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Stereoselective oxidation of alcohols using whole cells of *Rhizomucor miehei* CECT 2749

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Abstract

The oxidation of hydrophobic secondary alcohols catalyzed by immobilized *whole cells of Rhizomucor miehei* CECT 2749 is described for the first time. The biotransformation was performed with the fungus as resting cells and as immobilized catalyst using agarose and agars from different algae genera as matrix. The immobilized biocatalyst shows specificity in the oxidation of de *S* enantiomer of 1-(2-furyl)-ethanol and in the oxidation of *iso*-menthol, while *R*-furylethanol (+)-menthol and *neo*-menthol were not oxidized.

Keywords: *Rhizomucor miehei*; Stereoselective oxidation of alcohols; Immobilization; Agar; Agarose

1. Introduction

The fungus *Rhizomucor miehei* is well known in biotechnology as lipase producer [1] and its potential uses in organic synthesis are widely described in the literature [2–4]. In this paper, we show for the first time the stereoselective oxidation of hydrophobic secondary alcohols using immobilized whole cells of *R. miehei* CECT 2749. The oxidation of alcohols rarely exhibits insurmountable problems to the synthetic organic chemists, but there are some situations where the use of biocatalysts for the oxidation of alcohols could be of particular interest: (i) enzymatic oxidation of primary alcohols for the production of aldehydes [5,6] and (ii) the stereospecific oxidation of one enantiomer of a racemic secondary alcohol [7].

The fungus was immobilized by a new entrapment technique, described in the paper, using agarose or agar from different algae genera as matrix. These matrices are characterized by different methoxylation degrees, leading to different gelling temperatures.

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2. Experimental

R. miehei CECT 2749 was supplied by "Colección Española de Cultivos Tipo (CECT)", University of Valencia. Spain. The fungus was cultured in HAGGS medium [8]: glycine, 2 g/l; Tryptic soy broth, 6 g/l; starch, 20 g/l; mineral solution, 10 ml/l ($FeSO_4 \cdot 7H_2O$, 1 g/l; $MnSO_4 \cdot 4H_2O$, 1 g/l; $CuCl_2$, 0.025 g/l; $CaCl_2$, 0.10 g/l; H_3B0_3, 0.056 g/l; $ZnSO_4 \cdot 7H_2O$, 0.2 g/l; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.019 g/l). The pH of the medium was adjusted to 6.6 after sterilization.

2.1. Chemicals

All substrates were purchased from Sigma–Aldrich. The culture medium components were from Difco and Merck. Agarose D5 (Gelling point (g.p. (1.5%) = 36 ± 1.5 °C) and sulphate <0.12%. Three tailor-made agar types: Ref A27/03 (low methoxylation degree agar from *Pterocladia genus*, g.p. = 34 ± 2 °C), Ref. A28/03 (high methoxylation degree agar from *Gracilaria* g.p. = 42 ± 2 °C) and Ref. A-90 (medium methoxylation degree agar from *Gelidium* g.p. = 36 ± 2 °C) were provided by Hispanagar S.A. (Spain). The amount of sulphate groups in these agars was between 1.5% and 2.5%.

2.2. Culture conditions and immobilization procedure

The fungus was cultured in solid HAGGS medium. After 7 days, small cylinders (≈0.5 cm diameter) of mycelium grown in the solid medium were inoculated in 100 ml erlenmeyer flasks with 20 ml of HAGGS medium at 28 °C and 250 rpm. Fresh mycelium should be harvested after 7–8 days culture time in order to obtain active biomass. After 1 week, the filamentous fungi show a compact sphere-like shape (≈2 cm diameter). This growing shape makes that the traditional trapping immobilization techniques such as microbeads or microplates [7] cannot be used. Therefore, we have developed a new immobilization methodology, which we have called *immersion*. The fungus spherical structures were introduced in liquid agar or agarose at 45 °C, being coated by the thermogel. Afterwards, they were extracted by filtration and transferred to cool sunflower oil ($T < 25$ °C). The oil favours the gelification of agar or agarose around the biomass particles. Then, the coated spheres were washed with *n*-hexane to remove the excess of sunflower oil. Finally the remaining hexane can be removed with NaCl saline solution in order to avoid the deactivation of the biocatalyst by the hydrocarbon. The immobilized biocatalyst can be kept at 4 °C.

