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Aspergillus terreus CCT 3320 immobilized on chrysotile or cellulose/TiO_2 for sulfide oxidation

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Abstract

The increasing interest in applying chiral sulfoxides in asymmetric syntheses requires their preparation on a large scale, which can be obtained by enantioselective enzymatic oxidation of sulfides. We have focused on the preparation of sulfoxides **1**–**6** using *Aspergillus terreus* CCT 3320 cells to oxidize the precursor sulfides. These biotransformations lead to enantiomeric excesses (ee) better than 95%. In order to improve the biocatalytic process, the cells were immobilized on two supports, chrysotile and on cellulose/TiO_2. The immobilized cells showed a similar biocatalytic behavior in the conversion rate and in the sulfoxide enantiomeric excess. Scanning electron microscopy (SEM) micrographs show that the cells are intertwined with the fibers of both supports, allowing fast separation from the reaction media and easing the biocatalyst reuse. Supported cells stored for at least 3 months showed no loss of activity.

Keywords: *Aspergillus terreus*; Immobilization; Chrysotile; Cellulose/TiO_2; Enantioselective oxidation; Sulfide oxidation; Kinetic resolution

1. Introduction

In recent years, enantioselective oxidation of prochiral sulfides by biocatalytic methods has drawn considerable interest, with the goal of producing chiral sulfoxides which are useful tools in organic synthesis, for asymmetric carbon–carbon and carbon–heteroatom bond formation [1–5].

The international trend in the fine chemicals industry is towards the replacement of classical organic methods by catalytic alternatives that avoid hazardous reagents and produce enantiomerically pure compounds. Biocatalytic methods or enzyme based reactions certainly fulfill most of these requirements. This methodology involves the use of isolated enzymes or whole microbial cells which are either actively growing or at rest. The latter circumvents the problems associated with cofactors but care has to be taken in the maintenance of the microorganisms and the reproducibility of the reaction. When gram scale reactions are the goal, the use of large fermenting reactors is not always recommended. Thus, whole cell immobilization is an alternative to overcome these problems,

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allowing several months of storage and thus behaving as a normal reagent [6].

Adsorption of the biocatalyst onto a water-insoluble macroscopic carrier is the easiest and oldest method of immobilization. Numerous inorganic and organic materials have been used as carriers: activated charcoal, aluminum oxide, diatomaceous earth, cellulose, porous glass and synthetic resins [7,8]. Chrysotile fibers have been successfully used for adhesion of *Saccharomyces cerevisiae* for the gram scale production of alcohols from ketones [9] and ethanol from sugar cane [10].

This paper reports the immobilization of *Aspergillus terreus* CCT 3320 on chrysotile and on cellulose/TiO_2 for sulfide biotransformations.

2. Experimental

2.1. General

The reactions were monitored by silica gel TLC (Aluminum foil, 60 F_{254} Merck) and the visualization was obtained by spraying with *p*-anisaldehyde/sulfuric acid followed by heating at about 120 °C. Flash column chromatography was performed using Merck 60 silica (230–400 mesh). Optical rotation values were measured with a Polamat A polarimeter. The reported data refer to the Na-line value using a 1 dm cuvette. Enzymatic reactions were monitored by GC (FID) on a HP 5890 chromatograph, using hydrogen as carrier gas or by GC/MS with a HP 5890/5970 instrument at 70 eV with He carrier. Scanning speed was 0.84 scan/s from *m/z* 40 to 550. The fused silica capillary columns used were either a J&W Scientific DB-5 (25 m × 0.25 mm × 0.33 μm) or a chiral heptakis-(2,3-dimethyl-6-pentyl)-β-cyclodextrin (25 m × 0.20 mm × 0.33 μm).

2.2. Growth conditions for microorganism cultures

The microorganism *A. terreus* CCT 3320 was obtained from the Culture Collection, Fundação Tropical "André Tosello" (Brazil). The fungus was grown at 28 °C in culture shaker-flasks (500 ml Erlenmeyers) in 100 ml of Difco or Merck malt extract (2 g/l, 72 h). The cells were harvested by filtration and washed twice with phosphate buffer (Na_2HPO_4 and KH_2PO_4, pH 7.0, 0.1 mol l^{-1}). Sterile material was used to perform the experiments and the microorganisms were manipulated in a laminar flow cabinet.

