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Chemo- and stereoselective reduction of β-keto esters by spores and various morphological forms of *Mucor rouxii*

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Abstract

We report the efficient enantioselective reduction of a number of β-ketoesters by mycelial and yeast-like forms of the dimorphic fungus *Mucor rouxii* in a whole-cell process. Mycelial cells, grown in aerobiosis, were efficient in water, whereas the yeast-like cells, grown in anaerobic medium, were both efficient in water and in organic solvents. Almost 100% of conversion with 97% of enantiomeric excess of the (*S*) forms of the reduced β-ketoesters was obtained at 15 g biomass (wet weight)/mmol substrate in two hours. The fungal spores, which are the physiologically resistant form of the fungus, also catalyzed the reductive process efficiently and stereoselectively. The freeze-dried as well as the warm-air dried yeast-like cells, rehydrated in a small volume of water, maintained the same efficiency and selectivity of the reaction in organic solvents as the fresh biomass up to at least 4 months.

Keywords: Mucor rouxii; Yeast; Mycelial; Spores; β-Ketoesters.

1. Introduction

Optically pure hydroxyesters provide very versatile building blocks for chiral synthesis [1] and several methods for producing these synthons have been explored. Asymmetric reduction of ketoesters, either by chemical or enzymatic methods, has been the most extensively investigated route. Chemical approaches have included the use of chirally modified hydride reagents [2], transition metal catalysts [3], oxygenation of chiral imide enolates [4] and Lewis acid mediated borohydryde reductions [5].

Enantionselective biocatalytic reduction of prochiral ketoesters produces various compounds that can be used as building blocks of fine chemicals, including pharmaceuticals, flavors and fragances [6]. These reactions can be performed in two different ways: either by using isolated, purified enzymes [7] or by using whole cells containing the enzymes of interest.

There are advantages to using whole cells as biocatalysts rather than purified enzymes [8]. Many enzymatic reactions require the presence of expensive cofactors. Whole cells are an economic and continuous source of enzymes and cofactors, thus simplifying these reactions significantly. In recent years microbial reductions gained increasing importance in view of their application in asymmetric synthesis [9,10]. The application of baker's yeast oxidoreductases in reduction of carbonyl compounds such as aldehydes, ketones, ketoesters and ketoacids is well known [11]. Recently, their use in the presence of organic solvents has been reported [12]. Since many reactants are only slightly soluble in aqueous environments, organic media are particularly useful for such reactions [13]. Moreover, by varying the nature and/or composition of the organic medium it is possible to manipulate the stereoselectivity of the enzyme in order to obtain the desired products [14].

In order to extend the whole-cell methodology to other microorganisms, we have studied the behavior of the fungus *Mucor rouxii* in the reduction of polyfunctional carbonyl

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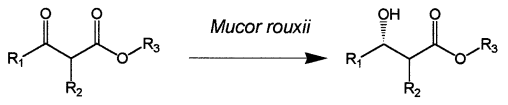

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Substrate	R_1	R_2	R_3
1a	CH_3	H	CH_3CH_2
1b	CH_3	H	$(CH_3)_2CH$
1c	CH_3	H	$CH_3OCH_2CH_2$
1d	CH_3	CH_3	CH_3CH_2
1e	$(CH_3)_2CH$	H	CH_3CH_2

Scheme 1.

compounds, such as β-ketoesters in aqueous and organic media (Scheme 1).

Mucor rouxii is a dimorphic fungus that can grow as a cenocytic mycelium under aerobic conditions or as yeast-like cells under anaerobiosis and high glucose levels in the culture medium. There are some reports in the literature characterizing biochemically, but not biotechnologically, oxidoreductase activities from different species of *Mucor*: a dihydroxyacetone reductase from *Mucor javanicus* [15]; a NAD-dependent glutamate dehydrogenase in *Mucor racemosus* [16]; a NADPH-dependent carbonyl reductase in *Mucor ambiguus*, with high specificity towards conjugated polyketones [17]; and very recently a NAD-dependent alcohol dehydrogenase in *Mucor rouxii*, which seems to work physiologically in the reduction of acetaldehyde to ethanol [18].

