investigations with purified forms of the recently discovered novel epoxide hydrolases will reveal the actual scope and limitations of these versatile enzymes to their use as biocatalysts.

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