Journal of Molecular Catalysis B: Enzymatic 84 (2012) 160–165

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Immobilization of marine fungi on silica gel, silica xerogel and chitosan for biocatalytic reduction of ketones

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ARTICLE INFO

Article history:
Available online 14 July 2012

Keywords:
Biocatalysis
Penicillium citrinum
Aspergillus sclerotiorum
Whole living cells
Scanning electron microscopy

ABSTRACT

The scanning electron microscopy (SEM) analysis showed that whole living hyphal of marine fungi *Aspergillus sclerotiorum* CBMAI 849 and *Penicillium citrinum* CBMAI 1186 were immobilized on support matrices of silica gel, silica xerogel and/or chitosan. *P. citrinum* immobilized on chitosan catalyzed the quantitative reduction of 1-(4-methoxyphenyl)-ethanone (**1**) to the enantiomer (S)-1-(4-methoxyphenyl)-ethanol (**3b**), with excellent enantioselectivity (*ee* > 99%, yield = 95%). Interestingly, ketone **1** was reduced with moderate selectivity and conversion to alcohol **3b** (*ee* = 69%, *c* 40%) by the free mycelium of *P. citrinum*. This free mycelium of *P. citrinum* catalyzed the production of the (*R*)-alcohol **3a**, the antipode of the alcohol produced by the immobilized cells. *P. citrinum* immobilized on chitosan also catalyzed the bioreduction of 2-chloro-1-phenylethanone (**2**) to 2-chloro-1-phenylethanol (**4a,b**), but in this case without optical selectivity. These results showed that biocatalytic reduction of ketones by immobilization hyphal of marine fungi depends on the xenobiotic substrate and the support matrix used.

1. Introduction

Biocatalytic processes can take advantage of the habitat-related properties of marine enzymes, such as salt tolerance, hyperthermostability, barophilicity and cold adaptivity, as well as the common enzyme characteristics of substrate specificity and affinity [1]. For a biocatalyst to be effective in a given process, it must be protected from interaction with the solvent in which the reaction is carried out, as this could lead to its inactivation, halting the catalytic reaction [2]. In this context, new immobilization techniques have been developed to provide stability to enzymes and facilitate their recovery and reutilization [3]. Immobilized enzymes are defined as enzymes that are physically confined or localized in a certain region of space with retention of catalytic activities that may be used continuously or repeatedly [4]. Enzymatic processes in the immobilization of biocatalysts offers advantages such as increased stability and improved process control, yield and purity of the final product, besides enabling the use of reactors of a variety of configurations [5]. One very simple immobilization technique is the adsorption of the enzyme on porous material, such as celite, silica or polyurethane foam. Immobilization has been achieved in a number of other ways, by covalent linkage, ion-coagulation, crosslinking and/or entrapment methods, on a variety of matrices [6,7]. Among the biocatalysts, purified enzymes and whole living cells of bacteria and yeasts are commonly immobilized in various supports and matrices, but the immobilization of filamentous fungi has been rarely described [8]. However, whole mycelia of filamentous fungi are often used in biocatalytic processes, especially in redox reactions with various organic compounds [9]. The morphology of fungi is very complex, with different cellular structures at each stage of their life cycle [10]. One way to preserve the morphological structure of these microorganisms is the immobilization of whole living cells, such that the desired catalytic activity is preserved, and the immobilized cells can then be used in the laboratory and on the industrial scale [11]. The immobilization of whole living cells of microorganisms is interesting in view of the increasing number of industrial applications of biocatalytic process in the pharmaceutical and food industries, especially those involving filamentous fungi with high production of enzymes [12].

