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Deracemization of aryl ethanols and reduction of acetophenones by whole fungal cells of *Aspergillus terreus* CCT 4083, *A. terreus* CCT 3320 and *Rhizopus oryzae* CCT 4964

João Valdir Comasseto, Leandro Helgueira Andrade, Álvaro Takeo Omori, Leonardo Fernandes Assis, André Luiz Meleiro Porto*

Instituto de Química, Universidade de São Paulo – USP, Av. Prof. Lineu Prestes, no. 748, CEP 05508-900, São Paulo, SP, Brazil

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Abstract

The enantioselective deracemization of a number of *p*-substituted aryl ethanols and the reduction of *p*-substituted acetophenones were carried out with whole fungal cells of *Aspergillus terreus* CCT 4083, *A. terreus* CCT 3320 and *Rhizopus oryzae* CCT 4964 giving the corresponding alcohols in enantiomeric excesses up to >99%.
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Keywords: Deracemization; Reduction; *Aspergillus terreus*; *Rhizopus oryzae*; Chiral secondary alcohols

1. Introduction

Enantiomerically pure alcohols are useful as building blocks for the synthesis of bioactive compounds such as pharmaceuticals [1] and agrochemicals [2]. The usual methods employed to prepare optically active alcohols are asymmetric reduction of ketones by chiral organometallic reagents [3] and biocatalytic methods [4]. In this last method microorganisms [5] can be employed to perform the reactions. This approach has attracted much attention in view of its high enantioselectivity and for being environmentally benign [5c]. Another method which has been used to prepare enantiomerically pure alcohols is the deracemization of mixtures of chiral alcohols [6]. This method presents advantages over the enzymatic resolution [7], since theoretically 100% of the racemate can be transformed into a single enantiomer, while in the enzymatic kinetic resolution only 50% of the racemic mixture can be transformed into the desired enantiomer [6a,6c]. Deracemization of secondary alcohols has been performed by combination of a transition metal-catalyzed racemization with an enantioselective lipase-catalyzed esterification [7a]. Two enzymes systems can also be used to promote the deracemization [6], but the more attractive method to achieve this goal is the use of microbial systems containing redox enzymes [6]. In this case, one of the two redox reactions shown in Scheme 1 has to be irreversible (Scheme 1). A limited number of microorganisms containing the enzymatic combination capable of effecting such transformations were reported to date [4a,6,8,9a]. Recently we initiated a program aiming to identify new microbial strains from the Brazilian rain forest with potential for synthetic transformations [5a,9]. One of these papers reported the reduction and deracemization of fluorocompounds by fungal strains [9a]. In this paper we reported our results concerning the redox potential of *Rhizopus oryzae* CCT 4964, *Aspergillus terreus* CCT 4083 and *A. terreus* CCT 3320 which efficiently reduce *p*-substituted acetophenones (**1a–6a**) to arylethanols with high enantionselectivity or promote the deracemization of arylethanols (**1–6**) in a Prelog or anti-Prelog fashion (Scheme 2).

* Corresponding author. Tel.: +55-11-3091-2287; fax: +55-11-3815-5579.
E-mail address: almporto@iq.usp.br (A.L.M. Porto).

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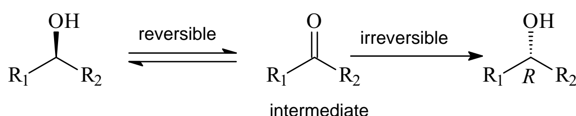


Scheme 1. Deracemization via microbial stereo-inversion of secondary alcohols.

