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Bioreduction of fluoroacetophenones by the fungi *Aspergillus terreus* and *Rhizopus oryzae*

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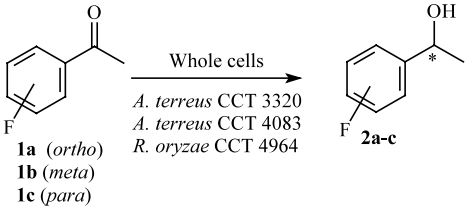
Abstract—The enantioselective bioreduction of a number of fluoroacetophenones was carried out with whole cells of *Rhizopus* oryzae CCT 4964, *Aspergillus terreus* CCT 3320 and *Aspergillus terreus* CCT 4083 giving the corresponding alcohols in good yield and high enantioselectivity. Initial results with these fungi indicated that some of them are promising biocatalysts for deracemization reactions of secondary alcohols.

1. Introduction

Biocatalysis is one of the most important methods for the highly stereoselective preparation of optically active compounds.[1] Of all biocatalysed reactions, the asymmetric reduction of ketones with whole cells or isolated enzymes is particularly noteworthy,[2,3] as it leads to enantiopure secondary alcohols. In view of the possible transformations of alcohols into other functionalities, these approaches are of great synthetic utility and interest. Economically, the use of microbial whole cells presents advantages over isolated dehydrogenases, which require expensive coenzymes and recycling systems. In view of this fact, the search for new microorganism strains with improved selectivity is of fundamental importance to this area of catalysis. Recently, we have explored new microorganism strains native to the Brazilian rain forests for synthetic purposes, for instance in the oxidation of sulfides[4] and the hydrolysis of epoxides.[5] The availability of the microorganisms[6] makes these synthetic methodologies widely accessible. As part of this program, we initiated the search for microorganisms of the tropical rain forests[6] with oxidoreductase activity. The substrates used in this study were chosen considering the importance of optically active fluorinated compounds.[7,8] The prochiral fluorinated ketones **1a**–**c** were submitted to whole cells cultures of *Rhizopus oryzae* CCT 4964, *Aspergillus terreus* CCT 3320 and *Aspergillus terreus* CCT 4083 (Scheme 1). Different selectivities were observed for each of the mentioned microorganisms.



Scheme 1. Reduction of fluoroacetophenones with whole cells of fungi.

2. Results and discussion

2.1. Reduction of *o*-fluoroacetophenone 1a

The microbial reduction of ketone **1a** was carried out with whole cells of *R. oryzae* CCT 4964, *A. terreus* CCT 3320 and *A. terreus* CCT 4083 in Sörensen buffer medium (see Section 4 for reaction and analysis conditions). The results are summarized in Table 1. The best result for the bioreduction was achieved when whole cells of *R. oryzae* CCT 4964 were used. The reaction occurred rapidly, yielding alcohol (*S*)-(−)-**2a** with high conversion rate (*c* 98%) and excellent enantiomeric excess (e.e. 99%). The observed enantioselectivity is in accord with Prelog's rule.[9] In addition, this reaction was performed on preparative scale using compound **1a** (561 mg) and whole cells of *R. oryzae* (12 g), to afford (*S*)-(−)-**2a** in 90% yield with 99% e.e. after 72 h.

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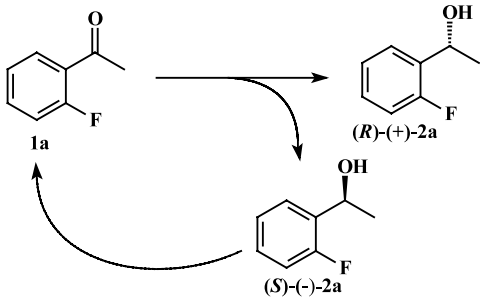
Table 1. Microbial reduction of *o*-fluoroacetophenone **1a** with whole cells of fungi

