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Research Article

Cloning of an epoxide hydrolase-encoding gene from *Rhodotorula mucilaginosa* and functional expression in *Yarrowia lipolytica*

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Abstract

Epoxide hydrolases (EHs), especially those of fungal origin, have the ability to catalyse the enantioselective hydrolysis of epoxides to their corresponding diols. Recombinant DNA technology has been used extensively to overproduce these catalysts for the efficient hydrolytic kinetic resolution of epoxides, which serve as high-value intermediates in the fine chemicals and pharmaceutical industries. Degenerate primers, based on data from available EH-encoding gene sequences, in conjunction with inverse PCR, were used to amplify the genomic EH-encoding gene from *Rhodotorula mucilaginosa*. The 2347 bp genomic sequence revealed a 1979 bp ORF containing nine introns. The cDNA sequence revealed an 1185 bp EH-encoding gene that translates into a 394 amino acid protein exhibiting low sequence homology towards the known EH proteins. The EH gene from *R. mucilaginosa* was functionally expressed in *Yarrowia lipolytica* using a constitutive integrative expression cassette. Whole-cell biotransformation of (2,3-epoxypropyl)benzene, using the recombinant EH, revealed activity and selectivity far superior to any other activity and selectivity reported in literature using wild-type organisms. The GenBank Accession No. for the *R. mucilaginosa* EH gene is AY627310.

Keywords: **epoxide hydrolase; *Yarrowia lipolytica*; recombinant expression; fungi**

Introduction

Epoxide hydrolases (EHs; E.C. 3.3.2.3) have the ability to catalyse the enantioselective hydrolysis of epoxides to the corresponding diols. EHs are found in a wide variety of organisms, including plants, bacteria, fungi, mammals and yeasts (Orru and Faber, 1999). The hydrolysis of an epoxide by these structurally and mechanistically similar enzymes proceeds via an enzyme–ester–substrate intermediate (Lacourciere and Armstrong, 1993), resulting in the formation of the corresponding vicinal *trans*-diol with either retention or inversion of configuration, depending on the regioselectivity of the enzyme and the substitutional pattern of the carbon atom involved (Orru and Faber, 1999) (Figure 1). The synthetic applications of enantioselective EHs as catalysts in the kinetic resolution of various epoxides for the fine chemicals industries have been reviewed in detail (Archelas and Furstoss, 2001; Steinreiber and Faber, 2001; De Vries and Janssen, 2003).

Recent advances in genome sequencing have produced a variety of putative EH-encoding genes from a diverse number of organisms, the majority being of bacterial origin (Barth *et al*., 2004). EH-related activity associated with several wild-type fungi, especially yeasts, indicated that several of these organisms were able to catalyse the enantioselective hydrolysis of various epoxides, making them the ideal sources for isolating EH-encoding genes for recombinant expression

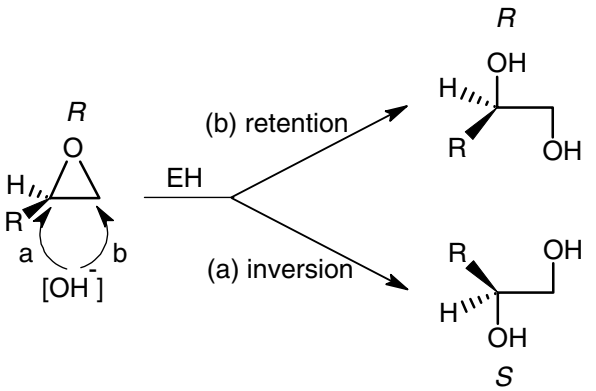

Figure 1. Epoxide hydrolase catalyses a reaction where chemically reactive epoxides are hydrated via the *trans* addition of water to the epoxide moiety, resulting in the formation of the corresponding vicinal *trans*-diol with either retention or inversion of configuration (adapted from Orru and Faber, 1999)

(Botes *et al*., 1999). Recently, a number of novel EHs were cloned from different fungi, including: *Xanthophyllomyces dendrorhous* (Visser *et al*., 1999); *Aspergillus niger* (Arand *et al*., 1999); *Rhodotorula glutinis* (Visser *et al*., 2000); *Rhodotorula araucariae* (Visser *et al*., 2002); and *Rhodosporidium paludigenum* (Labuschagne *et al*., 2004).

Utilization of these cloned genes requires the functional recombinant expression of these genes in a suitable host. Most of these fungal EH-encoding genes have been expressed as recombinant proteins in *Escherichia coli* (Visser *et al*., 1999, 2000, 2002). However, it was shown that the majority of the recombinant protein was present as insoluble aggregates in inclusion bodies (Visser *et al*., 2003). *Pichia pastoris* and *A. niger* were also successfully employed as alternative host systems for the production of recombinant EH-encoding genes from fungal origin to allow enantioselective hydrolytic resolution of epoxides (Lee *et al*., 2004; Monfort *et al*., 2004).

