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Original Research Paper

Chemoselective reduction of chalcones by whole hyphae of marine fungus *Penicillium citrinum* CBMAI 1186, free and immobilized on biopolymers

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ABSTRACT

Whole mycelia of marine fungal strain *Penicillium citrinum* CBMAI 1186, both free and immobilized on cotton (*Gossypium* sp.), fibroin (*Bombyx mori*) and a local kapok (*Ceiba speciosa*), catalyzed the chemoselective reduction of chalcones [(*E*)-3-(4-fluorophenyl)-1-phenylprop-2-en-1-one **3a**, (*E*)-3-(4-bromophenyl)-1-phenylprop-2-en-1-one **3b**, (*E*)-3-(4-methoxyphenyl)-1-phenylprop-2-en-1-one **3c**, (*E*)-3-(4-methoxyphenyl)-1-phenylprop-2-en-1-one **3d**, (*E*)-3-(3-nitrophenyl)-1-phenylprop-2-en-1-one **3e**] to dihydrochalcones [3-(4-fluorophenyl)-1-phenylpropan-1-one **4a**, 3-(4-bromophenyl)-1-phenylpropan-1-one **4b**, 3-(4-methoxyphenyl)-1-phenylpropan-1-one **4c**, 1-(4-methoxyphenyl)-3-phenylpropan-1-one **4d**, 3-(3-nitrophenyl)-1-phenylpropan-1-one **4e**] in good yields. The immobilized fungus and free whole mycelium showed a similar behavior in the conversion of the chalcones. The hyphae immobilized on biopolymers were active in biotransforming the chalcones after being preserved for 30 days in refrigerator. Scanning electron micrographs showed that the cells of marine fungus *P. citrinum* CBMAI 1186 were intertwined with the fibers of the supports, allowing fast separation from the reaction media and easing reuse of the biocatalyst. It is concluded that marine fungus *P. citrinum* CBMAI 1186 presents potential for the biotransformations of reduction of chalcones (**3a–e**). This paper describes the first reported use of immobilized marine fungus in reactions catalyzed by enoate reductases.

1. Introduction

Dihydrochalcones are common substructures in numerous natural products belonging to the chalcone family. These compounds have attracted attention through reported that possess they several biological activities, being cytotoxic (Anto et al., 1995; Ducki et al., 1998), antileishmanial (Boeck et al., 2006), antitumor (Cabrera et al., 2007), antibacterial (Joshi et al., 2001), anti-*Trypanosoma cruzi* (Aponte et al., 2008) and anti-HIV (Cheenpracha et al., 2006). Having such a variety of pharmacological activities, these molecules interest medicinal chemists and several strategies have been developed to synthesize them.

In general the reduction of carbon–carbon double bond involves chemical methods no chemoselective and less eco-friendly conditions, especially the use of metal salts and complexes, including copper (Nahra et al., 2013), palladium (Sommovigo and Howard, 1993), rhodium (Shiomi et al., 2009). So following the growing concern over environmental pollution and sustainable development, new methods chemoselective reduction of carbon–carbon double bond derived from enones are emerging (Oberdorfer et al., 2012; Tasnádi et al., 2012).

Interest in the immobilization of whole cells of microorganisms has been increasing, is fueled by the desire to replace the isolated enzymes used in industrial and laboratory processes (Peart et al., 2012). The immobilization of microorganisms can be defined as any technique that limits the free migration of the cells (Chibata et al., 1986; Chibata and Tosa, 1981). The cells can be immobilized in two basic ways, entrapment and attachment. In the first, the

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organisms are entrapped in the interstices of fibrous or porous materials or are physically restrained within or by a solid or porous matrix such as a stabilized gel or a membrane. In the latter, the microorganisms adhere to surfaces or other organisms by self-adhesion or chemical bonding (Couto et al., 2004).

In one study reported recently by our research group, whole marine fungi *Aspergillus sclerotiorum* CBMAI 849 and *Penicillium citrinum* CBMAI 1186, were immobilized on chitosan, silica xerogel and silica gel and used biocatalytic to promote reactions (Rocha et al., 2012). In others studies, natural polymers such as alginate (Cruz et al., 1998), cellulose (Porto et al., 2002) or derivatives (Stolarzewicz et al., 2011) were used as matrices for the immobilization of whole microbial cells for use in biocatalysis.

Natural fibers such as cotton (*Gossypium* sp.) have advantages over synthetic fiber in that their porous hydrophilic structures retain water, oxygen and nutrients and provide a perfect environment for the growth of microorganisms (Abdel-Halim et al., 2011; Nikolic et al., 2010).

Another interesting fiber is fibroin, obtained from *Bombyx mori* (silkworm) cocoons; this natural macromolecule is a fibrous protein primary structure consists largely of a repeating sequence of six aminoacids (Gly–Ala–Gly–Ala–Gly–Ser) (Zhang, 1998; Asakura et al., 1997). This robust biomaterial offers a wide range of properties making it suitable for biomedical applications, on account of its mechanical properties, environmental stability, biocompatibility and biodegradability (Sah and Pramanik, 2011; Zhang et al., 2005). Finally, kapok is a fiber derived from the fruits of the silk-cotton tree (*Ceiba speciosa*), mainly composed of cellulose and lignin; beside these constituents, a small amount of waxy material coats the fiber surface, making it very hydrophobic (Wang et al., 2012).

Whole cells of yeast and bacteria are frequently used to reduce organic compounds; This, α, β-unsaturated carbonyl compounds were used as substrates in biotransformation reactions mediated by three industrial *Saccharomyces cerevisiae* yeast strains in biphasic systems (Silva et al., 2010). However, the use of immobilized filamentous fungi for biocatalytic reactions is quite rare in the literature (Porto et al., 2002; Rocha et al., 2012).

In the present paper, we report the immobilization of whole mycelia of the marine fungal *P. citrinum* CBMAI 1186 on three natural support matrices to catalyze the reduction of chalcones (**3a–e**) to dihydrochalcones (**4a–e**) by fungal enoate reductases.

