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Novel fungi-catalyzed reduction of α-alkyl-β-keto esters

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ABSTRACT

A screening of 15 fungi and yeast strains was carried out in fermentation processes to perform the diastereo- and enantioselective reduction of ethyl 2-methyl-3-oxobutanoate, to the corresponding (*R**,*S**)-3-hydroxy-2-methyl esters. Overall, biotransformations led to excellent conversions, as well as good to excellent diastereo- and enantioselectivities. A strain of *Aureobasidium pullulans* (CCM H1) was found to be the most efficient biocatalyst in terms of conversion (100%), *syn*:*anti* ratio (3:97), and enantiomeric excess (94% *anti*-(2*S*,3*S*) isomer). This biotransformation was successfully carried out on a preparative level as well. Other microorganisms, such as *Fusarium graminearum* (CCM HH 224), *Aspergillus terreus* (BFQU 121), *Geotrichum candidum* (CCM H38), *Trichoderma koningii* (ATCC 76666), and *Aspergillus niger* (CCM H21) also showed excellent diastereo- and enantioselectivities, combined with high conversions (>95% conversion, ⩾95% ee, and excellent *syn*:*anti* ratios). Many of the strains used in this work had scarcely been described as oxido-reducing agents, or had never been used with the substrates reported herein.

1. Introduction

The use of enzymatic transformations with either whole microorganisms or isolated enzymes to carry out stereospecific and stereoselective reactions has grown steadily over the last few decades.[1] In particular, fungal or yeast-catalyzed reactions have been used as stereoselective alternatives for hydroxylations, sulfoxidations, epoxidations, Baeyer–Villiger oxidations, deracemizations, and stereo- and enantioselective reductions of ketones.[2]

The enzymatic reduction of prochiral ketones is a widely investigated method to produce chiral building blocks.[3–8] A broad range of structurally different chiral alcohols can be obtained depending on the substrate structure, as a consequence of the huge variety of enzymes available,[9–20] together with the catalytic promiscuity shown by many of them.[21–23] Likewise, recombinant microorganisms overexpressing reductases are also powerful biocatalysts that can provide excellent yields and enantiomeric excesses due to their high specificity.[24–30] Moreover, different non-conventional reaction systems can be used, such as ionic liquids as (co-)solvents,[31–35] which clearly enhance the practical applicability of these synthetic approaches.

Filamentous fungi or yeast-catalyzed reductions of carbonyl compounds have been reported in the past few years as an attractive methodology to prepare chiral alcohols, since it is not only an environmentally friendly procedure, but also an economic alternative compared to expensive commercial reductases.[13,36–49] The low cost of this whole cell-catalyzed process, often contrasts with the fact that side products are frequently obtained due to the presence of other competitive enzymes, or the low efficiency associated with the low permeability of the cell membrane toward the substrate.[50] As a consequence, the importance of finding new microorganisms that could catalyze highly stereoselective processes in an efficient manner remains open.

Herein we report our findings on the screening of different microorganisms, fungi and yeasts, as catalysts for the stereoselective reduction of ethyl 2-methyl-3-oxobutanoate. We were particularly interested in identifying new microorganisms that are able to perform diastereospecific reactions, thus generating two stereogenic centers within one biocatalytic turnover. This task is part of our research program for designing inexpensive as well as environmentally responsible methodologies to prepare chiral building blocks.

2. Results and discussion

Our model reaction was a diastereoselective synthesis starting from substrate **1**, which contains both a stereogenic and a prochiral center. In this reduction, up to four stereoisomers can be produced, due to the presence of two stereogenic centers in the product (Scheme 1).

