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Journal of Biotechnology 92 (2001) 21–26

JOURNAL OF Biotechnology

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Efficient and selective microbial esterification with dry mycelium of *Rhizopus oryzae*

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Received 10 April 2001; received in revised form 5 June 2001; accepted 20 June 2001

Abstract

The use of dry mycelium of *Rhizopus oryzae* as biocatalyst for ester production in organic solvent has been studied. Mycelia with notable carboxylesterase activity were produced when different Tweens (20, 40, 60 and 80) were employed as main carbon source for the growth. Dry mycelium of four strains of *Rhizopus oryzae* proved effective for efficiently catalysing the synthesis of different flavour esters (hexylacetate and butyrate, geranylacetate and butyrate) starting from the corresponding alcohol and free acid, including acetic acid. The esterification of the racemic mixture of 2-octanol and butyric acid proceeded with high enantioselectivity (*R*-ester produced with enantiomeric excess $\geqslant 97\%$) when *Rhizopus oryzae* CBS 112.07 and *Rhizopus oryzae* CBS 260.28 were employed.

Keywords: *Rhizopus oryzae*; Carboxylesterase; Lipase; Esterification; Enantioselectivity; Flavour esters

1. Introduction

Fungi of the genus *Rhizopus* were reclassified so that *R. delemar*, *R. liquefaciens*, *R. javanicus* and *R. niveus* are now ascribed to the species *R. oryzae* (Schipper and Stalpers, 1984). This microbiological classification is consistent with the observation that lipases isolated from *R. delemar*, *R. javanicus* and *R. niveus* have identical amino acid sequences and lipase from *R. oryzae* differs by two amino acids (Bornscheuer and Kazlauskas, 1999). Lipases from *R. oryzae* (ROL) are normally secreted extracellularly after cleaving of the prolipase and it has been suggested that the various lipase forms found in these microorganisms are due to the degree of the proteolytic action involved in the post-translational cleavage, rather than the involvement of different genes (Beer et al., 1998).

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0168-1656/01/$ - see front matter
PII: S0168-1656(01)00345-5

Extracellular or mycelium-bound ROL production is strongly affected by growth conditions (Nahas, 1988; Essamri et al., 1998; Fadiloğlu and Erkmen, 1999) and it has been shown that mycelium-bound ROL can be advantageously employed as biocatalyst in water and/or organic solvents (Gancet and Guignard, 1986; Salleh et al., 1993; Essamri et al., 1998; Razak et al., 1999). We have previously observed that *Rhizopus oryzae* CBS 112.07 showed interesting mycelium-bound activity when Tween 80 was employed as main carbon source (Molinari et al., 1995, 1998). This strain had been also used for efficiently resolving the racemic mixture of 2-octanol by esterification with butyric acid in heptane (Molinari et al., 1998).

In this paper we have studied the mycelium bound activity of four strains of *Rhizopus oryzae* (*Rhizopus oryzae* CBS 112.07, type of *Rhizopus oryzae*; *Rhizopus oryzae* CBS 260.28, formerly type of *Rhizopus liquefaciens*; *Rhizopus oryzae* CBS 328.47, formerly type of *Rhizopus delemar*; *Rhizopus oryzae* CBS 391.34, formerly type of *Rhizopus javanicus*), grown using different Tween as main carbon source. The synthesis of different flavour esters (hexylacetate and butyrate, geranylacetate and butyrate, 2-octylbutyrate) catalysed by dry mycelium of *Rhizopus oryzae* was evaluated.

2. Materials and methods

2.1. Chemicals and microorganisms

Chemicals were from Fluka Chimica, Milano (Italy). Four microorganisms were used: *Rhizopus oryzae* CBS (Centraal Bureau voor Schimmelcultures, Baarn, Holland) 112.07, *Rhizopus oryzae* CBS 260.28, *Rhizopus oryzae* CBS 328.47 and *Rhizopus oryzae* CBS 391.34. They were routinely maintained on malt extract (8 g l^{-1}, agar 15 g l^{-1}, pH 5.5).