#	*A. terreus* CCT 3320		*A. terreus* CCT 4083		*R. oryzae* CCT 4964[a]	
t (days)	*c* (%) **2a**	e.e. (%) **2a**	*c* (%) **2a**	e.e. (%) **2a**	*c* (%) **2a**	e.e. (%) **2a**
1	37	67 (*S*)	58	60 (*S*)	>98	98 (*S*)
2	57	68 (*S*)	85	59 (*S*)	>98	99 (*S*)
3	69	65 (*S*)	91	57 (*S*)	>98	99 (*S*)
4	63	53 (*S*)	92	53 (*S*)		
5	60	35 (*S*)	92	46 (*S*)		
6	61	25 (*S*)	93	43 (*S*)		
7	63	07 (*R*)	nd	nd		
8	65	07 (*R*)	nd	nd		
10	71	40 (*R*)	nd	nd		
11	75	55 (*R*)	nd	nd		
17	98	72 (*R*)	94	66 (*R*)		

t: time; *c*: conversion determined by GC; e.e.: enantiomeric excess
[a] Isolated yield: 90%; nd: not determined.

When the bioreduction of **1a** was performed with *A. terreus* CCT 3320 and *A. terreus* CCT 4083 different behaviour was observed. In the course of the first six days an increase in the conversion and a decrease in the enantiomeric excess attributed to (*S*)-(−)-**2a** were observed. After this time the conversion rate continued to increase, but the enantiomeric excess was now in favour of (*R*)-**2a**. A similar result was also observed when the bioreduction of **1a** was performed with *A. terreus* CCT 4083. An inversion of configuration from the (*S*)-**2a** to (*R*)-**2a** was detected along the reaction course (Table 1). According to these results, we can suggest that an oxido-reduction process occurred when **1a** was submitted to the action of *A. terreus* CCT 3320 and *A. terreus* CCT 4083. This process consisted of rapid oxidation of (*S*)-**2a** of the scalemic mixture initially formed, to regenerate the original ketone **1a**, which underwent further reduction yielding (*R*)-**2a**. This mechanism supposes that oxidation of (*R*)-**2a** does not occur under these conditions or is very slow (Scheme 2).



Scheme 2. Mechanistic pathway proposed for the biotransformation of **1a** by whole cells of *A. terreus*.

This process results in deracemization[10] of the initially formed secondary alcohol, an assumption that was confirmed when we exposed the racemic alcohol **2a** to whole cells of *A. terreus* CCT 3320 and *A. terreus* CCT 4083 (Table 2). As can be seen from Table 2, the mixture becomes enriched in the (*S*)-enantiomer during the reaction.

2.2. Reduction of *m*-fluoroacetophenone 1b

The bioreduction of ketone **1b** was also promoted by whole cells of *A. terreus* CCT 3320, *A. terreus* CCT 4083 and *R. oryzae* CCT 4964 (Table 3). As shown in Table 3, using the fungi *A. terreus* CCT 3320 and *A. terreus* CCT 4083, and monitoring the reaction course by GC analysis, we observed that after 24 h the e.e. of compound (*R*)-(+)-**2b** were low (up to 38%), but during the reaction the e.e. increased to 99% with anti-Prelog enantioselectivity. This increase in e.e. is in contrast to the definition of selectivity, which should be constant in enzymatic reactions.[1a] Most probably, a similar process to that observed for **1a** also occurred for **1b**. To confirm this assumption, racemic alcohol (±)-**2b** was submitted to the reaction with whole cells of *A. terreus* CCT 3320 and *A. terreus* CCT 4083 (Table 4), where we observed a similar deracemization process to that seen with (±)-**2a**. When cells of *A. terreus* CCT 3320 were used, the alcohol (*R*)-(+)-**2b** was obtained with e.e. >99% and 91% conversion. On the other hand, the fungus *A. terreus* CCT 4083 deracemized (±)-**2b** to afford (*S*)-(−)-**2b** (Table 4). GC analysis using a chiral column revealed that the (*S*)-(−)-**2b** enantiomer of the racemic mixture was selectively oxidized to yield the prochiral ketone **1b**, which was selectively reduced to (*R*)-(+)-**2b** (Fig. 1). Bioreduction of **1b** with *R. oryzae* CCT 4964 and *A. terreus* CCT 4083 led to the preferential formation of the alcohol **2b** with (*S*)-configuration (Table 3).