Yarrowia lipolytica has been shown to be one of the most promising yeasts for heterologous protein production (Müller *et al*., 1998) and has been used for the production of recombinant proteins from a wide variety of organisms (Madzak *et al*., 2004). The *Y. lipolytica* system allows for the use of a wide variety of selective markers, promoters and secretion signals and stable site-directed or random integration of either single- or multi-copy expression cassettes into the genome. This facilitates the cultivation of the recombinant organism without the need to continually apply selective pressure to prevent expression cassette loss (Madzak *et al*., 2004).

This report describes the isolation and cloning of an EH-encoding gene and its cDNA from *Rhodotorula mucilaginosa* and the functional expression of this gene in *Y. lipolytica.*

Materials and methods

Strains and culture conditions

R. mucilaginosa (CBS 8596), *Y. lipolytica* strain Po1h (*MAT***a**, *ura3-302, xpr2-322, axp-2, XPR2*^{p}*::SUC2*; Madzak *et al*., 2004) and Yl96-Rm (Po1h transformed with pKOV96 containing the EH ORF from *R. mucilaginosa*) were maintained on YPD medium (20 g/l peptone, 20 g/l glucose, 10 g/l yeast extract and 15 g/l agar).

Nucleic acid isolation and manipulation

Standard genetic techniques were used according to Sambrook and Russell (2001). Restriction and modifying enzymes were obtained from Fermentas and oligonucleotides from Inqaba Biotechnical Industries. PCR amplification was performed using Expand High Fidelity Plus or Expand Long Template High Fidelity PCR systems (Roche Applied Sciences). PCR and gel band purification was performed using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences). All subcloning of PCR products was performed using pGEM®-T Easy Vector (Promega).

Nucleic acids were isolated from *R. mucilaginosa* (CBS 8596) following cultivation in 50 ml YPD media at 30 °C for 48 h while shaking. Cells were harvested by centrifugation and the subsequent pellet was either frozen at −70 °C for RNA isolation or suspended to a final concentration of 20% (w/v) in 50 mM phosphate buffer, pH 7.5, containing 20% (v/v) glycerol and frozen at −70 °C for DNA isolation.

DNA isolation entailed addition of 500 µl lysis solution (100 mM Tris–HCl, pH 8.0, 50 mM EDTA, pH 8.0, 1% SDS) and 200 µl glass beads (425–600 µm diameter) to 0.4 g wet cells, followed by vortexing for 4 min, cooling on ice and addition of 275 µl ammonium acetate (7 M, pH

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7.0). After incubation at 65 °C for 5 min followed by 5 min on ice, 500 µl chloroform was added, vortexed and centrifuged (20 000 × *g*, 2 min at 4 °C). The supernatant was transferred and the DNA precipitated with 1 volume isopropanol and centrifuged (20 000 × *g*, 5 min at 4 °C). The pellet was washed with 70% (v/v) ethanol, dried and re-dissolved in 100 µl TE (10 mM Tris–HCl; 1 mM EDTA, pH 8.0).

Total RNA isolation entailed grinding 10 g wet cells under liquid nitrogen to a fine powder, 0.5 ml of the powder was added to a pre-cooled 1.5 ml polypropylene tube and thawed by the addition of TRIzol® solution (Invitrogen). The isolation of total RNA using TRIzol® was performed according to the manufacturer's instructions. The total RNA isolated was suspended in 50 µl formamide and frozen at −70 °C for further use.

Cloning of the EH-encoding gene from *R. mucilaginosa*

Degenerate primers, EPH1-3F and EPH1-3R (Table 1), were used to PCR-amplify an internal region of the EH gene from *R. mucilaginosa* (initial denaturation for 2 min at 94 °C; followed by 30 cycles of 94 °C for 30 s, 45 °C for 30 s, 72 °C for 2 min, and a final elongation of 72 °C for 7 min).

Primers Rm-probe-1F and Rm-probe-1R (Table 1) were used to PCR-amplify an internal region of the degenerate product to serve as a probe (Rm-probe) following DIG-labelling (Roche Applied Science). Southern hybridization was performed on *R. mucilaginosa* DNA after digestion with various restriction enzymes.

