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Enhanced enantioselectivity of *Bacillus coagulans* in the hydrolysis of 1,2-*O*-isopropylidene glycerol esters by thermal knock-out of undesired enzymes

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Abstract—The enantioselective hydrolysis of different (*RS*)-1,2-*O*-isopropylidene glycerol esters has been achieved with whole cells of *Bacillus coagulans* NCIMB 9365 furnishing the (*S*)-alcohol as the major enantiomer. The reaction is catalysed by a thermostable cell-bound carboxylesterase and improvement of the enantioselectivity has been achieved by heat treatment of the whole cells, which causes the knock-outs a non-enantioselective competing enzyme. Thermally-treated cells hydrolysed (*RS*)-1,2-*O*-isopropylidene glycerol esters with high enantioselectivity, the highest enantiomeric ratio (80–100) being observed for the benzoate. The biocatalyst displayed good stability and could be re-used after filtration for 12 cycles before showing significant loss of activity; repeated biotransformation batches allowed the recovery of 9.55 g/L of enantiomerically pure (*S*)-isopropylideneglycerol benzoate starting from 24.0 g/L of the racemic mixture.

1. Introduction

Both enantiomers of 1,2-*O*-isopropylidene glycerol are valuable chiral building blocks.[1,2] Enzymatic hydrolysis with a number of commercial lipases gives limited stereoselectivity.[3–7] Microbial esterases from *Bacillus* species and *Kluyveromyces marxianus* have proven more enantioselective.[8–10] Two cell-associated carboxylesterases occur in *Bacillus coagulans* NCIMB 9365: one is thermostable and enantioselective towards benzoate and butanoate, while the second one is thermolabile and not enantioselective.[8] Esterase(s) associated with the cells of *B. coagulans* have been employed for the preparation of enantiomerically enriched cyanohydrin acetates.[10] Herein we describe the thermal treatment of whole cells of *B. coagulans* NCIMB 9365 for knocking out the undesired enzyme and thus producing a biocatalyst with enhanced enantioselectivity in the hydrolysis of 1,2-*O*-isopropylidene glycerol esters.

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2. Results

B. coagulans NCIMB 9365 was grown in stirred tank reactors employing different cultural media (data not shown) and the activity was tested on 1,2-*O*-isopropylidene glycerol benzoate and naphthyl butanoate (NB) as substrates. Media containing protein extracts were suitable for the production of high cell-bound esterase activity and the addition of 1,2-*O*-isopropylidene glycerol benzoate did not significantly affect esterase production or enantioselectivity of the biotransformation of (*RS*)-1,2-*O*-isopropylidene glycerol benzoate. Cells from a 20 L reactor were lyophilised and employed in further experiments aimed at the improvement of the enantioselectivity towards (*RS*)-1,2-*O*-isopropylidene glycerol benzoate hydrolysis.

B. coagulans NCIMB 9365 possesses two cell-associated competing esterases: carboxylesterase 1 (BCE1) is enantioselective towards (*RS*)-1,2-*O*-isopropylidene glycerol benzoate and thermostable at 65 °C, while carboxylesterase 2 (BCE2) is not-enantioselective with a maximum activity at 35 °C. Whole cells were, therefore, exposed to

heat treatments combining different temperatures, times and buffers for obtaining a selective knock-out of the undesired carboxylesterase. Cells maintained in phosphate buffer (0.1 M, pH 6.8) at various temperatures were used for catalysing the hydrolysis of (*RS*)-1,2-*O*-isopropylidene glycerol benzoate at 45 °C, starting from 2.5 g/L of substrate and using 20 g/L (dry weight) of cells (Fig. 1).

Heat treatment improved the enantioselectivity and lowered the overall activity; the best compromise between initial rates and enantioselectivity (expressed as enantiomeric ratio, *E*) was encountered when cells were treated for 1 h at 65 °C. The zymograms of enzymatic extracts of thermally treated and untreated cells (Figure not shown) stained after reaction with naphthyl butanoate confirmed the selective knock-out of the non-enantioselective enzyme.

Thermally-treated cells were employed for optimising the conditions of the enantioselective hydrolysis of (*RS*)-1,2-*O*-isopropylidene glycerol benzoate; conventional parameters (pH, *T*, cell and substrate concentration) were simultaneously evaluated using the Multisimplex experimental design.[11] The profile of the kinetic resolution obtained under optimised conditions (pH 6.5, temperature 25 °C, 2.0 g L^{-1} substrate, 30 g L^{-1} dry cells) is shown in Figure 2.

