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Kinetic resolution of (*R*, *S*)-1,2-*O*-isopropylideneglycerol by esterification with dry mycelia of moulds

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Abstract

The esterification of (*R*, *S*)-1,2-*O*-isopropylideneglycerol catalysed by dry mycelia of different moulds in organic solvent was studied. Dry mycelium of *Rhizopus oryzae* CBS 112.07 and *Aspergillus oryzae* MIM gave the (*R*)-ester with moderate optical purity; the kinetic data of the esterification with butyric acid in *n*-heptane showed an enantiomeric ratio of 3.4 with *Rhizopus oryzae* and 8.0 with *Aspergillus oryzae*. The esterification catalysed by *Aspergillus oryzae* was performed on larger scale (operative volume 2.3 l) allowing for the production of 2.3 g l^{-1} of (*R*)-IPG butyrate with an e.e. = 56% and a molar conversion of 52%. The enantiomerically enriched ester was hydrolysed with thermally-treated cells of *Bacillus coagulans*: the (*S*)-ester was obtained as enantiomerically pure molecule, while the alcohol was recovered with e.e. = 90%.

Keywords: 1,2-*O*-Isopropylideneglycerol; Solketal; enzymatic esterification; *Rhizopus oryzae*; *Aspergillus oryzae*

1. Introduction

The resolution of racemic 1,2-*O*-isopropylideneglycerol (IPG or solketal) by enzymatic methods is an easy way to obtain valuable chiral building blocks usable in the synthesis of β-adrenoreceptor antagonists, prostaglandins, phospholipids and leukotrienes [1,2]. Enzymatic hydrolysis of solketal esters catalysed by microbial esterases have been assayed with commercial and newly isolated enzymes, sometimes with good enantioselectivity [3–11], but the most straightforward method is the enzymatic esterification of the racemic mixture of the alcohol which can be obtained in low water activity media. However, the use of commercial lipases was shown to be moderately enantioselective for the esterification of IPG esters [12,13], while esterases are not sufficiently active in organic solvents and need to be modified for achieving good activity/enantioselectivity [14]. Esterases/lipases still bound to whole cells can be exploited for catalysing the esterification of different alcohols and carboxylic acids; dry whole cells-preparations in hydrophobic organic solvents have proven an interesting alternative to extracellular enzymes for the production of flavour esters [15–20] or stereoselective transformations [21,22].

In this work we have studied the kinetic resolution of racemic IPG by esterification catalysed by lyophilized mycelium of different moulds in organic solvents. Kinetics of the biotransformation and systems for achieving enantiomerically pure IPG have been defined and developed.

2. Experimental

The chemicals used in this study were of reagent grade and were purchased from Fluka (Milano, Italy). Authentic samples of 1,2-*O*-isopropylideneglycerol esters (acetate, butyrate, caprylate) were prepared in our laboratory following the protocol described before [10].

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2.1. Microorganisms and growth conditions

Two strains of *Aspergillus oryzae* were from our collection (MIM, Microbiologia Industriale Milano), while the remaining strains of *Aspergillus oryzae* and *Rhizopus oryzae* were from CBS (Centraal Bureau voor Schimmelcultures, Baarn, Holland). The moulds were routinely maintained on malt extract (8 g l^{-1}, agar 15 g l^{-1}, pH 5.5). The microorganisms were cultured in 500 ml Erlenmeyer flasks containing 100 ml of medium and incubated for 48 h at 28 °C on a reciprocal shaker (100 spm). The liquid media contained a basal medium (Difco yeast extract 1 g l^{-1}, $(NH_4)_2SO_4$ 5 g l^{-1}, K_2HPO_4 1 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.2 g l^{-1}, pH 5.8) added with Tween 80 (0.5%). Suspensions of spores (1.6×10^4) were used as inoculum. Mycelium grown for 48 h in submerged cultures was harvested by filtration at 4 °C, washed with phosphate buffer (pH 7.0, 0.1 M) and lyophilized.

