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SPECIAL ISSUE ARTICLE

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Raw and waste plant materials as sources of fungi with epoxide hydrolase activity. Application to the kinetic resolution of aryl and alkyl glycidyl ethers

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ABSTRACT

The by-products of olive oil production can be used as sources of microbial strains. *Penicillium* sp., *Aspergillus terreus*, *Penicillium aurantiogriseum*, *Aspergillus tubingensis* and *Aspergillus niger* were selected on the basis of their epoxide-hydrolyzing activity towards racemic *rac*-glycidyl phenyl ether. We studied the effect on enzymatic activity of adding styrene oxide to the growth medium. It induced the biosynthesis of epoxide hydrolases and reduced cell growth. The resolution capacity of the five fungi was tested on *rac*-glycidyl phenyl ether, *rac*-benzyl glycidyl ether, *rac*-1,2-epoxyhexane and *rac*-1,2-epoxyoctane. The resolution of *rac*-glycidyl phenyl ether by *A. niger*, *rac*-benzyl glycidyl ether by *P. aurantiogriseum* and *A. terreus*, *rac*-1,2-epoxyhexane by *A. tubingensis* and *rac*-1,2-epoxyoctane by *A. terreus* provided (*S*)-3-phenoxy-1,2-propanediol (45.1% yield, 51.4% *ee*), (*R*)-3-benzyloxy-1,2-propanediol (40.8% yield, 43.3% *ee*), (*S*)-3-benzyloxy-1,2-propanediol (45.4% yield, 45.6% *ee*), (*R*)-1,2-hexanediol (70.4% yield, 24.4% *ee*) and (*R*)-1,2-octanediol (21.4% yield, 27.5% *ee*), respectively. The (*R*)-enantiopreference of the epoxide hydrolases from *P. aurantiogriseum* is unprecedented.

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Introduction

Chiral 1,2-diols are crucial building blocks for various synthetic applications. Therefore, the development of efficient and cost effective processes for the preparation of these chiral synthons in enantiopure form is of great interest (Archelas and Furstoss 2001). Chiral 1,2-diols can be prepared by several chemical procedures (most of which are based on the use of organometallic catalysis); however, these processes have several limitations, including low substrate-to-catalyst ratios, low efficiency and waste generation (Genzel et al. 2001). In this regard, increased efforts have been devoted to the search for mild and cleaner processes such as the biocatalytic hydrolytic kinetic resolution of racemic epoxides by epoxide hydrolases (EC 3.3.2.3). These enzymes offer several advantages since they are co-factor independent and can specifically hydrolyze one enantiomer of a racemic mixture, thus leading to the formation of an enantiopure diol and leaving the unreacted enantiomer unchanged in the medium (Beloti et al. 2013).

Epoxide hydrolases have been isolated from a wide range of organisms including bacteria, yeasts, moulds, plants, insects and mammals (Arand et al. 1994; Blée and Schuber 1995; Mischitz et al. 1995; Osprian et al. 1997; Beloti et al. 2013; Stephani de Oliveira et al. 2016). The discovery of new fungal strains with enantioselective epoxide hydrolases with broad substrate selectivity and high activity has attracted great interest because of their easy manipulation and numerous potential applications in the asymmetric hydrolysis of epoxides on a preparative scale (Monterde et al. 2004). Microorganisms able to produce epoxide hydrolases can be found in several habitats, including marine environments, polluted soil, biofilters and petroleum-polluted bioremediation sites (Pan and Xu 2003; Kotik et al. 2005; Martins et al. 2011; Bala et al. 2012).

The olive oil industry generates large amounts of waste containing a wide variety of microorganisms and compounds. The former can be isolated and evaluated for their potential as enzyme producers. Filamentous fungi such as *Aspergillus* and *Penicillium* spp. are commonly found in olive oil mill waste (Ntougias et al. 2013). The production of olive oil generates solid and aqueous wastes. The solid form (olive oil cake) is a combination of olive pulp and stones,

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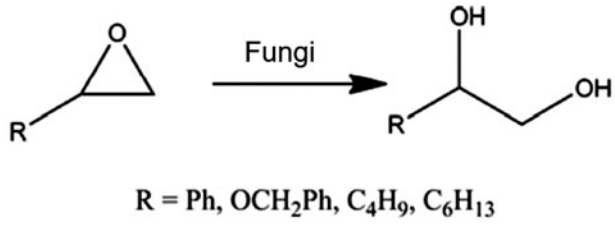

Figure 1. Hydrolysis of aryl and alkyl glycidyl ethers by epoxide hydrolases.

while aqueous liquor (olive oil waste water) comes from the vegetation water and the soft tissue of the olives plus the water added during processing (Dermeche et al. 2013).

Although several reports describe the production of various hydrolytic enzymes using olive oil by-products as substrates (Ramachandran et al. 2007), to the best of our knowledge, this is the first dedicated to epoxide hydrolase-producing fungi isolated from olive oil waste water and cake.

Our main objective was to use these wastes to isolate fungi with epoxide hydrolase activity. These epoxyhydrolases were used to hydrolyse two aromatic epoxides (*rac*-glycidyl phenyl ether and *rac*-benzyl glycidyl ether) and two aliphatic epoxides (*rac*-1,2-epoxyhexane and *rac*-1,2-epoxyoctane) (Figure 1). The effect on enzymatic activity of adding styrene oxide to the growth medium was also studied.

Materials and methods

Chemicals and reagents

Racemic epoxides (glycidyl phenyl ether; benzyl glycidyl ether, 1,2-epoxyhexane and 1,2-epoxyoctane), racemic diols (3-phenoxy-1,2-propanediol, 3-benzyloxy-1,2-propanediol, 1,2-hexanediol and 1,2-octanediol), adrenaline, sodium periodate, iodine, acetic acid and sodium sulphate were obtained from Sigma-Aldrich (Madrid, Spain). Acetonitrile, hexane, 2-propanol and ethyl acetate were supplied by Thermo Fisher (Madrid, Spain). Microbial media and buffer components were from Thermo Fisher and Panreac (Barcelona, Spain).

