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Interspecies differences in the enantioselectivity of epoxide hydrolases in *Cryptococcus laurentii* (Kufferath) C.E. Skinner and *Cryptococcus podzolicus* (Bab'jeva & Reshetova) Golubev

Adriana L. Botes[a,b], Jeanette Lotter[a,b], Owen H.J. Rhode[c], Alfred Botha[c,*]

[a]*CSIR Bio/Chemtek, Modderfontein, South Africa*
[b]*Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein, South Africa*
[c]*Department of Microbiology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa*

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Abstract

Isolates representing *Cryptococcus laurentii* and *Cryptococcus podzolicus*, originating from soil of a heathland indigenous to South Africa, were screened for the presence of enantioselective epoxide hydrolases for 2,2-disubstituted epoxides. Epoxide hydrolase activity for the 2,2-disubstituted epoxide (±)-2-methyl-2-pentyl oxirane was found to be abundantly present in all isolates. The stereochemistry of the products formed by the epoxide hydrolase enzymes from isolates belonging to the two species (11 isolates representing *C. laurentii* and 23 isolates representing *C. podzolicus*) was investigated. The enantiopreferences of the epoxide hydrolases for 2,2-disubstituted epoxides of these two species were found to be opposite. All strains of *C. laurentii* preferentially hydrolysed the (*S*)-epoxides while all *C. podzolicus* isolates preferentially hydrolysed the (*R*)-epoxides of (±)-2,2-disubstituted epoxides. These findings indicate that the stereochemistry of the products formed from 2,2-disubstituted epoxides by the epoxide hydrolase enzymes of these yeasts should be evaluated as additional taxonomic criterion within the genus *Cryptococcus*. Also, the selectivity of some epoxide hydrolases originating from isolates of *C. podzolicus* was high enough to be considered for application in biotransformations for the synthesis of enantiopure epoxides and vicinal diols.

Keywords: *Cryptococcus laurentii*; *Cryptococcus podzolicus*; Epoxides; Epoxide hydrolases; Soil

Introduction

It is well known that the *Cryptococcus laurentii* (Kufferath) C.E. Skinner and *Cryptococcus podzolicus* (Bab'jeva & Reshetova) Golubev are autochthonous soil yeasts since they are frequently isolated from this habitat [6]. However, very little is known about the niches these two basidiomycetous yeasts occupy in soil. Their nutritional abilities, which may be an indication of their niches [13], were recorded and it was found that both yeast species are characterized by a poor ability to utilize monomeric aromatic compounds as sole carbon sources [19]. This contrasts with the fact that both species, but especially *C. podzolicus,* have been isolated from podzolic soils [13], a soil-type characterized by the presence of monomeric aromatic acids [16]. Evidently, the ability to utilize these aromatic acids is not characteristic of these species, despite the occurrence of monomeric aromatic acids in soil, their natural

*Corresponding author. Fax: +27 21 808 5846.
E-mail address: abo@sun.ac.za (A. Botha).

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habitat. However, except for these aromatic acids, carbohydrates and short-chain organic acids may also find their way into soil as components of root exudates [11]. Both yeasts species are able to assimilate a wide range of carbohydrates including pentoses and hexoses, as well as organic acids [6], which may therefore enable them to grow in the rhizosphere of a number of plant species. Interestingly, evidence does exist for the presence of *C. laurentii* in the rhizosphere [4].

Another class of compounds that are produced by plant roots is epoxides. The biochemistry of these biologically active compounds was studied extensively in plants [10,14,15] and it is known that the ability to hydrolyse epoxides does occur among basidiomycetous yeasts [2,3,23]. Strains representing the genera *Rhodosporidium*, *Rhodotorula* and *Trichosporon* were found to enantioselectively hydrolyse monosubstituted 1,2-epoxides. While *gem*-2,2-dialkylsubstituted epoxides were hydrolysed by these epoxide hydrolases, chiral recognition was destroyed by a directing group on C-2 of the epoxide. The latter 2,2-disubstituted epoxides is a class of bulky epoxides, previously found to be selectively hydrolysed only by bacterial epoxide hydrolases [17].