2.2. Biotransformations

Ester synthesis was carried out in 10 ml screw capped test tubes by suspending lyophilized mycelium in organic solvent (5 ml) and then adding the alcohol and the acid. In the optimization phase, initial water activity (a_w) was measured. The values of the initial a_w of the different samples were set after equilibration with different amounts of water using a Rotronic-Hygroscop model BT-RS1 at 30 °C, which was previously calibrated using standard fixed water activity values 0, 0.35, 0.50, 0.75 and 0.85. Lyophilized mycelia were extensively dehydrated under vacuum in the presence of anhydrous P_2O_5 at room temperature and suspended in dry *n*-heptane. After substrates addition, the first a_w value was recorded, small volumes of water (2–8 μl) were added, and a_w was measured after reaching the equilibrium (1–2 h). The reaction mixtures were magnetically stirred at different temperatures. All the kinetic data were collected 5 min after the start of the reaction. The work-up of the esterification of IPG with butyric acid was performed by centrifugation to separate the mycelium, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography on silica gel pretreated with triethylamine (hexane:ethyl acetate = 6:4). The enantiomerically enriched (*S*)-IPG-ester was hydrolysed using thermally-treated cells of *Bacillus coagulans* as previously described [10].

2.3. Analytical methods

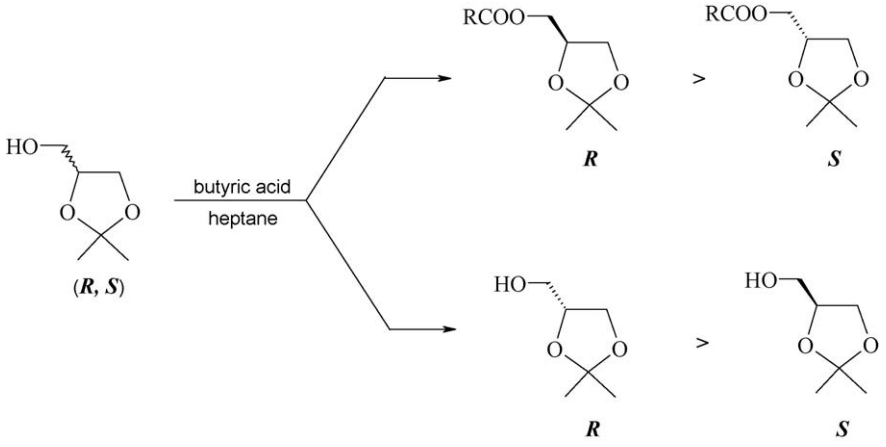
Samples (0.5 ml) were taken at intervals and centrifuged; 200 μl of the supernatant were added to an equal volume of a $CHCl_3$ solution containing an internal standard (2-phenyl-1-ethanol). The enantiomeric composition and conversion were routinely determined by gas chromatographic analyses of the esters using a chiral capillary column (DMePeBeta-CDX-PS086, MEGA, Legnano, Italy), having 0.25 mm-diameter, 25 m-length and 0.25 μm-thickness; column temperature was initially maintained at 90 °C for 10 min and then raised until 120 °C with a gradient of 4 °C min^{-1}. Under these conditions, typical retention times were 10.6 min for (*R*)-IPG, 11.3 for (*S*)-IPG, 19.9 min for (*S*)-IPG butyrate and 21.5 for (*R*)-IPG butyrate. The stereochemical outcome of the transformations was expressed as enantiomeric excess (e.e.) of the major enantiomer or as enantiomeric ratio (*E*) [23,24].

3. Results and discussion

The hydrolytic activity of different moulds belonging to the species *Aspergillus oryzae* and *Rhizopus oryzae* was evaluated using acetate, butyrate and caprylate esters as racemic substrates and different conditions of temperature, pH and substrate and biocatalyst concentrations. The reaction was always very fast being complete in all the cases within 2 h, no matter the strain employed, and very low enantioselectivity was observed.

Lyophilized mycelia of the same moulds were then employed in *n*-heptane to catalyse the esterification of racemic IPG with acetic and butyric acids. The best results were obtained with *Rhizopus oryzae* CBS 112.07 and *Aspergillus oryzae* MIM which catalysed the predominant formation of the (*R*)-ester, although with moderate enantioselectivity, according to the reaction schemes (Scheme 1).

Preliminary experiments performed at different temperatures showed that the highest enantioselectivity was observed at 50 °C with *R. oryzae* and at 30 °C with *A. oryzae*.



Scheme 1. Stereopreference observed in the esterification of IPG with butyric acid catalysed by *Rhizopus oryzae* and *Aspergillus oryzae*.

Table 1
Effect of biocatalyst concentration on the kinetics of IPG esterification catalysed by lyophilized mycelium of *Rhyzopus oryzae* in *n*-heptane

X_0 (g l^{-1})	2.0	5.0	10	20	30
S-IPG, v_0 (mmol$_P$ g^{-1} h^{-1})	0.046	0.110	0.197	0.365	0.421
R-IPG, v_0 (mmol$_P$ g^{-1} h^{-1})	–	0.212	0.459	0.727	0.940

S_o = 50 mM; T = 50 °C.