2.3. Enzymatic activity

Oxidations of **1**–**6** were performed in 125 ml bottles on an orbital shaker (120 rpm). To the bottles containing pH 7.0 phosphate buffer (0.1 mol l^{-1}, 25 ml) and washed cells (0.5 g wet weight), substrates **1**–**6** were added (20 μl). The mixture was shaken at 28 °C and the reaction was monitored by chiral GC. Upon reaching the appropriate degree of conversion, the cells were separated by filtration, the aqueous solution was extracted with ethyl acetate and the organic layer was dried with Na_2SO_4 anhydrous.

Enantiomeric excesses (ee) were determined by CG/FID on a HP 5890 equipped with a fused silica chiral capillary column having a heptakis-(2,3-dimethyl-6-pentyl)-β-cyclodextrin stationary phase.

2.4. Support preparation

Chrysotile 5R (Quebec Standard) obtained from SAMA Mineração de Amianto Ltda (Minaçu, GO, Brasil) was washed with tap water and activated by sonication at controlled pH (4.7) as described elsewhere [11].

The cellulose/TiO_2 composite was prepared through the reaction of 7.0 ml of titanium IV butoxide with 10.0 g of cellulose previously suspended in 250.0 ml of heptane, at room temperature and stirred for 1 h in dry-N_2 atmosphere. The product was vacuum-dried for 5 h, held in gaseous ammonia for 10 min, washed with deionized water, vacuum filtered and dried at 60 °C [12].

2.5. Immobilization

2.5.1. Chrysotile support

A. terreus CCT 3320 was grown as described above. The cells were harvested by filtration (0.5 g) and suspended in water (100 ml) containing chrysotile (1 g) in an Erlenmeyer flask (250 ml). The mixture was shaken for 24 h at 120 rpm at 28 °C. After filtration, the produced immobilized cells (1.5 g) were stored in a closed flask in the refrigerator (5 °C).

2.5.2. Cellulose/TiO_2 support

A. terreus CCT 3320 was grown as described previously. The cells were harvested by filtration and suspended in phosphate buffer (Na_2HPO_4 and KH_2PO_4, pH 7.0, 25 ml) in Erlenmeyers (125 ml) to which cellulose/TiO_2 was added. The cellulose/TiO_2/*A. terreus* CCT 3320 ratios were 1/1 and 2 g/2 g. The mixture was further shaken for 24 h at 120 rpm and 28 °C. The immobilized cells were harvested by filtration and stored in closed flasks in the refrigerator (5 °C).

2.6. Enzymatic activity of immobilized microorganism

2.6.1. Chrysotile/A. terreus CCT 3320 systems

The enzymatic activity was monitored immediately after preparation and after 3 and 12 months storage immobilized cells were suspended in phosphate buffer (Na_2HPO_4 and KH_2PO_4, pH 7.0, 25 ml) in Erlenmeyers (125 ml) and the sulfide **1** (20 μl) was added. Reaction progress was monitored every 24 h using 500 μl samples which were extracted with ethyl acetate, dried over Na_2SO_4 and analyzed by GC/FID (1 μl) on the fused silica chiral capillary column. Cell immobilization was observed by scanning electron microscopy (SEM).

2.6.2. Cellulose/TiO_2/A. terreus CCT 3320 systems

The enzymatic activity was monitored for 3 months by suspending the immobilized cells in phosphate buffer (Na_2HPO_4 and KH_2PO_4, pH 7.0, 25 ml) in Erlenmeyers (125 ml) and adding sulfide **5** (20 μl). The reaction progress was monitored every 24 h using 500 μl samples which were extracted with ethyl acetate, dried over Na_2SO_4 and analyzed by GC/FID (1 μl) on the fused silica chiral capillary column. Cell immobilization was observed by SEM.