To obtain cells for biocatalytic activity tests, 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 moulds were grown on media containing a basal medium (BM: Difco yeast extract 1 g l^{-1}, $(NH_4)_2SO_4$ 5 g l^{-1}, K_2HPO_4 1 g l^{-1}, $MgSO_4 7H_2O$ 0.2 g l^{-1}, pH 5.8) added with glucose or Tween 20, 40, 60 or 80 (5 g l^{-1}). Suspensions of spores (2×10^6 spores ml^{-1}) were used as inoculum.

2.2. Hydrolysis and esterification

Hydrolysis and esterification were performed as described previously (Molinari et al., 2000). Each experiment was carried out in triplicate.

2.3. Analytical methods

Alcohol and ester concentrations of the biotransformations in organic solvent were determined by gas-chromatographic (GC) analysis on a Carlo Erba Fractovap GC equipped with a hydrogen flame ionization detector. The column (3×2000 mm) was packed with Carbowax 1540 (10% on Chromosorb 80–100 mesh). The injector temperature was 200 °C. Samples (0.25 ml) were taken at intervals and added to an equal volume of an internal standard solution (1-octanol) in *n*-heptane. The enantiomeric composition of the 2-octyl esters were determined by chiral GC analysis using a capillary DmePeBeta-CDX-PS086 column (MEGA, Legnano, Italy).

3. Results

3.1. Hydrolytic activity

The four strains of *Rhizopus oryzae* were initially grown using Tween 80 or glucose as main carbon source and using different spore concentrations, ranging from 1.6×10^4 to 2×10^6 (spores ml^{-1}). Mycelia were harvested by filtration after 48 h and the filtrate directly employed for hydrolysis, the mycelium was washed with phosphate buffer and the pellets disrupted by homogenization at 4 °C to obtain suspensions suited for the test.

The mycelium-bound and extracellular activities were independently tested using α-naphtyl acetate, butyrate, caprylate and palmitate as cur-

rent substrates. Mycelia obtained from submerged cultures inoculated with the highest spore concentration (2×10^6 spores ml^{-1}) gave the highest mycelium-bound activity. Very sluggish hydrolytic activity could be found in mycelia grown on glucose. The overall activity (OA) and the relative distribution of the mycelium-bound (M) activity for cultures obtained using Tween 80 are reported in Table 1.

Hydrolytic activities were found in all the tested strains, being both A mycelium-bound and extracellular. The highest degree of activity was observed with butyrate and caprylate, indicating marked preference for medium length chains.

3.2. Esterifications

Mycelia obtained after growth on Tween 80 as main carbon source were lyophilized and used as dry biocatalyst for catalysing the synthesis of different flavour esters (geranylacetate and butyrate, hexylacetate and butyrate). Biotransformation conditions were chosen on the basis of previous results (Molinari et al., 1995): lyophilized mycelium (30 g l^{-1}) was resuspended in heptane and reactions were carried out starting from the free acid and the alcohol (equimolar 35 mM) at 50 °C. The time-courses of the reactions are reported in Fig. 1.

All the tested strains catalysed ester formation. As expected the esterification with butyric acid occurred faster than with acetic acid; however, the direct acetylation of hexanol and geraniol furnished high molar conversions (ranging from 65 to 90%) after 24 h.

Different Tweens (20, 40, 60) were used as main carbon source for growth and the synthesis of geranylacetate was chosen for comparing the mycelium-bound activity in organic solvent (Table 3).

The performances of lyophilized mycelia of *Rhizopus oryzae* CBS 112.07 was not significantly influenced by the type of Tween employed for growth, while marked differences were observed with the other strains. In particular, *Rhizopus oryzae* CBS 260.28 and CBS 328.47 showed the highest rates and molar conversions when grown on Tween 60 as main carbon source, while the best results with *Rhizopus oryzae* CBS 391.34 were obtained when grown on Tween 20.

Finally, the four microorganisms were evaluated as catalyst for the acylation of racemic 2-octanol. Different carboxylic acids were chosen to check the influence of the chain length on activity and enantioselectivity. Biotransformations were carried at 30 and 50 °C. Although reactions occurred generally faster at 50 °C, the best resolutions of the racemic mixture were obtained at 30 °C (Table 2). The acetate ester was produced only in little amounts, while butyrate and caproate esters were obtained often with good yields.