Table 2
Effect of biocatalyst concentration on the kinetics of IPG esterification catalysed by lyophilized mycelium of *Aspergillus oryzae* in *n*-heptane

X_0 (g l^{-1})	2.0	5.0	10	20	30
S-IPG, v_0 (mmol$_P$ g^{-1} h^{-1})	0.029	0.051	0.115	0.151	0.196
R-IPG, v_0 (mmol$_P$ g^{-1} h^{-1})	0.115	0.246	0.537	0.821	1.040

S_o = 50 mM; T = 30 °C.

The kinetics of the esterification of the single enantiomers of IPG with butyric acid was studied by carrying out independent batch tests on the commercially available *R*-IPG and *S*-IPG varying the biocatalyst concentration (X_0) in the range $1.0 \leq X_0 \leq 30$ g l^{-1}. Tests were performed at 50 °C with *R. oryzae* and 30 °C with *A. oryzae* using 50 mM substrates concentration in *n*-heptane and water activity (a_w) of 0.75. The study was performed utilizing the initial rates of esters formation (v_0) collected under conditions of negligible water formation (5 min after the start of the reaction). Satisfactory average yields (>75%) were obtained at $X_0 > 10$ g l^{-1} with both microorganisms. Initial rates (v_0) of both ester enantiomers almost linearly increased with X_0 up to 20 g l^{-1} (Tables 1 and 2).

To estimate the kinetic parameters of this system, additional experiments were carried out with a mycelium concentration of 30 g l^{-1} and varying S_o in the range 30–200 mM. According to the typical ping-pong mechanism proposed for the esterification in organic solvent [20], it was demonstrated that, at the start of the reaction, the system can be described by the Michaelis-Menten type-equation:

$$v_0 = \frac{k_{cat} S_o}{(K_m + S_o)}$$

where k_{cat} is the biocatalyst constant (mmol$_P$ g^{-1} h^{-1}), K_m a Michaelis-type constant (mM) and S_o is the equimolar substrate concentration (mM).

The results of the kinetic parameters obtained by Linewaver-Burk-type plots (Table 3) are always indicative of better kinetics of the *R*-enantiomer, being characterized by much lower K_m

Table 3
Kinetic parameters of *R*-IPG and *S*-IPG esterification with *Rhizopus oryzae* and *Aspergillus oryzae*

	Rhizopus oryzae		*Aspergillus oryzae*	
	R-IPG	*S*-IPG	*R*-IPG	*S*-IPG
K_m (mM)	87.2	284.4	125.2	3277.0
k_{cat} (mmol$_P$ g^{-1} h^{-1})	1.31	1.26	4.29	14.45
Enantiomeric ratio	3.4		8.0	

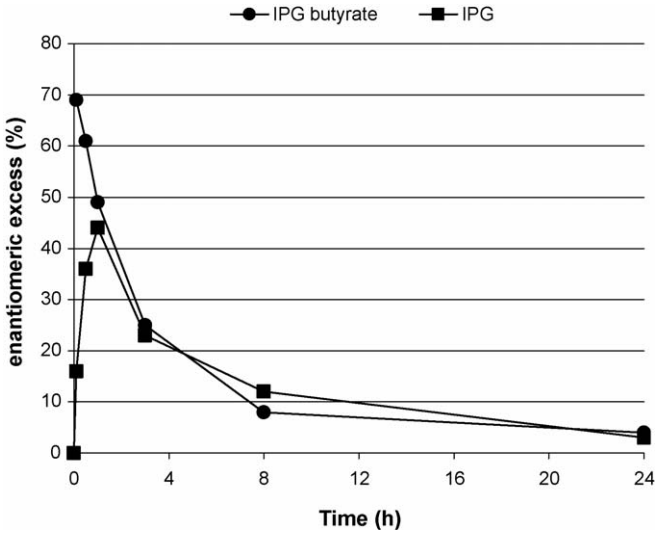


Fig. 1. Profile of the esterification with butyric acid of (*R*, *S*)-IPG under optimized conditions. T = 30 °C, concentration of the mycelium = 30 g l^{-1}, concentration of (*R*, *S*)-IPG = 3 g l^{-1}, equimolar concentration of butyric acid, solvent: *n*-heptane, a_w = 0.75, biocatalyst: liophilized cells of *A. oryzae*.

values, even if k_{cat} was lower in the case of *A. oryzae*. Marked deviations from the straight lines were observed for $S_o > 100$ mM for both microorganisms, owing to the occurrence of possible substrate inhibition only at relatively high concentrations.