2.7. Scanning electron microscopy (SEM)

2.7.1. Chrysotile/A. terreus CCT 3320 systems

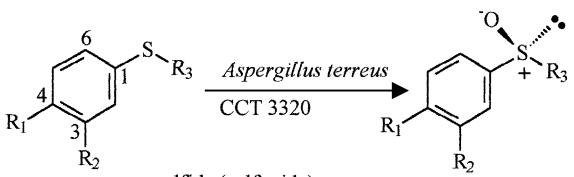
The *A. terreus* CCT 3320/chrysotile system was protected with a nylon gauze (ca. 0.5 mm opening) to avoid losing cells during handling. The samples were fixed in a phosphate buffered (0.1 mol l^{-1}, pH 7.0) 2.5% (v/v) glutaraldehyde solution by gently shaking for 2 h at 4 °C and rinsed in the same buffer. Postfixation was carried out in 1% osmium tetroxide in phosphate buffer by gently shaking for 2 h at room temperature. The samples were washed 10 times with distilled water and dehydrated with ethanol solutions of increasing concentration (50, 70, 80, 95, 100%, v/v), dried by the critical point method with CO_2, mounted on stubs and sputter coated (Edwards S150) with gold (4 nm). SEM observations were taken with a JEOL JSM-840A scanning electron microscope with an accelerating voltage of 25 kV.

2.7.2. Cellulose/TiO_2/A. terreus CCT 3320 systems

These samples were fixed and dried as above, except that the samples were recovered by centrifugation instead of protected with nylon gauze. The samples were dispersed on a double faces conductive tape on a copper support and coated with gold using BALZER MED SCD 050 equipment. Gold sputtering (3 min) resulted in a 10–20 nm layer. SEM was carried out in a JEOL JSM 5800 LV scanning electron microscope with an accelerating voltage of 10 kV.

*2.8. Syntheses of aryl alkyl thioethers **1**–**6***

Aryl alkyl thioethers **1**–**6** (Scheme 1 [13]): A round bottom flask (50 ml) containing the aryl alkyl thiophenol (0.5 ml,1 mol), CH_2Cl_2 (10–15 ml) and K_2CO_3 (3 mol) was stirred at 0 °C for 5 min. To this mixture, MeI or EtI was added, followed by triethyl amine (1 mol). The reactions were monitored by silica gel TLC (eluted with hexane). The reaction mixtures were extracted with CH_2Cl_2 (3 × 50 ml), the solvent was dried over anhydrous Na_2SO_4 and was evaporated under vacuum. Purification by silica gel chromatography



sulfide (sulfoxide)

1 - (**1a**) R_1= R_2 = H, R_3 = Ethyl
2 - (**2a**) R_1 = R_2 = H, R_3 = Methyl
3 - (**3a**) R_1 = Methyl, R_2 = H, R_3 = Ethyl
4 - (**4a**) R_1 = Methyl, R_2 = H, R_3 = Methyl
5 - (**5a**) R_1 = H, R_2 = Cl, R_3 = Ethyl
6 - (**6a**) R_1 = H, R_2 = Cl, R_3 = Methyl

Scheme 1. Sulfides used in the biotransformations with *A. terreus* CCT 3320.

(hexane) resulted in 90–95% yield of sulfides which were fully characterized by mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy (all data are available upon request).

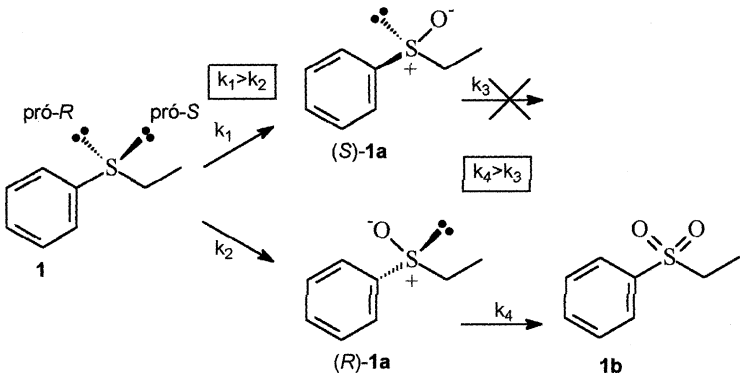
Aryl alkyl sulfoxides **1a–6a**: $NaIO_4$ (1 mol) was added to a stirred mixture of MeOH (5 ml), distilled water (5 ml) and aryl alkyl thioether (0.2 ml, 1 mol) in a round bottom flask (50 ml) at 0 °C. The reaction was further stirred at room temperature for 2–4 h. The reactions were monitored by silica gel TLC (hexane/20% ethyl acetate). The methanol was evaporated and the reaction mixture was extracted with ethyl acetate (3 × 50 ml) which was then dried over Na_2SO_4 and the solvent was evaporated in vacuum. The resulting sulfoxides were purified by column chromatography (hexane/ethyl acetate 20%) yielding the sulfoxides in 70–90% yield. These were fully characterized by mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy (all data are available upon request).