2. Experimental

2.1. General methods

Chemical reductions were monitored by silica gel TLC (Aluminum foil, 60 F_{254} Merck) and the visualization was obtained by spraying with *p*-anisaldehyde/sulfuric acid or vanillin followed by heating at about 120 °C. Flash column chromatography was performed using Merck 60 silica gel (230–400 mesh). Enzymatic reactions were monitored by GC (FID) in a Shimadzu GC-17A chromatograph, using hydrogen as a carrier gas or by GC–MS with a Shimadzu GC–MS P5050A with Helium carrier. The fused silica capillary columns used were either a J & W Scientific DB-5 (30 m × 0.25 mm × 0.25 μm) or a chiral Chirasil-Dex CB β-cyclodextrin (25 m × 0.25 mm). ^{1}H NMR spectra were recorded with a Bruker DPX300 (300.1 MHz). $CDCl_3$ was used as the solvent, with Me_4Si (TMS) as internal standard. ^{13}C NMR spectra were obtained with a Bruker DPX300 (75.5 MHz). Orbital shakers Tecnal TE-421 or Superohm G-25 were employed for the biocatalyzed transformations.

2.2. Synthesis of racemic alcohols

The racemic alcohols **1–6** were obtained by reduction of the corresponding ketones **1a–6a** (300 mg; 2.2 mmol) with sodium borohydride (20.5 mg; 0.5 mmol) in methanol (10 ml). After work-up with saturated aqueous solution of NH_4Cl (4 ml), the aqueous layer was extracted with ethyl acetate (3 × 50 ml), dried over $MgSO_4$ and the solvent was evaporated. The residue was purified by silica gel column chromatography using hexane and ethyl acetate (90:10 or 80:20) as eluent. The ^{1}H NMR and ^{13}C NMR spectra of these compounds were in agreement with those reported in the literature [5c,5d].

2.3. Growth conditions for the microorganisms cultures

The microorganisms *A. terreus* CCT 4083 (Pfenning, L., 08/94. Isolated from the soil. Amazon Forest, Brazil), *A. terreus* CCT 3320 (Attili, D.S., 09/93. Isolated from the soil. Rain Atlantic Forest, Brazil) and *R. oryzae* CCT 4094 (Costa, A.S., 04/96. Isolated from fermented Manihot esculenta, Amazon Forest, Brazil) were purchased from the Culture Collection of the André Tosello Foundation (Brazil) [10]. The fungi were grown in culture shaker-flasks (170 rpm, 250 ml Erlenmeyer) in 100–130 ml of Oxoid malt extract medium (20 g/l, 72–96 h) at 32 °C. The cells were harvested by filtration. Sterile material was used to perform the experiments and the microorganisms were manipulated in a laminar flow cabinet (Veco).

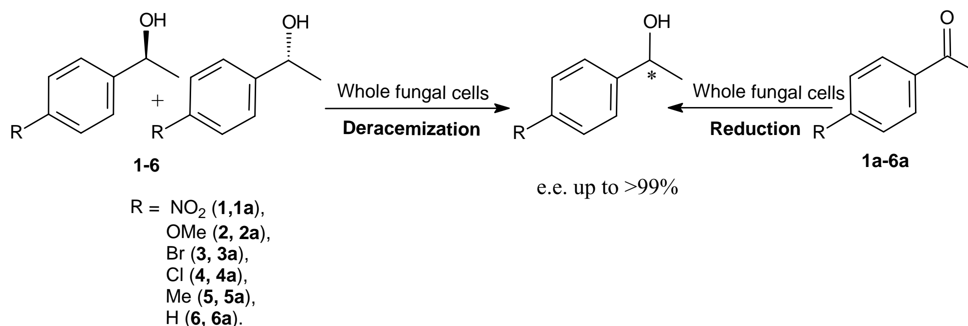
2.4. Standard procedure for small scale enzymatic deracemization and reduction reactions

The appropriate substrate (acetophenones **1a–6a** or alcohols **1–6**, 20 μl or 20 mg) was added to a buffer phosphate solution (Na_2HPO_4/KH_2PO_4, 50 ml) [11] containing a suspension of washed wet cells of *A. terreus* (5 g) or *R. oryzae* (3 g) in Erlenmeyer flasks (250 ml or 125 ml). The mixtures were stirred on orbital shaker (32 °C, 170 rpm) for the time indicated in Tables 1–5.