The reaction is carried out in erlenmeyer flasks with 100 ml of phosphate buffer pH 6.6, where the fungus is added. Then, the substrates are added to a concentration of 2.5 or 5.0 mM depending on the corresponding reactions. Samples are taken at different times in order to follow the course of the reaction. The temperature and the reaction rate are described in each figure. When a reaction cycle is finished, the fungus is recovered by filtration and washed with NaCl saline solution. Afterwards, the fungus is put into a new erlenmeyer flask and the process above described is repeated.

2.3. Oxidation of alcohols

The analysis of the oxidation of cyclohexanol was performed with a Shimadzu GC-14A with a FID detector and a column SPB^{TM}-1 sulphur (15 m, 0.32 mm, 0.25 μm) (Supelco Inc., Bellafonte, P.A. EEUU). Initial temperature = 90 °C; heating rate = 15 °C/min; final temperature = 200 °C. Carrier gas (N_2) flow = 25 ml/min.

The oxidation of the other alcohols was monitored using a Varian 3400CX-GC equipped with a FID detector and a chiral capillary column CP-7502 (25 m, 0.39 mm, 0.25 μm) from Sugelabor (Spain). Chromatographic conditions: $T_i = 90$ °C; $t_i = 5$ min; heating rate 5 °C/min; T_f: = 155 °C. Carrier flow (He) = 25 psi, and split ratio = 100. The analysis of the oxidation of 1-(2-furyl)-ethanol enantiomers was performed using the same equipment but adjusting the conditions. $T_i = 155$ °C; heating rate 4 °C/min; $T_f = 175$ °C. Carrier flow (He) = 25 psi and split ratio = 100.

3. Results and discussion

Different structures were tested to describe the substrate scope of the fungus in the oxidation of secondary alcohols, using cyclohexanol as non-chiral reference (**1**); *R* and *S*-2-furylethanol (**2**); *cis*-2-decalol (mixture of stereoisomers) (**3**); *trans*-2-decalol (mixture of estereoisomers) (**4**); *R* and *S*-2-tetralol (**5**); 2-adamantanol (**6**); menthol (**7**); *iso*-menthol (**8**) and *neo*-menthol (**9**). The results obtained using native cells are shown in Table 1 (Scheme 1).

Table 1
Oxidation of different alcohols using free and immobilized cells of *Rhizomucor miehei* [substrate] = 2.5 mM; reaction time = 72 h; $T = 28$ °C; stirring speed = 250 rpm