The enantioselectivity (*E* = 80–100) is among the highest reported so far, allowing for the recovery of enantiomerically pure (*S*)-1,2-*O*-isopropylidene glycerol benzoate when the overall conversion had reached 54%. Thermally-treated cells of *B. coagulans* were also tested for the hydrolysis of different 1,2-*O*-isopropylidene glycerol esters (Scheme 1); results are reported in Table 1.

Hydrolysis occurred with good enantioselectivity of butanoate and benzoate esters; the (*S*)-ester was obtained as the remaining enantiomer, except in the hydrolysis of phenylacetate, where a slight preference for hydrolysis of the (*S*)-ester was observed.

The whole cells were re-used after centrifugation for the hydrolysis of (*RS*)-1,2-*O*-isopropylidene glycerol benzoate under the same conditions of biotransformation; decrease of activity was observed after six cycles, due to the partial extracellular release of the BCE1. The re-

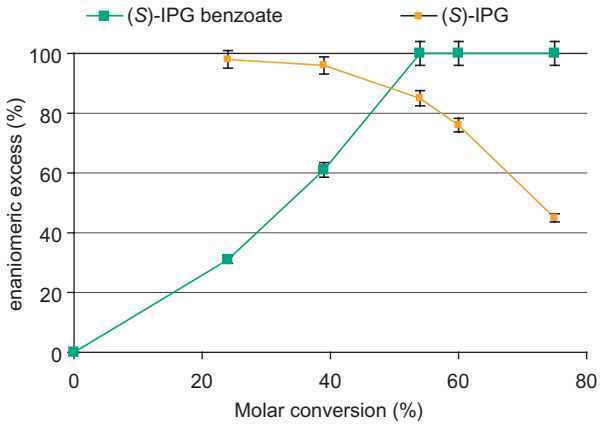


Figure 2. Hydrolysis of racemic 1,2-*O*-isopropylidene glycerol benzoate catalysed by thermally treated cells of *B. coagulans* NCIMB 9365 under optimised conditions.

peated-batch process was carried out recovering the biocatalyst by ultrafiltration (cut-off 10,000 Da) for maintaining both the cells and the released enzyme inside the reaction vessel. The membrane reactor proved suited for performing repeated batches of the biotransformation with significant loss of activity only after 11 cycles; enantiomerically pure substrate was recovered when the biotransformation had reached the desired conversion, allowing for the production of (*S*)-1,2-*O*-isopropylidene glycerol benzoate on multigram scale (9.55 g L^{-1} recovered from 24.0 g/L of racemic substrate).

In conclusion, the heat treatment of cells of *B. coagulans* caused a thermal knock-out of non-enantioselective enzymes furnishing an enantioselective catalyst for the kinetic resolution of different esters of 1,2-*O*-isopropylidene glycerol. The (*R*)-esters were predominantly hydrolysed giving the corresponding (*S*)-alcohols; the *E*-values for the hydrolysis of butanoate and benzoate esters were in the range 80–100.

3. Experimental

3.1. General

Chemicals were of reagent grade and purchased form Fluka, Milano, Italy.

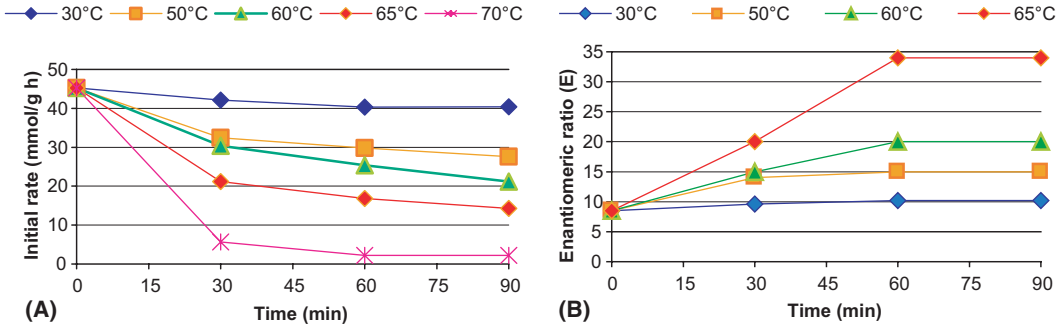
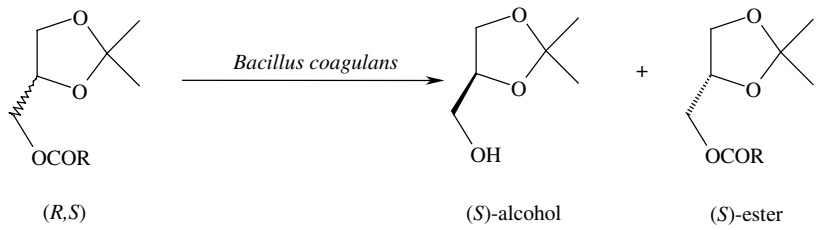