Since we are interested to elucidate the niches of *C. laurentii* and *C. podzolicus* in soil the focus of this study was on the interactions of these cryptococci with epoxides. Not only will such information provide an indication of the ability of these yeasts to catabolize epoxides in their natural habitat, but it is also relevant in biotechnology where novel microbial enzymes for the preparation of chiral epoxides and vicinal diols, are continually being screened for [2,17,23]. Consequently, we decided to screen for epoxide hydrolase activity for the 2,2-disubstituted epoxides (±)-2-methyl-2-pentyl oxirane and (±)-2-methyl-2-phenyl-1,2-epoxypropane, among *C. laurentii* and *C. podzolicus*, isolates obtained from soil.

Materials and methods

Isolation of yeasts

Podzols, the natural habitat of *C. podzolicus* [6] are often covered with heathlands [16]. One such vegetation type, in which soil *C. laurentii* has also been found [4], is fynbos, a unique collection of indigenous sclerophyllous evergreen shrubs, adapted for the Mediterranean climate of the southern tip of Africa [12]. This heathland consists of a huge diversity of plant species, many of which contain substantial amounts of oils, waxes and terpenes [22], making it an ideal habitat to search for novel microbial enzymes associated with this habitat.

The fynbos soil from which the isolates originated was classified as sandy loam soil of the Oakleaf form derived from a mixture of granite and quartzite [7,20]. Surface litter was removed to reduce contamination and soil samples to a depth of 5 cm were used to prepare soil dilution plates with a defined isolation medium containing a minimal concentration of nitrogen (Table 1). After 7 days of incubation at 25 °C, yeast colonies were isolated. Successive inoculation and incubation on yeast malt extract (YM) agar [21,24] at 25 °C were used to purify the isolates. Yeast cultures were subsequently maintained on YM slants at 25 °C.

Table 1. The composition of the isolation medium used to obtain the yeast isolates

Components per liter of distilled water.	
Carbon source	
Glucose (g)	5.00
Mineral salts	
$CaCl_2$ (g)	0.10
KH_2PO_4 (g)	1.00
$MgSO_4 \cdot 7H_2O$ (g)	0.50
NaCl (g)	0.10
Trace elements	
$AlK(SO_4)_2 \cdot 12H_2O$ (μg)	10
$CuSO_4 \cdot 5H_2O$ (μg)	40
$CoSO_4$ (μg)	100
$FeCl_3 \cdot 6H_2O$ (μg)	200
H_3BO_3 (μg)	500
KI (μg)	100
$MnSO_4 \cdot H_2O$ (μg)	40
$Na_2MoO_4 \cdot H_2O$ (μg)	200
$ZnSO_4 \cdot 7H_2O$ (μg)	400
Vitamins	
Biotin (μg)	1
Calcium pantothenate (μg)	200
Folic acid (μg)	1
Inositol (μg)	1000
p-Aminobenzoic acid (μg)	100
Pyridoxine hydrochloride (μg)	200
Riboflavin (μg)	100
Thiamine (μg)	500
Anti-bacterial agent	
Chloramphenicol (g)	0.20
Solidifying agent	
Agar	10.00
Final pH	5.2

Identification of yeast isolates

Preliminary identification was achieved by using morphological and physiological characteristics as determined by the methods by Van der walt and Yarrow [21] and Barnett [1]. The basidiomycetous nature of the isolates was confirmed by performing the Diazonium Blue B (DBB) colour test. Carbon source utilization was tested at 25 °C on a Tissue Culture Rollordrum rotating at 40 rph. Nitrogen source utilization was examined by the auxanographic method.

The identity of the yeast isolates, as determined using physiological and morphological characteristics was confirmed by sequence analyses of the D1/D2 region of the large subunit rDNA [5]. Yeast strains were grown for 24 h in YPD broth (2% glucose, 2% peptone, 1% yeast-extract). Genomic DNA was extracted according to the method of Hoffman and Winston [8]. Using the polymerase chain reaction (PCR), the DNA was amplified with universal fungal primers ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and LR6 (5′-CGC CAG TTC TGC TTA CC-3′) in a Perkin-Elmer thermal cycler [5]. The PCR products were purified with Nucleospin® (Seperations) chromatography columns.