2.3. Reduction of *p*-fluoroacetophenone, 1c

Compound **1c** was reduced with the three fungi *A. terreus* CCT 3320, *A. terreus* CCT 4083 and *A. terreus* CCT 4964 (Table 5). It was found that *A. terreus* CCT 3320 promoted the reduction of **1c** to (*S*)-**2c** with 95% enantiomeric excess and 30% conversion. When the reaction was carried out in a higher scale (15 g of whole cells, 100 μL substrate, 72 h) a better conversion was achieved (*c* 52%, e.e. 97%). Reaction of **1c** with *R. oryzae* CCT 4964 led to (*R*)-(+)-**2c** with 70% conversion and 74% e.e. with anti-Prelog enantioselectivity. The

Table 2. Deracemization of (*o*-fluorophenyl)ethanol (±)-**2a** with whole cells of fungi

#	*A. terreus* CCT 3320		*A. terreus* CCT 4083		
t (days)	*c* (%) **1a**	*c*[a] (%)	e.e. (%) **2a**	*c*[b] (%) **2a**	e.e. (%) **2a**
1	–	–	1 (*S*)	–	4 (*S*)
2	1	5	11 (*S*)	2	9 (*S*)
3	4	8	21 (*S*)	4	14 (*S*)
4	7	9	38 (*S*)	7	19 (*S*)
5	7	10	51 (*S*)	10	25 (*S*)
6	5	9	54 (*S*)	13	31 (*S*)
7	3	8	56 (*S*)	15	36 (*S*)
8	2	6	61 (*S*)	21	52 (*S*)

t: time; *c*: conversion determined by GC; e.e.: enantiomeric excess
[a] *ortho*-fluorophenol.[11]
[b] The ketone **1a** was not detected.

Table 3. Microbial reduction of *m*-fluoroacetophenone **1b** with whole cells of fungi

#	*A. terreus* CCT 3320[a]		*A. terreus* CCT 4083		*R. oryzae* CCT 4964	
t (days)	*c* (%) **2b**	e.e. (%) **2b**	*c* (%) **2b**	e.e. (%) **2b**	*c* (%) **2b**	e.e. (%) **2b**
1	19	38 (*R*)	33	2 (*S*)	91	57 (*S*)
2	32	58 (*R*)	57	6 (*S*)	92	61 (*S*)
3	45	76 (*R*)	63	26 (*S*)	92	62 (*S*)
4	69	90 (*R*)	65	46 (*S*)		
5	90	>99 (*R*)	66	65 (*S*)		
6	91	>99 (*R*)	67	72 (*S*)		
7			71	79 (*S*)		
8			75	83 (*S*)		

t: time; *c*: conversion determined by GC; e.e.: enantiomeric excess.
[a] Isolated yield: 48%.

Table 4. Deracemization of (*m*-fluorophenyl)ethanol (±)-**2b** with whole cells of fungi

#	*A. terreus* CCT 3320[a]		*A. terreus* CCT 4083[b]	
t (days)	*c* (%) **1b**	e.e. (%) **2b**	*c* (%) **1b**	e.e. (%) **2b**
1	6	2 (*R*)	6	1 (*S*)
2	23	20 (*R*)	14	42 (*S*)
3	18	68 (*R*)	14	75 (*S*)
5	12	>99 (*R*)	18	95 (*S*)
7	12	>99 (*R*)	16	97 (*S*)

t: time; *c*: conversion determined by GC; e.e.: enantiomeric excess.
[a] Isolated yield: 35%.
[b] Isolated yield: 59%.

deracemization of (±)-**2c** with *A. terreus* CCT 3320 and *A. terreus* CCT 4083 was attempted with no success. This result was already expected in view of the low yield of the reduction process with this specific substrate (Table 5).