2.5. Standard procedure for preparative-scale reduction enzymatic reaction

The previously filtered wet cell cultures of *A. terreus* (15 g) and *R. oryzae* CCT 4964 (7–12 g) were suspended in a buffer phosphate solution (150 ml or 200 ml) in 500 ml or 250 ml Erlenmeyer flasks, respectively. The acetophenones were added (100 μl) to the flasks. The mixtures were stirred on a rotary shaker (32 °C, 170 rpm) until the starting material was completely consumed.

- *A. terreus* CCT 3320: **1** (yield: 59%), **3** (yield: 65%).
- *A. terreus* CCT 4083: **2** (yield: 70%).
- *R. oryzae* CCT 4964: **4** (yield: 60%); **5** (yield: 66%).



Scheme 2. Deracemization of **1–6** and reduction of **1a–6a** by whole fungal cells of *A. terreus* CCT 4083, *A. terreus* CCT 3320 and *R. oryzae* CCT 4964.

Table 1
Microbial reduction of acetophenones (**1a–6a**) with whole cells of the fungus *R. oryzae* CCT 4964

t (days)	c (%) alcohol	e.e. (%) alcohol	Config.
1			
2	90	>99	*S*
4	90	>99	*S*
2			
1	50	82	*S*
3	58	77	*S*
3			
2	92	>99	*S*
4	92	>99	*S*
4			
1	38	>99	*S*
4	73	>99	*S*
5			
1	16	>99	*S*
3	73	>99	*S*
6			
1	65	78	*S*
3	76	68	*S*

t: time; c: conversion determined by GC; e.e.: enantiomeric excess; Config.: absolute configuration.

Table 2
Microbial reduction of acetophenones (**1a–6a**) with whole cells of the fungus *A. terreus* CCT 4083

t (days)	c (%) alcohol	e.e. (%) alcohol	Config.
1			
1	99	53	*R*
2	99	33	*R*
4	99	2	*S*
5	99	36	*S*
7	99	47	*S*
2			
3	19	>99	*R*
7	99	>99	*R*
3			
1	97	91	*R*
3	94	75	*R*
5	94	10	*R*
9	98	56	*S*
4			
2	69	88	*R*
4	58	35	*R*
5			
3	8	>99	*R*
6	8	>99	*R*
6			
1	57 (5[a])	83	*S*
6	>98 (38[a])	90	*S*
9	98 (71[a])	>99	*S*

t: time; c: conversion determined by GC; e.e.: enantiomeric excess; Config.: absolute configuration.
[a] Phenol.

Table 3
Microbial reduction of acetophenones (**1a–6a**) with whole cells of the fungus *A. terreus* CCT 3320

t (days)	c (%) alcohol	e.e. (%) alcohol	Config.
1			
2	98	82	*R*
3	98	92	*R*
5	99	97	*R*
2			
3	75	>99	*R*
7	99	>99	*R*
3			
1	90	63	*R*
3	51	79	*R*
5	57	70	*R*
4			
1	70	>99	*R*
7	54	>99	*R*
10	47	>99	*R*
5			
1	9	>99	*R*
3	12	>99	–
7	–	–	–
6			
1	13 (8[a])	14	*S*
3	100[a]	–	–

t: time; c: conversion determined by GC; e.e.: enantiomeric excess; Config.: absolute configuration.
[a] Phenol.

Table 4
Deracemization of phenyl ethanols (**1–6**) by whole cells of the fungus *A. terreus* CCT 4083

t (days)	c (%)	c (%)	e.e. (%)	Config.
	1a	**1**		
3	1	99	23	*S*
5	18	82	84	*S*
6	15	85	95	*S*
7	14	86	>99	*S*
	2a	**2**		
3	79	21	2	*R*
7	64	36	92	*R*
9	48	52	98	*R*
	3a	**3**		
3	12	88	6	*S*
5	12	88	8	*S*
17	21	79	>99	*S*
	4a	**4**		
2	27	73	15	*S*
6	14	86	12	*S*
	5a	**5**		
2	71	29	–	–
4	98	2	–	–
6	11	2	–	–
	6a	**6**		
3	8[a]	92	12	*S*
6	27[a]	72	41	*S*
12	42[a]	49	66	*S*

t: time; c: conversion determined by GC; e.e.: enantiomeric excess; Config.: absolute configuration.
[a] Phenol.