The sequence of the internal region obtained with the degenerate primers was used for the design of primers Rm inverse-3R and Rm inverse-3F for use in the subsequent inverse PCR (Ochman *et al*., 1988). Circular template was obtained according to the following procedure: 1 µg genomic DNA was digested with *Sal*I and *Pst*I in separate reactions, purified using phenol : chloroform : isoamyl alcohol (25 : 24:1, v/v) followed by alcohol precipitation. Self-circularization of the digested DNA was obtained using 50 ng digested DNA in the presence of T4 DNA ligase in a final volume of 20 µl, as described by the manufacturer. The inverse PCR was performed using a standard reaction mixture with 2 µl ligated DNA as template with primers Rm inverse-3R and Rm inverse-3F (Table 1) on the *Sal*I- and *Pst*I-religated DNA. PCR amplicons obtained in these reactions were subjected to sequence analysis by primer walking until the full-length genomic sequence was identified. To isolate the cDNA sequence, primers were designed according to the sequence data obtained and used in a two-step RT–PCR reaction as follows: First-strand cDNA synthesis was performed on total RNA, using Expand Reverse Transcriptase (Roche Applied Science) in combination with primer Rm cDNA-2R at 42 °C for 1 h, followed by heat inactivation for 2 min at 95 °C. The newly synthesized cDNA was amplified using primers Rm cDNA-2F and Rm cDNA-1R (initial denaturation for 2 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 67 °C for 30 s, 72 °C for 2 min and a final elongation of 72 °C for 7 min). PCR products were cloned into pGEM®-T Easy Vector, followed

Table 1. Primers used in this study

Primer name	Sequence in 5′ to 3′ orientation	Restriction sites introduced/comments
TEF-1F	ATC GAT AGA GAC CGG GTT GGC GG	*Cla*I
TEF-1R	AAG CTT TTC GGG TGT GAG TTG ACA AGG	*Hin*dIII
-sigP-1F	TCG GAT CCG GTA CCT AGG GTG TCT GTG	*Bam*HI
-sigP-1R	GAG GAT CCT TCG GGT GTG AGT TGA CAA GGA G	*Bam*HI
EPH1-3F	CAY GGN TGG CCN GGN CAY TTY GGN GAR TTY	Degenerate primer
EPH1-3R	NGG YTT YTC NAR NGC NGC RAA RTG NCC NCC	Degenerate primer
Rm-probe-1F	CTT CCA CTG GGC CAC AAG CTT TTG TC	Hybridization probe primer
Rm-probe-1R	AGA TTG CGA GGA TCG TGC CGA GG	Hybridization probe primer
Rm inverse-3R	GAA GGA TCT TGA TCG CTG TAT AGG TGT GCG	Inverse PCR primer
Rm inverse-3F	CTT CCA CTG GGC CAC AAG CTT TTG TC	Inverse PCR primer
Rm cDNA-2F	AGA TCT ATG CCC GCC CGC TCG CTC	*Bgl*II
Rm cDNA-1R	TCC TAG GCT ACG ATT TTT GCT CCT GAG AGA GAG	*Avr*II

Underlined characters in the primer sequence indicate the introduced restriction sites.

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by fluorescent DNA sequencing of two independent clones for both genomic DNA and cDNA products.

Construction of a constitutive non-defective *Y. lipolytica* integrative plasmid

The *TEF* promoter was PCR amplified from the *Y. lipolytica* Po1h genome using primers TEF-1F and TEF-1R (Table 1) and cloned into pGEM®-T Easy Vector, followed by sequence verification. The hp4d promoter in pINA1313 (Table 2) was replaced with the *TEF* promoter using the *Cla*I and *Hin*dIII restriction sites, followed by the PCR removal of the *LIP2* signal peptide using primers -sigP-1F and -sigP-1R (Table 1). The purified PCR mixture was treated with *Bam*HI and *Hin*dIII (where *Hin*dIII digested the template DNA but not the PCR product) to prevent recircularization of the template DNA, thereby preventing concomitant template contamination of transformation mix upon ligation. The PCR product was allowed to circularize using T4 DNA ligase to join the compatible *Bam*HI ends, resulting in plasmid pKOV96 (Table 2, Figure 6).

The amplified EH gene and the pKOV96 plasmid were digested with the appropriate restriction enzymes to create compatible cohesive ends suitable for ligation of the EH gene into the *Bam*HI–*Avr*II cloning sites of the plasmid, resulting in plasmid pKOV96-Rm.

Transformation of *Y. lipolytica*

Y. lipolytica (Po1h) was transformed according to the method described by Xuan *et al*. (1988) to facilitate genomic integration of the *Not*I linearized pKOV96-Rm expression cassette. The transformants were plated onto selective YNB casamino acid media (YNB without amino acids and ammonium sulphate 1.7 g/l, NH_4Cl 4 g/l, glucose 20 g/l, casamino acids 2 g/l, and agar 15 g/l), supplemented with 300 mg/l leucine and incubated at 28 °C. Colonies were isolated after 2–5 days of incubation as positive transformants containing the integrated expression cassette.