Esterification with butyric acid gave always the *R*-ester as major product and enantioselectivity resulted to be dependent on the Tween employed. The use of Tween 80 gave mycelia which showed almost complete enantioselectivity in the case of *Rhizopus oryzae* CBS 112.07 and CBS 260.28. The use of caproic acid as acylating agent generally furnished lower enantiomeric excesses with respect

Table 1
Mycelium-bound and extracellular activity (U 10^2) of strains of *Rhizopus oryzae* on α-naphtyl acetate, butyrate, caprylate and palmitate[a]

Rhizopus oryzae	Acetate		Butyrate		Caprylate		palmitate	
	Mycelium	Extra	Mycelium	Extra	Mycelium	Extra	Mycelium	Extra
CBS 112.07	0.6	1.5	4.3	4.0	2.2	2.2	0.8	1.7
CBS 260.28	4.0	3.8	4.9	12.5	5.3	4.8	0.2	2.4
CBS 328.47	3.2	1.4	9.8	6.4	7.1	2.5	1.7	0.2
CBS 391.34	1.5	1.4	8.8	5.0	8.4	1.3	1.9	0.2

[a] The enzymatic activity was expressed as the amount (U) of enzyme contained in 1 ml of filtered broth or in the mycelium filtered from 1 ml of the broth which catalysed the transformation of 1 μmole of substrate in 1 min at 45 °C

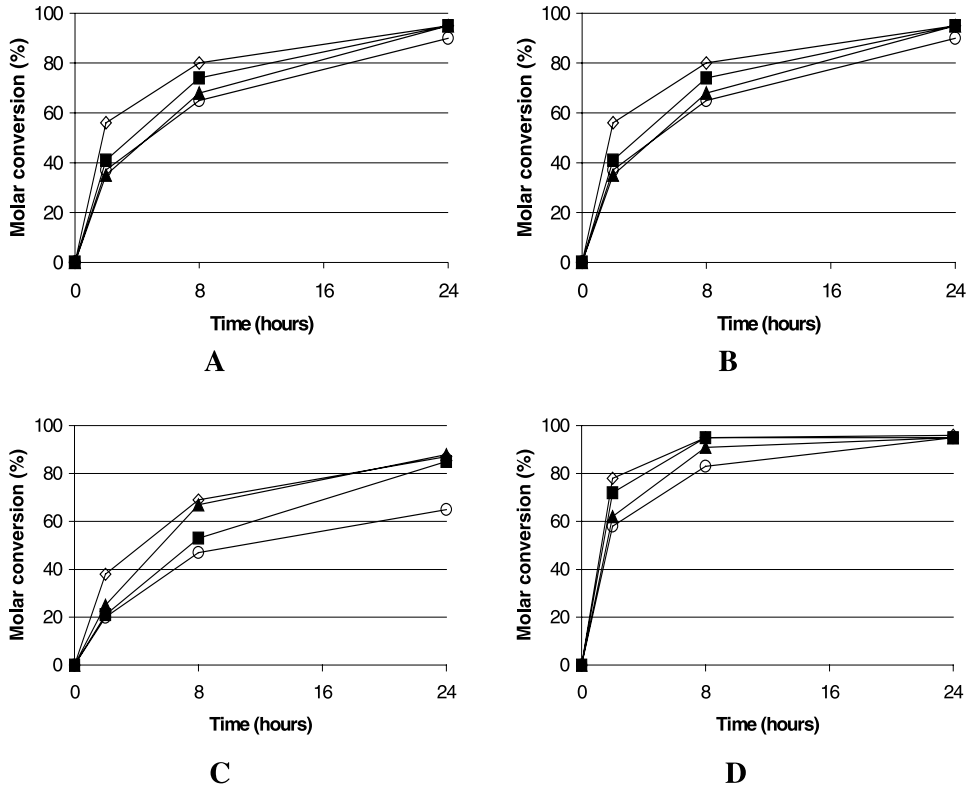

Fig. 1. Formation of hexylacetate (A), hexylbutyrate (B), geranylacetate (C), and geranylbutyrate (D) catalyzed by *Rhizopus oryzae* CBS 112.07 (◇), *Rhizopus oryzae* CBS 260.28 (○), *Rhizopus oryzae* CBS 328.47 (■) and *Rhizopus oryzae* CBS 391.34 (▲) grown on Tween 80 as main carbon source.

to butyric acid and in one case (*Rhizopus oryzae* CBS 260.28 grown on Tween 60) even the *S*-enantiomer was preferentially produced.