2. Material and methods

2.1. General methods

The dihydrochalcones **4a–e**, obtained by enzymatic processes, were purified by flash column chromatography (CC) over silica gel (0.035–0.075 mm) eluted with a mixture of *n*-hexane/EtOAc (19/1). The column fractions were monitored by TLC on precoated silica gel 60 F254 layers (aluminum backed, Sorbent).

All the manipulation involving marine fungus was carried out under sterile conditions in a Veco laminar-flow cabinet. A Technal TE-421 orbital shaker was used in the biocatalytic experiments. Gas chromatography-mass spectrometry: a Shimadzu GC2010plus gas chromatography system coupled to a mass selective detector (Shimadzu MS2010plus) in electron ionization (70 eV) with a DB-5 fused-silica column (J&WScientific, 30 m × 0.25 mm × 0.25 μm), and the following conditions were employed in the gas chromatography analyses: carrier gas, nitrogen (81.4 KPa); injector temperature, 250 °C; injector split ratio, 1:20; detector temperature, 200 °C; oven (temperature: initial 50 °C, final 270 °C (5 min); heating rate, 5 °C min^{-1}; total time of analysis, 49.0 min.

2.2. Chemicals

Acetophenone **1a** (99%), 4-methoxyacetophenone **1b** (99%), 4-fluorobenzaldehyde **2a** (99%), 4-bromobenzaldehyde **2b** (99%), benzaldehyde **2d** (99%), and 3-nitrobenzaldehyde **2e** (99%) were purchased from Sigma-Aldrich, 4-anisaldehyde **2c** (98%) from Vetec and sodium hydroxide (97%) and hydrochloric acid (37%) from Quemis. All compounds were used without further purification. The salts used for preparation of artificial seawater were purchased from Vetec and Synth.

2.3. Preparation of the chalcones *3a–e*

In two-necked round-bottomed flasks, mixtures of one of the ketones 1**a–b** (10 mmol), one of the benzaldehydes **2a–e** (11 mmol) and anhydrous EtOH (50 mL) were prepared. Each solution was stirred at room temperature for 5 min, after which 5 mL of NaOH was added (6 mol L^{-1}). The reaction was stirred at room temperature for 12 h, and then stopped by adding HCl (5 mL, 10%), yielding a yellow precipitate. The precipitate was filtered off and recrystallized from EtOH. The products (**3a–e**) were obtained in good yields and identified by comparing spectroscopic data (^{1}H NMR, GC–MS) (Supporting information) with data in the literature (Kanagarajan and Gopalakrishnan, 2012; Pawluc et al., 2009, 2011; Kim et al., 2012).

2.4. Marine fungus P. citrinum CBMAI 1186

The marine fungus *P. citrinum* CBMAI 1186 was isolated from the marine alga *Caulerpa* sp., which was collected by Prof. R. G. S. Berlinck in the town of São Sebastião, on the coast of the State of São Paulo, Brazil. The fungus was identified by both conventional and molecular methods at the Chemical, Biological and Agricultural Multidisciplinary Research Center (CPQBA) at the State University of Campinas (UNICAMP), SP, Brazil (Rocha et al., 2009). The fungi *P. citrinum* CBMAI 1186 is deposited in the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI).

2.5. Culture of marine fungus P. citrinum CBMAI 1186

Small slices of solid medium (0.5 × 0.5 cm) bearing mycelia of *P. citrinum* CBMAI 1186 were cut from the stock solid culture and used to inoculate 600 mL of liquid culture medium contained in Erlenmeyer flasks (2 L). The mycelia were incubated in the culture medium of 2% malt extract (Acumedia) in artificial seawater at 32 °C for 7 days in a rotary shaker (130 rpm). Composition of artificial seawater: $CaCl_2.2H_2O$ (1.36 g L^{-1}), $MgCl_2 \cdot 6H_2O$ (9.68 g L^{-1}), KCl (0.61 g L^{-1}), NaCl (30.0 g L^{-1}), Na_2HPO_4 (0.014 mg L^{-1}), Na_2SO_4 (3.47 g L^{-1}), $NaHCO_3$ (0.17 g L^{-1}), KBr (0.1 g L^{-1}), $SrCl_2 \cdot 6H_2O$ (0,040 g L^{-1}), H_3BO_3 (0.030 g L^{-1}) at pH 8 (Rocha et al., 2010).

2.6. Biocatalytic reduction of chalcones *3a-e* by marine fungus P. citrinum CBMAI 1186

The mycelia of *P. citrinum* CBMAI 1186 were harvested by Buchner filtration and suspended in a 100 mL of buffer solution (0.1 mol L^{-1}, Na_2HPO_4/KH_2PO_4, pH = 7) in 250 mL Erlenmeyer flasks. The biocatalytic reductions were carried out with 2.5 g (wet weight) of mycelia and 50 mg of chalcones **3a–e**, previously dissolved in 400 μL of dimethyl sulfoxide, mixed into the buffer solution. The reaction mixtures were incubated for 6 days in an orbital shaker at 32 °C and 130 rpm, the reactions being monitored by TLC every 24 h. After that, 1.0 mL samples were extracted with 1.0 mL EtOAc by mixing on a vortex and centrifuging at 6000 rpm

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for 6.0 min in a HERMLE Z-200 A, centrifuge and analyzed by GC–MS.

2.7. Isolation of dihydrochalcones **4a–e** produced by fungus P. citrinum CBMAI 1186

The reaction was filtered after 6 days of incubation, the filtrate was extracted with EtOAc (3 × 50 mL), and the organic phase was dried over anhydrous Na_2SO_4, filtered, evaporated under vacuum and analyzed by GC–MS. The extracts obtained were purified by flash CC over silica gel to yield the dihydrochalcones **4a–e**. The spectroscopic data of compounds **4a–e** were confirmed by analysis of ^{1}H NMR and GC–MS (Supporting information) and consistent with those reported in the literature (Shang et al., 2012; Feng et al., 2012).

2.8. Preparation of support matrices

Bombyx mori silk cocoons were cut into small pieces and immersed in a boiling aqueous solution (100 °C) of 0.2% (w/v) of sodium carbonate (99% pure) for 30 min with magnetic stirring. After boiling, the material was repeatedly washed with distilled water (3 × 1.0 L) to remove the glue-like sericine protein and dried in a hot air oven for 24 h. Later, the resultant fibroin was sterilized at 121 °C for 20 min before use (Meechaisue et al., 2007). *Bombyx mori* silk cocoons were donated by local farmer.