Most of the fungi contain chitin in the cell wall, forming 22–40% of the wall constituents [13]. The presence of chitin, together with that of other polysaccharides, has been used as a criterion in fungal taxonomy. This polysaccharide, which forms fibrils of different lengths, depending on the species and the cellular location [10,14], is a homopolymer and it has a broad spectrum of distribution in the biosphere, being formed in the shells of

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http://dx.doi.org/10.1016/j.molcatb.2012.05.025

Table 1
Reduction of ketones **1** and **2** by free and immobilized whole living cells of marine fungi (9 days, 120 rpm, 32 °C).[a]

1-(4-Methoxyphenyl)ethanone (**1**)

Entry	Fungi marine	Supports matrices	*c* **1**	*c* **3a**; **3b**	*ee* **3a**; **3b** (ac)	Yield (%)
1	*Penicillium citrinum*	Not immobilized	60	40	69 (*R*)	35
2		silica gel	100	0	–	–
3		silica xerogel	100	0	–	–
4		chitosan	–	100	>99 (*S*)	95
5	*Aspergillus sclerotiorum*	Not immobilized	–	100	>99 (*S*)	90
6		silica gel	–	100	>99 (*S*)	97
7		silica xerogel	100	0	–	–

2-Chloro-1-phenylethanone (**2**)

Entry	Fungi marine	Supports matrices	*c* **2**	*c* **4a**; **4b**	*ee* **4a**; **4b** (ac)	Yield (%)
8	*Penicillium citrinum*	Not immobilized	30	70	31 (*R*)	66
9		silica gel	35	65	25 (*S*)	62
10		silica xerogel	100	0	–	–
11		chitosan	–	100	0	98
12	*Aspergillus sclerotiorum*	Not immobilized	11	89	68 (*R*)	85
13		silica gel	–	100	65 (*R*)	97
14		silica xerogel	100	0	–	–

[a] See Section 2.
Yield, obtained after purification by column chromatography; *c* (%), concentration determined by GC analyses using chiral column; *ee* (%), enantiomeric excess; ac, absolute configuration.

crustaceans, such as crab, shrimp and lobster, and the exoskeletons of marine zoo-planktons such as coral and jellyfish, and insects such as butterflies and ladybirds [15,16]. Chitosan, the *N*-deacetylated derivative of chitin, is a β(1-4)-linked copolymer built of 2-amino-2-deoxy-D-glucopyranose (GlcN) and 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) units which is characterized by its average degree of acetylation (DA), expressing the average content of GlcNAc units along its chains [17,18].

In the present study, we report the immobilization of whole living mycelial cells of the marine fungi *Penicillium citrinum* CBMAI 1186 and *Aspergillus sclerotiorum* CBMAI 849 on silica gel, xerogel and chitosan to catalyze the asymmetric reduction of 1-(4-methoxyphenyl)-ethanone (**1**) and 2-chloro-1-phenylethanone (**2**).

2. Experimental

2.1. General methods

The substrates 1-(4-methoxyphenyl)-ethanone (**1**) and 2-chloro-1-phenylethanone (**2**) were purchased from Sigma–Aldrich. Sodium borohydride, sodium hydroxide and ethanol were purchased from Vetec or Synth. All manipulations involving the fungi *A. sclerotiorum* CBMAI 849 and *P. citrinum* CBMAI 1186 were carried out under sterile conditions in a Veco laminar flow cabinet. Technal TE-421 or Superohm G-25 orbital shakers were employed in the biocatalysed conversion experiments. The products of the reduction reactions were purified out by column chromatography (CC) over silica gel (230–400 mesh). The column was eluted with mixtures of *n*-hexane and ethyl acetate (Hex:EtOAc – 9:1 and 8:2) and monitored by TLC, using pre-coated silica gel 60 F254 layers (aluminum-backed: Sorbent). The alcohols were analyzed in a Shimadzu GC2010 gas chromatograph, equipped with a Varian Chiral column, CP-Chiralsil-DEX, β-Cyclodextrin (25 m × 0.25 mm i.d.; 0.39 μm), an AOC 20i auto injector and a flame ionization detector (FID). The chromatography analysis were carried out employing the following conditions: oven temperature initially 120 °C (2 min) at 165 °C (8 min) and increased at 2 °C/min; total run time 32.5 min; injector temperature 200 °C; detector temperature 200 °C; injector split ratio 1:20; nitrogen carrier gas at a pressure of 69.2 kPa. The enantiomeric excess (*ee*) of 1-(4-methoxyphenyl)-ethanol (**3a,b**) and 2-chloro-1-phenylethanol (**4a,b**) were determined by GC-FID analysis. The retention times of the alcohols (*S*)-enantiomer **3b**, (*R*)-enantiomer **3a**, (*S*)-enantiomer **4b** and (*R*)-enantiomer **4a** were 15.2 min, 15.8 min, 16.5 min, and 17.0 min, respectively. For carrying out the gas chromatography-mass spectrometry analysis, a Shimadzu GC 2010 plus gas chromatography system with a 30 m × 0.25 mm × 0.25 DB5 fused silica column (J&W Scientific) coupled to a mass selective detector (Shimadzu MS 2010 plus) in electron ionization (EI, 70 eV) mode was used.