Figure 1. Time course of changes of the initial rate (A) and enantioselectivity (B) of cells of *Bacillus coagulans* NICIM 9365 towards (*RS*)-1,2-*O*-isopropylidene glycerol benzoate exposed after different thermal treatments.



1 R = H
2 R = CH_3
3 R = CH_2CH_3
4 R = $CH_2CH_2CH_3$
5 R = $CH_2CH_2CH_2CH_2CH_3$
6 R = CH_2Ph
7 R = Ph
8 R = 4-NO_2Ph
9 R = 4-CH_3OPh

Scheme 1. Hydrolysis of racemic 1,2-*O*-isopropylidene glycerol esters.

Table 1. Hydrolysis of different 1,2-*O*-isopropylidene glycerol esters catalysed by thermally treated cells of *B. coagulans* NCIMB 9365

Substrate	Ee substrate (%)	Ee product (%)	Molar conv. (%)	*E*	Time (h)
1	12–13 (*S*)	10–11 (*S*)	55–56	1.4	3
2	<5 (*S*)	35–36 (*S*)	10–11	2.2	5
3	62–63 (*S*)	86–87 (*S*)	42–43	24–26	7
4	95–96 (*S*)	79–80 (*S*)	54–55	32–34	7
5	27–28 (*S*)	88–89 (*S*)	23–24	20–22	7
6	15–16 (*R*)	9–10 (*R*)	62–63	1.4	24
7	70–71 (*S*)	95–96 (*S*)	42–43	80–100	9
8	97–98 (*S*)	85–86 (*S*)	52–53	50–60	8
9	62–63 (*S*)	92–93 (*S*)	40–41	45–50	24

Formate ester gave 2–3% spontaneous hydrolysis after 2 h.

3.2. Microorganism, growth and biotransformation conditions

B. coagulans NCIMB 9365 (National Collection of Industrial and Marine Bacteria, Aberdeen, UK) was routinely maintained on Difco nutrient broth (8 g L^{-1}, agar 15 g L^{-1}, pH 7) and cultured in 2.0 L Erlenemeyer flasks containing 200 mL of CYSP broth (casitone 15 g L^{-1}, yeast extract 5 g L^{-1}, soytone 3 g L^{-1}, peptone 2 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 15 mg L^{-1}, $FeCl_3$ 115 mg L^{-1}, $MnCl_2$ 20 mg L^{-1}, pH 7.0) and incubated for 24 h at 45 °C on a reciprocal shaker (120 spm). The dry weights were determined after centrifugation of 100 mL of cultures: cells were washed with distilled water and dried at 110 °C for 24 h.

Biotransformations were carried out in 10 mL screw capped test tubes with cells suspended in 5 mL of different aqueous media. The substrates were added in 10% acetone solution and the incubation continued under magnetical stirring at different temperatures.

A stirred ultrafiltration cell (Model 8050, capacity 50 mL) containing 20 mL of biotransformation medium was employed as membrane reactor for repeated-batch biotransformation. The membrane had a cut-off of 10,000 Da. The reaction was stopped when the enantiomeric excess of the substrate was 100% by filtering the suspension under nitrogen pressure. The recovered filtrate was extracted with ethyl acetate for recovering both the unreacted (*S*)-1,2-*O*-isopropylidene glycerol benzoate and enantiomerically enriched (*S*)-1,2-*O*-isopropylidene glycerol. The organic extracts were dried over Na_2SO_4 and the solvent removed under reduced pressure. Flash chromatography on silica gel pretreated with triethylamine (hexane/ethyl acetate, 75:25) afforded (*S*)-1,2-*O*-isopropylidene glycerol benzoate $[\alpha]_D^{25} = -8.6$ (*c* 1, $CHCl_3$).