Cotton fiber was purchased from a commercial source (APOLO®, Brazil), being sterilized at 121 °C for 20 min before use.

The kapok fiber was collected from the fruit of *Ceiba speciosa*, on March 20, 2013, in the city of São Carlos, SP, Brazil. The kapok fiber was washed with 500 mL of phosphate buffer (0.1 mol L^{-1}, Na_2HPO_4/KH_2PO_4, pH = 7) to remove the impurities and then the material was sterilized at 121 °C for 20 min for later use.

2.9. Immobilization of whole P. citrinum CBMAI 1186 hyphae on biopolymers

P. citrinum CBMAI 1186 was grown as described in Section 2.5. The hyphae were harvested by filtration (2.5 g wet), and resuspended in 100 mL phosphate buffer (0.1 mol L^{-1}, Na_2HPO_4/KH_2PO_4, pH = 7) with 0.5 g of the respective biopolymer (fiber cotton, fibroin or kapok) in an Erlenmeyer flask (250 mL). The mixtures were incubated for 48 h in an orbital shaker at 32 °C and 130 rpm. After Buchner filtration, the immobilized fungus was used immediately in the biocatalytic reactions in chalcone **3a**.

2.10. Effect of mass of substrate **3a** on the conversion by P. citrinum CBMAI 1186

Preliminary the experiments were conducted to determine the best mass of substrate for the bioconversion of chalcone **3a**. Fungal mycelia (wet weight 2.5 g) were suspended in 100 mL phosphate buffer (0.1 mol L^{-1}, Na_2HPO_4/KH_2PO_4, pH = 7) in a 250 mL Erlenmeyer flask 25, 50, 75 or 100 mg of chalcone **3a**, previously solubilized in dimethyl sulfoxide (400 μL) was added. The reactions were incubated for 6 days in an orbital shaker at 32 °C and 130 rpm.

2.11. Bioconversion of chalcone **3a** by immobilized mycelia of P. citrinum CBMAI 1186

The bioreduction of chalcone **3a** was carried out with fungal mycelia (2.5 g) immobilized in silk fibroin (0.50 g), cotton fiber (0.50 g) or kapok fiber (0.50 g). The chalcone **3a**, dissolved in dimethyl sulfoxide (400 μL), was placed in a 250 mL Erlenmeyer flask containing 100 mL of phosphate buffer solution (0.1 mol L^{-1}, Na_2HPO_4/KH_2PO_4, pH = 7). The flask was shaken at 32 °C and 130 rpm in an orbital shaker in 6 days. After that the reaction was filtered, the filtrate was extracted with EtOAc (3 × 50 mL), and the organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum. The extract obtained was analyzed by GC–MS. Purified by flash CC over silica gel to yield the dihydrochalcone **4a**.

2.12. Scanning electron microscopy (SEM)

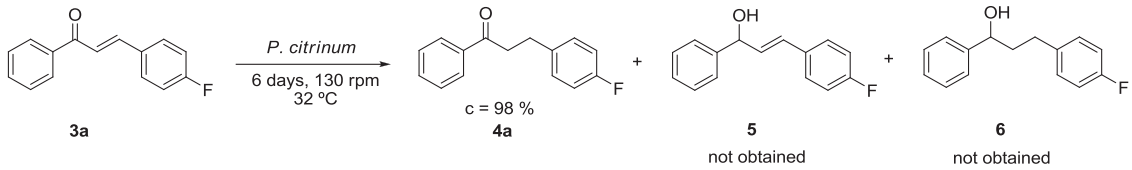
For SEM analysis, the surfaces of samples of immobilized mycelia of fungus *P. citrinum* CBMAI 1186 were washed with water to remove the non-adhering support matrix. The samples were then dehydrated in a graded series of water–ethanol solutions in (10 mL of 10%, 25%, 40%, 50%, 70%, 80%, 90% and 100% ethanol, in a 50 mL Erlenmeyer flask) for 15 min at each step (Porto et al., 2002). Samples were air-dried at room temperature, and coated with 8–10 nm of gold by argon ion sputtering using a Baltec MED 020 model sputter coater. Micrographs of the surface were taken with a Leica-Zeiss LEO 440 scanning electron microscope with an accelerating voltage of 20 kV.

3. Results and discussion

Whole mycelia of marine fungi have been used in the biotransformation of organic compounds and the selection of an appropriate microorganism is an important part of this process. Recently, we investigated the use of filamentous fungi isolated from marine environments to reduce prochiral ketones (Rocha et al., 2012). In view of the excellent results obtained in the biocatalytic reduction of ketones with free hypahe of marine fungus *P. citrinum* CBMAI 1186, we proceeded with the immobilization of whole mycelium on biopolymer supports of low cost such as cotton, fibroin and kapok and its application to the reduction of chalcones **3a–e**.

Whole free mycelia of *P. citrinum* CBMAI 1186 were tested first and succeeded in reducing the carbon–carbon double bond of chalcone **3a** in 6 days, yielding 98% conversion dihydrochalcone **4a**. The compounds (*E*)-3-(4-fluorophenyl)-1-phenylprop-2-en-1-ol **5** and 3-(4-fluorophenyl)-1-phenylpropan-1-ol **6**, which can result from reduction of the carbonyl group (catalyzed by alcohol dehydrogenase), were not detected obtained (Scheme 1). Therefore, reduction of chalcone **3a** by the whole fungus showed excellent chemoselectivity.

In addition, the effect of the amount of chalcone **3a** added to the mycelia in culture medium on the conversion of the substrate was investigated. In all these experiments, 2.5 g of whole mycelium of *P. citrinum* CBMAI 1186 was used. In the first experiment, 25.0 mg of chalcone **3a** was used and total reduction of the



Scheme 1. Possible products that might be obtained in the bioreduction of chalcone **3a** by whole free or immobilized *P. citrinum* CBMAI 1186 mycelia.

carbon–carbon double occurred bond occurred, yielding the product **4a** (Scheme 1).