Isolation of microorganisms

Waste water and cake from an olive oil mill located in Lleida, Spain, were used to isolate fungi. Ten grams of olive oil cake was aseptically transferred to a bottle containing 90 mL of sterile distilled water with Tween 80 (0.02%, v/v), homogenously shaken and allowed to settle for 10 min. This procedure gave a 10^{-1} dilution. Aliquots (100 µL) of diluted olive oil cake was spread evenly, by means of a sterile Drigalski spatula, on a solid medium prepared with 5 g of mycological peptone, 3 g of yeast extract and 15 g of agar per liter of distilled water, and adjusted to pH 5 with NaOH (1 M). Olive oil waste water was directly spread on agar plates containing the same medium. Thereafter, the plates were incubated at 25 °C for 5–7 d until the appearance of fungal colonies. The isolates were further sub-cultured on a solid medium containing 15 g of corn steep liquor, 10 g of fructose, 1 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4{\cdot}7H_2O$, 0.01 g of $FeSO_4{\cdot}7H_2O$ and 15 g of agar per litre of distilled water, and adjusted to pH 5 with NaOH (1 M). The plates were incubated at 25 °C for 5–7 d and stored at 4 °C.

Culture conditions

The isolated fungi were grown in cotton-plugged Erlenmeyer flasks containing 100 mL of a liquid medium containing 15 g of corn steep liquor, 10 g of fructose, 1 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4{\cdot}7H_2O$ and 0.01 g of $FeSO_4{\cdot}7H_2O$ per litre of distilled water, and adjusted to pH 5 with NaOH (1 M) and autoclaved at 121 °C for 15 min. Two fungal plugs of approximately 0.5 cm in diameter were transferred aseptically from the plates to each Erlenmeyer flask and incubated at 27 °C in an orbital shaker at 100 rpm for 8 d. Fungal mycelia were harvested by vacuum filtration and washed twice with phosphate buffered saline (PBS) and twice with distilled water. After freeze-drying for 24 h, the lyophilized mycelia were ground and stored at –20 °C.

Screening for hydrolytic capacity using rac-glycidyl phenyl ether

The enzymatic hydrolyses of *rac*-glycidyl phenyl ether were performed in 2 mL low protein-binding Eppendorf tubes containing 10 mg of lyophilized mycelium pre-incubated at 30 °C for 5 min in 980 µL of PBS (pH 7.4) and 20 µL of a 5 mg/mL solution of racemic epoxide in dimethyl sulphoxide (DMSO). The reactions were carried out in a thermo-mixer (Eppendorf, Madrid, Spain) at 30 °C and 1400 rpm for 1 h. After the extraction of the residual epoxide with hexane (2 × 1 mL), the diol was extracted with ethyl acetate (2 × 1 mL). Reaction mixtures were vortexed for 15 s and centrifuged at 9000 rpm for 5 min at 4 °C between extractions. Ethyl acetate extracts were evaporated under a stream of nitrogen and dry residues were dissolved in 1 mL of Milli-Q water/acetonitrile (ACN) (30:10, v/v). All experiments were performed in duplicate.

A stock solution of *rac*-3-phenoxy-1,2-propanediol was prepared in Milli-Q water/ACN (30:10, v/v) at a concentration of 1 mg/mL. Calibration standards ranging from 0.001 to 0.1 mg/mL were obtained from the stock solution by serial dilution with Milli-Q water/ACN (30:10, v/v). The content of 3-phenoxy-1,2-propanediol was quantified by HPLC-UV.

Effect of styrene oxide on biomass production and epoxide hydrolase activity

To evaluate the effect of styrene oxide on biomass production (g of lyophilized fungal mycelium/100 mL of culture) and epoxide hydrolase activity (in terms of hydrolysis yield by time unit), we performed three assays (A, B and C). Styrene oxide was filtered through sterile 0.22 μm syringe filters (Millex®-GV, Carrigtwohill, Ireland) and added aseptically to Erlenmeyer flasks immediately after the inoculation (assay A), 24 h after the inoculation (assay B) and 24 h before the inoculation (assay C) to reach a concentration of 0.4% (v/v). In order to compare assays, three replicates were performed in each experiment. The results were subjected to an analysis of variance. When the analysis was statistically significant ($p < .05$), Bonferroni's multiple range test for means was performed.

Kinetic resolution of rac*-glycidyl phenyl ether,* rac*-benzyl glycidyl ether,* rac*-1,2-epoxyhexane and* rac*-1,2-epoxyoctane*