The D1/D2 600–650 bp region of the large subunit of ribosomal DNA (rDNA) was subsequently amplified by subjecting the above mentioned PCR products to another round of cycle sequencing [5]. The forward primer F63 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and reverse primer LR3 (5′-GGT CCG TGT TTC AAG ACG G-3′) were used in the reactions. Sequences representing the D1/D2 region of the rDNA from the strains were obtained using an ABI PRISM model 3100 genetic sequencer. The forward and reverse sequences were aligned with DNAMAN for WINDOWS Version 4.13 (Lynnon Biosoft). The yeast strains were identified by comparing the sequencing results with known sequences using the BLAST program (www.ncbi.nlm.nih.gov./blast).

Depositing of isolates and origin of reference strains

The isolates (Table 2) were deposited in the Yeast Culture Collection of the University of the Free State (UFS) in Bloemfontein (South Africa). The reference strains studied were obtained from the Centraalbureau voor Schimmelcultures (CBS) in Utrecht (The Netherlands) and from the Yeast Culture Collection of the UFS.

Syntheses of epoxide reference compounds

The epoxides (±)-2-methyl-2-pentyl oxirane and (±)-2-methyl-2-phenyl-1,2-epoxypropane were synthesized as previously described [2]. The corresponding diols were synthesized by acid catalyzed hydrolysis of the corresponding epoxide in THF-H_2O. The crude products were purified by silica-gel chromatography ($CHCl_3$/ETOAc 1:1). Absolute configurations of the remaining epoxide and formed diols of the biotransformation reactions were determined by analogy of the established elution order of 2,2-disubstituted epoxides and their corresponding diols on cyclodextrin columns (see Fig. 1).

Preparation yeast cells to be screened for epoxide hydrolase activity

Yeasts were grown at 30 °C in 1 L shake-flask cultures containing 200 ml YM medium supplemented with 1% glucose (w/v). At late stationary phase (48–72 h) the cells were harvested by centrifugation (10 000*g*, 10 min, 4 °C), washed with phosphate buffer (50 mM, pH 7.5), centrifuged and frozen in phosphate buffer containing glycerol (20%) at −20 °C as 20% (w/v) cell suspensions. The cells were stored for several months without significant loss of activity.

Screening for epoxide hydrolase activity

Epoxide (10 µl of a 1 M stock solution in EtOH) was added to a final concentration of 20 mM to 500 µl cell suspension (20% w/v) in phosphate buffer (50 mM, pH 7.5). The reaction mixtures were incubated at 30 °C for 2 h (2-methyl-2-pentyl oxirane) or 5 h (2-methyl-2-phenyl-1,2-epoxypropane). The reaction mixtures were extracted with EtOAc (300 µl) and centrifuged. Diol formation was evaluated by TLC (silica gel Merck 60 F_{254}). Compounds were visualized by spraying with vanillin/concentrated H_2SO_4 (5 g/l). Reaction mixtures that showed substantial diol formation were evaluated for asymmetric hydrolysis of the epoxide by chiral GLC analysis. Some reactions were repeated over longer or shorter times and with more dilute cell suspensions (10% w/v) in order to analyse the reactions at suitable conversions.

Kinetic resolution of 2-methyl-2-pentyl oxirane (±)1a and 2-methyl-2-phenyl-1,2-epoxypropane (±)2a

Frozen cells were thawed, washed with phosphate buffer (50 mM, pH 7.5) and resuspended in buffer. Cell suspensions (10 ml, 20 or 50% w/v) were placed in 20 ml glass bottles with screw caps fitted with septa. The substrate (100 or 250 µl of a 2 M stock solution in ethanol) was added to final concentrations of 20 mM or 50 mM. The mixtures were agitated on a shaking water bath at 30 °C. The course of the bioconversions of epoxides was followed by withdrawing samples (500 µl)

Table 2. Enantioselectivities and absolute configuration of products formed from 2,2-disubstituted epoxides (±)1a and (±)2a by *C. laurentii* and *C. podzolicus*