When 50.0 mg of chalcone **3a** was used, 98% conversion to the desired product **4a** was obtained. However, on adding 75.0 mg of chalcone **3a**, only 60% conversion occurred, and with 100.0 mg of substrate only 52% of dihydrochalcone **4a** was found (Fig. 1). Accordingly, we decided to perform the chemoselective reduction of this carbon–carbon double bond by *P. citrinum* CBMAI 1186 with 2.5 g of mycelia and 50 mg of each substrate (**3b–e**). Other conditions were maintained: 6 days of incubation in 100 mL of phosphate buffer solution (0.1 mol L^{-1}, Na_2HPO_4/KH_2PO_4, pH=7) in an orbital shaker at 32 °C.

The reduction of the double bond is directly influenced by substituent groups attached to ring B in chalcones **3a–e**, by means of the conjugation of π-bonds on this side of the molecule. Thus electron-withdrawing groups on ring B are expected to increase the reactivity of the β-carbonyl carbon, whereas electron-withdrawing or electron-donating groups on ring A should not interfere in the reaction (Silva et al., 2010).

In this study, the electronic effect and type of substituent groups attached to ring B in chalcones **3a–e** were analyzed. Surprisingly, all the chalcones **3a–e** containing groups electron-withdrawing or electron-donating were chemoselectively reduced to dihydrochalcones **4a–e** in good conversions by whole cells of *P. citrinum* CBMAI 1186 (Scheme 2).

The chalcone **3b**, containing a bromine atom in the in *para* position of ring B, showed half the conversion (49%) of **3a**, with a fluorine atom attached in the *para* position of ring B (98%). This results show the importance of halogen groups and their properties such as relative lipophilicity and electronegativity.

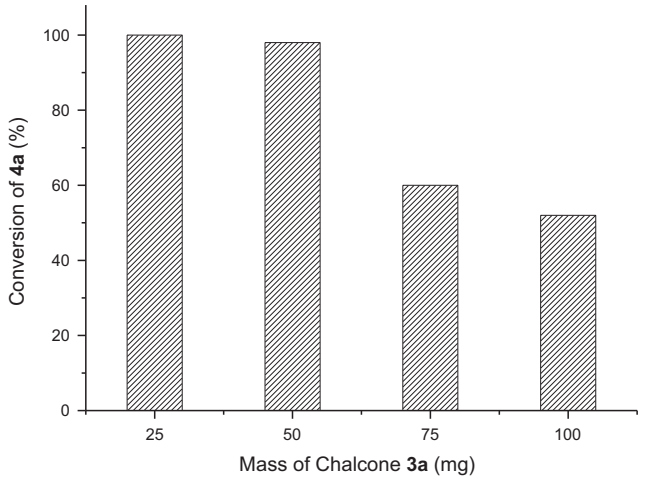


Fig. 1. Assessing the quantity of chalcone **3a** used in the reduction with for *P. citrinum* CBMAI 1186 (2.5 g of fungus, phosphate buffer solution (0.1 mol L^{-1}, Na_2HPO_4/KH_2PO_4, pH=7) at 32 °C and 130 rpm for an orbital shaker in 6 days.

Higher lipophilicity facilitates the permeation of substrates across the cell membrane to the site of action, the enzyme, alloroing greater the substrate conversion to the product (Kayser and Kiderlen, 2001). In this case, fluorine is more lipophilic than bromine and **3a** penetrates the hypha more readily interacting with intracellular enzymes.

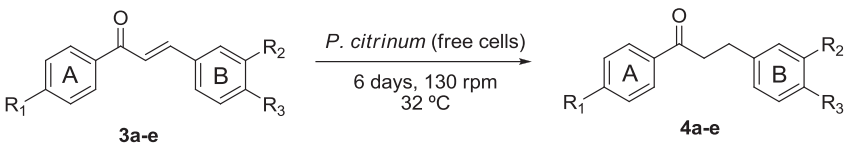
By using whole mycelia of *P. citrinum* CBMAI 1186 chalcone **3c** containing the methoxy group in the *para* position on ring B, underwent 72% conversion to the corresponding dihydrochalcone **4c**, while 91% of chalcone **3d**, containing the methoxy group in the *para* position on ring A, was converted to the corresponding dihydrochalcone **4d** with 91%. The methoxy group attached at the *para* position to ring B of chalcone **3c** decreases the reactivity of the β-carbonyl carbon and consequently decreases the reduction of the carbon–carbon double bond. Therefore, the reduction of chalcone **3c** is less favored than that of chalcone **3a**, which contains a fluorine atom the while the higher conversion of chalcone **3d** (91%), compared to chalcone **3c**, may be due to the smaller electronic influence of the ring A methoxy group on the reactivity of the β-carbonyl carbon.

Finally, the bireduction of chalcone **3e**, containing the nitro substituent, the strongest electron-withdrawing group attached in the *meta* position to ring B, was tested. As expected, high conversion to dihydrochalcone **4e** (97%) was achieved. These results for biocatalytic reduction with the fungus *P. citrinum* CBMAI 1186 confirm the strong influence of substituent groups attached to ring B.

In the case, of chalcone **3e**, an excellent result was obtained by the action of two enzymes to produce a single product, the dihydrochalcone **4e**. Thus, the action of enoate reductase promoted quantitatively the reduction of the carbon–carbon double bond and the nitro group was reduced by the action of nitroreductase. Action of two enzymes on a single substrate, and yielding a single product in hight conversion is unusual by enzymatic methods.

In fact, the nitro group is enzymatically reduced by nitroreductases, by a mechanism that involves a multi-enzyme sequence of oxidation–reduction reactions. In aerobic medium, the nitroaromatic radical anion $Ar–NO_2^-$ generated in the first step of enzyme reduction can interact with the oxygen dissolved in the medium, in the so-called metabolic futile cycle, bearing reoxidized to $ArNO_2$ and forming the radical O_2^-. This radical suffers action of enzymes such as superoxide dismutase, forming peroxide hydrogen (H_2O_2), which may disrupt biological membranes and ferredoxins and also react with enzymes, releasing reactive species ($OH^•$) (Tocher, 1997). There is evidence that the one-electron process of reduction of the nitro group shown by the pair $Ar–NO_2/Ar–NO_2^-$ is responsible for the primary biological action of most nitro compounds to amine group.