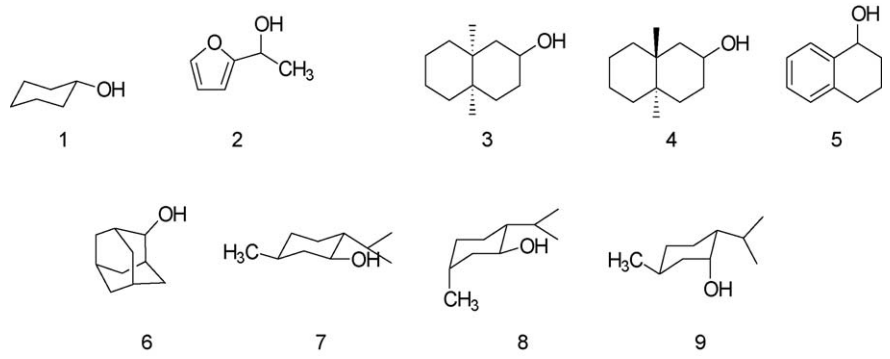
No.	Substrate	Biocatalyst	Yield (%)
1	Cyclohexanol	Resting cells	>99
2[a]	(*R*)-1-(2-Furyl)-ethanol	Resting cells	0
2[b]	(*S*)-1-(2-Furyl)-ethanol	Resting cells	99
		Immobilized in Agar A28/03	99
		Immobilized in Agar A27/03	99
		Immobilized in agarose D5	79.2
3	*cis*-Decalol	Resting cells	25
4	*trans*-2-Decalol	Resting cells	0
5[a]	(*R*)-1-Tetralol	Resting cells	99
		Immobilized in Agar A28/03	99
		Immobilized in Agar A27/03	99
		Immobilized in agarose D5	99
5[b]	(*S*)-1-Tetralol	Resting cells	99
		Immobilized in Agar A28/03	99
		Immobilized in Agar A27/03	99
		Immobilized in agarose D5	99
6	2-Adamantanol	Resting cells	0
7	(+) Menthol[c]	Resting cells	0
8	*iso*-Menthol[c]	Resting cells	99
		Immobilized in Agar A28/03	99
		Immobilized in Agar A27/03	99
		Immobilized in agarose D5	42.0
9	*neo*-Menthol[c]	Resting cells	0

Free cells wet weight, 3.8 g; catalyst wet weight: 4.2 g. Superscripts a and b show the difference between *R* or *S* enantiomers at the same kind of reaction.
[c] Reaction time: 120 h.

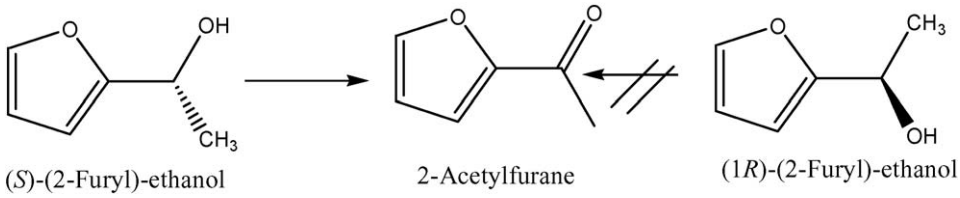
From these results we can deduce that the fungus is very stereoselective with small secondary alcohols such as **2**. Only **2** (*S*) isomer was oxidized (Table 1) without degradation of furane ring (Scheme 2).

The stereoselectivity observed is higher than the ones described for the active isolated yeasts of *Williopsis* genus (e.g. 40%) [7].

