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Effect of cell immobilization and organic solvents on sulfoxidation and steroid hydroxylation by *Mortierella isabellina*

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SUMMARY

The effects of calcium alginate bead immobilization and the presence of organic solvents on two bioconversion reactions carried out by *Mortierella isabellina* ATCC 42613 have been investigated. These reactions, the 14α-hydroxylation of progesterone and the sulfoxidation of thioanisole, both proceed in high yield using resting-cell bioconversions, but are not carried out by alginate bead preparations in the absence of an organic co-solvent, the best results being obtained with 5 or 10% aqueous methanol. The stereoselectivity of sulfoxidation of thioanisole was found to be dependent upon the nature and concentration of organic co-solvent.

INTRODUCTION

Although the use of immobilized cells for bioconversion of steroids has received considerable attention, the majority of reported examples are concerned with dehydrogenation reactions [6]. Of these, immobilization of *Arthrobacter simplex* has been extensively investigated [4], and similarly other reported cases almost all involve bacterial cells [6]. The use of immobilized fungal preparations for bioconversion of steroids is less frequently reported, and of the examples which are known, most involve spore preparations. Only a small number employ immobilized fungal cells for hydroxylation [6], a surprising phenomenon in view of the importance of fungal hydroxylation in the preparation of the corticosteroids and related compounds. A related process, sulfoxidation by microbial bioconversion, has been developed as an efficient procedure for the preparation of chiral sulfoxides [2], but the application of cell immobilization procedures to this process has not been reported. We now report the use of calcium alginate bead preparations of the fungus *Mortierella isabellina* ATCC 42613 for both steroid hydroxylation and sulfoxidation.

The application of non-aqueous environments for enzyme catalyzed reactions has received much attention recently, in particular the use of non-aqueous solvents to control both the stereochemistry and position of equilibrium in reactions catalyzed by esterase and related enzymes [1,5,7]. In view of the inaccessibility of hydroxylase sulfoxidase enzymes of microbial origins, and the complexity of analyzing the effects of organic solvents on growing organisms, we have also used alginate bead preparations of *M. isabellina* to study the effects of non-aqueous water miscible solvents on both the steroid hydroxylation and sulfoxidation reactions carried out by this microorganism.

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MATERIALS AND METHODS

Starting materials were commercial samples (thioanisole from the Aldrich Chemical Co., and steroids from the Sigma Chemical Co.), and products were identified by spectral (NMR, IR, MS) comparisons with authentic samples. *Mortierella isabellina* ATCC 42613 was stored and grown as described previously [3].

Preparation of alginate beads

M. isabellina harvested from 15 1-l Erlenmeyer flasks (100 g of wet cells) was suspended in 100 ml of distilled water, and then mixed with 300 ml of an autoclaved 3% (w/v) sodium alginate solution. The alginate cell mixture was placed in a separatory funnel and added dropwise to a stirred solution of calcium chloride (0.07 M) at 5 °C. The resulting beads (approx. 5 mm diameter) were washed with distilled water, and either used directly or stored at 4 °C until required.

Bioconversion using alginate beads

Results are summarized in the tables. Alginate beads prepared from *M. isabellina* (200 g) were suspended in 1.5 l of aqueous solvent (see tables), and the mixture distributed evenly over 7 1-l Erlenmeyer flasks. To this suspension was added a solution of substrate (total 0.5 g) in ethanol (95%, total 15 ml). The flasks were placed on a rotary shaker at 80–100 rpm for 72 h, and the beads then separated from the medium by vacuum filtration. The aqueous solvent was extracted (methylene chloride, continuous extractor, 72 h), the extract evaporated, and the residue purified by column chromatography. Yields quoted in the tables refer to combined, homogeneous column fractions of product. The recovered beads were discarded or reused (see tables).

RESULTS AND DISCUSSION

The reactions which we have studied are the 14α-hydroxylation of progesterone (**3**), and 11-deoxycorticosterone (**4**) giving **5** and **6**, respectively, and the conversion of thioanisole (**1**) to the sulfoxide **2** [3]. The latter reaction also afforded an opportunity to study the effects of immobilization and organic solvents on the stereoselectivity of the bioconversion, as the product is formed predominantly as the (*R*)-(+) enantiomer in an enantiomeric excess of 55% under resting cell conditions [3].

The results of bioconversions carried out in this study are summarized in Tables 1 and 2. When bioconversions were carried out with calcium alginate immobilized cells in 100% aqueous medium, very low yields of product were obtained, and in the case of thioanisole oxidation the low optical purity of the product suggested the presence of a degree of autooxidation. The failure of immobilized preparations of active fungal cells to carry out steroid hydroxylations has been noted previously [6], and is presumably attributable to transport and/or permeability limitations.