3.3. Esters of 1,2-O-isopropylideneglycerol

The chemicals used in this study were of reagent grade and were purchased from Fluka (Milano, Italy). 1,2-*O*-isopropylideneglycerol esters (acetate, butanoate, valerate, isovalerate, heptanoate, benzoate) were prepared in our laboratory. The preparation of (*RS*)-acetyl-1,2-*O*-isopropylideneglycerol is reported as an example: acetic anhydride (6.0 mL) was slowly added to a stirred solution of (*RS*)-1,2-*O*-isopropylideneglycerol (4.0 g) and dry pyridine (8.0 mL) in 30 mL of dry benzene at 4 °C. After 24 h under magnetic stirring at room temperature the reaction was quenched with 100 mL of 5% $NaHCO_3$ solution. The product was extracted with ethyl acetate. The organic layers were washed with water, dried over Na_2SO_4. Then the solvent was removed under reduced pressure. The crude product was purified (78% yield) by flash chromatography (hexane/ethyl acetate, 4:6) using silica gel pretreated with triethylamine. The butanoate, valerate, isovalerate, heptanoate and benzoate were prepared by a similar procedure, except 1,2-*O*-isopropylideneglycerol benzoate which was purified by distillation under vacuum (bp 120 °C/3 mm).

1,2-*O*-Isopropylideneglycerol formate: ^{1}H NMR ($CDCl_3$, 200 MHz): 1.36 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 3.68–3.76 (m, 2H, CH_2), 4.01–4.18 (m, 2H, CH_2), 4.20–4.29 (m, H, CH), 8.08 (s, 1H, CHO); 1,2-*O*-isopropylideneglycerol acetate: ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.38 (s, 3H, CH_3), 1.44 (s, 3H, CH_3), 2.12 (s, 3H, CH_3), 3.36–3.41 (m, 2H, CH_2), 4.01–4.11 (m, 2H, CH_2), 4.13–4.19 (m, 1H, CH); 1,2-*O*-isopropylideneglycerol propionate: ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.15 (t, 3H, CH_3), 1.26 (s, 3H, CH_3), 1.32 (s, 3H, CH_3), 2.35 (q, 2H, CH_2), 3.64–3.69 (m, 2H, CH_2), 4.04–4.15 (m, 2H, CH_2), 4.14–4.22 (m, 1H, CH); 1,2-*O*-isopropylideneglycerol butanoate: ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.98 (t, 3H, CH_3), 1.23–1.36 (m, 2H, CH_2), 1.29 (s, 3H, CH_3), 1.38 (s, 3H, CH_3), 1.48–1.59 (m, 2H, CH_2), 2.29 (t, 2H, CH_2), 3.50–3.59 (m, 2H, CH_2), 4.04–4.15 (m, 2H, CH_2), 4.14–4.22 (m, 1H, CH); 1,2-*O*-isopropylideneglycerol hexanoate: ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.89 (t, 3H, CH_3), 1.22–1.33 (m, 4H, CH_2), 1.34 (s, 3H, CH_3), 1.40 (s, 3H, CH_3), 1.50–1.60 (m, 2H, CH_2), 2.32 (t, 2H, CH_2), 3.54–3.63 (m, 2H, CH_2), 4.00–4.12 (m, 2H, CH_2), 4.19–4.27 (m, 1H, CH); 1,2-*O*-isopropylideneglycerol phenylacetate: ^{1}H NMR ($CDCl_3$, 200 MHz): 1.32 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 3.40 (s, 2H, CH_2), 3.48–3.56 (m, 2H, CH_2), 4.08–4.15 (m, 2H, CH_2), 4.11–4.24 (m, 1H, CH), 7.12–7.25 (m, 5H, Ph); 1,2-*O*-isopropylideneglycerol benzoate: ^{1}H NMR ($CDCl_3$, 200 MHz): 1.35 (s, 3H, CH_3), 1.45 (s, 3H, CH_3), 3.42–3.49 (m, 2H, CH_2), 4.06–4.14 (m, 2H, CH_2), 4.10–4.22 (m, 1H, CH), 7.50–8.12 (m, 5H, Ph); 1,2-*O*-isopropylidene glycerol 4-NO_2-benzoate: ^{1}H NMR ($CDCl_3$, 200 MHz): 1.29 (s, 3H, CH_3), 1.44 (s, 3H, CH_3), 3.83–3.90 (m, 2H, CH_2), 4.13–4.20 (m, 2H, CH_2), 4.36–4.50 (m, 1H, CH), 8.20–8.34 (m, 4H, Ph); 1.37 (s, 3H, CH_3), 1.46 (s, 3H, CH_3), 3.70 (s, 3H, OCH_3), 3.79–3.84 (m, 2H, CH_2), 4.18–4.26 (m, 2H, CH_2), 4.42–4.60 (m, 1H, CH), 6.70–6.81 (m, 2H, Ph), 7.79–7.86 (m, 2H, Ph).