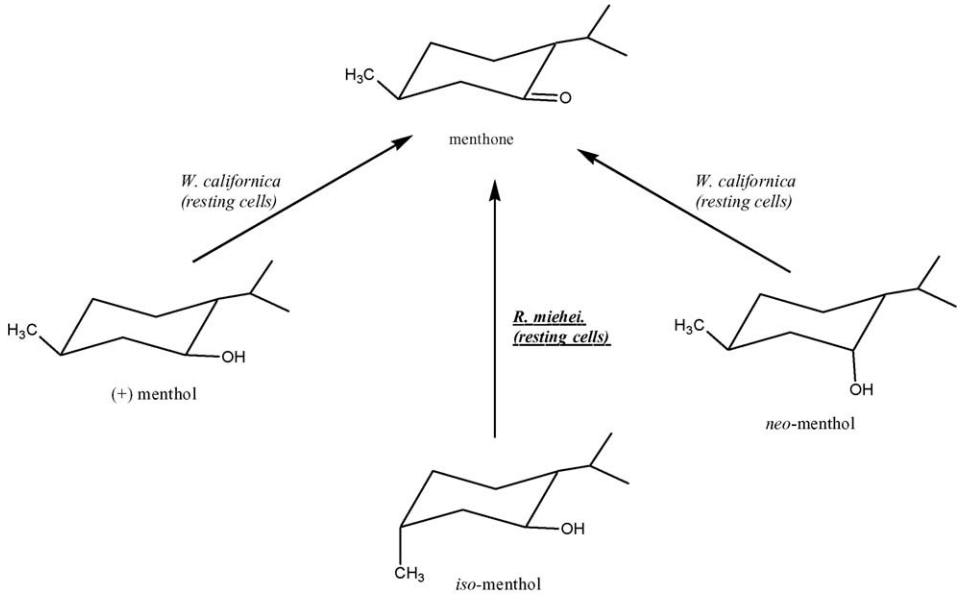
R. miehei CECT 2749 does not show any selectivity in the oxidation of 1–tetralol enantiomers. This behaviour is opposite to the one observed with yeasts of *Williopsis* and *Pachysolen* genera that displayed a marked *S* enantiopreference. [7]. Thus, we can see that *R. miehei* always shows a complementary behaviour, being of great interest in this kind of reactions. *cis*-Bicyclic alcohol such as **3** is slowly oxidized (25% yield 72 h). On the other hand, the *trans*-isomer **4** was not oxidized. A very large alcohol such as **6** was not oxidized. These results indicate that the alkyl-bone of the alcohols is determinant for the stereocontrol. *Rh. miehei* does not show stereoselectivity in the oxidation of (*S*) or (*R*) **5,** where both stereomers are oxidized reaching the



Scheme 1.



Scheme 2.



Scheme 3.

maximum yield at 72 h (see Table 1). The oxidation of menthol isomers was as well interesting. The biocatalyst oxidizes *iso*-menthol to menthone while the other isomers are not oxidized. This behaviour is opposite to the ones described for *Williopsis californica*, *W. saturnus* and *Pachysolen tannophilus* [7]. These oxidize menthol (**7**) and *neo*-menthol (**9**) to menthone but do not oxidize *iso*-menthol (Scheme 3).

The *R. miehei* cells immobilized in different supports were used in the oxidation of cylohexanol. In Fig. 1 we show the initial reaction rate obtained in the different cycles. This parameter was analytically obtained from the reaction curve profile. The experimental data were fitted to a first order reaction model equation, indicating the diffusion control of the process. We can

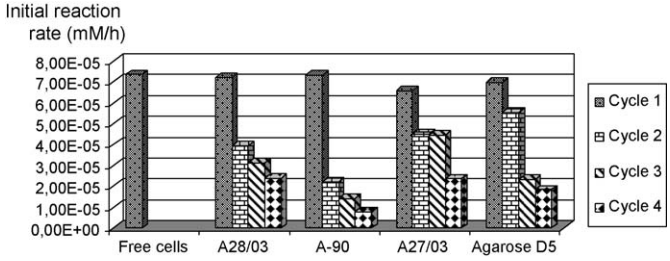


Fig. 1. Initial oxidation rates (mM/h) using *R. miehei* fungus immobilized in different supports. *Reaction conditions:* [cyclohexanol] = 5 mM; temperature, 28 °C; cycle reaction time, 72 h; free cells wet weight, 3.8 g; catalyst wet weight, 4.2 g.

see that the initial reaction rates for free and immobilized cells are similar but the immobilization enables the re-use of the biocatalyst, protecting the cells by controlling the diffusion of the hydrophobic substrates. Agar and agarose of different methoxylation degrees were used to control the rate of diffusion trying to optimize the conditions. From our analysis, agarose D5 and Agar from *Pterocladia* genus (with low methoxylation degree) were the most interesting supports.

The source of the agar has a crucial influence on the reaction rate, as well as on the number of biotransformation cycles carried out. In this way, when either the agar A27/03 or agarose D5 were used, the best results were achieved. Therefore, the lower the gelling point, the more reusable the biocatalyst is. In addition, Fig. 1 shows that the immobilization of fungus is necessary to reuse the biocatalyst, specially in the case of the agar A27/03 and agarose D5.