2.2. Absolute configuration

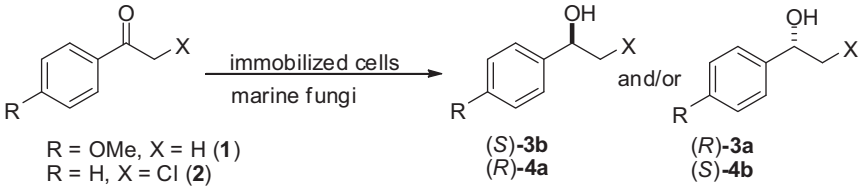
The optical rotation of the purified products 1-(4-methoxyphenyl)-ethanol (**3**) and 2-chloro-1-phenylethanol (**4**) were measured in a Perkin-Elmer (Waltham, MA, USA) 241 polarimeter with a 1 dm cuvette, at the sodium D-line. The absolute configurations of the alcohols **3a,b** and **4a,b** were determined by comparing the measurements of its specific rotations with those reported in the literature [19,20].

2.3. Chemical synthesis of 1-(4-methoxyphenyl)-ethanol (*3a,b*) and 2-chloro-1-phenylethanol (*4a,b*)

The racemic 1-(4-methoxyphenyl)-ethanol (**3a,b**) and 2-chloro-1-phenylethanol (**4a,b**) were synthesized by reduction of the 1-(4-methoxyphenyl)-ethanone (**1**) (100.0 mg, 0.70 mmol) and 2-chloro-1-phenylethanone (**2**) (100.0 mg, 0.65 mmol) with sodium borohydride (30.0 mg, 0.77 mmol) in 25 mL methanol [21]. The spectroscopic data (^{1}H and ^{13}C NMR, MS and IR) of alcohols **3a,b** and **4a,b** were in agreement with those reported in the literature [19,20].

2.4. Marine fungi

The marine fungi *P. citrinum* CBMAI 1186 and *A. sclerotiorum* CBMAI 849 were isolated from the marine alga *Caulerpa* sp. and a *Brazilian cnidarian* species, namely *Palythoa variabilis*, respectively, which were collected by Prof. R.G.S. Berlinck in the town of São Sebastião, on the coast of the State of São Paulo, Brazil. The fungi used in this work were identified by both conventional and molecular methods at the Chemical, Biological and Agricultural



Scheme 1. Reduction of ketones **1** and **2** by immobilized marine fungi.

Pluridisciplinary Research Center (CPQBA) at the State University of Campinas (UNICAMP), SP, Brazil [20,22,23].

2.5. Biocatalytic reduction of ketones *1* and *2* by marine fungi

The marine fungi were grown in culture media and artificial sea water [20]. Small slices of solid medium (0.5 × 0.5 cm) bearing mycelia of *A. sclerotiorum* and *P. citrinum* were cut from the stock solid culture and used to inoculate 1 L of liquid culture medium contained in Erlenmeyer flasks (2 L). The mycelia were incubated at 32 °C for 9 days in a rotatory shaker (120 rpm). Following, the mycelia were harvested by Buchner filtration and suspended in a buffer solution contained in Erlenmeyer flasks (250 mL). The biocatalytic reductions were carried out with 5.0 g (wet weight) of mycelium and 0.50 mmol of ketones **1** or **2**, previously dissolved in 300 μL of dimethyl sulfoxide, and mixed into 100 mL phosphate buffer solution (Na_2HPO_4/KH_2PO_4, pH = 7, 0.1 M). The mixtures were incubated for 9 days in an orbital shaker at 32 °C and 120 rpm. After 9 days the samples were extracted with 2.0 mL ethyl acetate by mixing on a vortex and centrifuging at 6000 rpm for 6.0 min in a HERMLE Z-200 A, and analyzed by GC-FID and GC–MS. The products were purified by column chromatography over silica gel to yield the alcohols **3a,b** or **4a,b** (Table 1 and Scheme 1). All the reactions were performed in duplicates.