Quantitative whole-cell EH activity analysis

Chiral quantitative analysis for EH activity was performed on transformants cultured in liquid YPD media for 48 h. The cells were harvested, washed with and suspended in 50 mM phosphate buffer, pH 7.5, to a final concentration of 6.67% (w/v). Biotransformation reactions were started with the addition of (2,3-epoxypropyl)benzene to a final concentration of 100 mM, and incubated in a carousel stirrer at 25 °C. Samples (300 µl) were taken at regular intervals, extracted with 500 µl ethyl acetate after saturation with NaCl, and centrifuged at 10 000 × *g* for 10 min, after which the organic layers were removed, dried using $MgSO_4$ and analysed by chiral gas chromatography (GC) as described by Lotter *et al*. (2004).

Results

Cloning of the EH-encoding gene from *R. mucilaginosa*

Degenerate primers, based on conserved consensus sequences between EHs from mammalian and fungal origin, were used to amplify a 1433 bp fragment from the genomic DNA of *R. mucilaginosa*. Sequence information obtained from this PCR product was subjected to BLAST searches (**http://www.ncbi.nlm.nih.gov/BLAST/**) and indicated homology towards other known EH-encoding genes.

By hybridizing a DIG-labelled probe, based on an internal region of the degenerate PCR product, to digested genomic DNA from *R. mucilaginosa*,

Table 2. Description of plasmids used in this study

Plasmid	Description	Source/reference
pINA1313	Single copy integrative shuttle vector containing Kan^R and *ura3d1* selective markers. Random integration into Po1h genome through the ZETA transposable element. The plasmid contains the synthetic promoter, hp4d, and the *Y. lipolytica LIP2* signal peptide	Nicaud *et al*., 2002
pKOV96	Similar to pINA1313, with hp4d replaced with TEF promoter and *Y. lipolytica LIP2* signal sequence removed	This study
pKOV96-Rm	pKOV96 harbouring the EH ORF from *R. mucilaginosa*	This study
pGEM®-T Easy	*E. coli* vector for cloning and gene maintenance	Promega

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it was possible to identify restriction profiles that could yield useful products during inverse PCR (data not shown).

The inverse PCR was performed on genomic DNA that was digested with *Sal*I, followed by ligation of the restricted DNA in an attempt to obtain regions flanking the degenerate PCR region. Sequence analysis of the inverse PCR product obtained revealed the region flanking the 3′-end of the degenerate PCR region, including the termination codon. Sequence data indicated a still incomplete region upstream of the degenerate region. Inverse PCR on *Pst*I-digested and self-ligated DNA revealed the total reading frame upstream of the degenerate PCR product, including two putative initiation codons. Combined sequence data obtained from the degenerate and inverse PCR products assembled into a genomic contig of 2347 bp (Figure 2). The contig consisted of a 1979 bp genomic EH sequence, flanked by 217 bp upstream of the translational start (ATG) and 151 bp downstream of the putative termination codon. Promoter prediction, for eukaryotic sequences, revealed no conclusive results when the 217 bp upstream region served as template.

Two putative initiation codons were observed in the same reading frame. Primers complementary to both regions were used in attempts to amplify the cDNA of the gene. Only the primer combination using the second downstream ATG produced a positive result. This was supported by prediction of the second ATG as the start of the EH gene using GENSCAN 1.0 (**http://genes.mit.edu/GENSCAN.html**).

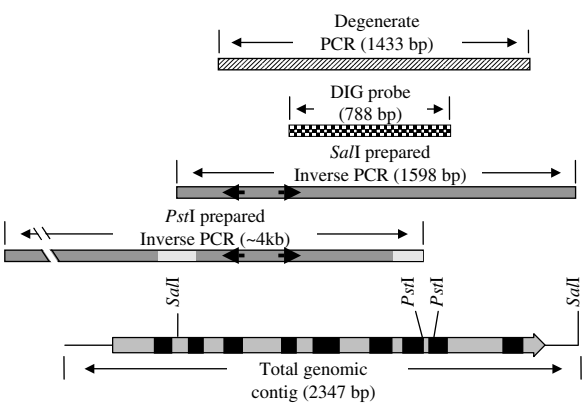

Figure 2. Graphical representation of the 2347 bp genomic contig containing the EH-encoding region. The degenerate PCR product is represented by the hatched bar, with the DIG-labelled Rm-probe represented by the checked bar. Restriction sites used during preparation of inverse PCR template are also indicated, with the arrows representing the inverse PCR primers used to obtain the flanking regions. The genomic region encoding the intron containing the EH gene is indicated by the grey arrow, with the introns (black) separating the coding regions

The cDNA sequence (GenBank Accession No. AY627310) revealed an ORF of 1185 bp encoding a predicted EH consisting of 394 amino acids, with a theoretical molecular weight (MW) of 44 kDa and an iso-electric point (pI) of 5.6. Comparison of the genomic EH-encoding sequence and the cDNA sequence revealed the presence of nine introns varying between 67 bp and 120 bp in size. All of the intron–exon boundaries conformed to the GT–AG canonical rule of intron splicing.