Table 5
Deracemization of phenyl ethanols (**1–6**) by whole cells of the fungus *A. terreus* CCT 3320

t (days)	c (%)	c (%)	e.e. (%)	Config.
	1a	**1**		
3	93	7	–	–
4	98	2	–	–
6	100	0	–	–
	2a	**2**		
3	64	36	77	*R*
7	18	82	>99	*R*
4	32	68	70	*R*
6	37	63	74	*R*
	4a	**4**		
1	21	79	38	*R*
7	35	65	>99	*R*
	5a	**5**		
2	75	25	>99	*R*
6	87	13	>99	*R*
	6a	**6**		
2	8[a]	92	3	*S*
4	19[a]	81	7	*S*
6	26[a]	74	14	*S*

t: time; c: conversion determined by GC; e.e.: enantiomeric excess; Config.: absolute configuration.
[a] Phenol.

2.6. General procedure for the extraction of the alcohols

The reactions were monitored by GC, and after appropriate conversion, the mixture was filtered and the aqueous phase was extracted with ethyl acetate (4 × 150 ml). The yellow organic phase was dried over $MgSO_4$ and evaporated. The residue was purified by silica gel column chromatography using hexane and ethyl acetate (90:10 or 80:20) as eluent to yield the desired alcohols.

2.7. Analysis of the reactional course of the biocatalytic reactions

The reaction progress was monitored every 24 h by collecting 2 ml samples. These samples were extracted by stirring with ethyl acetate (0.5 ml) followed by centrifugation (6000 rpm, 5 min). The organic phase was analyzed by GC/FID (1 μl) in a fused silica chiral capillary column. The products of the biocatalyzed reactions were compared with a racemic mixture previously obtained by chemical reduction (see Section 2.2).

2.8. GC analysis conditions (chiral column Chirasil-Dex CB β-cyclodextrin)

- Injector 200 °C; detector 220 °C; Column Press (Kpa) 100; split ratio (1:20).
- 1-(*p*-nitro-phenyl)-ethanol **1**: rate 0 °C/min; oven 165 °C (20 min); retention time [(*R*)-isomer: 9.5 min; (*S*)-isomer: 10.4 min)].
- 1-(*p*-methoxy-phenyl)-ethanol **2**: rate 0 °C/min; oven 120 °C (20 min); retention time [(*R*)-isomer: 14.6 min; (*S*)-isomer 15.4 min)].
- 1-(*p*-bromo-phenyl)-ethanol **3**: rate 0 °C/min; oven 140 °C (20 min); retention time [(*R*)-isomer: 9.3 min; (*S*)-isomer 10.4 min)].
- 1-(*p*-chloro-phenyl)-ethanol **4**: rate 1 °C/min; oven 100–180 °C (30 min); retention time [(*R*)-somer: 22.5 min; (*S*)-isomer: 24.8 min)].
- 1-(*p*-methyl-phenyl)-ethanol **5**: rate 1 °C/min; oven 100–180 °C (20 min); retention time [(*R*)-isomer: 13.5 min; (*S*)-isomer 14.2 min)].
- 1-(phenyl)-ethanol **6**: rate 1 °C/min; oven 100–180 C (20 min); retention time [(*R*)-isomer: 8.8 min; (*S*)-isomer 9.6 min)].

2.9. Assignment of the absolute configuration of the alcohols *1*–*6*

Optical rotation values were measured in a Jasco DIP-378 polarimeter. The reported data refer to the Na-line value using a 1 dm cuvette. The absolute configurations were determined by comparison of the sign of the measured optical rotation with those of the literature values [3d,4g,5c,5d].