The chalcone **3e**, however, was probably reduced by an oxygen independent nitroreductases in three consecutive two-electron transfers.



3a-R_1= R_2=H, R_3=F
3b-R_1= R_2=H; R_3=Br
3c-R_1= R_2=H, R_3=OCH_3
3d-R_1= OCH_3,R_2= R_3= H
3e-R_1= R_3=H, R_2=NO_2

4a- 98[a] (79)[b]
4b- 49[a] (20)[b]
4c- 72[a] (37)[b]
4d- 91[a] (50)[b]
4e-R_1= R_3=H, R_2=NH_2 97[a] (30)[b]

Scheme 2. Bioreduction reactions of chalcones **3a-e** by whole free cells of *P. citrinum* CBMAI 1186. [a]Conversion obtained by GC–MS; [b]Yield isolated obtained after purification by column chromatography.

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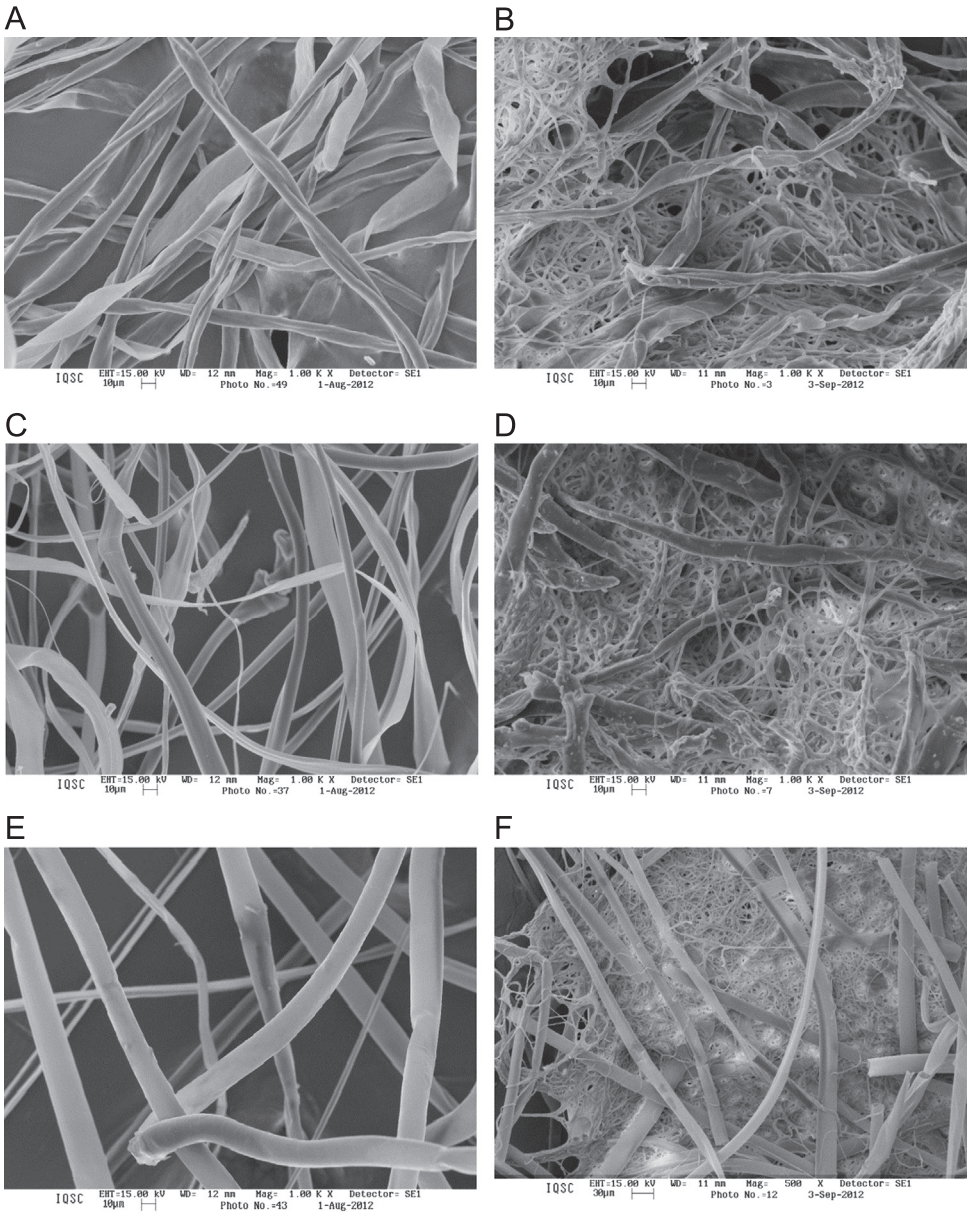

Fig. 2. Scanning electron micrographs: (A) Cotton fibers. (B) Whole hyphae of *P. citrinum* CBMAI 1186 immobilized on cotton fiber. (C) Fiber of fibroin. (D) Whole *P. citrinum* CBMAI 1186 immobilized on fibroin fiber. (E) Fiber of kapok. (F) Whole *P. citrinum* CBMAI 1186 immobilized on kapok fiber.

The results suggest that the enzymes involved in the reduction of the carbon–carbon double bond of chalcones **3a–e** are enoate reductases from *P. citrinum* CBMAI 1186. The catalytic mechanism of the asymmetric reduction of alkenes catalyzed by enoate reductases has been studied detail and it has been shown that a hydride (derived from FDH_2) is stereoselectively transferred to C_β, whereas a Tyr residue adds a proton (which is ultimately derived from the solvent) to C_α from the opposite side (Supporting information). As a consequence of the stereochemistry of this mechanism, the overall addition of [H_2] proceeds in a *trans*-fashion with absolute stereospecificity (Toogood et al., 2008; Yanto et al., 2010). In this case was more effective in competition with the alcohol dehydrogenases for the type of substrate used in this study.