The characterization of the four possible isomers was carried out. A diastereomeric mixture of ethyl α-methyl-β-hydroxy esters was used as standard for GC analysis. Such a diastereomeric mixture was obtained by $NaBH_4$ reduction of the corresponding racemic ethyl α-methyl-β-keto ester according to the procedure described in the Experimental (Section 4.4.1). By GC analysis, two pairs of peaks of different areas were observed, which corresponded to the two pairs of enantiomers, and which is consistent with a

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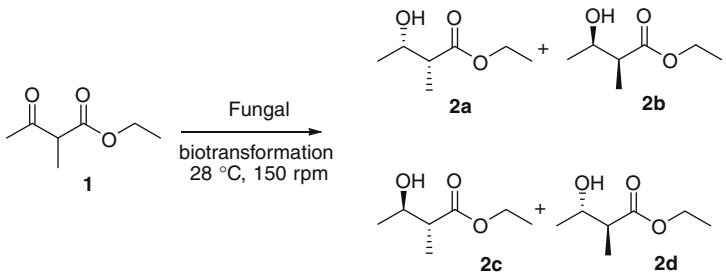

Scheme 1. Fungi-catalyzed reduction of racemic ethyl 2-methyl-3-oxobutanoate.

diastereo- but non-enantioselective chemical reduction mediated by $NaBH_4$. To assist in the assignment of the absolute configuration, the (2*R*,3*S*)- and (2*S*,3*S*)-isomers, **2a** and **2d**, respectively, were prepared as standards. *syn*-Isomer **2a** was obtained (97.2% de; >99% ee) using a recombinant *Escherichia coli* JM 105 strain expressing Gcy1p aldo keto reductase, according to the procedure described by Rodríguez et al.[30] This compound was then assigned in the GC analysis (**2a**, t_R = 29.5 min). Considering that the $NaBH_4$-mediated reduction of a racemic compound is not enantioselective, the enantiomer of **2a** was identified as the only other peak with the same area in the chromatogram. Hence, ethyl (2*S*,3*R*)-2-methyl-3-hydroxybutanoate, **2b**, was assigned as the peak with t_R = 31.9 min.. The *anti*-isomer **2d** was prepared (81% conversion; *anti*/*syn* ratio 89/11; 64% ee) according to the literature,[28] using the recombinant *E. coli* strain BL21 (DE3) *ΔyqhE* (pPP4), allowing us to assign (2*S*,3*S*)-2-methyl-3-hydroxybutanoate as the peak with t_R = 36.0 min. Finally, the remaining isomer **2c** (t_R = 33.2 min) was assigned as ethyl (2*R*,3*R*)-2-methyl-3-hydroxybutanoate (Fig. 1). This assignment of chromatographic peaks was further confirmed by transesterification of the diastereomeric mixture with vinyl acetate using *Candida antarctica* B lipase (CaL B) as a biocatalyst. Only isomers **2b** and **2c** were acetylated, and no consumption of isomers **2a** and **2d** was observed. The acetylated compounds had retention times of 35.5 and 35.7 min. In agreement with Kazlauskas rule,[51] only the (3*R*)-alcohols were transesterified, and the assignment of **2b** [t_R = 31.9 min as the (2*S*,3*R*)-isomer and **2c** (t_R = 33.2 min) as ethyl (2*R*,3*R*)-2-methyl-3-hydroxybutanoate] was confirmed.

Once the analytic characterization was finished, 15 different strains of fungi and yeasts were screened as biocatalysts for the stereoselective reduction of racemic ethyl 2-methyl-3-oxobutanoate (Scheme 1). The reactions were performed using culture media described in Section 4.1., in an orbital shaker at 28 °C and 150 rpm, according to the general procedures described in the Experimental (Section 4.4.1). Aliquots were taken at 24, 48, 72, and 96 h. The results are summarized in Table 1.