BLAST analysis, using the putative EH from *R. mucilaginosa* as query, revealed hits towards EH and hypothetical proteins from various sources. The best hit (93% identity) was obtained with the partial putative EH-encoding sequence from *R. mucilaginosa* that was submitted by Visser and co-workers to GenBank under Accession No. AY062024. Submission of the partial putative EH-encoding sequence (AY062024) occurred subsequent to the cDNA cloning and sequence analysis of the full EH-encoding ORF described in the present paper.

Phylogenetic analysis representing EH proteins from fungal origin (Figure 3) indicates clustering of the *R. mucilaginosa* EH with the confirmed EH from *A. niger* and the putative EHs from *Cryptococcus neoformans* and *Aspergillus nidulans*, with amino acid similarities of 51%, 51% and 48%, respectively. Multiple sequence alignments between the EHs from fungal origin indicate the presence of the conserved motifs found in the family of α/β-hydrolase fold EHs (Figure 4).

The putative members of the catalytic triad were deduced from the alignment to be Asp^{183} (nucleophile), Glu^{338} (acid) and His^{364} (base). It seems that only *A. niger* contains an Asp in place of the acidic residue of the catalytic triad, in contrast to the Glu residues found in most of the other mEHs. The two conserved tyrosine residues (Figure 4, shaded in grey) implicated in activation of the epoxide during catalysis were also present in the EH from *R. mucilaginosa*.

The consensus sequence of the nucleophile motif (sm–x–nu–x–sm–sm, where sm represents a small amino acid, nu the nucleophile and x any

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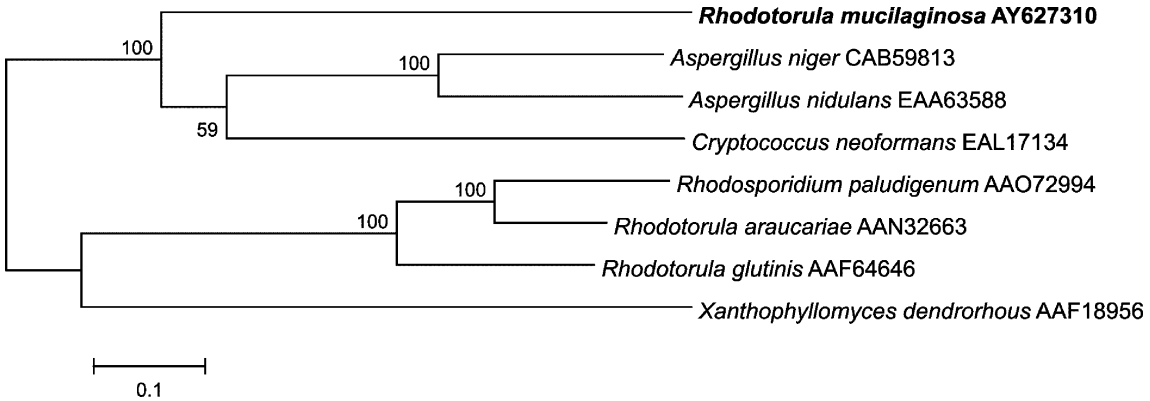

Figure 3. Phylogeny of the confirmed and putative fungal EHs which gave the highest BLAST scores when the EH from *R. mucilaginosa* (bold) was used as query. Entrez protein Accession numbers are also indicated. Sequences for phylogenetic analysis were aligned using CLUSTALX 1.83 (Thompson *et al*., 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al*., 2004), using the neighbour-joining method with the Kimura two-parameter distance measure. Confidence values were estimated from bootstrap analysis of 1000 replicates. The bar length corresponds to 10% amino acid dissimilarity

amino acid) and the HGXP motif that contains the oxyanion hole of the α/β-hydrolase fold enzymes (Ollis *et al*., 1992) could also be observed in the deduced sequence (Figure 4).