The kinetic resolution experiments were carried out at an initial epoxide concentration of 6 mM (for *rac*-glycidyl phenyl ether and *rac*-benzyl glycidyl ether) and 9 mM (for *rac*-1,2-epoxyhexane and *rac*-1,2-epoxyoctane). For *rac*-glycidyl phenyl ether or *rac*-benzyl glycidyl ether, the production of enantioenriched diols by selected mycelium-bound epoxide hydrolases was achieved by mixing 10 mg of lyophilized mycelium pre-incubated at 30 °C for 5 min in 880 μL of PBS (pH 7.4) and 20 μL of a 270 mM solution of racemic epoxide in DMSO in 2 mL low protein-binding Eppendorf tubes. Conversely, for *rac*-1,2-epoxyhexane or *rac*-1,2-epoxyoctane, we used 10 mg of lyophilized mycelium pre-incubated at 30 °C for 5 min in 880 μL of PBS (pH 7.4) and 20 μL of a 405 mM solution of racemic epoxide in DMSO. Reactions were performed in a thermomixer at 30 °C and 1400 rpm the time required to yield ca. 50% of diol. Samples were filtered through 20 μm polypropylene frits fitted into 3 mL cartridges and mycelia were washed applying positive pressure to the cartridge inlet. The remaining epoxide in reaction mixture or adsorbed onto fungal mycelia was extracted with hexane (3 × 1 mL) and organic extracts were discarded. The diol was subsequently extracted with ethyl acetate (3 × 1 mL). Before extractions, reaction mixtures were vortexed for 15 s and centrifuged at 9000 rpm for 5 min at 4 °C. Ethyl acetate extracts were evaporated under a stream of nitrogen and the dry residues were dissolved in 900 μL of the corresponding solvent. Milli-Q water/ACN (30:10, v/v) was used for HPLC-UV or UV–vis spectrophotometry, hexane/2-propanol (85:15, v/v) for the analysis by chiral HPLC-UV and ethyl acetate for the analysis by chiral GC-FID. 3-Phenoxy-1,2-propanediol and 3-benzyloxy-1,2-propanediol were quantified by HPLC-UV and their enantiomeric excesses were determined by chiral HPLC-UV. 1,2-Hexanediol and 1,2-octanediol were quantified by UV–vis spectrophotometry and their enantiomeric excesses were obtained from their corresponding 1,2-diacetates, analysed by chiral GC-FID. All experiments were performed in duplicate.

Pre-extraction spiked calibration standards were prepared in triplicate from five spiking solutions containing the racemic diol in DMSO (67.5–405 mM). Twenty microlitres of the corresponding spiking solution was added to Eppendorf tubes containing 10 mg of lyophilized mycelium and 880 μL of PBS (pH 7.4) and then extracted. Dry extracts were dissolved in 900 μL of Milli-Q water/ACN (30:10, v/v) to be analysed by HPLC-UV or UV–vis spectrophotometry. All samples and calibration standards were diluted 1.5 times for UV–vis spectrophotometry analyses (300 μL of solution and 150 μL of Milli-Q water/ACN (30:10, v/v)) and 30 times for HPLC-UV analyses (30 μL of solution and 870 μL of Milli-Q water/ACN (30:10, v/v)). Samples were filtered through 0.22 μm syringe filters before analysis.

HPLC-UV analysis

The HPLC-UV analyses were performed using an Alliance 2695 liquid chromatograph and a 2487 dual absorbance detector coupled to MassLynx data acquisition software (version 4.1) (Waters, Milford, MA). The injection volume was 10 μL and the detection wavelength 210 nm. The mobile phase was composed of Milli-Q water (solvent A) and ACN (solvent B), freshly prepared, filtered through a 0.45-μm membrane filter and degassed prior to use. The solvents were mixed in a linear gradient as follows: 0 min – 90% A and 10% B, 4 min – 80% A and 20% B, 8 min – 70% A and 30% B, 12–14 min – 0% A and 100% B, 14.1–16 min – 90% A and 10% B. The flow rate was 0.5 mL/min. An Atlantis dC_{18} (150 mm × 2.1 mm, 5 μm) column (Waters, Milford, MA) was used for the analysis of 3-phenoxy-1,2-propanediol and 3-benzyloxy-1,2-propanediol.

The samples were analysed at 35 °C and the total run time was 16 min. The retention times for these diols were 4.6 and 5.1 min, respectively.

Chiral HPLC-UV analysis

The chiral HPLC-UV analyses were performed using an Alliance 2695 liquid chromatograph and a 2996 photodiode array detector (200–300 nm) coupled to Empower data acquisition software (Waters, Milford, MA). The injection volume was 10 μL. The mobile phase was composed of hexane and 2-propanol (85:15, v/v), freshly prepared, filtered through a 0.45 μm membrane filter and degassed prior to use. The flow rate was 1 mL/min. A Chiralcel OD (250 mm × 4.6 mm, 10 μm) column (Chiral Technologies Europe, Illkirch Cedex, France) was used for the analysis of 3-phenoxy-1,2-propanediol and 3-benzyloxy-1,2-propanediol. The samples were analysed at 35 °C and the total run time was 20 min: 3-phenoxy-1,2-propanediol: $\lambda = 219$ nm, (*R*)-3-phenoxy-1,2-propanediol, $t_r = 9.9$ min; (*S*)-3-phenoxy-1,2-propanediol, $t_r = 16.4$ min. 3-Benzyloxy-1,2-propanediol: $\lambda = 210$ nm, (*R*)-3-benzyloxy-1,2-propanediol, $t_r = 8.5$ min; (*S*)-3-benzyloxy-1,2-propanediol, $t_r = 9.5$ min.

Chiral GC-FID analysis

The enantiomeric discriminations of 1,2-hexanediol and 1,2-octanediol were performed through their corresponding 1,2-diacetates (Oromí et al. 2012). An Agilent 7890A gas chromatograph coupled to a flame ionization detector and a Chrompack Chirasil-Dex CB β-cyclodextrin (25 m × 0.25 mm, 0.25 μm) column were used. Chiral GC-FID analyses were conducted using a H_2 flow rate of 2 mL/min, split mode (50:1), and the following temperature program: initial temperature of 100 °C (1 min) increasing to 135 °C at 5 °C/min and holding for 7 min. The injector and detector temperatures were both set at 250 °C. 1,2-Hexanediol 1,2-diacetate: (*R*)-1,2-hexanediol 1,2-diacetate, $t_r = 6.1$ min; (*S*)-1,2-hexanediol 1,2-diacetate, $t_r = 6.3$ min. 1,2-Octanediol 1,2-diacetate: (*R*)-1,2-octanediol 1,2-diacetate, $t_r = 10.2$ min; (*S*)-1,2-octanediol 1,2-diacetate, $t_r = 10.4$ min.