2.6. Support matrix preparation

The support matrices of silica gel 60 F254 layer (aluminum-backed: Sorbent) and chitosan [24] of high molecular weight (159,000 g/mol, average degree of acetylation 25%, lot code: 407490/123500), on the reagent tetraethylorthosilicate (TEOS), were purchased from a commercial source (Sigma–Aldrich). All reagents were utilized without washing or purification. The xerogel support was prepared by mixing the following chemicals in a Teflon® reactor TEOS (26.8 mL, 0.12 mol), ethanol (100.0 mL), deionized water (1.5 mL, resistivity: 18.2 MΩ cm^{-1}) and 1.0 mL of solution NaOH 0.1 mol L^{-1}. This mixture was heated to 60 °C and kept at this temperature for 20 min under constant magnetic stirring. Finally, the container with the mixture was left at room temperature until complete drying and the xerogel was collected. Hereafter, this silica is going to be named xerogel [25].

2.7. Immobilization of whole living cells of A. sclerotiorum and P. citrinum on the support matrices

The whole living cells of *A. sclerotiorum* and *P. citrinum* were grown as described in Section 2.5. The mycelia were harvested by filtration and 5.0 g were suspended in 100 mL of phosphate buffer solution (Na_2HPO_4/KH_2PO_4, pH = 7, 0.1 M) in a 250 mL Erlenmeyer flask. The supports matrices of commercial silica gel (10.0 g), synthesized silica xerogel (10.0 g) or chitosan (3.0 g) were added to each Erlenmeyer flask. The mixtures were incubated for 24 h in an orbital shaker maintained at 32 °C and 120 rpm. After filtration, the immobilized mycelia were immediately used in the biocatalytic reductions. The supported fungi were analyzed by scanning electron microscopy as described in the following section (Figs. 1 and 2).

2.8. Scanning electron microscopy (SEM)

For SEM analysis, the surfaces of samples of immobilized mycelia of fungi *A. sclerotiorum* and *P. citrinum* were washed with water to remove the non-adhering support matrix. Samples were dehydrated in a graded series of water–ethanol solutions (10%, 25%, 40%, 50%, 70%, 80%, 90% and 100%), 10 mL in 50 mL Erlenmeyer flasks, for 15 min at each step. Samples were air dried at room temperature, and coated with 8–10 nm of gold by argon ion sputtering using a Baltec MCS 010 model sputter. Images were obtained at a magnification of 200–5.00 K in a scanning electron microscope (Leica-Zeiss LEO 440) operating at an accelerating voltage of 20 kV (Figs. 1 and 2) using a Secondary Electron Detector positioned among 13–25 away from the sample.

2.9. Reduction of ketones *1* and *2* by immobilized mycelia of A. sclerotiorum and P. citrinum

Biocatalytic reduction reactions were carried out with fungal mycelia immobilized on (wet weight) 12.0 g silica gel, 12.0 g silica xerogel or 7.0 g chitosan. The ketones **1** and **2**, previously dissolved in 300 μL of dimethyl sulphoxide, were added to 100 mL of 0.1 M phosphate buffer solution (Na_2HPO_4/KH_2PO_4, pH = 7). The mixtures containing the substrates and the immobilized mycelia were incubated for 9 days on an orbital shaker at 32 °C and 120 rpm. The cultures were extracted with ethyl acetate (1.0 mL) by vortexing followed by centrifugation (6000 rpm for 6.0 min in a HERMLE Z-200 A centrifuge) and analyzed by GC-FID and GC–MS. The products were purified by CC over silica gel, to yield the alcohols **3a,b** and **4a,b** (Table 1 and Scheme 1).

3. Results and discussion

Whole living cells of marine fungi are sometimes preferred for biocatalytic applications and the selection of an appropriate fungus is an important part of this process. Recently we have investigated the use of filamentous fungi from marine environments for the reduction of prochiral ketones [20,26]. Due to the excellent results obtained in the biocatalytic reduction of ketones with free cells, in this study we investigated the immobilization of living mycelia of marine fungi on various support matrices.