3.4. Optimisation by the sequential simplex method

The simplex optimisation method was based on sequential experimental trials guided by the systematic search strategies of the Multisimplex® 2.0 program (Multisimplex AB, Karlskrona, Sweden).[12] The starting experiments were selected with levels of each control variable (substrate concentration, pH, temperature and biomass concentration) within the following ranges: pH 4–8, temperature 20–26 °C, substrate concentration 1–10 g/L, biomass concentration 10–45 g/L. The control responses to be optimised were the molar conversion after 3 h and the corresponding enantiomeric ratio (*E*). Each experiment was carried out in triplicate.

3.5. Analytical methods

Alcohol and ester concentrations were determined by gas-chromatographic (GC) analysis on a Carlo Erba Fractovap GC gas-chromatograph equipped with a hydrogen flame ionisation detector. The column (3×2000 mm) was packed with Carbowax 1540 (10% on Chromosorb 80–100 mesh). Samples (0.2 mL) were taken at intervals and added to an equal volume of an internal standard solution (1-hexanol 2 g/L) in water; the resulting solution was extracted with ethyl acetate and analysed. The enantiomeric composition of 1,2-*O*-isopropylidene glycerol, 1,2-*O*-isopropylidene glycerol acetate, 1,2-*O*-isopropylidene glycerol propionate, 1,2-*O*-isopropylidene glycerol butanoate, 1,2-*O*-isopropylidene glycerol pentanoate, 1,2-*O*-isopropylidene glycerol hexanoate and 1,2-*O*-isopropylidene glycerol benzoate was determined by gas chromatographic analysis using a chiral capillary column (diameter 0.25 mm, length 25 m, thickness 0.25 μm, DMePeBeta-CDX-PS086, MEGA, Legnano, Italy), using the following temperature program: 10 min at 90 °C, increased to 150 °C over 5 min and then held at 150 °C for 30 min. The retention times of the enantiomers of 1,2-*O*-isopropylidene glycerol and its esters under these conditions were: (*R*)-1,2-*O*-isopropylidene glycerol = 8.2 min, (*S*)-1,2-*O*-isopropylidene glycerol = 9.0 min; (*R*)-1,2-*O*-isopropylidene glycerol formate = 10.4 min, (*S*)-1,2-*O*-isopropylidene glycerol formate = 9.9 min; (*R*)-1,2-*O*-isopropylidene glycerol acetate = 13.1 min, (*S*)-1,2-*O*-isopropylidene glycerol acetate = 12.4 min; (*R*)-1,2-*O*-isopropylidene glycerol propionate = 17.4 min, (*S*)-1,2-*O*-isopropylidene glycerol propionate = 16.4 min; (*R*)-1,2-*O*-isopropylidene glycerol butanoate = 22.3 min, (*S*)-1,2-*O*-isopropylidene glycerol butanoate = 21.2 min; (*R*)-1,2-*O*-isopropylidene glycerol pentanoate = 25.8 min, (*S*)-1,2-*O*-isopropylidene glycerol pentanoate = 24.9 min; (*R*)-1,2-*O*-isopropylidene glycerol hexanoate = 30.2 min, (*S*)-1,2-*O*-isopropylidene glycerol hexanoate = 29.6 min; (*R*)-1,2-*O*-isopropylidene glycerol benzoate = 38.2 min, (*S*)-1,2-*O*-isopropylidene glycerol benzoate = 37.4 min. The enantiomeric composition of 4-MeO- and 4-NO_2-benzoates was analysed using a Chiralcel OD column (Daicel Chemical Industries); the mobile phase was hexane/isopropanol (9:1) at a flow rate of 0.5 mL min^{-1}. The retention times were: (*S*)-*p*-MeO-benzoate 17.0 min, (*R*)-*p*-MeO-benzoate 30.1, (*R*)-*p*-NO_2-benzoate 20.8 min (*S*)-*p*-NO_2-benzoate 22.0 min.

The absolute configurations of 1,2-*O*-isopropylidene glycerol were determined by comparison with commercially available enantiomerically pure samples, while enantiomerically pure esters were synthesised starting from enantiomerically pure 1,2-*O*-isopropylidene glycerol following the procedure described above.

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