3. Results and discussion

Although microbial sulfoxidation of alkyl aryl sulfides to yield enantiopure sulfoxides has been known for more than three decades, its major limitation lies in the specificity of the microorganisms, i.e. a particular strain will produce only one type of sulfoxide with high enantiomeric excess. Therefore, a large number of different strains are required to produce different enantiomerically pure sulfoxides and this is certainly cumbersome. With a view towards gram scale chiral sulfoxide production we focused on detecting microbial strains with the ability of producing several different sulfoxides and then, on a method that would allow its application and storage like a common chemical reagent. The selection of appropriate fungi is part of a project on the enzymatic evaluation of Brazilian microorganisms [15], using the production of phenyl ethyl sulfoxide 1 as a probe.

Phanerochaete chrysosporium CCT 1999, *Emericella nidulans* CCT 3119 and *A. terreus* CCT 3320 (isolated from the Atlantic Rain Forest, Brazil) are among the best microorganisms for this oxidation [13,14]. The stereochemical outcome of the reactions with *E. nidulans* CCT 3119 and *A. terreus* CCT 3320 were certainly unexpected, with low initial and high final enantiomeric excess. These results were rationalized as a double step process with moderate enantiotopic differentiation in the first step, followed by an efficient kinetic resolution of the resulting sulfoxides (Scheme 2). The efficiency of the kinetic resolution was confirmed by using a racemic mixture of (±)-sulfoxide **1a** (Table 1). The mixture was converted into sulfone **1b** (50%), leaving enantiomerically pure (−)-(*S*)-**1a** (see data in Table 1).

This biocatalytic reaction would be easier if the microorganism is supported. The choice of chrysotile as support was based on previous data of the immobilization of *S. cerevisae* [9,10]. Immobilization of *A. terreus* was performed following the previously established protocol [10]. Fig. 1 shows SEM micrographs of a free *A. terreus* CCT 3320 colony. These filamentous fungi form circular colonies. Fig. 2 shows SEM micrographs of *A. terreus* adhered on chrysotile fibrils, of which samples were taken immediately



Scheme 2. Kinetic path proposed for the oxidation of sulfide **1** using *A. terreus* CCT 3320.

Table 1
Activity monitoring of sulfide **1** oxidation to sulfoxide **1a** with *A. terreus* CCT 3320 supported on chrysotile

Parameters	Free cells			Just supported			After 3 months		
Time (h)	24	48	96	24	48	72	24	48	72
Conversion	6	10	100	69	92	100	–	100	100
Sulfoxide yield (%)	6	10	76	69	69	71	–	67	74
Sulfone yield (%)	–	–	24		23	29	–	33	26
ee[a] (−)-(*S*)-**1a** (%)	26	26	>99	14	93	>99	–	>99	>99

[a] ee: enantiomeric excess of sulfoxide.