		Epoxide (±)1a					Epoxide (±)2a			
Cryptococcus strains	Culture collection no	Conv[a] (%)	ee_s[b] (%)	ee_p[c] (%)	Abs. conf. of products 1a	1b	ee_s[b] (%)	ee_p[c] (%)	Abs. conf. of products 2a	2b
C. laurentii[d] *var. laurentii* CBS 2174	UOFS Y-1349	1.2	0.5	50.6	*R*	*S*	1.3	28.4	*R*	*S*
C. laurentii[d] TVN 329	UOFS Y-0135	2.6	0.8	65.3	*R*	*S*	5.5	23.7	*R*	*S*
C. laurentii	UOFS Y-0509	34.9	22.0	37.3	*R*	*S*				
C. laurentii	UOFS Y-0514	56.8	38.3	33.7	*R*	*S*	7.6	20.1	*R*	*S*
C. laurentii	UOFS Y-1880	71.6	48.0	26.4	*R*	*S*	7.4	15.5	*R*	*S*
C. laurentii	UOFS Y-1884	62.1	43.3	30.3	*R*	*S*	7.3	14.7	*R*	*S*
C. laurentii	UOFS Y-1885	69.0	51.9	27.2	*R*	*S*	10.0	15.2	*R*	*S*
C. laurentii	UOFS Y-1886	42.7	26.4	37.6	*R*	*S*	8.2	17.5	*R*	*S*
C. laurentii	UOFS Y-1887	59.7	41.9	30.9	*R*	*S*	5.0	15.9	*R*	*S*
C. laurentii	UOFS Y-1888	53.6	36.9	33.9	*R*	*S*	6.9	16.0	*R*	*S*
C. laurentii	UOFS Y-1892	44.8	35.2	46.4	*R*	*S*				
C. podzolicus	UOFS Y-1889	34.7	49.7	93.7	*S*	*R*				
C. podzolicus	UOFS Y-1897	38.9	57.3	94.0	*S*	*R*	4.5	65.6	*S*	*R*
C. podzolicus	UOFS Y-1904	43.2	70.9	93.9	*S*	*R*				
C. podzolicus	UOFS Y-1890	42.6	66.7	90.1	*S*	*R*				
C. podzolicus	UOFS Y-1893	42.2	58.4	93.7	*S*	*R*				
C. podzolicus	UOFS Y-1896	52.0	70.2	89.7	*S*	*R*				
C. podzolicus	UOFS Y-1912	50.4	70.7	92.4	*S*	*R*				
C. podzolicus	UOFS Y-1881	27.7	33.4	93.9	*S*	*R*				
C. podzolicus	UOFS Y-1899	25.3	27.3	93.5	*S*	*R*				
C. podzolicus	UOFS Y-1902	14.9	2.2	23.1	*S*	*R*	6.5	0.8	*S*	*R*
C. podzolicus	UOFS Y-1906	42.6	45.9	92.6	*S*	***R***				
C. podzolicus	UOFS Y-1907	27.8	22.5	90.4	*S*	*R*	10.1	94.1	*S*	*R*
C. podzolicus	UOFS Y-1908	23.2	19.5	89.9	*S*	*R*				
C. podzolicus	UOFS Y-1910	36.8	39.5	86.1	*S*	*R*				
C. podzolicus	UOFS Y-1882	43.0	52.2	94.3	*S*	*R*				
C. podzolicus	UOFS Y-1883	50.0	85.5	92.7	*S*	*R*				
C. podzolicus	UOFS Y-1895	39.3	41.4	94.4	*S*	*R*				
C. podzolicus	UOFS Y-1898	10.1	6.7	84.4	*S*	*R*				
C. podzolicus	UOFS Y-1900	19.3	2.8	20.8	*S*	***R***	6.8	2.8	*S*	*R*
C. podzolicus	UOFS Y-1911	55.2	17.2	22.6	*S*	*R*				
C. podzolicus	UOFS Y-1913	42.7	36.5	92.4	S	*R*	11.1	92.1	*S*	*R*
C. podzolicus	UOFS Y-1914	46.6	57.6	92.8	*S*	*R*	12.8	97.0	*S*	*R*
C. podzolicus	UOFS Y-1894	38.7	45.4	94.4	*S*	*R*	4.7	88.0	*S*	*R*

[a]Conv (%), percentage conversion of substrate.
[b]ee_s, enantiomeric excess of remaining epoxide.
[c]ee_p, enantiomeric excess of formed diol.
[d]Reference strains of Cryptococcus laurentii from CBS and UFS culture collections.