Construction of a constitutive *Y. lipolytica* integrative vector and heterologous expression of recombinant *R. mucilaginosa* EH

The mono-integrative vector, pINA1313 (Nicaud *et al*., 2002), was modified by replacing the quasi-constitutive hp4d promoter (Madzak *et al*., 2000) with the *TEF* promoter (described by Müller *et al*., 1998). This replacement still allows high level expression of the cloned EH-encoding gene (driven by the *TEF* promoter), but with less growth phase dependence, as observed with the hp4d promoter.

Previous results indicated poor secretion and different activity and selectivity profiles for various EH-encoding genes expressed by *Y. lipolytica* when targeted to the extracellular environment (Labuschagne, 2003). The poor secretion and differences between the intracellular and the extracellular fractions, as well as the absence of any extracellular secretion signals observed for known fungal EHs, prompted the removal of the *LIP2* secretion signal via PCR, resulting in the plasmid pKOV96 (Figure 5).

Initial TLC activity screening of strain Yl96-Rm (*Y. lipolytica* strain Po1h transformed with pKOV96-Rm) indicated good activity towards (2,3-epoxypropyl)benzene as substrate (data not shown). The negative control Yl96 (Po1h transformed with the empty pKOV96 plasmid) did not exhibit any hydrolytic activity towards the substrate. Almost complete hydrolysis of 50 mM (*R*)-(2,3-epoxypropyl)benzene was obtained after incubation of the racemic substrate with 6.67% (w/v) cells for 1 h (Figure 6).

The distinct preference towards hydrolysis of the (*R*)-epoxide was in correlation with previous studies on fungal EHs (Botes *et al*., 1999; Orru and Faber, 1999) and yielded (*S*)-(2,3-epoxypropyl)benzene with an enantiomeric excess of substrate (ee_s) of 97% and a yield of 55%. The activity, selectivity and yield of the recombinant organism was far better than data obtained from various wild-type yeasts studied by Lotter (2000) for the hydrolytic kinetic resolution of (2,3-epoxypropyl)benzene.

The slope from a plot of $\ln[R]_0/[R]_t$ vs. $\ln[S]_0/[S]_t$ (Moussou *et al*., 1998) describes the enantiomeric ratio (E). The E value is a parameter characterizing the enantioselectivity of the catalyst that must remain constant if the catalyst exhibits absolute regioselectivity. The kinetic data from strain Yl96-Rm yielded a non-linear slope ($R^2 = 0.7879$) for the $\ln [R]_0/[R]_t$ vs. $\ln[S]_0/[S]_t$ plot, where R and S represent the concentrations of the remaining (*R*)- and (*S*)-epoxides at different time intervals (Figure 7), indicating a low level of regioselectivity, making it impossible to determine E accurately with available data.

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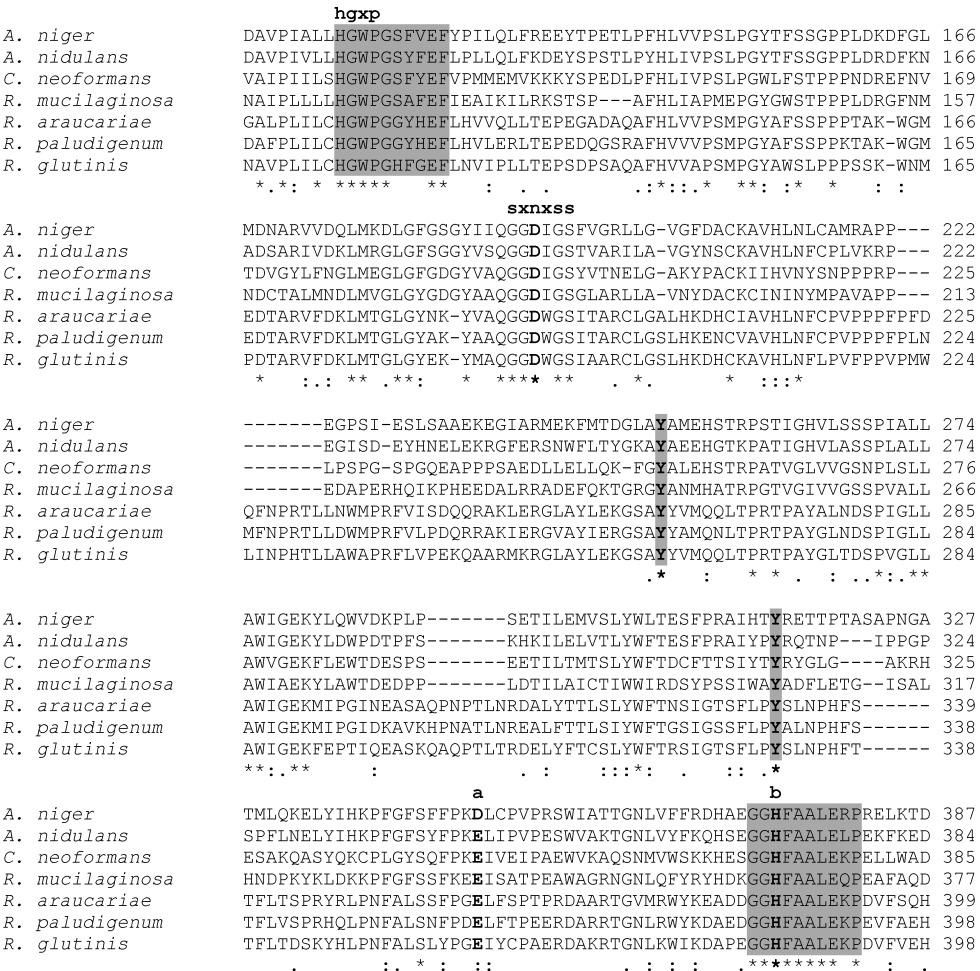