In most cases, biotransformations led to good to excellent conversions, except for *Botrytis cinerea* (entry 9, no conversion observed), and for *Rhizopus* sp. and *Phanerochaete chrysosporum* strains, in which only moderate conversions were obtained (entries 13 and 15). All microorganisms preferentially afforded the *anti*-isomers, except for *P. chrysosporum*, which gave a *syn*:*anti* ratio 70:30 (entry 15), together with excellent enantiomeric excesses. The highest diastereoselectivity was obtained with *Aureobasidium pullulans* (entry 2, *syn*:*anti* 3:97; 100% conversion), displaying excellent enantioselectivities (for both *syn* and *anti* isomers) as well. The biotransformation was successfully conducted at preparative scale (0.5 g of substrate, 70% isolated yield), showing the same diastereo- and enantioselectivities. In addition, other strains such as *Fusarium graminearum*, *Aspergillus terreus*, *Geotrichum candidum*, *Trichoderma koningii*, and *Aspergillus niger*

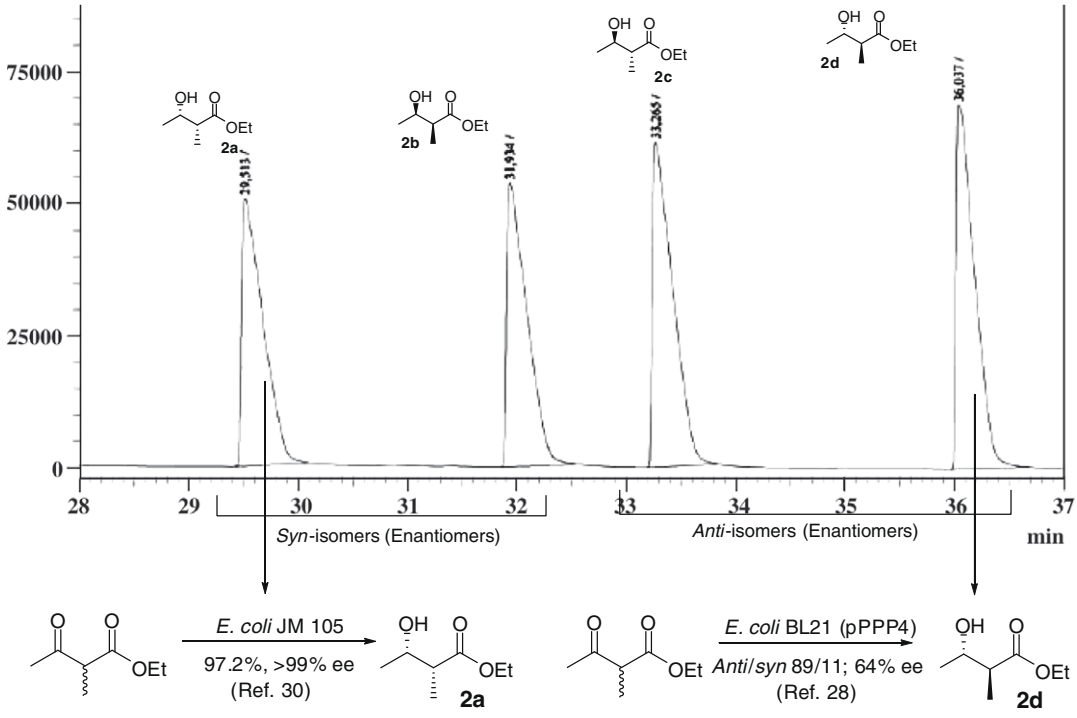

Figure 1. Characterization of diastereomers.

Table 1
Reduction of Ethyl 2-methyl-3-oxobutanoate by fungi and yeast strains

Entry	Strain	Reaction time (h)	Conversion[a,b] (%)	Ethyl 3-hydroxy-2-methylbutanoate		
				syn:anti	ee[b] (%)	
					syn-(2*R*,3*S*)	*anti*-(2*S*,3*S*)
1	*Penicilium expansum* (CCM P17)	96	91	12:88	90	94
2	*Aureobasidium pullulans* (CCM H1)	24	100	3:97	>99	94
3	*Penicilium italicum* (CCM L7)	72	82	30:70	64	84
4	*Aspergillus ochraceus* (CCM H22)	96	93	13:87	>99	96
5	*Fusarium graminearum* (CCM H224)	24	95	6:94	86	96
6	*Aspergillus terreus* (BFQU 121)	48	100	6:94	86	96
7	*Geotrichum candidum* (CCM H38)	96	96	10:90	>99	97
8	*Trichoderma koningii* (ATCC 76666)	96	100	7:93	60	96
9	*Botrytis cinerea* (CCM P18)		No conversion			
10	*Alternaria* sp. (CCM A3)	48	93	27:73	>99	97
11	*Kluyveromyces marxianus* (NRRL Y-8281)	24	100	19:81	>99	90
12	*Trichoderma atroviride* (CCM M2)	24	100	25:75	98	96
13	*Rhizopus* sp. (CCM H43)	72	46	11:89	93	97
14	*Aspergillus niger* (CCM H21)	96	100	8:92	>99	96
15	*Phanerochaete chysosporum* (CCM B4)	96	53	70:30	96	>99