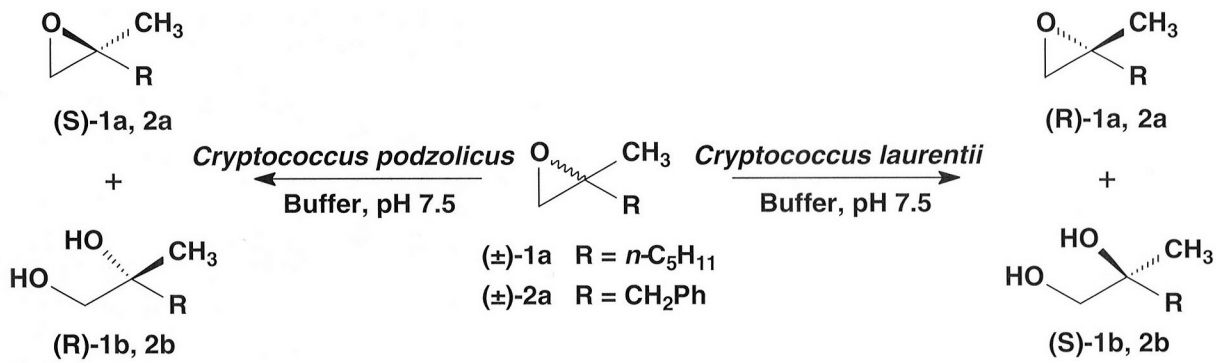

Fig. 1. Absolute configuration of the remaining epoxide and formed diol during epoxide hydrolase catalysed hydrolysis of 2,2-disubstituted epoxides by all strains of *C. podzolicus* and *C. laurentii* used in this study.