UV–vis spectrophotometric method

The concentrations of 1,2-hexanediol and 1,2-octanediol were determined with the adrenaline test for enzymes (Fluxá et al. 2008). To perform the test, aliquots (25 μL) of standard and sample solutions were transferred to F-bottom polypropylene 96-well microplates (Eppendorf, Madrid, Spain). Then, 125 μL of sodium periodate (1.3 mM) in sodium acetate buffer pH 5.5 (0.1 M) was added to each well using a multichannel pipette. The microplates were sealed, shaken and incubated in the spectrophotometer (Thermo Scientific Multiskan GO controlled by SkanIt software, Waltham, MA) for 75 min at 25 °C. The seal was removed and 50 μL of adrenaline (4 mM) in HCl (0.02 M) was added to each well using a multichannel pipette. The microplates were briefly shaken for 5 min and absorbance was measured immediately at 490 nm. Background absorbance was averaged and these values were subtracted from data readings.

Hydrolysis yields

The hydrolysis yields were calculated as the quotient between the concentration of the formed diol and the initial concentration of the racemic epoxide; that is, Y_{diol} (%) = ($C_{diol}/C_{initial\ epoxide}$) × 100%. For each experiment, the chemical hydrolysis was determined and subtracted from the total hydrolysis to obtain the enzymatic hydrolysis. Additionally, solvent and pre-extraction spiked blanks were analysed.

Absolute configuration and enantiomeric excess of the products

The absolute configurations of the products were deduced from the data reported in the literature (Berkessel and Ertürk 2006; Bala et al. 2010; Oromí et al. 2012). The enantiomeric excesses of the diols were calculated according to the following expression: ee_{diol} (%) = ([(*R*)-diol] − [(*S*)-diol])/([(*R*)-diol] + [(*S*)-diol]) × 100%, where (*R*)-diol and (*S*)-diol are the integrated areas of (*R*)-diol and (*S*)-diol, respectively.

DNA extraction, amplification and identification of Aspergillus niger *and* Aspergillus tubingensis

DNA was extracted (Querol et al. 1992) and concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). All PCR assays were performed using a mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany).

Amplification reactions were carried out in volumes of 25 μL containing 200 ng of template DNA, 1 μL of each primer (20 μmol/L), 2.5 μL of 10 × PCR buffer, 1 μL of $MgCl_2$ (50 mmol/L), 0.2 μL of dNTPs (100 mmol/L) and 0.15 μL of Taq DNA polymerase (5 U/μL) supplied by the manufacturer (Bio-tools, Madrid, Spain).

After DNA extraction, genomic DNAs were tested for suitability for PCR amplification using primers 5.8S1/5.8S2 (Gil et al. 2009). PCR products were detected in 2% agarose ethidium bromide gels in TAE 1 × buffer (Tris-acetate 40 mmol/L and ethylenediaminetetraacetic acid (EDTA) 1.0 mmol/L). A 100 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania) was used as molecular size marker.

DNA extraction, amplification and identification of Penicillium *species*

Cultures were grown in 500 μL of malt extract broth (2% w/v of malt extract, 0.1% w/v of peptone and 2% w/v of glucose) for 2 d at 26 °C. The mycelial extract was recovered after 10 min centrifugation at 17500 × g and 300 μL of DNA extraction buffer (200 mmol/L of Tris-HCl pH 8.5, 250 mmol/L of NaCl, 25 mmol/L of EDTA and 0.5% w/v of sodium dodecyl sulphate) was added. The mycelial suspension was lysed by vortexing with five 2.8mm Precellys metal beads (Bertin Technologies, Montigny-le-Bretonneux, France) for 10min. After centrifugation at 17 500 × *g* for 10min, 150 μL of sodium acetate (3mol/L, pH 5.2) was added to the supernatant. The supernatant was stored at –20 °C for 10 min and then centrifuged (17500 × g, 10 min). The DNA-containing supernatant was transferred to a new tube and nucleic acids were precipitated by addition of 1 volume of isopropanol. After a 5 min incubation time at room temperature, the DNA suspension was centrifuged (17,500 × *g*, 10min). The DNA pellet was washed with 70% ethanol to remove residual salts. Finally, the pellet was air-dried and the DNA was resuspended in 50 μL of TE buffer (10 mmol/L Tris-HCl pH 8, 1 mmol/L EDTA).

To identify *Penicillium* isolates, primer pairs BT2A/BT2B (Glass and Donaldson 1995) and EF-1/EF-2 (O'Donnell et al.1998) were used to obtain partial sequences of the β-tubulin and elongation factor genes. Amplification reactions were carried out in volumes of 50 μL containing 50 ng of DNA, 50 mmol/L of KCl, 10 mmol/L of Tris-HCl, 250 μmol/L of dNTP, 1 μmol/L of each primer, 2 mmol/L of $MgCl_2$ and 0.5 U of DFS-Taq DNA polymerase (BIORON, Ludwigshafen am Rhein, Germany). PCR assays were conducted in a GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA) under the following conditions: initial denaturation at 95 °C for 5min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C (BT2A/BT2B) or 53 °C (EF-1/EF-2) for 45 s, and extension at 72 °C for 60 s with a final extension of 10min. PCR products were cleaned with the Ultra Clean PCR clean-up DNA purification kit (MoBio, Carlsbad, CA). The PCR purified products were sequenced by the company Macrogen Europe (Amsterdam, Netherlands). Blast *n*-searches in GenBank allowed the identification of the level of identity with known sequences, and subsequently the species corresponding to the isolates.