3. Conclusion

In conclusion, we have reported an efficient method for the reduction of fluoroacetophenones to the corre-

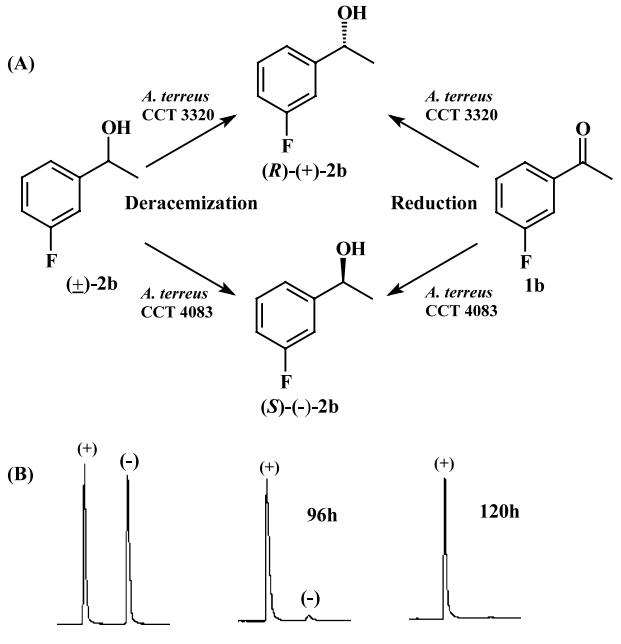


Figure 1. (A) Deracemization of (±)-**2b** and reduction of **1b** by whole cells of *A. terreus* CCT 3320 and *A. terreus* CCT 4083. (B) Chromatograms of the deracemization reaction of (±)-**2b** with *A. terreus* CCT 3320.

Table 5. Microbial reduction of *p*-fluoroacetophenone **1c** with whole cells of fungi

#	*A. terreus* CCT 3320		*A. terreus* CCT 4083		*R. oryzae* CCT 4964	
t (days)	*c* (%) **2c**	e.e. (%) **2c**	*c* (%) **2c**	e.e. (%) **2c**	*c* (%) **2c**	e.e. (%) **2c**
1	33	88 (*S*)	40	82 (*S*)	23	76 (*R*)
2	42	95 (*S*)	58	78 (*S*)	34	77 (*R*)
3	35	94 (*S*)	52	70 (*S*)	48	76 (*R*)
4	30	95 (*S*)	44	58 (*S*)	57	75 (*R*)
5	28*	96 (*S*)	37	42 (*S*)	68	74 (*R*)
6	28	96 (*S*)	36	33 (*S*)	70	74 (*R*)
7	28	95 (*S*)	33	20 (*S*)	79	69 (*R*)
8	29	97 (*S*)	31	22 (*S*)	84	69 (*R*)

t: time; *c*: conversion; e.e.: enantiomeric excess.
* Isolated yield: 18%.

sponding alcohols using whole cells of fungi. *R. oryzae* CCT 4964 proved to be an excellent biocatalyst in the reduction of *o*-fluoroacetophenone **1a**. The fungus *A. terreus* CCT 3320 yielded the best results for the bioreduction of *m*-fluoroacetophenone **1b** and *p*-fluoroacetophenone **1c**. In addition, *A. terreus* CCT 3320 and *A. terreus* CCT 4083 promoted the efficient deracemization of (*m*-fluorophenyl)ethanol **2b**. The results show that these microorganisms have great potential to perform bioenzymatic reduction and deracemization reactions. The enantioselectivity was anti-Prelog in some cases. Further studies with microbial cells of native Brazilian fungi are currently in progress within our research group.

4. Experimental

4.1. 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 followed by heating at about 120°C. Flash column chromatography was performed using Merck 60 silica (230–400 mesh). Enzymatic reactions were monitored by GC (FID) in a Shimadzu GC-17A chromatograph, using hydrogen as a carrier gas or by GCMS with a Shimadzu GCMS 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).

4.2. Synthesis of substrates

Alcohols (±)-**2a**, (±)-**2b** and (±)-**2c** were obtained by reduction with sodium borohydride (20.52 mg; 0.54 mmol) of the corresponding ketones **1a**, **1b** and **1c** (300 mg; 2,17 mmol) in methanol (10 mL). After work-up with saturated aqueous solution of NH_4Cl (4 mL), the organic layer was extracted with ethyl acetate (3×50 ml) dried over $MgSO_4$ and the solvent was evaporated. The residue was purified on a silica gel column using as eluent hexane and ethyl acetate (80:20). The ^{1}H NMR and ^{13}C NMR spectra of these compounds were in agreement with those reported in the literature.[7a]