Dihydrochalcone derivatives were obtained by reduction of carbon–carbon bond from chalcones, using Zn metallic (10 equiv.) and HOAc (200 equiv.) and assisted by ultrasound for 6 min reaction (Zhang et al., 2008). Hantzsch ester was used as a source of hydride in reducing the carbon–carbon double bond of enone derivatives by refluxing in xylene for 30 min at 130 °C (Xu et al., 2008). It was also described an method for the reduction of α,β-unsaturated enones with *N*, *N*-dimethylbenzylimidazole (1.5 equiv.) as a source of

Table 1
Biosynthesis of dihydrochalcone (**4a**) by whole free and immobilized mycelia of *P. citrinum* CBMAI 1186 (6 days, 130 rpm, 32 °C).

Entry	Matrices	Yield of **4a** by fresh biocatalyst (%)	Yield of **4a** after one month biocatalyst storage (%)
1	Free mycelia	98[a](79)[b]	15[a](8)[b]
2	Cotton fiber	92[a](75)[b]	30[a](25)[b]
3	Fibroin fiber	80[a](68)[b]	22[a](19)[b]
4	Kapok fiber	93[a](73)[b]	36[a](27)[b]

[a] Conversion determined by GC–MS.
[b] Yield isolated after purification by column chromatography.

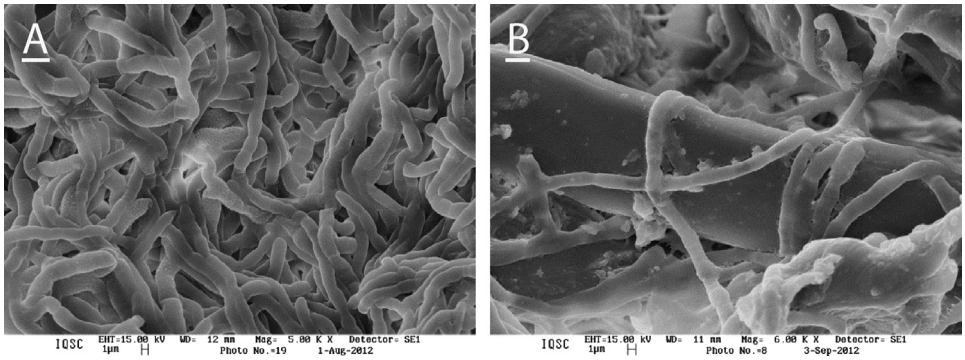

Fig. 3. Scanning electron micrographs: (A) Free mycelium of *P. citrinum*. CBMAI 1186 and (B) Whole hyphae *P. citrinum* CBMAI 1186 immobilized on fibroin fiber.

hydride ion and $Mg(ClO_4)_2$ (10 mol%) as catalyst in toluene for 24 h at 80 °C (Feng et al., 2012). In some cases these chemical methods afforded good yields, but under conditions less efficient and less eco-friendly compared to our proposed method using whole cells.

Following the excellent results in the reduction of chalcones **3a–e** with free *P. citrinum* CBMAI 1186 new experiments were performed in which whole mycelia immobilized on various natural biopolymers (cotton, kapok and fibroin) were tested (Fig. 2). In these tests, chalcone **3a** was the substrate summarizes the results (Table 1).

The experiments with *P. citrinum* CBMAI 1186 hyphae immobilized on cotton yielded the dihydrochalcone **4a** with 92% conversion (75% isolated yield); on fibroin, 80% conversion (78% isolated yield) and on kapok, 93% conversion (73% isolated yield) (Table 1). The same experiment was simultaneously conducted with free whole mycelia, achieving 98% conversion (79% isolated yield) to **4a** by reduction of the carbon–carbon double bond. These studies demonstrated that the immobilized whole mycelia of *P. citrinum* CBMAI 1186 presented excellent biocatalytic activity on natural supports.

In order to assess the reuse of immobilized whole cells hyphae on cotton, fibroin and kapok, the bioreduction of chalcone **4a** was performed after one month storage of supports at 4 °C in the refrigerator. It must be stressed that after the immobilized fungus was used in the first cycle, the supported mycelium was filtered, washed with ethyl acetate and preserved in the refrigerator one month. It was observed that a decrease occurred in the formation of the desired product for all supports used (Table 1).

However, the immobilized cells preserved the enzymes, since they were active after being stored for one month. The experiment performed with free whole *P. citrinum* CBMAI 1186 mycelia exhibited a more pronounced decrease in conversion of **3a**–**4a**, compared to the immobilized cells after month, thus demonstrating the fungus had superior activity when supported on the natural materials. These results demonstrate that the natural biopolymers (cotton, fibroin and kapok) are excellent supports to immobilize filamentous fungi, such as *P. citrinum* CBMAI 1186 (Table 1).

It is known that the fibroin consists essentially of common short side-chain amino acids (glycine, serine and alanine). This amino acid composition results in a very stable crystal structure of β-pleated sheet over about 60% of the molecule. This secondary structure of fibroin is very resistant to enzymatic hydrolysis (Seves et al., 1998). In addition, SEM micrographs of the fungus *P. citrinum* CBMAI 1186 immobilized on fibroin fibers show evidence of strong adhesion of the mycelium to the fiber surface, possibly indicating a microbial attack on the fiber, which can serve as a source of nitrogen and carbon for the fungus (Fig. 3). This strong binding of the mycelia with fibroin may hinder the interaction between the microorganism and substrate of interest since the area of contact of the fungus with the medium is reduced. This hypothesis might explain the lower conversion of substrate **4a** (80%) with the fibroin support than that obtained when *P. citrinum* CBMAI 1186 was immobilized on cotton (92%) or kapok fiber (93%), as reported in Table 1.

By SEM it was observed that the fibers of cotton and kapok are also intertwined with the hyphae of the fungus, but without the strong adhesion observed with the fibroin. These biopolymers are excellent supports for the immobilization of the fungus *P. citrinum* CBMAI 1186 maintaining its activity well and moreover they are low cost.