- (*R*)-(+)-(*p*-nitrophenyl)-ethanol **1**: $[\alpha]_D^{20}$ +48.0° (c 1.43, $CHCl_3$), e.e. 93%.
- (*R*)-(+)-(*p*-methoxyphenyl)-ethanol **2**: $[\alpha]_D^{20}$ + 48.3° (c 2.03, $CHCl_3$), e.e. 74%.
- (*R*)-(+)-(*p*-bromophenyl)-ethanol **3**: $[\alpha]_D^{20}$ + 21.70° (c 4.37, $CHCl_3$), e.e. 95%.
- (*S*)-(−)-(*p*-chlorophenyl)-ethanol **4**: $[\alpha]_D^{20}$ − 3.93° (c 8.14, $CHCl_3$), e.e. 70%.
- (*S*)-(−)-(*p*-methylphenyl)-ethanol **5**: $[\alpha]_D^{20}$ − 35.5° (c 2.03, $CHCl_3$), e.e. 95%.
- (*S*)-(−)-phenylethanol **6** [9b].

3. Results and discussion

3.1. Bioreduction of p-substituted-acetophenones *1a*–*6a*

The microbial reduction of acetophenones **1a–6a** was carried out with whole fungal cells of *R. oryzae* CCT 4964, *A. terreus* CCT 4083 and *A. terreus* CCT 3320 in a buffer phosphate solution (Scheme 2).

The best results for the bioreduction were obtained when whole cells of *R. oryzae* CCT 4964 were used. The reaction occurred with good conversion (c 70–90%) and excellent enantiomeric excess (e.e. >99%, compounds **1a**, **3–5a**, Table 1). The observed enantioselectivity is in accordance with Prelog's rule [12] in all cases. In addition, the reaction with the fungus *R. oryzae* was carried out in a preparative scale (12 g of wet cells of *R. oryzae* and 100 μl of compound

4a) affording the (S)-(−)-(*p*-chlorophenyl)-ethanol **4** in 70% isolated yield with 99% e.e. after 120 h (see Section 2.5). The acetophenones **2a** and **6a** were reduced with the fungus *R. oryzae* in moderate conversion and enantioselectivity (Table 1).

The reduction of acetophenones **1a**–**6a** was also promoted by whole cells of *A. terreus* CCT 4083. As can be observed in Table 2, when this fungus was used, the reaction with *p*-nitro-acetophenone **1a** and *p*-bromo-acetophenone **3a** was very fast and initially the anti-Prelog [(*R*)-enantiomer)] predominated, but as the reaction progressed the enantiomeric excess decreased and after some time the observed enantiomeric excess favored the Prelog's product [(*S*)-enantiomer]. This behavior can be rationalized as a dynamic process where the starting acetophenones are reduced in favor of the (*R*)-arylethanols (compound **1**, 53% e.e. and compound **3**, 91% e.e.). Along the reaction the (*R*)-enantiomers are reoxidized to the corresponding ketones. As a consequence, after some time the mixture becomes enriched in the (*S*)-enantiomer. This process is closely related to the deracemization sequence discussed in Section 1 (Scheme 1) and led us to explore the fungi under study in deracemization reactions as will be discussed later (Section 3.2).

The fungus *A. terreus* CCT 3320 reduced compounds **1a** and **2a** to the corresponding (*R*)-arylethanols **1** and **2** with good conversion and enantiomeric excess. Compounds **3a**–**5a** were also reduced with anti-Prelog enantioselectivity, but with low conversion, although **4a** and **5a** showed high enantioselectivity (Table 3).

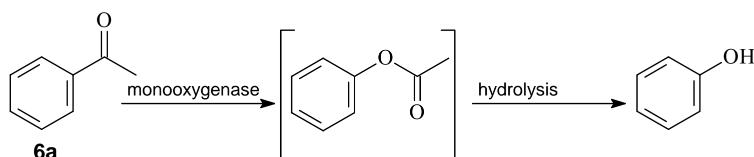
Acetophenone **6a** was transformed into phenol by the fungi *A. terreus* CCT 4083 and *A. terreus* CCT 3320 (Tables 2 and 3). The formation of phenol probably occurred via on oxidation reaction promoted by monooxygenase present in these fungi (Scheme 3). The phenol was identified by GC–MS analysis. The structure of this product was confirmed after comparison with Mass Spectral Database (CLASS-5000/Wiley) and co-injection with authentic sample. This result revealed that these microorganisms have potential to perform bioenzymatic Baeyer–Villiger oxidation and are presently being explored in our group for this purpose.