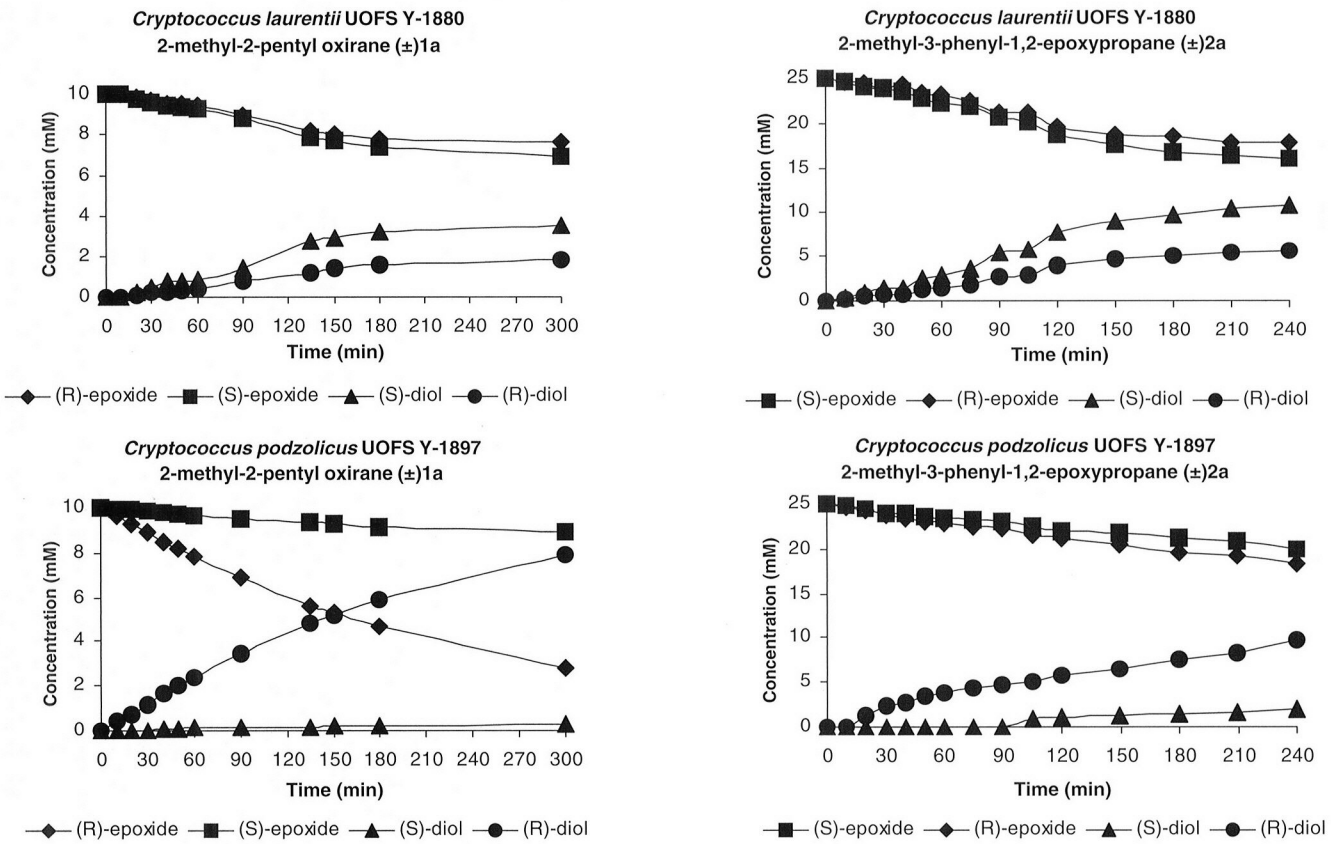

Fig. 2. Opposite enantiopreferences for 2,2-disubstituted epoxides displayed by *C. laurentii* and *C. podzolicus* strains.

at appropriate time intervals. Samples were extracted with 300 µl EtOAc. After centrifugation (3000*g*, 2 min), the organic layer was dried over anhydrous $MgSO_4$ and the products analyzed by chiral GLC.

Chiral analysis of products formed during biotransformation reactions

Gas chromatography (GLC) was performed on a Hewlett-Packard 6890 gas chromatograph equipped with FID detector and using H_2 as carrier gas. Determination of the enantiomeric excesses of products was performed by GLC using a fused silica β-DEX 225 (for 2-methyl-2-pentyl oxirane) and β-DEX 110 (for 2-methyl-2-phenyl-1,2-epoxypropane) cyclodextrin capillary column (Supelco) (30 m length, 25 mm ID and 25 µm film thickness). For chiral analysis of 2-methyl-2-pentyl oxirane, the initial temperature of 75 °C was maintained for 5.6 min, increased at a rate of 8 °C per minute to 115 °C, and maintained at this temperature for 8.5 min. The retention times (min) were as

follows: R_t (*R*)-epoxide = 5.3, R_t (*S*)-epoxide = 5.6, R_t (*S*)-diol = 17.9, R_t (*R*)-diol = 18.4. For chiral analysis of 2-methyl-2-phenyl-1,2-epoxypropane, the initial temperature of 80 °C was maintained for 22 min, increased at a rate of 4 °C per minute to 160 °C, and maintained at this temperature for 1 min. The retention times (min) were as follows: R_t (*S*)-epoxide = 31.9, R_t (*R*)-epoxide = 32.1, R_t (*S*)-diol = 47.7, R_t (*R*)-diol = 48.0. Concentrations of epoxides and diols were derived from calibration curves obtained from extractions of the epoxide and diol from buffer without cells.

Results

Using the keys and descriptions of Barnett [1], as well as of Van der walt and Yarrow [21], the isolates (Table 2) were identified using morphological and physiological characteristics as either belonging to the species *C. laurentii* (Kufferath) C.E. Skinner or *C. podzolicus* (Bab'jeva & Reshetova). The identity of the yeast isolates was subsequently confirmed by sequence analyses of the D1/D2 region of the large subunit rDNA [5].

All strains of *C. laurentii* and *C. podzolicus* isolated from the fynbos soil displayed epoxide hydrolase activity for 2-methyl-2-pentyl oxirane [Table 2, (±)1a] and were evaluated for enantioselectivity by chiral GC analysis. Reaction mixtures of those strains that displayed high activities for 2-methyl-2-phenyl-1,2-epoxypropane [Table 2, (±)2a] as judged by TLC were also analysed by chiral GC (Table 2). All the tested *C. laurentii* strains preferentially hydrolysed the (*S*)-enantiomer of (±)1a and (±)2a, while all *C. podzolicus* strains preferentially hydrolysed the (*R*)-enantiomer of (±)1a and (±)2a (Table 2). Typical time-course reactions of the hydrolysis of two selected 2,2-disubstituted epoxides (±)1a and (±)2a by representative strains from the two species, *C. podzolicus* UOFS Y-1897 and *C. laurentii* UOFS Y-1880, illustrate the opposite enantioselectivities observed for all strains of these two species (Fig. 2).