4. Conclusions

In summary, in this paper is presented the first investigation of the use of whole mycelia of the marine fungus *P. citrinum* CBMAI 1186 in the chemoselective biotransformation of chalcones. The substrates **3a–e** were biotransformed into dihydrochalcones **4a–e** with excellent conversions catalyzed by enoate reductases in the marine fungus. In addition, the filamentous fungus was efficiently immobilized on natural biopolymers as low-cost supports. All reactions conducted with the immobilized fungus *P. citrinum* CBMAI 1186 showed better results than free mycelia after month of storage. Finally, this study shows the potential use of an immobilized filamentous fungus for the chemoselective reduction of chalcones by enoate reductase.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bcab.2014.04.001.

References

Abdel-Halim, E.S., Abdel-Mohdy, F.A., Fouda, M.M.C., El-Sawy, S.M., Hamdy, I.A., Al-Deyab, S.S., 2011. Antimicrobial activity of monochlorotriazinyl-beta-cyclodextrin/chlorohexidin diacetate finished cotton fabrics. Carbohydr. Polym. 86, 1389–1394.

Anto, R.J., Sukumaran, K., Kuttan, G., Rao, M.N.A., Subbaraju, V., Kuttan, R., 1995. Anticancer and antioxidant activity of synthetic chalcones and related-compouds. Cancer Lett. 97, 33–37.

Aponte, J.C., Verastegui, M., Malaga, E., Zimic, M., Quiliano, M., Vaisberg, A.J., Gilman, R.H., Hammond, G.B., 2008. Synthesis, cytotoxicity, and anti-*Trypanosoma cruzi* activity of new chalcones. J. Med. Chem. 51, 6230–6234.

Asakura, T., Minami, M., Shimada, R., Demura, M., Osanai, M., Fujito, T., Imanari, M., Ulrich, A.S., 1997. H-2-Labeling of silk fibroin fibers and their structural characterization by solid-state H-2 NMR. Macromolecules 30, 2429–2435.

Boeck, P., Falcao, C., Leal, P.C., Yunes, R.A., Cechinel, V., Torres-Santos, E.C., Rossi-Bergmann, B., 2006. Synthesis of chalcone analogues with increased antileishmanial activity. Bioorg. Med. Chem. 14, 1538–1545.

Cabrera, M., Simoens, M., Falchi, G., Lavaggi, M.L., Piro, O.E., Castellano, E.E., Vidal, A., Azqueta, A., Monge, A., De Ceráin, A.L., Sagrera, G., Seoane, G., Cerecetto, H., González, M., 2007. Synthetic chalcones, flavanones, and flavones as antitumoral agents: biological evaluation and structure-activity relationships. Bioorg. Med. Chem. 15, 3356–3367.

Cheenpracha, S., Karalai, C., Ponglimanont, C., Subhadhirasakul, S., Tewtrakul, S., 2006. Anti-HIV-1 protease activity of compounds from Boesenbergia pandurata. Bioorg. Med. Chem. 14, 1710–1714.

Chibata, I., Tosa, T., 1981. Use of immobilized celles. Annu. Rev. Biophys. Bioeng. 10, 197–216.

Chibata, I., Tosa, T., Sato, T., 1986. Biocatalysis — Immobilized cells and enzymes. J. Mol. Catal. B: Enzym. 37, 1–24.

Couto, S., Sanroman, M.A., Hofer, D., Gubitz, G.M., 2004. Stainless steel sponge: a novel carrier for the immobilisation of the white-rot fungus Trametes hirsuta for decolourization of textile dyes. Bioresour. Technol. 95, 67–72.

Cruz, R., Cruz, V.D., Belini, M.Z., Belote, J.G., Vieira, C.R., 1998. Production of fructooligosaccharides by the mycelia of *Aspergillus japonicus* immobilized in calcium alginate. Bioresour. Technol. 65, 139–143.

Ducki, S., Forrest, R., Hadfield, J.A., Kendall, A., Lawrence, N.J., McGown, A.T., Rennison, D., 1998. Potent antimitotic and cell growth inhibitory properties of substituted chalcones. Bioorg. Med. Chem. Lett. 8, 1051–1056.

Feng, H., Li, Y., Eycken, E.V.V.der, Peng, Y., Song, G., 2012. Synthesis of polysubstituted pyridines under combined microwave and ultrasound irradiation: K_2CO_3 promoted tandem addition/cyclization/hydrogen shift process. Tetrahedron Lett. 53, 1160–1162.

Joshi, A.S., Li, X.C., Nimrod, A.C., ElSohly, H.N., Walker, L.A., Clark, A.M., 2001. Dihydrochalcones from Piper longicaudatum. Planta Med. 67, 186–188.

Kanagarajan, V., Gopalakrishnan, M., 2012. Pyrimidino piperazinyl acetamides: innovative class of hybrid acetamide drugs as potent antimicrobial and antimycobacterial agents. Pharm. Chem. J. 46, 26–34.

Kayser, O., Kiderlen, A.F., 2001. in vitro leishmanicidal activity of naturally occurring chalcones. Phytother. Res. 15, 148–152.

Kim, Y.S., Kumar, V., Trepel, J.B., 2012. Methoxychalcone inhibitors of androgen receptor translocation and function. Bioorg. Med. Chem. Lett. 22, 2105–2109.

Meechaisue, C., Wutticharoenmongkol, P., Waraput, R., Huangjing, T., Ketbumrung, N., Pavasant, P., Supaphol, P., 2007. Preparation of electrospun silk fibroin fiber mats as bone scaffolds: a preliminary study. Biomed. Mater. 2, 181–188.

Nahra, F., Macé, Y., Lambin, D., Riant, O., 2013. Copper/palladium-catalyzed 1,4 reduction and asymmetric allylic alkylation of α,β-unsaturated ketones: enantioselective dual catalysis. Angew. Chem. 52, 3208–3212.

Nikolic, T., Kostic, M., Praskalo, J., Pejic, B., Petronijevic, Z., Skundric, P., 2010. Sodium periodate oxidized cotton yarn as carrier for immobilization of trypsin. Carbohydr. Polym. 82, 976–981.

Oberdorfer, G., Gruber, K., Faber, K., Hall, K., 2012. Stereocontrol strategies in the asymmetric bioreduction of alkenes. Synlett 13, 1857–1864.