3.2. Deracemization of 1-(p-substituted-phenyl)-ethanols 1–6

As mentioned in Section 3.1 two of the fungi used in this study showed to be able to promote an oxido-reduction process leading to the deracemization of a mixture of (*R*) and (*S*) alcohols. In view of these results the deracemization of the secondary alcohols **1**–**6** by whole cells of *A. terreus* CCT 4083 and *A. terreus* CCT 3320 was investigated (see Sections 2.4 and 2.7) for reaction and analysis conditions. The results shown in Tables 4 and 5 indicate that the racemic mixtures of the alcohols **1**–**5** were effectively deracemized by the two fungi investigated. The deracemization reaction showed different behavior for each microorganism employed. In this way, the reactions were performed using different incubation periods in order to optimize the process for maximum levels of conversion and enantiomeric excess. When a racemic mixture of (*p*-nitrophenyl) ethanol **1** was submitted to whole cells of *A. terreus* CCT 4083 a deracemization process occurred leading to (*S*)-(*p*-nitrophenyl)-ethanol **1** in 99% e.e. and 86% conversion. Initially (*R*)-**1** was oxidized to the arylacetophenone **1a**, while (*S*)-**1** was not oxidized. Then the acetophenone **1a** was reduced to (*S*)-**1**, which is not reoxidized to **1a**. In this way along the reaction time the mixture becomes enriched in (*S*)-**1**, as show the chromatograms of Fig. 1. Contrary to this result the fungus *A. terreus* CCT 3320 did not promote the deracemization of **1**. The only product detected was *p*-nitroacetophenone (Table 5).

The deracemization of (±)-**5** with *A. terreus* CCT 3320 occurred with high enantiomeric excess (>99%) but with low conversion (29%). The *A. terreus* CCT 4083 did not deracemize (±)-**5**, and only the oxidation product **5a** was obtained (Table 4). The microbial deracemization of (±)-(*p*-methoxyphenyl)-ethanol **2** was promoted with high enantiomeric excess (>99%) and conversion (>80%) by both fungal strains (Tables 4 and 5). The obtained alcohol **2** showed the (*R*) configurations, attributed by comparisons of the sign of the specific rotation with that reported in the literature [5c]. Phenylethanol **6** was not efficiently deracemized by both fungi (Tables 4 and 5). As commented before, a competitive reaction took place leading to phenol.

When the deracemization of (*p*-bromophenyl)-ethanol **3** was performed with *A. terreus* 4083 and *A. terreus* 3320 a different behavior was observed in each case. When cells of *A. terreus* CCT 3320 were used, the alcohol (*R*)-(+)-**3** was obtained with moderate enantiomeric excess (e.e. 74%) and conversion (63%) (Table 4). In contrast, the fungus *A. terreus* CCT 4083 deracemized (±)-**3** to afford the (*S*)-(−)-**3** enantiomer with high enantiomeric excess (e.e. >99%) and conversion (80%) (Table 4). These results for deracemization with *A. terreus* CCT 4083 and *A. terreus* CCT 3320 are in accordance with the studies by Pérez et al. [8a].



Scheme 3. Proposed sequence for oxidation of the acetophenone **6** with *A. terreus*.