Figure 4. Protein sequence alignment (ClustalW 1.82) of cloned fungal epoxide hydrolases indicating the conserved regions. The region of the oxy-anion is indicated by hgxp, and sxnxss indicates the genetic motif found in α/β-hydrolase fold enzymes. The predicted catalytic residues are in bold type, with n, a and b representing the nucleophillic, acidic and basic members of the catalytic triad, respectively. The putative tyrosine residues responsible for epoxide polarization prior to nucleophyllic attack are in bold type and shaded in grey. Regions corresponding to the degenerate primers are boxed and shaded in grey

Discussion

The isolation of the EH-encoding gene from *R. mucilaginosa* (CBS8596) contributed to the relatively little molecular information available for EH-encoding genes from fungal origin. This gene differs from the three previously isolated EH-encoding genes from the genara *Rhodotorula* and *Rhodosporidium*, exhibiting only 32% protein homology towards these encoded proteins. The clustering of the EH from *R. mucilaginosa* with the EH from *A. niger* indicated that the *R. mucilaginosa* EH could be another member of the microsomal class of soluble EHs first described by Arand and co-workers (1999). This clustering was confirmed by studies performed by Kotik *et al*. (2005), which indicated that the EH from *R. mucilaginosa* is not membrane-associated.

The low percentage of homology between the EH from *R. mucilaginosa* and other known EHs might indicate an additional region on the edge of sequence space represented by proven EHs. Recently, Zhao and co-workers (2004) further increased the available sequence information of EHs by isolating and expressing more than 50 novel EH-encoding genes from environmental

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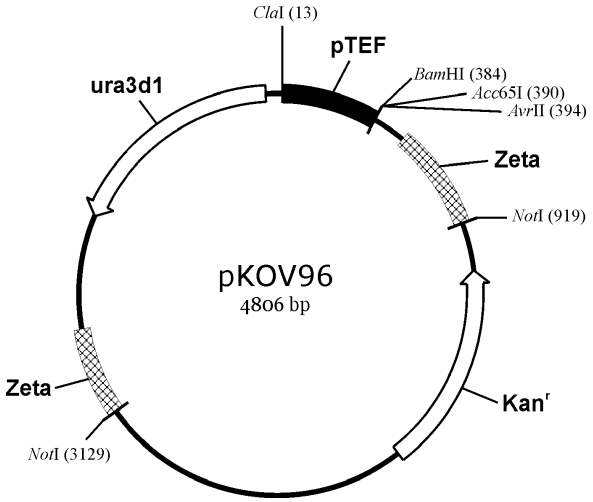

Figure 5. Graphical representation of the pKOV96 integrative vector driving constitutive intracellular expression of the *R. mucilaginosa* epoxide hydrolase gene. Following linearization using *Not*I, plasmid DNA was transformed into *Y. lipolytica* strain Po1h

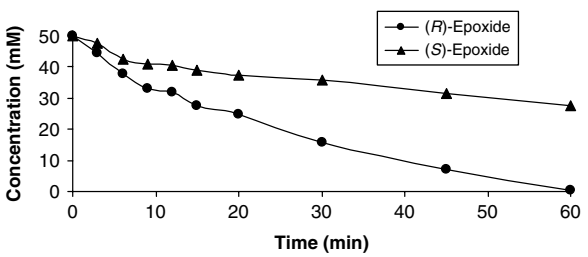

Figure 6. Enantioselective hydrolysis of (2,3-epoxypropyl)-benzene by the recombinant YI96-Rm indicating functional expression of this stereoselective recombinant enzyme in *Y. lipolytica*