In this report we describe the use of fresh cells from *Mucor rouxii* cultures, derived from aerobic (mycelium) or anaerobic (yeast-like) growth conditions, as source of reductase activity for β-ketoesters. We also characterize the stability of the described activity by using freeze-dried and warm-air dried fungal cells, as well as fungal spores, the physiological-resistant forms of the fungus.

2. Materials and methods

2.1. Materials

Chemicals and solvents used were of the highest purity available from Aldrich Chemical Co. (Milwaukee, WI) and Sigma Chemical Co. (St. Louis, MO).

2.2.1. Strain, growth conditions and dessication methods

Mucor rouxii (NRRL 1894) was used throughout this work. Spores, obtained and stored as described [19], were inoculated at a concentration of 1×10^6 or 2×10^6 spores ml^{-1} for aerobic or anaerobic cultures respectively. Cells were grown in YPG medium [20]. Flasks were incubated in a rotatory shaker at 120 r.p.m. and at 28°C for different times, as indicated. Anaerobic growth was perfomed in stoppered Erlenmeyer flasks continually flushed with 100% CO_2.

For freeze-drying procedures, cultures were collected on paper filters, weighed and frozen in liquid nitrogen, either directly or after resuspending the biomass in a small volume of a solution of 20% (w/v) trehalose in sterile water. Frozen material was lyophilized in aliquots for 90 min. Dried cultures were weighed and stored in flasks under vacuum at 4–10°C.

For warm air-drying procedures, cultures were collected on filter papers, weighed and dried using a current of air at 38°C overnight. Dried cells were weighed and stored in flasks under vacuum at 4–10°C.

From the weight ratio of fresh to dry biomass we could estimate the amount of dry biomass needed in order to perform experiments equivalent to those performed with fresh cultures.

Oxidoreductase activity was assayed immediately after harvesting of either spores or biomass from anaerobic or aerobic cultures.

2.2.2. Substrate reduction

Biomass (2 g) obtained from cultures at different growth stages was incubated with 5 ml of organic solvents such as ethyl acetate, toluene, hexane, etc, alone or in biphasic systems mixed with 2 ml sterile water, in 25 ml sterile Erlenmeyer flasks stoppered and sealed. Water incubations were performed in 5 ml sterile water alone. Spores (number of spores indicated in each case) and dessicated biomass (0.5 g) were resuspended respectively in 1 or 2 ml sterile water, previous to the addition of the organic solvents. The substrates were added to these systems (0.1–0.25 mmol for standard assays) and incubated at 28°C in a rotatory shaker at 120 r.p.m. for different times. The reactions were stopped by centrifugation at 10,000 × g; the supernatants were removed and, when applied, water phases were extracted with ethyl acetate. All experiments were performed in duplicate.

2.2.3. Analytical methods

Aliquots (1 μl) of extracts were injected into a gas chromatograph (Model 5890, Hewlett-Packard, Palo Alto, CA) equipped with a Carbowax 20H-022 capillary column (30 m, i.d. 0.2 mm, film thickness 0.2 μm) for determination of percentage of conversion and diasteromeric ratio. The initial temperature (80°C) was maintained for 1 min and then increased at 2°C/min to a final temperature of 200°C. For enantiomeric excess determination HP Chiraldex G-TA capillary column (40 m, i.d. 0.32 mm) was used. The initial temperature (80°C) was maintained for 11 min and then increased at 2°C/min to a final temperature of 100°C. Compounds were identified by comparison to peaks obtained with known standards and by analysis of spectra.