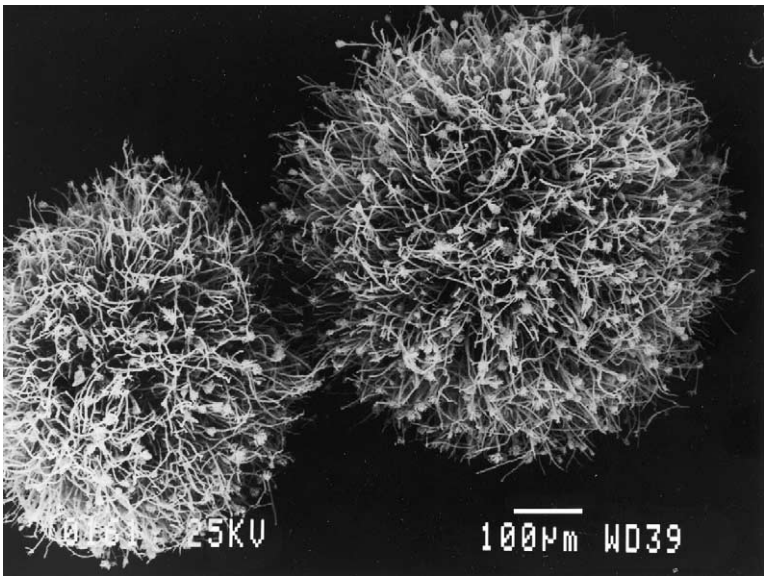


Fig. 1. SEM of *A. terreus* CCT 3320 colonies.

after enzymatic activity monitoring. Cells adhered to the chrysotile fibrils are still distinguishable. Cells observed immediately after the first enzymatic monitoring showed a small chrysotile/cell interaction (Fig. 2A). After 3 months (Fig. 2B) the interaction is higher and the shape of *A. terreus* CCT 3320 (Fig. 1) has changed due to interaction with the chrysotile fibrils. Similar results were observed by SEM after 12 months of storage in a refrigerator followed by enzymatic activity monitoring (micrographs not shown).

Table 2
Oxidation of the aryl alkyl sulfides **2**, **3**, **4** and **6** with *A. terreus* CCT 3320 free cells

Parameters	Sulfide **2**			Sulfide **3**		Sulfide **4**			Sulfide **6**		
Time (h)	24	72	96	48	96	24	72	96	24	72	96
Conversion	40	96	96	80	98	19	91	92	43	77	82
Sulfoxide (%)	38	25	24	53	66	18	27	24	41	71	71
Sulfone (%)	2	71	72	27	32	1	64	68	2	6	15
Time (h)	–	–	≥95	–	≥95	–	>98	>98	–	15	17
Conversion	24	72	96	48	96	24	72	96	24	72	96
ee[a] (%)			≥95		≥95		>98	>98		15	17

[a] ee: enantiomeric excess determined by NMR; method to be published [17].