Results and discussion

Isolation of fungi and epoxide hydrolase activity

Epoxide hydrolase-based reactions are attractive alternatives to more expensive chemical methods. For this reason, the demand for new epoxide hydrolases, particularly those of microbial origin, have attracted great interest from the chemical and pharmaceutical industries (Yildirim et al. 2013). The present study focused on the detection of novel fungal epoxide hydrolases with application for appropriate substrates. In this regard, we chose *rac*-glycidyl phenyl ether, *rac*-benzyl glycidyl ether, *rac*-1,2-epoxyhexane and *rac*-1,2-epoxyoctane epoxides because, to date, only very few fungal biocatalysts have provided satisfactory enantiomeric excesses for the hydrolytic products of these epoxides (Bala and Chimni 2010).

We achieved the isolation of epoxide hydrolase-producing fungi from olive oil by-products. A total of 19 isolates were obtained (7 from olive oil waste water and 12 from olive oil cake). These isolates and three additional fungi (strains 20, 21 and 22) were screened for epoxide hydrolase activity using *rac*-glycidyl phenyl ether as a substrate. Under our experimental conditions, 17 within the 22 fungi tested yielded less than 20% of the corresponding diol. All these strains were discarded for further experiments. Strains 5 and 12 (20–30% diol), strain 17 (50–60% diol), and strains 20 and 21 (80–90% diol) were selected (Figure 2).

Fungal strains 5, 12 and 17 were identified and added to the collection of the Department of Food Technology of the University of Lleida as *Penicillium* sp. BCT 051 (molecular identification, sequenced gene: betatubulin; <90% identity, the specie could not be determined), *Aspergillus terreus* BCT 052 (morphological identification) and *Penicillium aurantiogriseum* BCT 053 (molecular identification, sequenced gene: ITS; 99% identity), respectively. *Penicillium* sp. BCT 051 was isolated from olive oil waste water, and *Aspergillus terreus* BCT 052 and *Penicillium aurantiogriseum* BCT 053, from olive oil cake. Fungal strains 20 and 21, which had been previously isolated by our group from sunflower seeds, were reidentified as *A. niger* BCT 038 and *A. tubingensis* BCT 037.

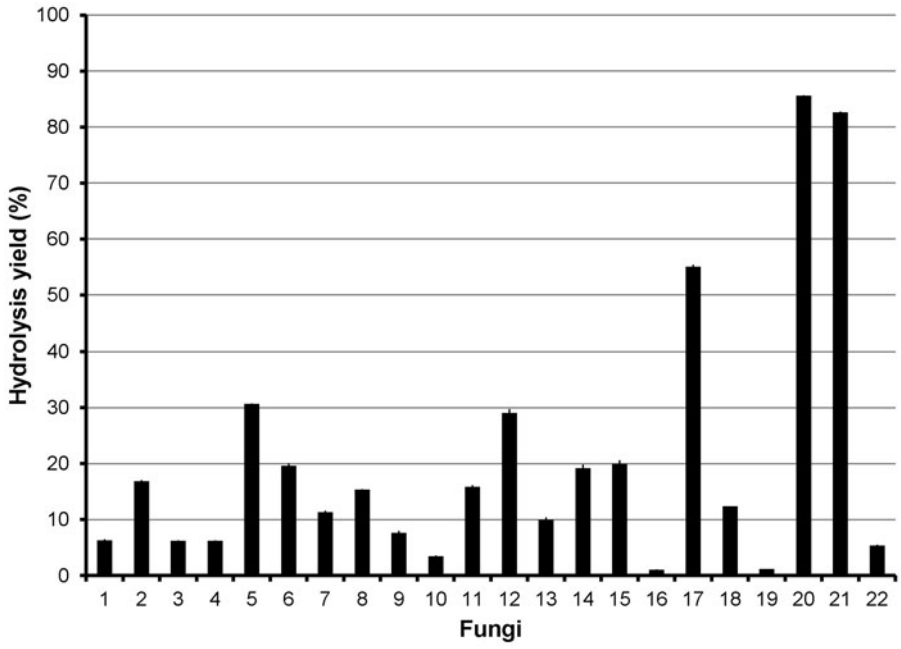

Figure 2. Hydrolysis of *rac*-glycidyl phenyl ether by epoxide hydrolases of isolated fungi. Reaction conditions: 980 µL PBS (pH 7,4), 20 µL of a solution of *rac*-glycidyl phenyl ether in DMSO (5 mg/mL), 10 mg of mycelium, 30 °C, 1400 rpm and 1 h. Each bar represents the average of two independent replications.

Effect of styrene oxide on biomass production and epoxide hydrolase activity improvement

Regarding the influence of styrene oxide on biomass production and epoxide hydrolase activity, we observed various behaviours depending on the time point at when the inducer was added to the growth medium. Styrene oxide was relatively toxic to the fungi when added after the inoculation, as reflected by a considerable reduction in biomass production. Moreover, epoxide hydrolase activity was inhibited. However, addition 24 h before inoculation enhanced this activity. The addition of styrene oxide to the growth medium 24 h before the inoculation (assay C) provided an increase in the biomass for *A. niger*, *A. tubingensis* and *A. terreus* cultures (Figure 3). The epoxide was transformed into 1,2-phenylethanediol during this pre-inoculation period (data not shown), thus reducing its inhibitory effect. However, the addition of the inducer immediately after inoculation (assay A) or 24 h after (assay B) led to a decrease in the biomass compared to the control (no inducer). For *P. aurantiogriseum* and *Penicillium* sp. cultures, the growth was inhibited in assays A and B, and the biomass production in assay C was lower than in the control.

The addition of styrene oxide to the growth medium 24 h before the inoculation (assay C) increased epoxide hydrolase activity in all cases. This effect was also observed in mycelium-bound epoxide hydrolases from *A. terreus* when the inducer was added immediately after inoculation (assay A) or 24 h after (assay B). However, the epoxide hydrolase activity of mycelium-bound epoxide hydrolases from *A. niger* and *A. tubingensis* strains decreased slightly (Figure 4).