^{1}H-NMR and ^{13}C-NMR spectra were measured at 200

Table 1
Mucor rouxii mycelium-reduction of β-ketoesters

Substrate	Solvent	Biomass/ Substrate (g/mmol)	Time (h)	Conversion (%)	ee (%)
1a	water	12	17	100	60 (*S*)
1a	hexane	25	36	6	–
1a	toluene	25	36	10	–
1a	toluene-water	25	36	4	–
1a	ethyl acetate	25	36	0	–
1a	ethyl acetate-water	25	36	0.7	–
1b	water	9	17	100	62 (*S*)
1c	water	8	24	100	67 (*S*)
1d	water	7	24	90[a]	99 (*S*)[b]

Reaction conditions are described in Materials and Methods. The biomass used was obtained from 6–8 h aerobic cultures.
[a] Diasteromeric ratio of (*2R, 3S*):(*2S, 3S*) was 2:1.
[b] For (*2R, 3S*) anti-isomer.

Table 2
Mucor rouxii yeast-like cells-reduction of isopropyl acetoacetate **1b**, solvent effect

Solvent	Biomass/ Substrate (g/mmol)	Time (h)	Conversion (%)	ee (%)
water	8	24	100	54 (*S*)
hexane	17	24	100	97 (*S*)
hexane-water	17	24	100	81 (*S*)
toluene	17	24	63	72 (*S*)
toluene-water	17	24	76	62 (*S*)
dioxane	17	36	0	–
dioxane-water	17	36	0	–
ethyl acetate	17	36	0.3	–
ethyl acetate-water	17	36	0.9	–

Reaction conditions are described in Materials and Methods. The biomass obtained from 18–20 h anaerobic cultures.

MHz and at 50 MHz respectively using a Bruker AC-200 spectrometer. Chemical shifts are reported in δ units relative to tetramethylsilane (TMS) as internal standard, using $CDCl_3$ and CD_3OD as solvents. EIMS were obtained at 70eV using a TRIO-2 VG Masslab and Shimadzu QP-5000 mass spectrometers, in m/z (%).

Optical purity of products was determined by specific rotation using $CDCl_3$ as solvent with a Perkin Elmer 343 polarimeter.

The absolute configuration of alcohols (**2a**, **2b** and **2c**) was determined by comparison with the sign of reported specific rotation [21], and **2d** by GC. Retention time of hydroxyester **2d** obtained by *Mucor rouxii* reduction was compared to the diasteromeric hydroxyesters prepared by baker's yeast reduction according to reported methodology [22]. The mixture of four stereoisomers was obtained through reduction with $NaBH_4$.

3. Results and discussion

3.1. Fresh cultures reduction

3.1.1. Mycelial and yeast-like cells reduction; solvent effect

β-Ketoesters bearing various carboxylic and alkoxy chains (Scheme 1) were submitted to reduction by *Mucor rouxii* whole cells. The results using fresh cultures of either the mycelial or the yeast-like forms of the fungus are shown in Tables 1, 2 and 3. It was observed that the carbonyl group of the ketoesters was chemoselectively reduced to give the corresponding hydroxyesters, while the ester carbonyl group remained unchanged. This behavior was observed by working with both mycelium (Table 1) and yeast-like cells (Table 2) in aqueous medium. Table 1 shows that oxidoreductases in mycelium are not active either in pure organic solvent such as hexane, toluene or ethyl acetate or in mixtures of water and these organic solvents. In water, the substrates were completely converted by mycelial cells, but with moderate stereoselectivity. On the other hand, yeast-like cells were active both in organic media and in biphasic systems (see Table 2).

The results obtained in the yeast-catalyzed reduction of isopropyl acetoacetate **1b** in various media are described in Table 2. The best results were obtained in low polar organic solvent such as pure hexane, with 100% conversion to hydroxyester and high enantioselectivity of (*S*) enantiomer. In the presence of more polar solvents such as dioxane or ethyl acetate only starting material was recovered. Conversion decreased with increasing solvent polarity.

The use of water:organic solvent media was only effective in mixtures hexane:water and toluene:water, but although the degree of conversion obtained was similar or even better than with the organic solvent alone, the stereoselectivity was decreased (Table 2). The (*S*)-alcohol was the isolated enantiomer in all cases. Under our experimental conditions *Mucor rouxii* reductases behave, regarding stereoselectivity, in accordance with reports on baker's yeast reductions [23].