[a] Conversion is expressed as % substrate transformed into alcohol product.
[b] Determined by chiral GC analysis. Culture collections: http://www.atcc.org/, http://nrrl.ncaur.usda.gov/, http://mail.fq.edu.uy/~microbio/.

(entries 5–8, 14) performed the bioreduction with excellent conversions (higher than 95%), *syn*:*anti* ratios (10:90–6:94) and enantiomeric excesses (higher than 95% for the *anti* isomer). To the best of our knowledge, microbial reductions using *P. chrysosporum* and *F. graminearum* have been scarcely described for the reduction of xenobiotics.[41,52–55]

Considering our screening results, in terms of diastereo- and enantioselectivities, herein we add a new promising group of enzymes that can be worth trying for further cloning, over-expressing, and deeper biochemical and biocatalytic characterization. Moreover, despite reductions with *A. ochraceus* were briefly described sometime ago,[56,57] no recent progress in this topic has been reported in the literature. Reductions of aromatic ketones and keto-acid derivatives using *Aureobasidium* spp. were described as well, showing moderate to good performances;[58–60] 4-chloro-3-oxoesters were successfully reduced using different reaction systems,[61] but no reports are known of α-alkyl-β-ketoesters reduction with *Aureobasidium* spp.

Moreover, our reductions using the *T. koningii* (ATCC 76666) strain led preferentially to the *anti*-(2*S*,3*S*) enantiomer (entry 8, with *syn*:*anti* ratio 7:93; 94% ee), instead of the *syn*-(2*S*,3*R*) enantiomer previously reported by Iwamoto et al. (*anti*:*syn* 28:72).[41]

3. Conclusion

We conducted the screening of fifteen microbial strains of fungi and yeasts. Most of the microorganisms showed a strong preference for the *anti*-isomer, except for *P. chrysosporum* where the *syn*-isomer was predominant, displaying a *syn*:*anti* ratio of 70:30. Promising results were obtained with *A. pullulans*, which gave complete conversion (100%) in short reaction times (24 h) via a highly diastereo- and enantioselective process (*syn*:*anti* 3:97; 94% ee of *anti*-(2*S*,3*S*) isomer). These results were confirmed on a preparative scale. Moreover, five other strains (*F. graminearum*, *A. terreus*, *G. candidum*, *T. koningii*, and *A. niger*) performed this biocatalytic reduction with excellent yields (>95%), showing a remarkable preference for the *anti*-isomer and excellent enantioselectivities (>95% ee for the *anti*-isomer). When using *T. koningii* (ATCC 76666) we found opposite results compared to those reported by Iwamoto et al.,[41] preferentially obtaining the *anti*-(2*S*,3*S*) enantiomer with high enantioselectivity, instead of the *syn*-(2*S*,3*R*).

Considering the excellent selectivities uncovered, the findings reported herein are a valuable contribution to the challenge of discovering new biocatalysts for the highly stereoselective reduction of prochiral building blocks. Preliminary studies on the scale-up of the process were successfully carried out, and further experiments on this topic are currently in progress.

4. Experimental

4.1. Fungal biotransformations

Fifteen strains of fungi and yeasts were screened for their reductase activity. All microorganisms belong to the collection of the Microbiology Laboratory of the Biosciences Department (Facultad de Química, UdelaR; http://mail.fq.edu.uy/~microbio/) and are freely available upon request.