Pawluc, P., Hreczycho, G., Szudkowska, J., Kubicki, M., Marciniec, B., 2009. New one-pot synthesis of (*E*)-beta-aryl vinyl halides from Styrenes. Org. Lett. 11, 3390–3393.

Pawluc, P., Szudkowska, J., Hreczycho, G., Marciniec, B., 2011. One-pot synthesis of (E)-styryl sktones from styrenes. J. Org. Chem. 76, 6438–6441.

Peart, P.C., Chen, A.R.M., Reynolds, W.F., Reese, P.B., 2012. Entrapment of mycelial fragments in calcium alginate: a general technique for the use of immobilized filamentous fungi in biocatalysis. Steroids 77, 85–90.

Porto, A.L.M., Cassiola, F., Dias, S.L.P., Joekes, I., Gushikem, Y., Rodrigues, J.A.R., Moran, P.J.S., Manfio, G.P., Marsaioli, A.J., 2002. *Aspergillus terreus* CCT 3320 immobilized on chrysotile or cellulose/TiO_2 for sulfide oxidation. J. Mol. Catal. B: Enzym. 19, 327–334.

Rocha, L.C., Ferreira, H.V., Pimenta, E.F., Berlinck, R.G.S., Seleghim, M.H.R., Javaroti, D. C.D., Sette, L., Bonugli, R., Porto, A.L.M., 2009. Bioreduction of alpha-chloroacetophenone by whole cells of marine fungi. Biotechnol. Lett. 31, 1559–1563.

Rocha, L.C., Ferreira, H.V., Pimenta, E.F., Berlinck, R.G.S., Rezende, M.O.O., Landgraf, M.D., Seleghim, M.H.R., Sette, L.D., Porto, A.L.M., 2010. Biotransformation of α-bromoacetophenones by the marine fungus *Aspergillus sydowii*. Mar. Biotechnol. 12, 552–557.

Rocha, L.C., de Souza, A.L., Rodrigues, U.P., Campana, S.P., Sette, L.D., Porto, A.L.M., 2012. Immobilization of marine fungi on silica gel, silica xerogel and chitosan for biocatalytic reduction of ketones. J. Mol. Catal. B: Enzym. 84, 160–165.

Sah, M.K., Pramanik, K., 2011. Preparation, characterization and in vitro study of biocompatible fibroin hydrogel. Afr. J. Biotechnol. 10, 7878–7892.

Seves, A., Romano, M., Maifreni, T., Sora, S., Ciferri, O., 1998. The microbial degradation of silk: a laboratory investigation. Int. Biodeterior. Biodegrad. 42, 203–211.

Shang, J.Y., Li, F., Bai, X.F., Jiang, J.X., Yang, K.F., Lai, G.Q., Xu, L.W., 2012. Malononitrile-assisted highly chemoselective bismuth triflate catalyzed conjugate reduction of α,β-unsaturated ketones. Eur. J. Org. Chem. 2012, 2809–2815.

Shiomi, T., Adachi, T., Toribatake, K., Zhou, L., Nishiyama, H., 2009. Asymmetric β-boration of α,β-unsaturated carbonyl compounds promoted by chiral rhodium-bisoxazolinylphenyl catalysts. Chem. Commun. 2009, 5987–5989.

Silva, V., Stambuk, B., Nascimento, M., 2010. Efficient chemoselective biohydrogenation of 1,3-diary1-2-propen-1-ones catalyzed by Saccharomyces cerevisiae yeasts in biphasic system. J. Mol. Catal. B: Enzym. 63, 157–163.

Sommovigo, M., Howard, A., 1993. Mild reduction of α,β-unsaturated ketones and aldehydes whit an oxygen-activated palladium catalyst. Tetrahedron Lett. 34, 59–62.

Stolarzewicz, I., Bialecka-Florjanczyk, E., Majewska, E., Krzyczkowska, J., 2011. Immobilization of Yeast on polymeric supports. Chem. Biochem. Eng. Q. 25, 135–144.

Tasnádi, G., Winkler, C.K., Clay, D., Sultana, N., Fabian, W.M.F., Hall, M., Ditrich, K., Faber, K., 2012. A Substrate-driven approach to determine reactivities of α, β-unsaturated carboxylic esters towards asymmetric bioreduction. Chem. Eur. J. 18, 10362–10367.

Tocher, J., 1997. Reductive activation of nitroheterocyclic compounds. Gen Pharmacol. 28, 485–487.

Toogood, H., Fryszkowska, A., Hare, V., Fisher, K., Roujeinikova, A., Leys, D., Gardiner, J., Stephens, G., Scrutton, N., 2008. Structure-based insight into the asymmetric bioreduction of the C=C double bond of alpha, beta-unsaturated Nitroalkenes by pentaerythritol tetranitrate reductase. Adv. Synth. Catal. 350, 2789–2803.

Wang, J.T., Zheng, Y., Wang, A.Q., 2012. Effect of kapok fiber treated with various solvents on oil absorbency. Ind. Crops Prod. 40, 178–184.

Xu, H.J., Liu, Y.C., Fu, Y., 2008. Metal-free uncatalyzed reduction of α, β-unsaturated ketones mediated by Hantzsch 1,4-dihydropyridine. Chin. J. Chem. 26, 599–601.

Yanto, Y., Hall, M., Bommarius, A.S., 2010. Nitroreductase from Salmonella typhimurium: characterization and catalytic activity. Org. Biomol. Chem. 8, 1826–1832.

Zhang, L., Zhang, W., Wang, X., Bao, K., Lu, G., Lin, J., 2008. Ultrasound Assisted selective reduction of chalcones to dihydrochalcones by Zn/HOAc. Lett. Org. Chem. 5, 370–373.

Zhang, Y.Q., 1998. Natural silk fibroin as a support for enzyme immobilization. Biotechnol. Adv. 16, 961–971.

Zhang, Y.Q., Zhou, W.L., Shen, W.D., Chen, Y.H., Zha, X.M., Shirai, K., Kiguchi, K., 2005. Synthesis, characterization and immunogenicity of silk fibroin-L-asparaginase bioconjugates. J. Biotechnol. 120, 315–326.

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