Considering that hexane was the best solvent in the case of substrate **1b,** we studied the course of its conversion and enantiomeric excess by *Mucor rouxii* yeast-like cells under these conditions. Results can be observed in Fig. 1. Conversion reached the maximum value after 2h. An enatiomeric excess of around 90–97% was obtained throughout, independently of the degree of conversion.

3.1.2. Ratio biomass-substrate influence

The influence of the ratio biomass/substrate was also studied and is shown in Fig. 2. Both conversion and enantiomeric excess increased with this ratio; maximal performance was attained at 15 g of biomass/mmol substrate. The performance of the whole-cell system in organic solvents was quite good since the ratio of productivity in water *vs* organic solvents was of two-fold.

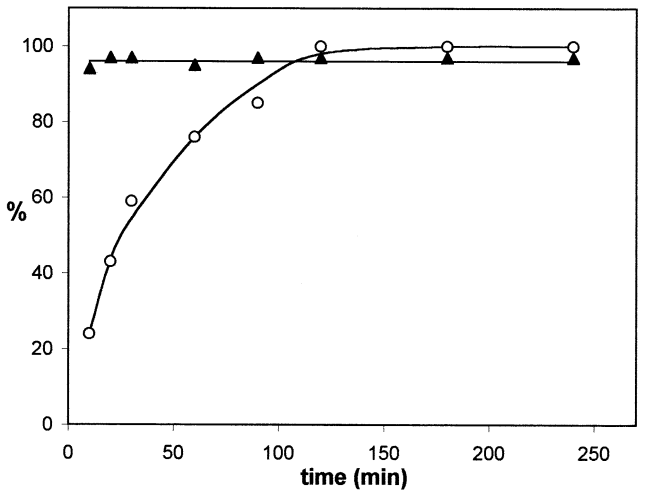

Fig. 1. Time course of *Mucor rouxii* yeast-like cells catalyzed reduction of isopropyl acetoacetate in hexane at a biomass/substrate ratio of 17 g/mmol. The biomass was obtained from 18–20 h anaerobic cultures. Symbol (▲) denotes enantiomeric excess (%) and (o) conversion (%).

The formation of (*S*) product and enantiomeric excess is increased considerably when biomass/substrate ratio increased. This result suggests the co-existence of more than one enzymatic activity in *Mucor rouxii* with difference in stereoselectivity, turnover, substrate affinity (K_m) and accesibility in the assay.

3.1.3. Substrate effect

Table 3 shows the results of yeast-catalyzed reduction of the rest of the substrates (**1a**–**1e**), using a suspension of cells in hexane. The data show that the alcoxy chain affected neither the degree of conversion of substrates nor the enantiomeric purity of the products, as can be observed in substrates **1a**, **1b** and **1c**. With substrates having various carboxylic moieties, yeast-like cells showed a different behavior. A methyl group in carbon two, as in compound **1d**, did not influence the degree of conversion. Reaction was enantioselective with 99% ee and diasteroselective (diasteromeric ratio: 8:1). This was proven by GC analysis comparing the retention time of **1d** with those of the diasteromeric hydroxyesters prepared by commercial baker's yeast reduction and of the mixture of four stereoisomers obtained through reduction with $NaBH_4$, as explained in the experimental part.

The cellular reductase activity was sensitive to the presence of a bulky group as substituent in R_1 such as isopropyl and only 20% of conversion was observed.

Table 3
Mucor rouxii yeast-like cells catalyzed reduction of β-keto esters

Substrate	Biomass/ Substrate (g/mmol)	Time (h)	Conversion (%)	ee (%)
1a	17	24	100	95 (*S*)
1b	17	24	100	97 (*S*)
1c	17	24	100	94 (*S*)
1d	15	24	65[a]	99[b] (*S*)
1e	17	40	20	–

Reaction conditions are described in Materials and Methods. The biomass was obtained from 18–20 h anaerobic cultures. Solvent: hexane.
[a] Diasteromeric ratio of (*2R, 3S*):(*2S, 3S*) was 8:1.
[b] For (*2R, 3S*) anti-isomer.