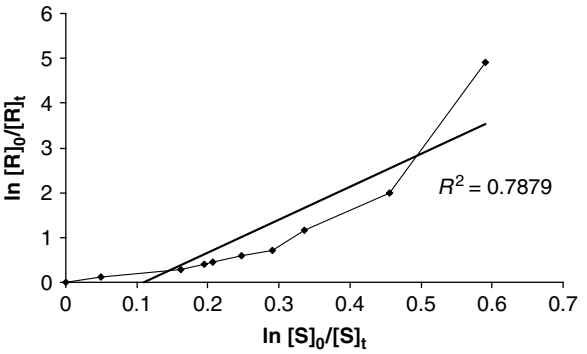

Figure 7. $\ln[R]_0/[R]_t$ vs. $\ln[S]_0/[S]_t$ plot, indicating the absence of regioselectivity for the hydrolytic kinetic resolution of (2,3-epoxypropyl)benzene during hydrolysis catalysed by YI96-Rm

libraries. Only seven of these sequences were made public and occupied sequence space regions other than what was obtained for the newly isolated EH from *R. mucilaginosa*, making the contribution of molecular data from *R. mucilaginosa* even more valuable.

The intracellular expression of the EH gene product would allow the recombinant enzyme to be associated with membranes during whole cell biotransformation. This is likely to have a more stable effect on the recombinant enzyme, since 76% of the native EH activity from *R. toruloides* was found to be membrane-associated (Botes, 1999) and it was shown that detergents forming micelles (thereby mimicking membranes) had a stabilizing effect on the purified EH from *R. glutinis* (Kronenburg and De Bont, 2001). The problem with interfacial inactivation (observed for purified or cell-free enzyme) by high concentrations of the product (Baldascini and Janssen, 2005) could also be addressed by whole cells, even though impaired movement of substrate and product could be expected over the cell membrane. Despite this limitation, whole-cell biotransformation was shown to be efficient (Botes *et al*., 1999; Lee *et al*., 2004). A low level of EH activity has been reported towards styrene oxide in another wild-type *Y. lipolytica* strain (Fantin *et al*., 2001). However, no EH activity could be detected in *Y. lipolytica* strains derived from the W29 wild-type strain, including Po1h, for the substrate evaluated in the present study. Furthermore, no activity could be detected when all three of the putative EHs present in the genome of *Y. lipolytica* were overexpressed in *Y. lipolytica* using a multicopy system (AL Botes, personal communication). This makes whole-cell biotransformations with *Y. lipolytica* strains, expressing other EHs, feasible and potentially useful.

Functional expression of the gene in *Y. lipolytica* yielded recombinant protein exhibiting unsurpassed selectivity and yield towards the hydrolytic kinetic resolution of (2,3-epoxypropyl)benzene. *Y. lipolytica* thus seems to be an attractive host for the production of enantioselective EHs. *R. mucilaginosa* (CBS 8596) was previously evaluated for the hydrolytic kinetic resolution of (2,3-epoxypropyl)benzene, but was omitted

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Table 3. Comparison of data on hydrolytic kinetic resolution of (2,3-epoxypropyl)benzene by different yeast biocatalysts

	(*S*)-Epoxide		
Yeast strain	**ee_s (%)**	**Yield (%)[a]**	**Reference**
YI96-Rm	97	55	This study
Thrichosporon mucoides (UOFS Y-2041)	49	38	Lotter, 2000
Thrichosporon sp. (UOFS Y-0861)	53	37	Lotter, 2000
Rhodotorula sp. (UOFS Y-2043)	62	36	Lotter, 2000
Rhodotorula sp. (UOFS Y-0448)	65	33	Lotter, 2000

[a] Yields were calculated as the percentage of remaining (*S*)-epoxide after the specified ee_s has been reached.

from further study since no promising results could be obtained for the wild-type organism towards this substrate (see Table 3; Lotter, 2000). Isolation of the EH-encoding gene from this strain followed by recombinant expression of the gene in *Y. lipolytica* allowed the best-ever recorded hydrolytic resolution of (2,3-epoxypropyl)benzene, emphasizing the fact that a good catalyst can be masked by low levels of wild-type activity during initial screening.

However, due to the lack of published data on the native *R. mucilaginosa* EH, no direct comparison could be made between the properties of the native and recombinant enzymes. With the data presented here, *Y. lipolytica* becomes a strong candidate for the recombinant production of enantioselective EHs, thereby joining the ranks of *E. coli* (Visser *et al*., 2003), *P. pastoris* (Lee *et al*., 2004) and *A. niger* (Monfort *et al*., 2004) as microbial producers of enantioselective recombinant EHs for hydrolytic kinetic resolution of epoxides to serve as chiral precursors in the fine chemicals industries.

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