Immobilized fungus and free cells show the same stereoselectivity in the oxidation of alcohols (Table 1). As an example, we show in Fig. 2 the reaction profiles in the oxidation of (1*S*) 1-(2-furyl)-ethanol and of *iso*-menthol. We think that the more the steric hindrance around OH, the longer the reaction time is (Fig. 2a and b). However, in all cases near 100% yield can be achieved. In addition we can observe that the immobilized biocatalyst in agar A27/03 is as active as the free cell and more active than the immobilized biocatalysts in agarose. The greater the log *P* of the alcohol (**2** (log *P* = −0.57); **8** (log *P* = +2.78)), the lower the catalytic yields achieved using agarose biocatalysts. These results can be related to the greater amount of included water and the smaller pore size of the agarose D5 compared to A27/03 matrix [9].

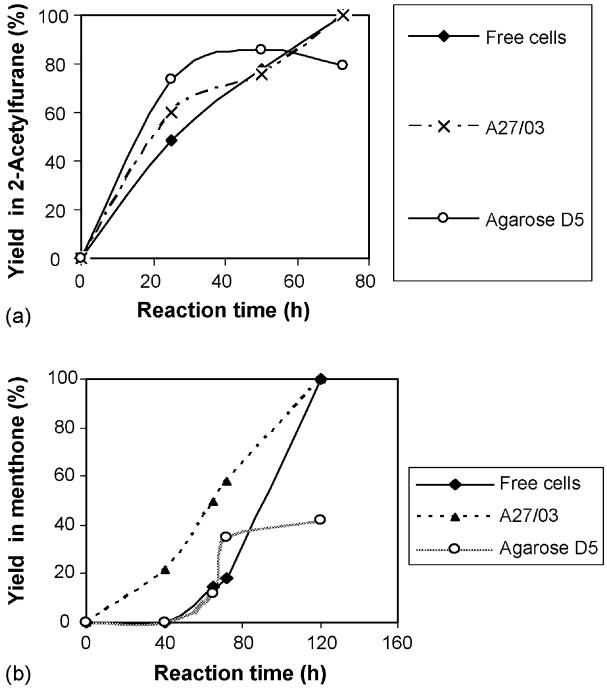


Fig. 2. Oxidation reaction profiles of (a) (1*S*) 1-(2-furyl) ethanol, **2**[b] and (b) *iso*-menthol, **8**. Using free and immobilized cells of fungus *R. miehei*. [Alcohol] = 2.5 mM; reaction time: 72 h (a) and 120 h (b); temperature: 28 °C; stirring speed = 250 rpm; free cells wet weight: 3.8 g; catalyst wet weight: 4.2 g.

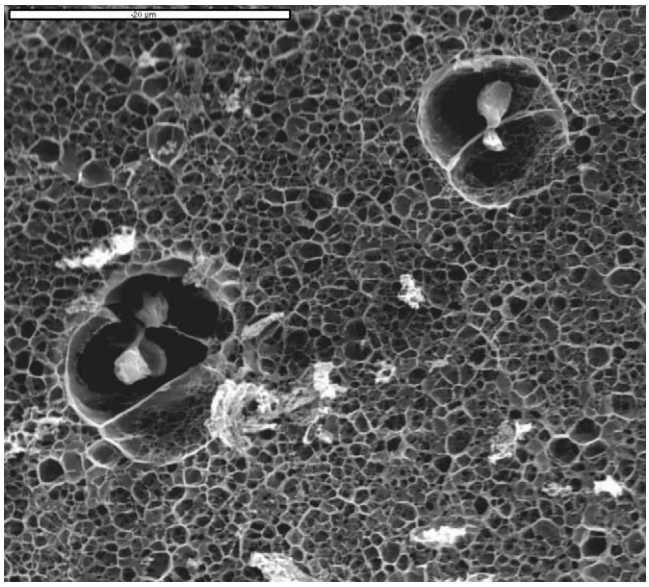


Fig. 3. *W. californica* immobilized in agar A27/03. Microphotographs obtained by low temperature electron microscopy (LTEM) Oxford. Model. CT1500.