The biocatalytic reductions of 1-(4-methoxyphenyl) ethanone (**1**) and 2-chloro-1-phenylethanone (**2**) were attempted with immobilized whole cells of the marine fungi *P. citrinum* and *A. sclerotiorum* (Scheme 1). Control reactions were performed with free fungal mycelia without the support matrices. The results are shown in Table 1. The immobilization of marine fungi was effected with three support matrices, namely silica gel, silica xerogel and chitosan. The choice of these three supports was based on previous reports and data about the immobilization of the fungi *Aspergillus* sp. and *Monascus kaoliang* [3,6,18,27].

The whole living cells of *P. citrinum* and *A. sclerotiorum* were successfully immobilized as shown on the SEM micrographs (Figs. 1 and 2). Both silica matrices present irregular dense particles with average particle size for silica larger than for the xerogels, respectively 77 ± 20 μm and 41 ± 24 μm. It can be observed that

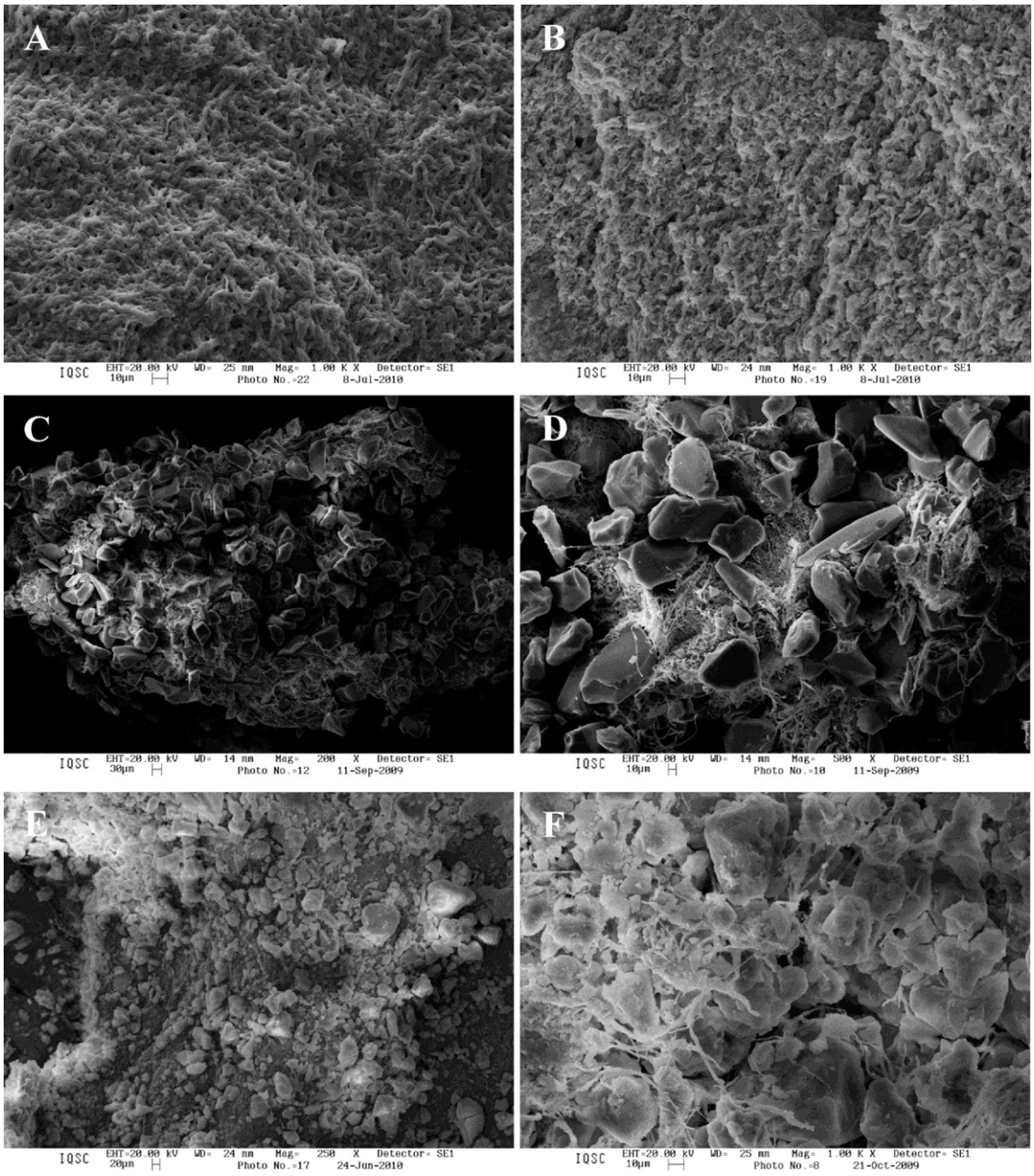


Fig. 1. Scanning electron microscopy micrographs. (A) Free cells of *A. sclerotiorum*. (B) Free cells of *P. citrinum*. (C and D) Whole cells of *P. citrinum* immobilized on silica gel. (E and F) Whole cells of *P. citrinum* immobilized on silica xerogel.

the support matrices of silica gel, silica xerogel and chitosan are closely intertwined with hyphae (Figs. 1 and 2). The hypha diameter in both silicas are statically the same, 3.7 ± 2.1 μm for silica gel and 2.6 ± 2.2 μm for the xerogels.