4.3. Growth conditions for the microorganism cultures and reaction with 1a–c and 2a–c

The microorganisms *Rhizopus oryzae* CCT 4094 (Costa, A. S., 04/96. Isolated from fermented Manihot esculenta, Amazon Forest, Brazil), *Aspergillus terreus* CCT 3320 (Attili, D. S., 09/93. Isolated from the soil. Rain Atlantic Forest, Brazil) and *Aspergillus terreus* CCT 4083 (Pfenning, L., 08/94. Isolated from the soil. Amazon Forest, Brazil) were purchased from the Culture Collection of the André Tosello Foundation (Brazil).[6] The fungi were grown in culture shaker-flasks (140–170 rpm, 250 mL erlenmeyers) in 100–130 mL of Oxoid malt extract (20 g/L, 72–96 h) at 28–32°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.

4.3.1. Small scale reactions. The appropriate fluoroacetophenone **1a–c** (20 μL) and whole cells of *A. terreus* (5 g) or *R. oryzae* (3 g) were mixed in erlenmeyer flasks (125 mL) containing phosphate buffer solution (50 mL). Alternatively, reactions using 100 μL of ketone **1a** were performed in 250 mL flasks containing whole cells of *A. terreus* (15 g) or *R. oryzae* (5 g).

4.3.2. Preparative-scale reduction reaction. *o*-Fluoroacetophenone (500 μL) and *R. oryzae* cells previously filtered (12 g) were mixed in a 500 ml erlenmeyer flask containing phosphate buffer solution (200 mL). The mixture was agitated on a rotary shaker (28–32°C, 170 rpm) until the starting material was completely depleted.

4.3.3. Deracemization reaction of 2a–c. The appropriate (fluorophenyl)ethanol **2a–c** (20 μL) and whole cells of *A. terreus* CCT 3320 and *A. terreus* CCT 4083 (5 g) were mixed in erlenmeyer flasks (125 mL, 5 flasks) containing phosphate buffer solution (50 mL). The reaction was monitored by chiral GC analysis.

4.3.4. General procedure for the extraction of alcohols 2a–c. The reactions were monitored by GC and, after appropriate conversion, the mixture was filtered and the aqueous phase was extracted with ethyl acetate (4×50 mL). The yellow organic phase was dried over $MgSO_4$ and evaporated. The residue was purified on a silica gel column using as eluent hexane and ethyl acetate (80:20) to yield compounds **2a–c**.

4.4. Determination of the enzymatic activity of the fungi

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 from chemical reduction (see Section 4.5). GC conditions: oven 100°C; injector 200°C; detector 220°C; rate 1°C/min; retention time for **2a** ($R=10.4$ min.; $S=11.5$ min.), **2b** ($R=11.3$ min.; $S=12.7$ min.) and **2c** ($R=10.9$ min; $S=12.4$ min).

4.5. Assignment of the absolute configuration of 2a–c

Specific rotation values were measured in a Jasco DIP-378 polarimeter. The reported data refer to the Na-line value using a 1 dm cuvette. (*S*)-(−)-(*o*-fluorophenyl)ethanol **2a**: $[\alpha]_D^{20}$ −34.7 (*c* 3.63, CH_2Cl_2), e.e. 99%; (*R*)-(+)-(*m*-fluorophenyl)ethanol **2b**: $[\alpha]_D^{20}$ +13.6 (*c* 1.25, CH_2Cl_2), e.e. 72%; (*S*)-(−)-(*m*-fluorophenyl)ethanol **2b**: $[\alpha]_D^{20}$ −26.3 (*c* 7.0, CH_2Cl_2), e.e. 90%; (*S*)-(−)-(*p*-fluorophenyl)ethanol **2c**: $[\alpha]_D^{20}$ −21.2 (c 0.33, CH_2Cl_2) e.e. 96%; The absolute configurations were determinated by comparison of the sign of the measured specific rotation with those reported in the literature.[7a]

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11. The compound was identified by GC-MS analysis. The structure was confirmed after comparison with Mass Spectral Database (CLASS-5000/WILEY).