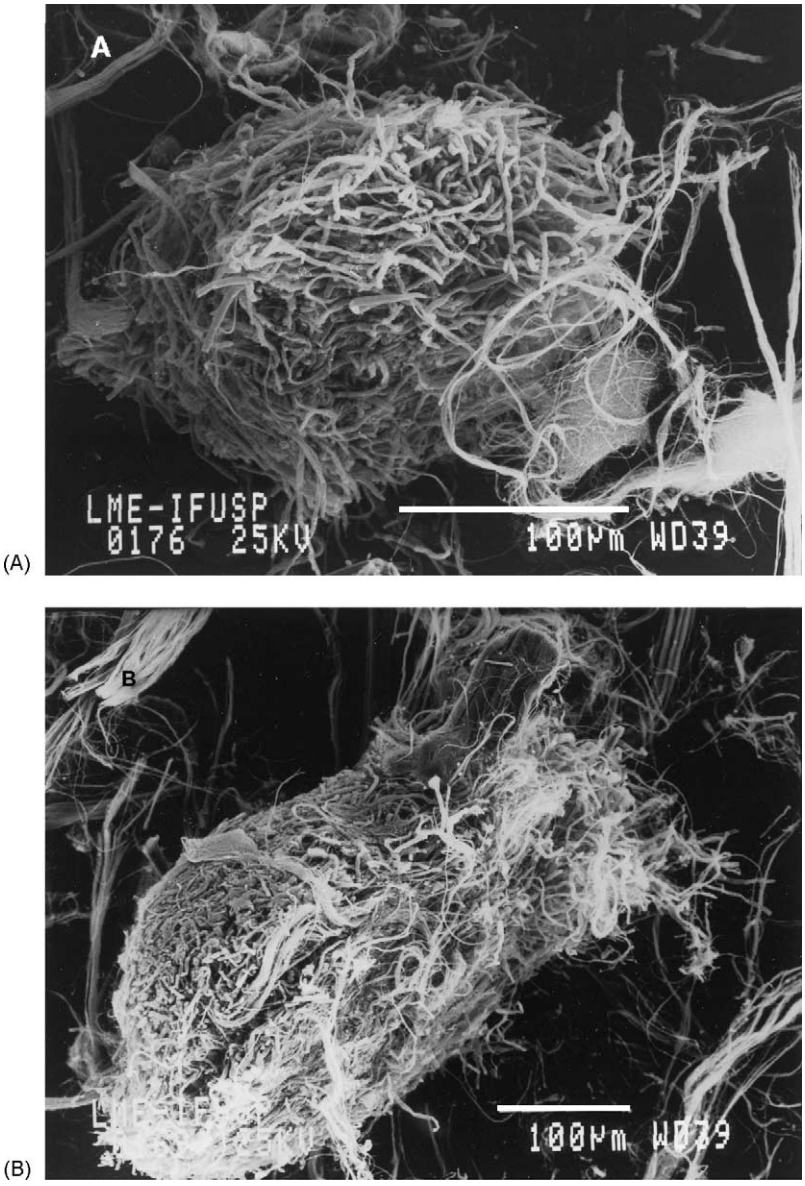


Fig. 2. SEM micrographs of *A. terreus* CCT 3320 immobilized on chrysotile fibers: (A) recently prepared, and after enzymatic monitoring; (B) after 3 months storage, again after enzymatic monitoring.

The supported cells could be stored for long periods in a common refrigerator without added nutrients. These results are similar to those obtained by Joekes et al. [10] using chrysotile as a support for *S. cerevisiae*.

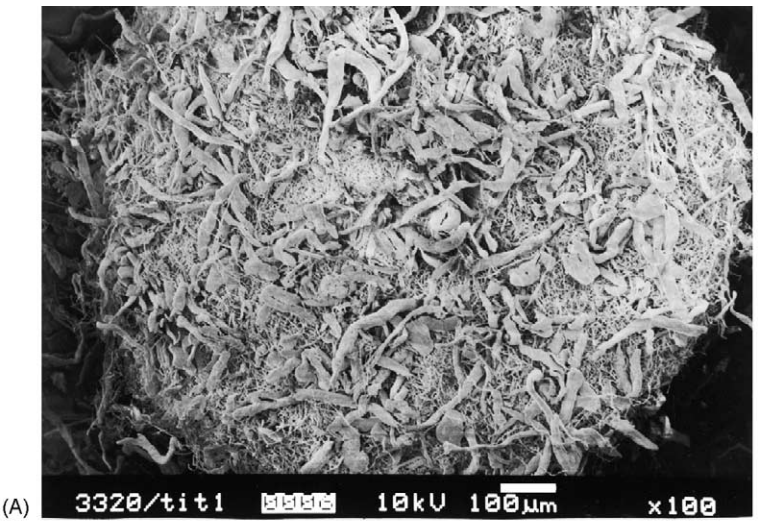
Table 2 shows that sulfoxidation of **1** by cells immobilized on chrysotile immediately after preparation was similar to that with the free cells. After storage at 5–10 °C for 3 months, the activity was almost the same as that observed immediately after preparation. Oxidation of **1** was also monitored after 12 months storage (qualitative data, not shown); in this case, the reaction still occurred but was slow.