However, when reactions were performed in the presence of low concentrations (5%–10%) of methanol, isolated yields and (in the case of sulfoxidation) product stereochemistry were comparable with those obtained from resting cell (replacement culture) bioconversions. This effect of the organic co-solvent appears to be specific for methanol, as comparable concentrations of ethanol led to low stereoselectivity of reaction (for sulfoxidation) and

TABLE 1

Summary of thioanisole incubations with *Mortierella isabellina*

Substrate	*M. isabellina*	Co-solvent (%)	Product (%)	E.E. (%)
1	Resting cells	None	**2** (65)	55
1	Alginate beads	None	**2** (5)	17
1	Alginate beads	MeOH (5)	**2** (72)	50
1	Alginate beads[a]	MeOH (5)	**2** (70)	48
1	Alginate beads[b]	MeOH (5)	**2** (65)	52
1	Alginate beads	MeOH (10)	**2** (68)	49
1	Alginate beads	MeOH (15)	**2** (32)	6
1	Alginate beads	MeOH (20)	None	
1	Alginate beads	EtOH (10)	**2** (61)	28
1	Alginate beads	n.PrOH (10)	None	
1	Alginate beads	i.PrOH (10)	None	
1	Alginate beads	n.BuOH (10)	None	
1	Alginate beads	DMF (10)	**2** (58)	3
1	Alginate beads	DMSO (10)	None	
1	Alginate beads	ACN (10)	None	
1	Alginate beads	THF (10)	None	

E.E. = enantiomeric excess. Structures **1** and **2** refer to Fig. 1.
[a] Following storage at 4°C for 21 days.
[b] Following use and recovery of beads by filtration.

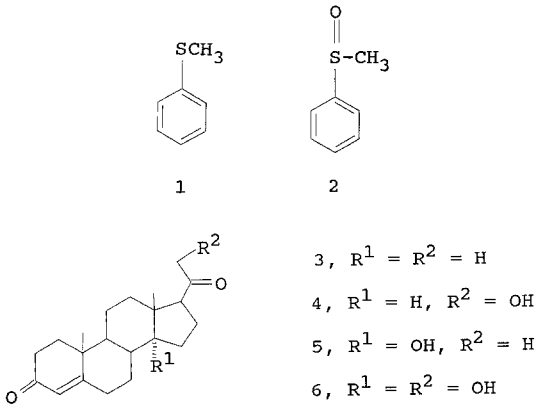

Fig. 1. Structures of thioanisole (**1**), thioanisole sulfoxide (**2**), steroid substrates **3** and **4**, and bioconversion products **5** and **6**.

TABLE 2

Summary of steroid incubations with *Mortierella isabellina*

Substrate	*M. isabellina*	Co-solvent (%)	Product (%)	E.E. (%)
3	Resting cells	None	**5** (57)	
3	Alginate beads	None	**5** (5)	
3	Alginate beads	MeOH (5)	**5** (65)	
3	Alginate beads[a]	MeOH (5)	**5** (69)	
3	Alginate beads[b]	MeOH (5)	**5** (62)	
3	Alginate beads	EtOH (5)	**5** (15)	
4	Resting cells	None	**6** (66)	
4	Alginate beads	MeOH (5)	**6** (70)	

Structures **3** to **6** refer to Fig. 1. See also footnote to Table 1.

low yield for steroid hydroxylation. No bioconversion was observed in the presence of other low molecular weight primary alcohols, or other water soluble solvents, although the use of dimethylformamide did result in sulfoxidation, but with drastically altered stereoselectivity.

For both sulfoxidation and steroid hydroxylation, beads could be recovered and re-used (up to at least three times) without loss of activity, and storage of beads for periods of up to at least 21 days was without detriment to bioconversion efficiency.

At this time we do not have a satisfactory explanation for the effect of methanol on bioconversions carried out in the above manner. However, we speculate that this effect may be attributable to an alteration in the permeability of the alginate bead to substrate. That the effect is not simply due to increased solubility of substrate in the bioconversion medium is shown by the differences between the uses of methanol and ethanol as co-solvent which are apparent from the Tables.

Both ethanol and DMF at concentrations of 10% have an effect on the stereoselectivity of sulfoxidation, as does the presence of higher levels (15%) of methanol. In all of these cases, the stereoselectivity is reduced when compared with that observed in resting cell bioconversions, indicating that the effect of organic solvent is not to cause the selective destruction of enantiocomplementary enzymes, but may simply be to enhance non-stereoselective oxidation, either enzymic or atmospheric. At the present time, therefore, although the use of organic co-solvents to increase the stereoselectivity of oxidative bioconversions appears not to be indicated, we do suggest the addition of 5–10% methanol to the bioconversion medium in those cases where immobilization as alginate beads appears to lead to loss of bioconversion ability.

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