In Figs. 3 and 4, the structural characteristics of agar A27/03 and agarose D5 gels are shown. The combined evaluation of the structural properties of the matrices based on electron microscopy images and the experimental results obtained in the reactions enhance our knowledge about the immobilized derivatives. Thus, the smaller pore size of the agarose D5 matrix could explain, the lower yields obtained in the oxidation of some of the substrates (*iso*-menthol and (*S*)-1-(2-furyl)-ethanol) in comparison with the ones obtained using agar A27/03 as support. This fact could be directly related with the differences of diffusion between both matrices.

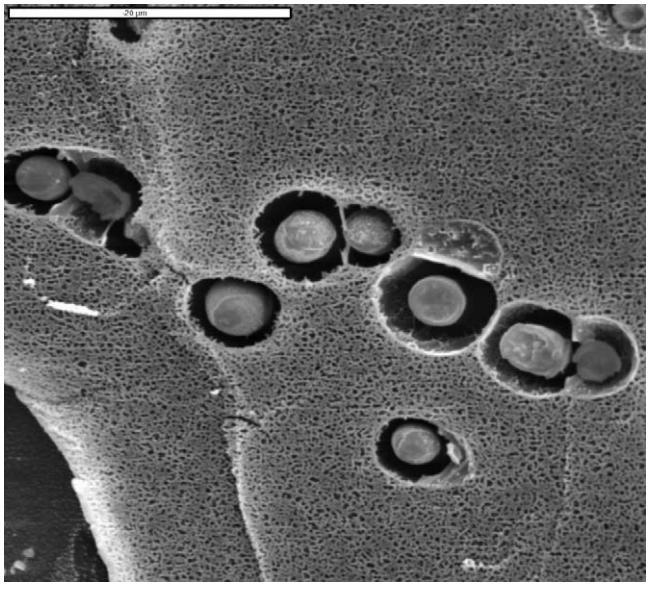


Fig. 4. *W. californica* immobilized in agarose D5. Microphotographs obtained by low temperature electron microscopy (LTEM) Oxford. Model. CT1500.

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References

[1] E. Boel, B. Huge-Jensen, M. Christensen, L. Thim, N.P. Fill, Lipids 23 (1988) 701–706.
[2] M.T. López-Belmonte, A.R. Alcántara, J.V. Sinisterra, J. Org. Chem. 62 (1997) 1831–1840.
[3] A.R. Alcántara, E.I. Fuentes, J.V. Sinisterra, Chem. Phys. Lipids 93 (1998) 169–184.
[4] L. Peng, X. Xu, H. Mu, C.-E. Hoy, J. Alder-Niersen, Enzyme Microb. Technol. 31 (2002) 523–532.
[5] F. Molinari, R. Villa, M. Manzoni, F Aragozzini, Appl. Microbiol. Biotechnol. 43 (1995) 989–994.
[6] R. Gandolfi, N. Ferrara, F. Molinari, Tetrahedron Lett. 42 (3) (2001) 513–514.
[7] J.D. Carballeira, C. García-Burgos, M.A. Quezada, E. Alvarez, J.V. Sinisterra, Biotechnol. Bioeng. 87 (5) (2004) 632–640.
[8] M.J. Vázquez, A.M. Roa, F. Reyes, A. Vega, A. Rivera-Sagrado, D.R. Thomas, E. Diez, J.A. Hueso-Rodriguez, J. Med. Chem. 46 (2003) 5117–5120.
[9] M.A. Quezada, C.A. García-Burgos, J.D. Carballeira, E. Alvarez, J.V. Sinisterra, BIOTEC, Oviedo, Poster 9P-20, ISBN 84-609-1771, August 2004.