A. terreus CCT 3320 oxidizing activity was monitored using the sulfides **2**–**6** [16]. Results in Tables 2

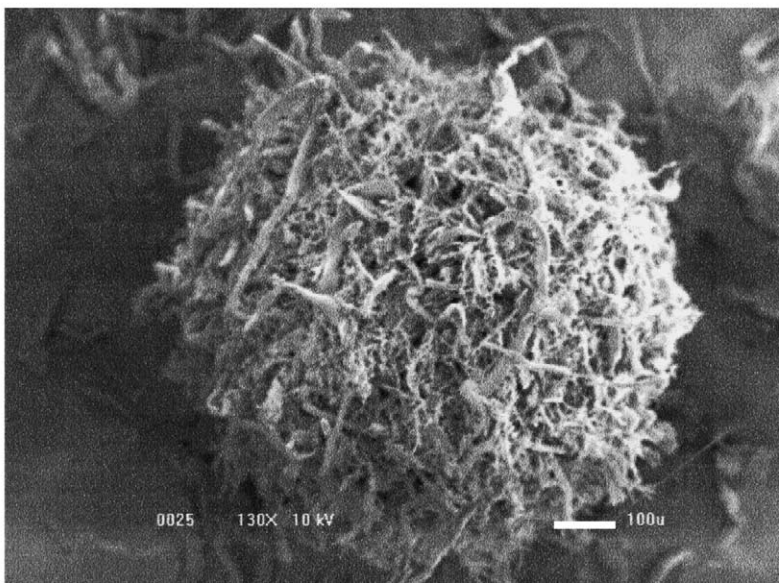
Table 3
Activity monitoring of sulfide **5** oxidation with *A. terreus* CCT 3320 supported on cellulose/TiO_2

Parameters	Free cells			Just supported			After 3 months			
Time (h)	24	48	72	24	48	72	24	48	72	96
Conversion	94	100	100	94	99	100	66	71	77	82
Sulfoxide yield (%)	78	76	70	69	69	69	51	47	45	49
Sulfone yield (%)	16	24	30	25	30	31	15	24	32	33
ee[a] (%)	>98	>98	>98	>98	>98	>98	73	85	95	>98

2 g of free cells or 2 g of cells on 1 g of support.
[a] ee: enantiomeric excess.



(A)



(B)

Fig. 3. SEM micrographs of *A. terreus* CCT 3320 immobilized on cellulose/TiO2: (A) recently prepared, and after enzymatic monitoring; (B) after 3 months storage, again after enzymatic monitoring.

and 3 clearly indicate that it is appropriate for the sulfide oxidation of those substrates and recommended for the production of compounds **2a**, **3a**, **4a** and **5a** with a high enantiomeric excesses, but strict control of the sulfone production is required. *A. terreus* CCT 3320 immobilized on chrysotile was tested and did not change the sulfoxide/sulfone ratio. Therefore, an alternative support was tested, namely cellulose fibers coated with TiO_2 [18].

The immobilization of *A. terreus* CCT 3320 on cellulose/TiO_2 was successfully accomplished. Fig. 3 shows the SEM micrographs. It can be observed that both cellulose/TiO_2 fibers and *A. terreus* are closely intertwined (compare Fig. 3 to Fig. 1) soon after supporting (Fig. 3A), and no appreciable difference can be seen in the interaction after 3 months of storage (Fig. 3B).

Several experiments were performed with compound **5** and *A. terreus* CCT 3320 immobilized on cellulose/TiO_2, mainly aiming a better conversion to sulfoxide. Table 3 shows that in all experiments the sulfoxide was produced in good enantiomeric excess but the production of sulfone was a little higher than with the non-immobilized cells.

4. Conclusions

Results show that *A. terreus* CCT 3320 can be an enantioselective biocatalyst for oxidation of prochiral sulfides, resolving racemic sulfoxides with enantiomeric excess of at least 95%. Results obtained with the cells immobilized on chrysotile or cellulose/TiO_2 are comparable with those obtained with the free cells for the preparation of enantiopure chiral compounds.

The immobilization of the cells was straightforward, rendering supported biocatalysts with activity for at least 3 months. This is important given the advantages that can be presumed by the use of these supported cells in a scale enlargement of the sulfide oxidation process.

However, the increasing sulfone production by oxidation of sulfide **5** with the immobilized cells on cellulose/TiO_2 points to an increasing oxidative activity, which may not be desired in the present reaction but might be benefic in reactions with Baeyer–Villigerases, which will be tested shortly.

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