C. podzolicus strains in general displayed higher enantioselectivity for 2-methyl-2-pentyl oxirane [Fig. 2, (±)1a] than *C. laurentii* strains. In cases where *C. podzolicus* strains displayed low enantioselectivity for 2-methyl-2-pentyl oxirane, the (*R*)-diol was still obtained in high enantiomeric excess (Fig. 3). Nonetheless, opposite absolute configurations of the epoxides and/or diols formed from 2,2-disubstituted epoxides were consistently observed for the two different species.

Discussion

Despite occurring in the same habitat and showing similar physiology by being able to grow on the selective

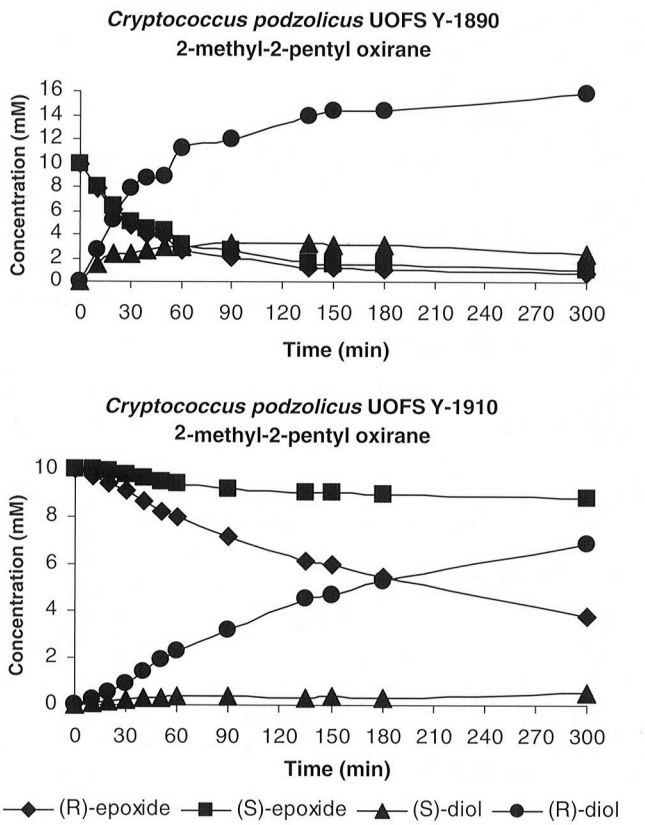

Fig. 3. Hydrolysis of 2-methyl-2-pentyl oxirane (±)1a by *C. podzolicus* strains gives access to the (*R*)-diol in high enantiomeric excess, even in cases where the enantioselectivity for the two antipodes of the epoxide is low. *C. podzolicus* UOFS Y-1890 and UOFS Y-1910 attack the two antipodes of the epoxide with opposite regioselectivites, leading to enantioconvergent diol formation.

medium and having similar nutritional requirements [6], *C. laurentii* and *C. podzolicus* differ notably regarding enantioselectivity of their epoxide hydrolases for 2,2-disubstituted epoxides. The stereochemistry of the products formed from 2,2-disubstituted epoxides by the epoxide hydrolase enzymes of these yeasts should therefore be evaluated as an additional taxonomic criterion within the genus *Cryptococcus*. The role of these and other hydrolases in the interactions of these two yeast species with other soil organisms should be investigated, especially since it is known that filamentous soil fungi, such as *Acremonium*, *Fusarium* and *Penicillium*, of which some are known endophytes, are able to produce biologically active epoxide containing alkaloids [9,18].

Although all the isolates showed asymmetric hydrolysis of the epoxides they were challenged with, intraspecific differences were observed in enantioselectivity (Table 2). Nevertheless, the selectivity of some epoxide hydrolases originating from isolates such as *C. podzolicus* UOFS Y-1883 and UOFS Y-1894 were comparable to that of bacterial epoxide hydrolases,

but with opposite enantiopreference [17]. These findings pave the way for further screening of cryptococci occurring in fynbos for the presence of enantioselective epoxide hydrolases with applications in biocatalysis.

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