Cell-bound epoxide hydrolases are considered to be induced enzymes. Consequently, epoxide hydrolase production is affected by growth conditions. Liu et al. (2007) described that the addition of *cis*-epoxysuccinate before inoculation increased both the biomass production and epoxide hydrolase activity of *Rhodococcus* sp. ML-0004. In contrast, Wei et al. (2012) showed that the addition of *rac*-benzyl glycidyl ether before inoculation does not improve the epoxide hydrolase activity of *Talaromyces flavus* ZJUTZQ159. Our experiments showed both behaviours indicating that the increase of the epoxy hydrolase activity depended on the microorganism and the time point. Moreover, the biomass production showed the same behaviour. In fact, the initial medium was always able to induce epoxide hydrolase activity without the addition of styrene oxide.

Kinetic resolution of rac*-glycidyl phenyl ether;* rac*-benzyl glycidyl ether;* rac*-1,2-epoxyhexane and rac-1,2-epoxyoctane*

This study is the first report on the hydrolysis of aryl and alkyl glycidyl ethers by the mycelium-bound epoxide hydrolases from fungi isolated from olive oil waste water, olive oil cake and sunflower seeds. The use of

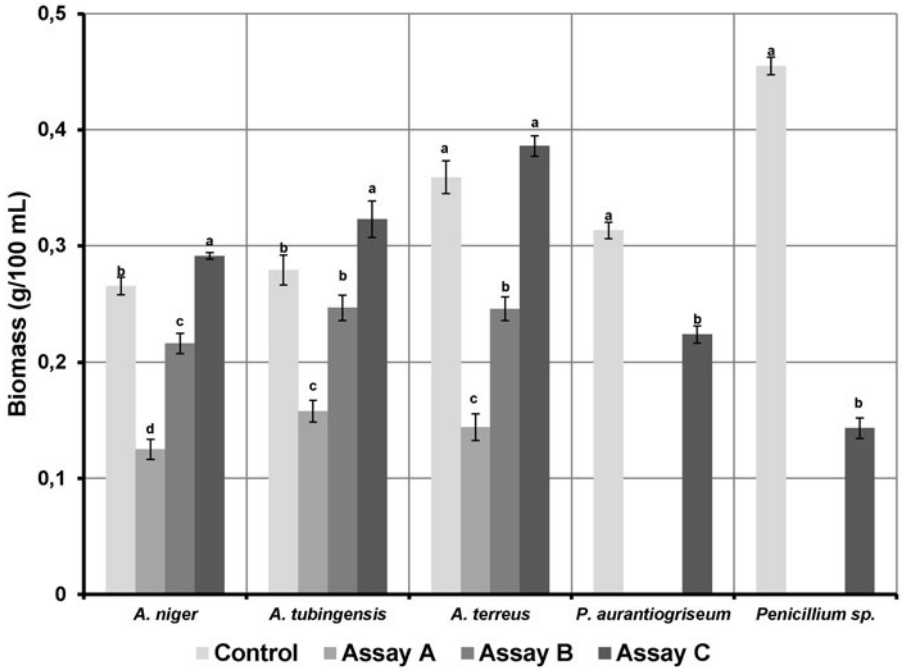

Figure 3. Effect of styrene oxide on the biomass production of *A. niger*, *A. tubingensis*, *P. aurantiogriseum*, *Penicillium* sp. and *A. terreus* cultures. Each bar represents the average of three independent replications and different letters indicate significant differences between assays according to Bonferroni's test (95% confidence interval).

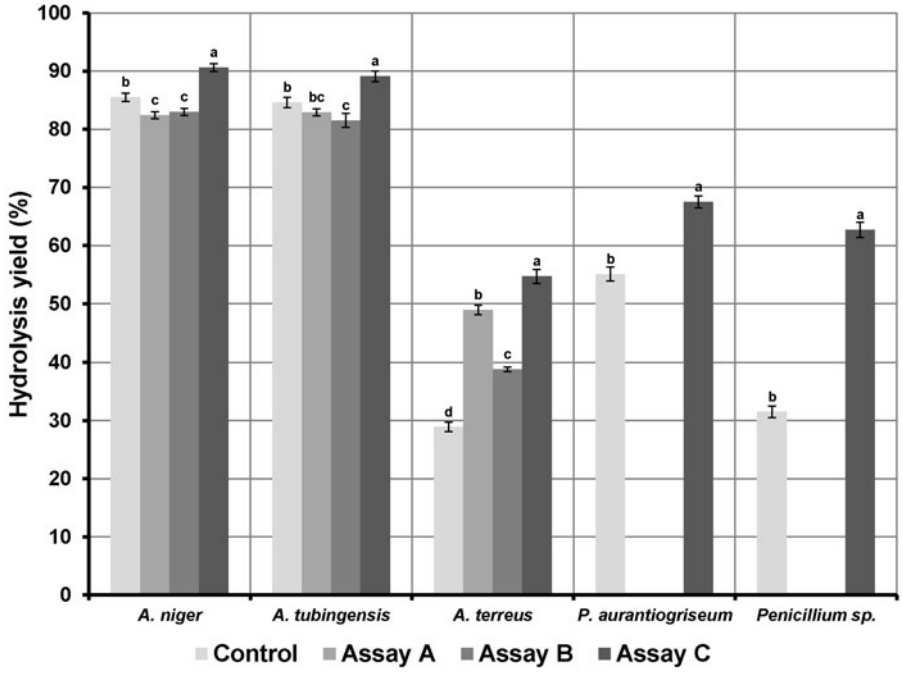

Figure 4. Effect of styrene oxide on the hydrolysis yield of mycelium-bound epoxide hydrolases from *A. niger*, *A. tubingensis*, *P. aurantiogriseum*, *Penicillium* sp. and *A. terreus*. Each bar represents the average of three independent replications and different letters indicate significant differences between assays according to Bonferroni's test (95% confidence interval).

fungal mycelia as a source of naturally immobilized catalysts, such as mycelium-bound epoxide hydrolases, is cost-effective because the biomass can be directly used as biocatalyst, thus eliminating the complex procedures of enzyme isolation, purification, and immobilization processes that often result in loss of enzymatic activity. Furthermore, the cell structure may act as natural matrix able to protect the enzymes from the negative action of external agents (León et al. 1998).