3.2. Spores reduction

We have studied the biocatalyzed reduction using *Mucor rouxii* spores, which are the natural bodies the fungus produces for resistance and preservation. Table 4 shows the results obtained with isopropyl acetoacetate **1b** as substrate and different solvents. As with mycelium and yeast-like cells, the hexane was the best solvent for high conversion and chemoselectivity. Once again, as it was observed with the other forms of the fungus, the reductive reaction did not

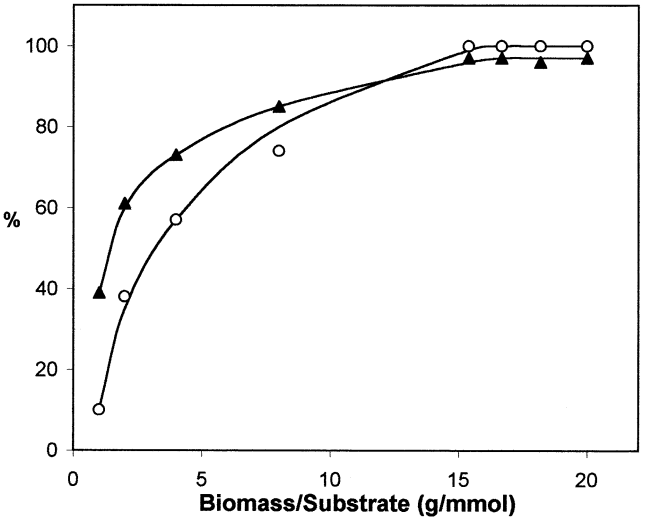

Fig. 2. Influence of biomass:substrate ratio on enantiomeric excess (%) (▲) and conversion (%) (o) in *Mucor rouxii* yeast-like cells catalyzed reduction of isopropyl acetoacetate in hexane. The biomass was obtained from 18–20 h anaerobic cultures. Incubation time: 24 h. Variation of the ratio biomass: substrate was achieved by fixing the amount of biomass in 2 *g* in 5 ml of solvent and increasing the amount of substrate between 0.1–2 mmoles.

Table 4
Mucor rouxii spores catalyzed-reduction of isopropyl acetoacetate **1b**

Solvent	Biomass/substrate ($\times 10^8$ spores/mmol)	Conversion (%)	ee (%)
Water	150	100	70 (*S*)
Hexane	350	97	95 (*S*)
Toluene	350	24	72 (*S*)
Ethyl acetate	350	0	–
Dioxane	350	0	–

Reaction conditions are described in Materials and Methods. Incubation time: 24 h. Number of spores used: 30×10^8 for water, 70×10^8 for organic solvents.

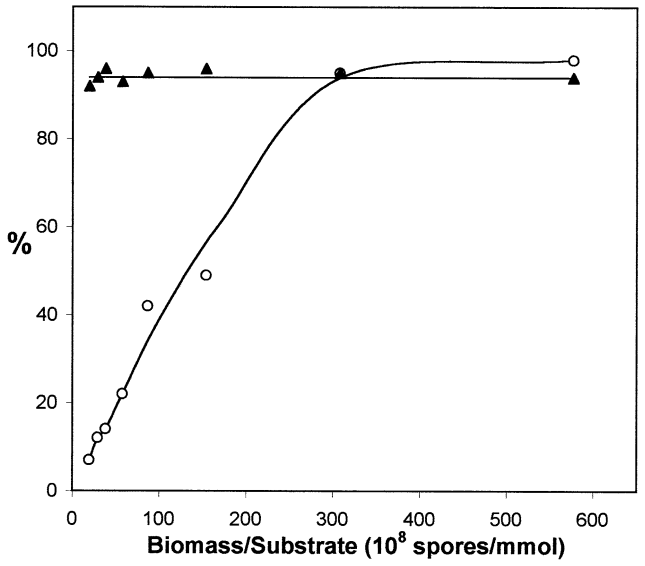

Fig. 3. Influence of biomass:substrate ratio on enantiomeric excess (%) (▲) and conversion (%) (o) in *Mucor rouxii* spores catalyzed reduction of isopropyl acetoacetate in hexane. Incubation time: 24 h. Variation of the ratio biomass: substrate was achieved by fixing the amount of substrate in 0.2 mmol in 5 ml of solvent and increasing the number of spores between 2×10^8 to 120×10^8.