The free (not immobilized) live mycelia of *P. citrinum* catalyzed the anti-Prelog stereospecific reduction of 1-(4-methoxyphenyl)-ethanone (**1**), and after 9 days the (*R*)-1-(4-methoxyphenyl)-ethanol (**3a**) was produced with moderate optical purity and conversion (*ee* = 69%, *c* 40%, entry 1, Table 1). The (*R*)-alcohol **3a** was isolated with 35% yield after purification by column chromatography over silica gel. When the mycelia of the fungus *P. citrinum* were immobilized on silica gel and silica xerogel, the reduction of ketone **1** failed to occur (entries 2 and 3, Table 1).

As can be seen in the micrographs of silica gel and silica xerogel, the fungal cells were covered by the support materials (Fig. 1C–F). It is possible that the total coverage of the mycelia must have hindered the access of the substrate to the reductases, preventing the reduction of the ketone **1** under these conditions.

However, the fungus *P. citrinum* immobilized on chitosan catalyzed quantitatively the reduction of 1-(4-methoxyphenyl)-ethanone (**1**) to alcohol (*S*)-**3b** (*c* 100%). Interestingly, in this case, the reduction was greatly enhanced by this type of organic support matrix. The reaction with the fungus immobilized on chitosan led to a total inversion of the configuration produced by the free mycelium, yielding the (*S*)-alcohol **3b** (*ee* > 99%) instead of the (*R*)-alcohol **3a** (Entry 1, Table 1). The inversion of configuration of the alcohol (*R*)-**3a** (not immobilized) to (*S*)-**3b** (immobilized) showed strong influence of immobilization in according to type of substrates used (supports and/or ketones). However, is not possible to confirm in this study the inversion of configuration, because during immobilization maybe favored the action of another dehydrogenase, and not necessarily is the same enzyme that catalyzed the