Table 1. Kinetic resolution of *rac*-glycidyl phenyl ether, *rac*-benzyl glycidyl ether, *rac*-1,2-epoxyhexane and *rac*-1,2-epoxyoctane by mycelium-bound epoxide hydrolases from *A. niger*, *A. tubingensis*, *P. aurantiogriseum*, *Penicillium* sp. and *A. terreus*.

Strain	Entry	Epoxide	Reaction time	ee_{diol}[a] (%)/a.c.[b]	$Yield_{diol}$[c] (%)
A. niger	1	*rac*-glycidyl phenyl ether	15 min	51.4/(*S*)	45.1 ± 0.1
	2	*rac*-benzyl glycidyl ether	15 min	34.9/(*S*)	49.5 ± 0.1
	3	*rac*-1,2-epoxyhexane	1 h	8.5/(*R*)	45.4 ± 2.2
	4	*rac*-1,2-epoxyoctane	1 h	5.3/(*R*)	93.7 ± 1.7
A. tubingensis	5	*rac*-glycidyl phenyl ether	1 h	36.9/(*S*)	59.2 ± 0.1
	6	*rac*-benzyl glycidyl ether	2 h	29.4/(*S*)	54.9 ± 0.1
	7	*rac*-1,2-epoxyhexane	4 h	24.4/(*R*)	70.4 ± 0.7
	8	*rac*-1,2-epoxyoctane	4 h	13.7/(*R*)	49.7 ± 1.5
P. aurantiogriseum	9	*rac*-glycidyl phenyl ether	1 h	28.5/(*S*)	51.4 ± 0.2
	10	*rac*-benzyl glycidyl ether	1 h 30 min	43.3/(*R*)	40.8 ± 0.1
	11	*rac*-1,2-epoxyhexane	4 h	17.9/(*R*)	19.1 ± 0.7
	12	*rac*-1,2-epoxyoctane	4 h	8.8/(*R*)	32.4 ± 1.4
Penicillium sp.	13	*rac*-glycidyl phenyl ether	2 h	30.7/(*S*)	48.3 ± 0.1
	14	*rac*-benzyl glycidyl ether	3 h	14.4/(*S*)	46.8 ± 0.2
	15	*rac*-1,2-epoxyhexane	4 h	10.8/(*R*)	14.9 ± 0.9
	16	*rac*-1,2-epoxyoctane	4 h	9.4/(*R*)	29.6 ± 3.1
A. terreus	17	*rac*-glycidyl phenyl ether	24 h	11.6/(*S*)	33.2 ± 0.1
	18	*rac*-benzyl glycidyl ether	24 h	45.6/(*S*)	45.4 ± 0.6
	19	*rac*-1,2-epoxyhexane	6 h	19.5/(*R*)	5.5 ± 0.2
	20	*rac*-1,2-epoxyoctane	6 h	27.5/(*R*)	21.4 ± 0.2

[a]ee_{diol}: enantiomeric excess of the main diol enantiomer.
[b]a.c.: absolute configuration of the main diol enantiomer.
[c]Yield was calculated as the quotient between the concentration of the diol and the initial concentration of the racemic epoxide.

In the last 20 years, several strains of *Aspergillus* sp. with the capacity to produce epoxide hydrolases have emerged. These strains include the following: *Aspergillus oryzae* IAM 2750, *Aspergillus sojae* IAM 2631, *Aspergillus wentii* (Moussou et al. 1998a), *A. niger* LCP 521 (Reetz et al. 2004), *A. terreus* CBS 116-46 (Moussou et al. 1998a, 1998b), *A. niger* M200 (Kotik et al. 2005), *Aspergillus sydowii* Gc12 (Martins et al. 2011) and *Aspergillus brasiliensis* CCT 1435 (Beloti et al. 2013). Only four epoxide hydrolases from *Penicillium* sp., namely *P. lilacinum* ATCC 10114, *P. simplicissimum* VKM F 16 (Moussou et al. 1998a), *P. raistrickii* Ce16 and *P.* miczynskii Gc5 (Martins et al. 2011) have been described. Nevertheless, to the best of our knowledge, neither *A. tubingensis* nor *P. aurantiogriseum* have been described as epoxide hydrolase producers.

Table 1 shows the enantiomeric excesses of the diols when mycelia from the five fungi were used as biocatalysts for the hydrolysis of *rac*-glycidyl phenyl ether, *rac*-benzyl glycidyl ether, *rac*-1,2-epoxyhexane and *rac*-1,2-epoxyoctane. Reaction times were determined with the aim to keep hydrolysis yields below 50% to maximize the enantiomeric excess.