The culture medium contained (g/l): sucrose (50), $(NH_4)_2SO_4$ (5), KH_2PO_4 (2), $CaCl_2{\cdot}2H_2O$ (0.25), and $MgSO_4{\cdot}7H_2O$ (0.25), except for the culture of *Trichoderma*, where Czapec medium (pH 7.3) was used, according to the literature.[41]

4.2. Chemicals and analysis

Solvents were purified and dried by conventional methods. Commercial reagents were purchased from Sigma–Aldrich Inc.

The purity of the reactants, as well as the development of the reactions, were controlled using analytical TLC on silica gel (Kieselgel HF254 from Macherey-Nagel) and visualized with UV light (254 nm) and/or *p*-anisaldehyde in acidic ethanolic solution. Further analyses were performed by chiral gas chromatography (GC) in Shimadzu 2010 equipment, with an FID detector and a Megadex DET-TBS (25 m × 0.25 mm × 0.25 μm) column. Temperature program: 60 °C/1 °C/min/70 °C (10 min)/2 °C/min/150 °C (5 min). T_{SPLIT}: 220 °C, T_{FID}: 250 °C. The % ee and absolute configuration of the reduction products were determined by chiral gas chromatography, comparing with the standards, as described in the text. Column chromatography was performed using silica gel flash (Kieselgel 60, EM reagent, 230–240 mesh.) from Macherey-Nagel. NMR spectra (^1H and ^{13}C) were carried out in a Bruker Avance DPX 400 MHz equipment. All experiments were carried out at 30 °C, $CDCl_3$ was used as solvent and TMS was used as an internal standard. Optical rotations were measured at 25 °C on a Zuzi 412 polarimeter using a 0.5 dm cell. $[\alpha]_D$ values are given in units of deg cm^2 g^{-1} and concentration values are expressed in g/100 mL.

Biotransformations were conducted in orbital shakers from Thermoforma (model 420) and Sanyo (model IOX400.XX2.C).

4.3. Racemic ethyl 2-methyl-3-oxobutanoate 1

Ethyl 2-methyl-3-oxobutanoate was prepared from commercially available ethyl 3-oxobutanoate by alkylation with methyl iodide, according to a procedure described in the literature.[62] Ethyl 3-oxobutanoate (0.2 g, 1.54 mmol) was dissolved in anhydrous acetone (2 mL) under a nitrogen atmosphere. Dried potassium carbonate (0.199 g, 1.44 mmol) was added with magnetic stirring. The solution was maintained at room temperature for 10 min, and methyl iodide (0.12 mL, 1.89 mmol) was added with a syringe. The reaction was refluxed for 5 h. After the reaction was complete, diethyl ether was added (3 mL), and the mixture was filtered. The solvent was evaporated under vacuum, and the residue was purified by flash column chromatography (silica gel, Hex/AcOEt, v/v, 95:5) giving compound **1** (0.211 g, 95%). ^{1}H NMR: δ (ppm) = 4.24–4.19 (m, 2H, CH_2), 3.49 (q, J = 7.2 Hz, 1H, CH), 2.26 (s, 3H, CH_3), 1.35 (d, J = 7.2 Hz, 3H, CH_3), 1.29 (t, J = 7.1 Hz, 3H, CH_3). ^{13}C NMR: δ (ppm) = 204.0, 170.9, 61.7, 54.0, 28.7, 14.4, 13.1. GC: t_R = 27.9, 28.0 min.