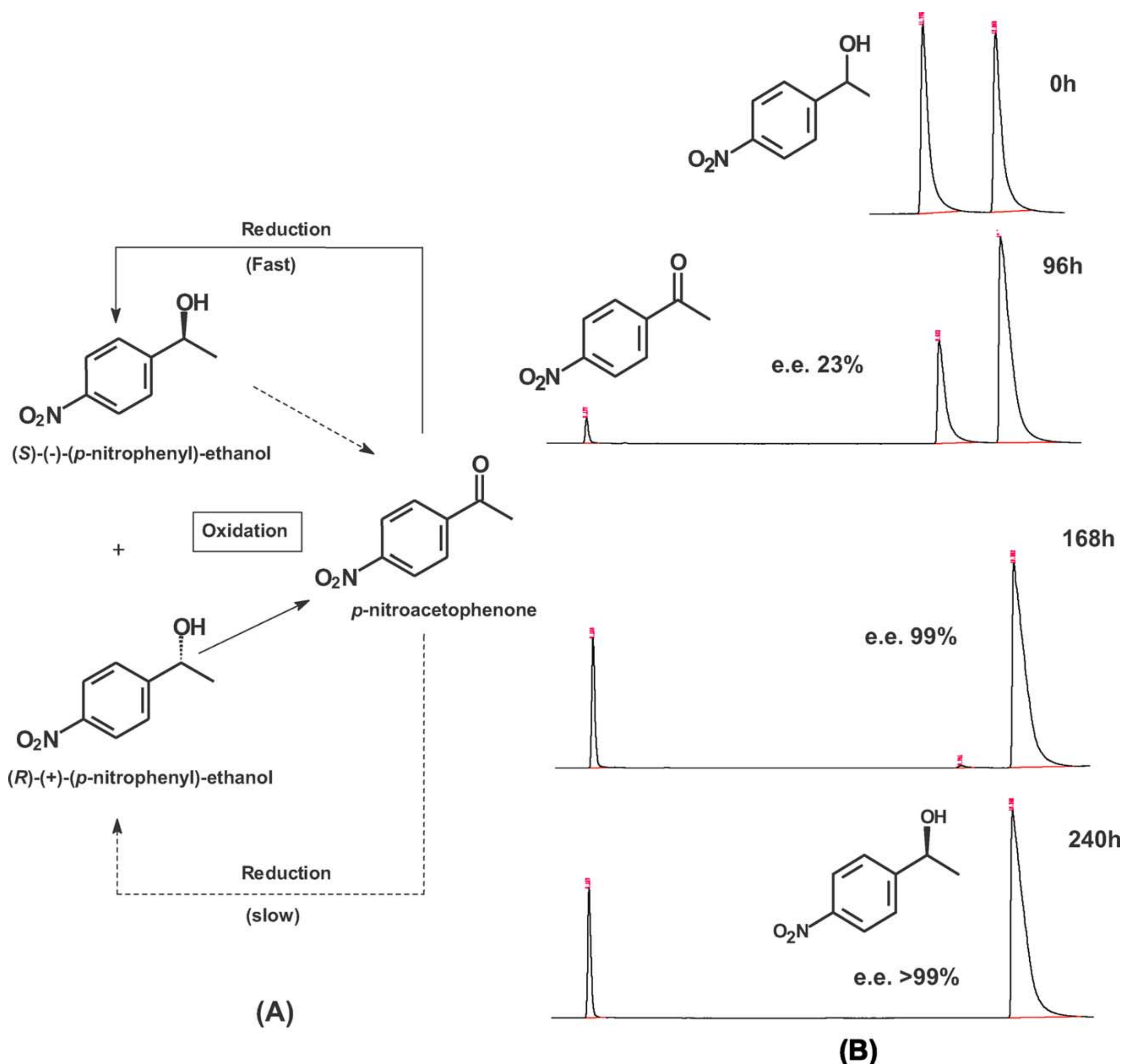


Fig. 1. (A) Deracemization of (±)-**1** by whole fungal cells of *A. terreus* CCT 4083. (B) Chromatograms of the deracemization reaction of (±)-**1** with *A. terreus* CCT 4083.

4. Conclusion

In conclusion, whole cells of *A. terreus* CCT 4083 and *A. terreus* CCT 3320 efficiently deracemize *p*-substituted-(phenyl)-ethanols (**1**–**4**) with high enantiomeric excess (up to e.e. >99%). In addition, *p*-substituted-acetophenones are reduced by whole cells of the fungus *R. oryzae* 4964 to the corresponding chiral alcohols (**1**–**5**) with high enantiomeric excess (up to >99%). The Prelog and anti-Prelog enantioselectivity are dependent of the microorganisms employed. Further studies with microbial whole cells of fungi are currently in progress in our group for the reaction scaling up, as well as for shortening the reaction time of the deracemization by means of additives.

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