The mycelium-bound epoxide hydrolases from *A. niger*, *A. tubingensis*, *P. aurantiogriseum*, *Penicillium* sp. and *A. terreus* showed high activity towards aryl glycidyl ethers and provided diols with enantiomeric excesses ranging from 11% to 51% *ee* (Supplementary material contains some HPLC chromatograms). The enantioselectivity of the mycelium-bound epoxide hydrolases from *A. niger*, *A. tubingensis* and *Penicillium* sp. decreased with the presence of a CH_2 between the phenyl and oxirane rings (entries 2, 6 and 14). In contrast, the mycelium-bound epoxide hydrolases from *P. aurantiogriseum* and *A. terreus* showed greater enantioselectivity (entries 10 and 18). In general, epoxide hydrolases displayed higher enantiopreference for aromatic epoxides since low to moderate enantiomeric excesses (5–27% *ee*) were obtained with aliphatic epoxides, although this preference was less pronounced in the mycelium-bound epoxide hydrolases from *P. aurantiogriseum* and *A. terreus*. The mycelium-bound epoxide hydrolases from *A. niger* and *A. terreus* showed the shortest and the largest hydrolysis times, respectively, compared with the other catalysts. The absolute configuration of the diols depended on the substrate structure ((*S*)-diol from aromatic epoxides and (*R*)-diol from aliphatic epoxides). The only exception was the mycelium-bound epoxide hydrolase from *P. aurantiogriseum*, which gave (*R*)-3-benzyloxy-1,2-propanediol from *rac*-benzylglycidyl ether (entry 10).

All the biocatalysts tested yielded aliphatic diols with low to moderate enantiomeric excesses. Moussou et al. (1998a) reported that only a few epoxide hydrolases, such as those from *A. niger* LCP 521, *A. oryzae* IAM 2750, *A. sojae* IAM 2631, *A. terreus* CBS 116-46, *A. wentii*, and *P. lilacinum* yielded (*R*)-1,2-hexanediol with 24–62% *ee*. The biocatalyst with the most outstanding performance in terms of conversion and enantiomeric excess in our set was *A. tubingensis*,

which yielded 70% of diol with 24.4% *ee*. The hydrolysis of *rac*-1,2-epoxyoctane by epoxide hydrolases from *Diplodia gossypina* ATCC 10936, *F. solani* DSM 62416 and *Glomerella cingulate* ATC 10534 (Mischitz et al. 1995), *Ulocladium atrum* CMC 3280 and *Zopfiella karachiensis* CMC 3284 (Grogan et al. 1996), *A. niger* LCP 521, *A. terreus* CBS 116-46, *Chaetomium globosum* LCP 679, *Cunninghamella elegans* LCP 1543 and *Syncephalastrum racemosum* MUCL 28766 (Moussou et al. 1998b), and *B. bassiana* ATCC 7149 (Moussou et al. 2000) also yielded (*R*)-1,2-octanediol with very low enantiomeric excesses. This was the case of our biocatalysts; only *A. terreus* yielded a diol with 27.5% *ee* but with only 21.4% conversion. Only epoxide hydrolases from *A. niger* CCT 2760, *A. niger* CCT 3086 and *A. niger* CCT 4846 (Cagnon et al. 1999) yielded (*R*)-1,2-octanediol, with 27%, 45% and 56% *ee*, respectively.

When the mycelium-bound epoxide hydrolases were tested with aliphatic epoxides, the absolute configuration of the main diol product was determined to be *R* for all strains (Supplementary material contains some CG chromatograms), as observed for other fungal epoxide hydrolases (Moussou et al. 1998a, 1998b; Cagnon et al. 1999; Moussou et al. 2000).

The enantiomeric excess of (*S*)-3-phenoxy-1,2-propanediol produced by the mycelium-bound epoxide hydrolase from *A. niger* (entry 1: 45% yield, 51% *ee*) was similar to that obtained with the epoxide hydrolase from *A. niger* LCP 521 (yield not reported, 56% *ee*) described by Reetz et al. (2004). Moreover, the enantiomeric excesses of (*R*)- and (*S*)-3-benzyloxy-1,2-propanediol by the mycelium-bound epoxide hydrolases from *P. aurantiogriseum* (entry 10: 41% yield, 43% *ee*) and *A. terreus* (entry 18: 45% yield, 46% *ee*), respectively, are as far as we know, the highest ever reported among wild-type fungal epoxide hydrolases. To the best of our knowledge, the enantiopreference of a fungal epoxide hydrolase, like that from *P. aurantiogriseum*, for the (*R*)-benzyl glycidyl ether is unprecedented. Other fungal epoxide hydrolases, such as those identified in *Fusarium solani* DSM 62416 (Mischitz et al. 1995), *A. niger* M200 (Kotik et al. 2005), *A. sydowii* Gc12, *P. raistrickii* Ce16, *P. miczynskii* Gc5, *Trichoderma* sp. Gc1 (Martins et al. 2011) and *T. flavus* ZJUTZQ159 (Wei et al. 2012), have been used in the hydrolysis of *rac*-benzyl glycidyl ether. However, the enantiomeric excesses of diol were very low or not determined.

Conclusions

In conclusion, five within the 22 fungi tested yielded more than 20% of the corresponding diol using *rac*-glycidyl phenyl ether as a substrate. The mycelium-bound epoxide hydrolases from *A. niger* and *A. tubingensis* should to be promising enzymes for the preparation of enantiopure (*S*)-3-phenoxy-1,2-propanediol, which is an intermediate for the synthesis of several drugs, including guaifenesin, an expectorant, mephenesin, a muscle relaxant, and clorphene, an antifungal agent (Sareen and Kumar 2011). In addition, our results indicate that the mycelium-bound epoxide hydrolases from *P. aurantiogriseum* and *A. terreus* are valuable enzymes for the preparation of enantioenriched (*R*)- and (*S*)-3-benzyloxy-1,2-propanediol, respectively. These compounds are key intermediates in the synthesis of various bioactive molecules, such as the anti-herpes virus agent Vistide, the migrastatin analogue for treating colon and/or ovarian cancer and the subtype-selective LPA3 receptor antagonist (Jia et al. 2011). We observed that the influence of styrene oxide on biomass production and epoxide hydrolase activity depend on the time point at when the inducer was added to the growth medium and to the fungus in some cases.

Disclosure statement

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