These results confirm that lyophilized mycelium of *A. oryzae* was always more enantioselective than *R. oryzae*, and it was, therefore, used for the subsequent tests. Fig. 1 illustrates the profile of the esterification with butyric acid of (*R*, *S*)-IPG under optimal conditions (30 °C, concentration of the mycelium = 30 g l^{-1}, concentration of (*R*, *S*)-IPG = 3 g l^{-1} and equimolar butyric acid in *n*-heptane at a_w 0.75) catalysed by *Aspergillus oryzae*.

It should be noted that the reaction was largely reversible, with enantiomeric excess (e.e.) of the substrate reaching a maximum after 1 h and progressively diminishing as the reaction progressed further, while the best resolution was obtained after 15 min (e.e. of the product = 68–70%).

Transesterification reactions were also accomplished by using trichloroethyl butyrate (TCB), isopropenyl butyrate (IPB), ethylbutyrate (ETB) and tributyrin (TB). The profiles were similar to the one observed with direct esterification, indicating that the water initially present in the reaction medium led to equilibrium reactions also in those cases where water was not produced during the biotransformation. Direct esterifications with butyric acid and transesterification with TCB and IPB were then performed at different initial water activities, ranging from 0.15 to 0.95. No significant differences in the initial rates and the overall profile of the kinetic resolution were observed (data not shown). These results suggest that water present even at very low a_w was sufficient to establish a fast equilibrium.

The direct esterification between butyric acid and (*R*, *S*)-IPG was also performed on larger scale (2.3 l) under optimised conditions (30 °C, concentration of the mycelium = 30 g l^{-1}, concentration of (*R*, *S*)-IPG = 3 g l^{-1} in *n*-heptane at a_w 0.75) giving 23.9 g l^{-1} (analytical data, 2.3 g l^{-1} after purification)

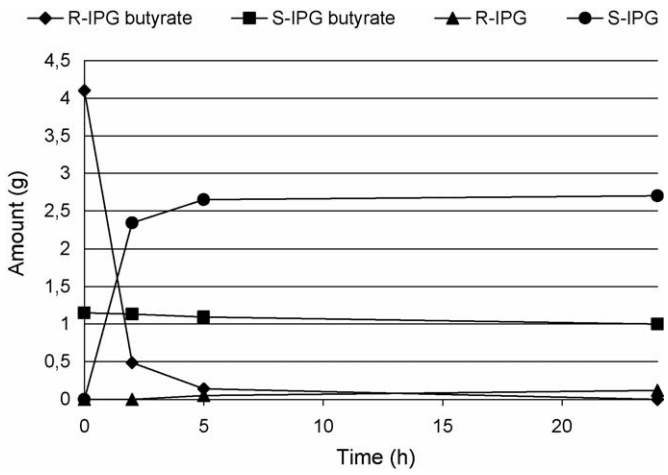


Fig. 2. Results of (*R*)-IPG butyrate and (*S*)-IPG butyrate hydrolysis using thermally-treated cells of *Bacillus coagulans*.

of (*R*)-IPG butyrate with an e.e. = 56% and a molar conversion of 52%.

The enantiomerically enriched ester (5.3 g in 2.5 l) was recovered and hydrolysed with thermally-treated cells of *Bacillus coagulans* able to catalyse the enantioselective hydrolysis of *R*-esters of IPG (Fig. 2).

The (*S*)-ester (1.04 g) was obtained enantiomerically pure, while 2.82 g of the (*S*)-alcohol were recovered with e.e. = 90% (Fig. 2).

4. Conclusions

Lyophilized mycelium of *Rhizopus oryzae* CBS 112.07 and *Aspergillus oryzae* MIM can be advantageously used as biocatalysts for the direct esterification or interesterification of 1,2-*O*-isopropylideneglycerol in organic solvent. The kinetics and the whole profile of the esterification of were investigated under optimised conditions. The reaction was fast and reversible causing a marked decrease of the enantiomeric ratio (*E*) along the reaction time as previously described for other enzymatic reactions [25,26]; only the kinetic data about the esterification of single enantiomers of IPG collected at the start of the reaction allowed for the determination of the *E*. Enantioselectivity was quite moderate with preference for the transformation of the *S*-alcohol, the highest enantioselectivity was observed with *Aspergillus oryzae* MIM (*E* = 8.0): this value is similar to the ones observed with commercial lipases [13]. The low enantiomeric excess of the (*R*)-IPG butyrate (e.e. = 56%) was "corrected" by using a catalyst able to catalyse the enantioselective hydrolysis of this enantiomer, such as thermally-treated cells of *Bacillus coagulans.*

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