4.4. Ethyl 3-hydroxy-2-methylbutanoates 2a–d

4.4.1. Chemical reduction

Sodium borohydride (0.244 g, 0.646 mmol) was added in dry ethanol (15 mL) under a nitrogen atmosphere, and the mixture was cooled to 0 °C and stirred for 10 min. A solution containing ethyl 2-methyl-3-oxobutanoate (0.2 g, 1.4 mmol) in dry ethanol (5 mL) was then added dropwise. After stirring for 3 h at 0 °C, the reaction was quenched with saturated ammonium chloride, after which ethanol was distilled under reduced pressure. Next 15 mL of water was added, and then extracted thrice with ethyl acetate (3 × 15 mL). The organic layer was dried over $MgSO_4$, and the solvent was distilled under vacuum. The residue was purified by flash column chromatography (silica gel, Hex/AcOEt, v/v, 8:2) giving compound **2a–d** (diastereomeric mixture) (0.192 g, 95%). ^{1}H NMR: δ (ppm) = 4.18–4.13 (q, J = 7.1, 4H, CH_2), 4.05–4.02 (m, 1H, CH), 3.89–3.84 (m, 1H, CH), 2.50–2.39 (m, 2H, CH), 1.26 (t, J = 7.1, 6H, CH_3), 1.21–1.13 (m, 12H, CH_3). ^{13}C NMR: δ (ppm) = 176.1, 69.7, 68.0, 61.0, 60.6, 47.4, 45.5, 21.0, 19.7, 14.5, 14.2, 11.5, 11.0. GC: t_R = 36.0 min (27.3%), t_R = 33.1 min (26.5%), t_R = 31.9 min (23.2%) and t_R = 29.5 min (22.9%).

4.4.2. Fungal reduction. Screening of microorganisms

The corresponding culture medium (50 mL) was inoculated in a 125 mL flask, with 5 mL of spores suspension (aprox. 10^7 cfu/mL), obtained from fresh cultures in PDA slants at 25 °C. Cultures were incubated for 3 days at 28 °C with orbital shaking (150 rpm), then 50 mg of ethyl 2-methyl-3-oxobutanoate was added and incubated for 96 h at 28 °C with shaking. Reduction products of ethyl 2-methyl-3-oxobutanoate were extracted from the culture medium with ethyl acetate (3 × 5 mL), and the organic layer was dried over anhydrous $MgSO_4$. The desiccant agent was filtered, and ethyl acetate was distilled under vacuum on a rotary evaporator at 30 °C. The residue was taken in CH_2Cl_2, and aliquots containing 1 mg/mL of reduction products were analyzed by GC using the conditions described in Section 4.2.

4.4.3. Preparative microbial reduction with *A. pullulans*

The culture medium (500 mL) was inoculated in a 1 L flask, with 10 mL of spores suspension (ca. 10^7 cfu/mL), obtained from fresh cultures in PDA slants at 25 °C. Cultures were incubated for 3 days at 28 °C with orbital shaking (150 rpm). Then 500 mg of ethyl 2-methyl-3-oxobutanoate was added and incubated for 96 h at 28 °C with shaking. Reduction products of ethyl 2-methyl-3-oxobutanoate were extracted from the culture medium with ethyl acetate (3 × 40 mL), and the organic layer was dried over anhydrous $MgSO_4$, filtered, and distilled under vacuum at 30 °C. The residue was purified by column chromatography (silica gel, Hex/AcOEt, v/v, 8:2), affording **2d**. Isolated yield: 70%. $[\alpha]_D^{20} = +22.5$ (c 1.20, $CHCl_3$).

4.5. CaL B-catalyzed transesterification of a diastereomeric mixture of ethyl 3-hydroxy-2-methylbutanoate

The experiments were carried out in orbital shaker at 30 °C and 200 rpm. Vinyl acetate was used as the acyl donor, *C. antarctica* B lipase (CaL B, Novozym 435) was the catalyst of choice and the diastereomeric mixture of ethyl-3-hydroxy-2-methylbutanoate acted as the nucleophile. The reactions were performed using 50 mg of the diastereomeric substrate, the lipase/hydroxy ester ratio (w/w) was 0.3, and the acyl donor/alcohol ratio (mol/mol) was 0.6. Hexane was used as solvent. The reaction was complete in 3 h, the enzyme was filtered-off through a short silica-gel column, and the solvent was distilled under vacuum. Chiral CG analysis of the crude was performed, showing complete consumption of the (3*R*)-isomers.

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