take place in dioxane and ethyl acetate. The influence of biomass/substrate ratio was analyzed. Fig. 3 shows the values of conversion and enantiomeric excess as a function of spore biomass expressed in number of spores. There is a remarkable dependence of the degree of conversion with biomass, being 350×10^8 spores/mmol substrate an appropriate ratio to get almost complete conversion. Enantioselectivity was excellent at all the assayed ratios. The use of spores avoids the practical limitation of having to use fresh cultures to generate biomass for each reaction. For this fungus, the production and storage of spores is a necessary step to generate aerobic or anaerobic biomass.

3.3. Dessicated yeast-like cells reduction

As an alternative to the use of spores, two methods of dessication of yeast-like cells were used in order to assay the effect of storage on biomass reductase activity. Table 5 shows the results of freeze-drying without or with trehalose and of warm air drying. When freeze-dried yeast cells, stored for two days, were suspended in pure hexane no reductase activity could be detected; only starting material was recovered. However previous rehydration of the yeast-like cells with water before addition of the organic solvent circumvented this problem and complete conversion with high enantioselectivity was observed. The preservation of reductase activity in freeze-dried cells was excellent, since biomass stored up to 120 days displayed the same activity and selectivity.

Considering the well known advantages of addition of trehalose in drying processes of microorganisms [24] we planned the dessication scheme including a set of cells freeze-dried under these conditions (Table 5). However no conclusions on the benefits of using trehalose could be derived from the established scheme, since during this time, the stability of the enzymatic activity in the cells freeze-dried without trehalose was maximal.

Table 5
Mucor rouxii dessicated yeast-like cells catalyzed reduction of isopropyl acetoacetate **1b**

Dessication method	Solvent	Storage time (days)	Conversion (%)	ee (%)
Freeze-drying	hexane	2	0	–
	hexane-water	2	100	96 (*S*)
	hexane-water	60	99	94 (*S*)
	hexane-water	120	98	95 (*S*)
Freeze-drying with trehalose	hexane-water	6	100	96 (*S*)
	hexane-water	20	100	95 (*S*)
	hexane-water	50	97	96 (*S*)
	hexane-water	120	96	97 (*S*)
Warm air drying	hexane-water	6	100	95 (*S*)
	hexane-water	20	100	96 (*S*)
	hexane-water	50	83	94 (*S*)

Reaction conditions are described in Materials and Methods. Incubation time: 24 h. Biomass/substrate: 20 g/mmol. The dehydrated biomass was obtained from 18–20 h anaerobic cultures and added to the reaction system according to the fresh:dehydrated ratio explained in Materials and Methods.

Finally, yeast-like cells dessicated through warm air drying also showed good performance (Table 5) regarding enantioselectivity, but lower activity than freeze-dried cells.

The efficiency and selectivity obtained with reconstituted dessicated cells was identical to the one obtained with fresh cultures. In all the cases complete chemo- and enantioselective reduction of the keto group to alcohol in β-ketoesters was achieved.

4. Conclusion

In conclusion, *Mucor rouxii* showed good performance in whole cells biocatalysis in aqueous and organic media. Both morphologies, mycelium and yeast, displayed an interesting reductase activity. Yeast-like cells and spores gave best results in a non polar medium using hexane as solvent. The good performance displayed by spores and stored des-

sicated yeast-like cells makes this procedure a simple and efficient method to attempt whole-cell biotransformations in aqueous and organic media.

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