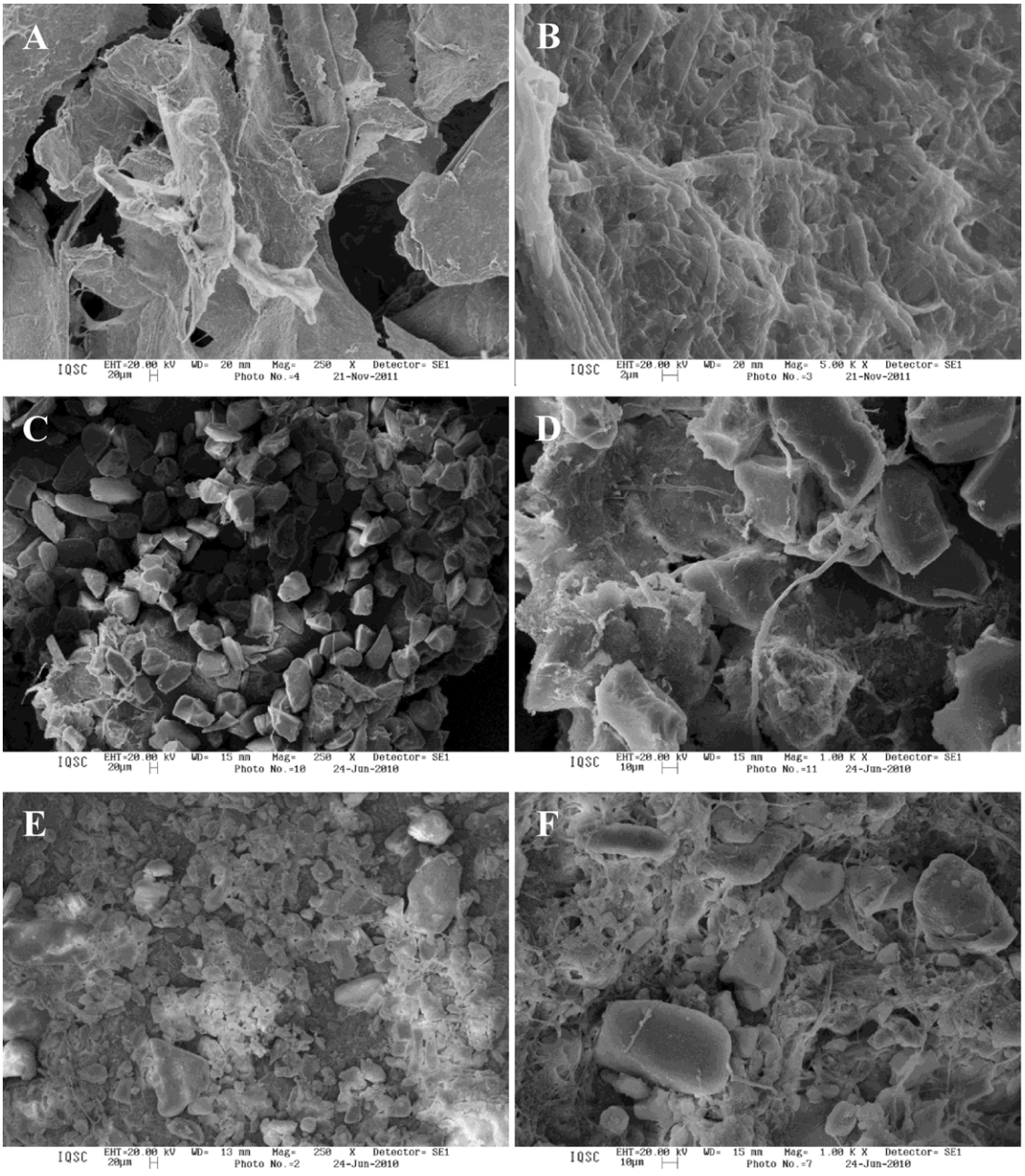


Fig. 2. Scanning electron microscopy micrographs. (A) Whole cells of *P. citrinum* immobilized on chitosan. (B) Whole cells of *P. citrinum* immobilized on chitosan. (C and D) Whole cells of *A. sclerotiorum* immobilized on silica gel. (E and F) Whole cells of *A. sclerotiorum* immobilized on silica xerogel.

reduction in the absence of support. Free and immobilized whole living hyphal of *P. citrinum* were efficient yielding both enantiomers by using a single microorganism. As can be seen in the SEM micrographs, mycelium of *P. citrinum* was completely intertwined with the chitosan matrix (Fig. 2A–B). SEM images of the *P. citrium* immobilized on chitosan show a higher dispersion than over inorganic matrices. Such entanglement among hypha and the chitosan is probably related to the surface charges on the surface of the matrices. The interactions between the organic matrix and mycelium probably enhanced the affinity with ketone **1** and preserved the activity of the enzyme that catalyzed the reduction of ketone **1** to enantiomerically pure (*S*)-alcohol-**3b**. It was therefore concluded that chitosan was an excellent support matrix for the immobilization of filamentous fungi, such as *P. citrinum*, with a beneficial influence on enzyme activity.

The whole cells of *A. sclerotiorum* immobilized on silica gel and the free mycelium catalyzed the reduction of ketone **1** to alcohol (*S*)-**3b** with excellent activity and selectivity (*c* 100%, *ee* > 99%, entries 5 and 6, Table 1). However, when the mycelia of *P. citrinum* were immobilized on silica xerogel there was no reduction of ketone **1**. Possibly, this was due to the silica xerogel adhering strongly to the surface of the hyphae and hindering access of the substrate to the enzymes (entry 7, Table 1).

The whole free cells of *P. citrinum* catalyzed the bioreduction 2-chloro-1-phenylethanone (**2**) to (*R*)-alcohol **4a** in accordance to Prelog's rule after 9 days and with low optical purity (*ee* = 31%, *c* 70%, Entry 8, Table 1). When the live mycelium of *P. citrinum* was immobilized on silica gel, it gave similar results to those obtained with free cells in the reduction of ketone **2** (*ee* = 25%, *c* 65%, entry 9, Table 1), except for a stereopreference for (*S*)-alcohol **4b**

following anti-Prelog's rule. In this case the support matrix used did not impair enzyme activity, but altered the chiral selectivity of the reduction reaction.

In addition, when the *P. citrinum* was immobilized on the silica xerogel matrix ketone **2** was not reduced. This result, as expected, confirmed that the silica xerogel matrix formed a strong barrier on the surface of the mycelium hindering the access of substrate to one or more enzymes involved in the reduction (entry 10, Table 1 and Fig. 1E–F).

Again, the whole cells of *P. citrinum* immobilized on chitosan catalyzed the reduction of ketone **2** to alcohol **4a,b** in excellent yield (98% after purification by CC), but without selectivity (entry 11, Table 1). However, when cells of *P. citrinum* were immobilized on chitosan the conversion to alcohol (*RS*)-**3a,b** was dramatically increased (entry 11, Table 1).

In these studies, we have observed that chitosan has clear advantages when used as a matrix for the immobilization of filamentous fungi. Probably, the reactive hydroxyl and amino groups present in the repeating units of the polymer chains interact with the mycelium of the fungus and promote strong interaction matrix/mycelia. In addition, as chitosan is an organic polymer matrix, it probably facilitates the interaction of the substrate with the immobilized hyphae, favoring the reactions to go to completion.

The use of whole cells of *A. sclerotiorum*, both free and immobilized on silica gel, catalyzed the reduction of ketone **2** to (*R*)-alcohol **4a** in accordance to Prelog's rule, with good optical purity (entries 12 and 13, Table 1). Previously, the fungus *A. sclerotiorum* was immobilized on silica xerogel and it did not catalyzed the reduction of ketone **2** (entry 14, Table 1). The fungus *A. sclerotiorum* was not immobilized in chitosan.

Taken together, these results show that the use of whole immobilized hyphae for the reduction of 1-(4-methoxyphenyl)-ethanone (**1**) and 2-chloro-1-phenylethanone (**2**) depends on both, the xenobiotic substrate and the type of support matrix used.

4. Conclusion

In this study, we reported the immobilization of whole mycelia of the *P. citrinum* CBMAI 1186 and *A. sclerotiorum* CBMAI 849 for the biocatalytic reduction of ketones. The selected strains of marine-derived fungi showed a potential for optically asymmetric reduction of the carbonyl group depending on the support matrix. Silica gel proved to be an interesting support matrix to immobilize cells of filamentous fungi. The silica xerogel matrix was of interest because it adhered strongly to the mycelium of the filamentous fungi. However, exactly because of the strong adhesion, the reactions could not take place, the barrier of the support preventing the access of substrate to enzyme. The use of chitosan as a support for the immobilization of filamentous fungi is certainly very advantageous. The reduction reactions of ketones **1** and **2** tested in this study with hyphae of *P. citrinum* immobilized on chitosan were enhanced as compared to the reactions carried out with the free cells. The SEM micrographs showed a strong interaction between chitosan and the mycelium of *P. citrinum*.

Acknowledgements

The authors wish to thank Prof. R.G.S. Berlinck (Instituto de Química de São Carlos – USP) for providing the marine fungal strains and wish to thank Prof. Timothy John Brockson (Universidade Federal de São Carlos – UFSCar) for optical rotation measurements in a Perkin-Elmer 241 polarimeter (Waltham, MA, USA). We also acknowledge to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support. We also thanks to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship grant to LCR.

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