4. Discussion

The use of mycelium-bound carboxylesterases as biocatalyst may have technical and economical advantages, such as low costs for biocatalyst production and improved stability of the enzymes. This is a notable simplification compared with purifying the enzymes involved.

Different strains of *Rhizopus oryzae* produced mycelium-bound carboxylesterases when grown on Tween 80 as main carbon source. These enzymes catalysed preferentially the hydrolysis of medium chain (butyrate and caprylate) esters. The production of mycelium-bound carboxylesterases was also achieved by employing other Tweens (20, 40 and 60) in the growth medium and dry mycelia can be exploited for efficiently catalysing ester synthesis in organic solvent. *Rhizopus oryzae* CBS 112.07 had been already used for direct acylation of primary alcohols biotransformation (Molinari et al., 1995), but the conditions of growth used in this work (inoculum from a suspension of 2×10^6 spores ml^{-1}) furnished higher mycelium-bound activities.

The esterification of primary alcohols proceeded with good rates and good conversions also with acetic acid, which proved quite toxic for the enzymatic activity (Langrand et al., 1990; De Castro et al., 1997). It was previously shown that dry mycelium of other fungi are suited for direct acetylation of ethanol and geraniol (Molinari et al., 2000).

Table 2
Molar conversions (m. c.), enantiomeric excesses (e.e.) of (*R*)-2-octyl esters and enantiomeric ratio (*E*) obtained by esterification of (*R*, *S*)-2-octanol with butyric and caproic acid catalysed by lyophilized mycelium of different strains of *Rhizopus oryzae* in *n*-heptane at 30 °C after 5 days

Strain	Tween	Butyrate			Caproate		
		m. c. (%)	e.e. (%)	*E*	m. c. (%)	e.e. (%)	*E*
CBS 112.07	20	<5	n.d.	–	<5	n.d.	–
	40	40	80	17	45	<5	<1
	60	45	85	25	55	<5	<1
	80	35	97	110	20	12	1.3
CBS 260.28	20	<5	n.d.	–	12	55	3.7
	40	20	90	23	32	35	2.4
	60	15	65	5.3	45	35 (*S*)	2.7
	80	15	>97	>200	20	25	1.8
CBS 328.47	20	<5	n.d.	–	<5	n.d.	–
	40	30	90	27	25	90	25
	60	29	85	17	25	92	32
	80	<5	n.d.	–	<5	n.d.	–
CBS 391.34	20	20	75	8.4	15	75	8
	40	38	45	3.4	30	50	3.7
	60	35	55	4.6	27	55	4.2
	80	45	50	4.4	45	55	5.3

Dry mycelia of *Rhizopus oryzae* seemed to be less sensitive to the effect of the water produced during the direct esterification, being able to mostly shift the thermodynamic equilibrium towards quantitative ester synthesis. It should be pointed out that in this work a small amount (30 g l^{-1}) of dry mycelium was employed for catalysing the esterifications, indicating a notably high specific activity of the biocatalyst.

Table 3
Formation of geranylacetate after 24 h catalysed by *Rhizopus oryzae* strains grown on Tween 20, Tween 40, Tween 60

Strain	Tween	Molar conversion (%)
Rhizopus oryzae CBS 112.07	20	84
	40	>95
	60	>95
Rhizopus oryzae CBS 260.28	20	62
	40	38
	60	>95
Rhizopus oryzae CBS 328.47	20	85
	40	38
	60	10
Rhizopus oryzae CBS 391.34	20	95
	40	60
	60	80

These biocatalysts were also able to resolve the racemic mixture of (*R*, *S*)-2-octanol by esterification with different carboxylic acids. The reaction rates were much slower than what observed with primary alcohols. Butyric acid resulted in being the best one for achieving enantioselective esterification: *Rhizopus oryzae* CBS 112.07 and CBS 260.28 gave high enantioselection ($E = 110$ and > 200 respectively). No regular trends could be derived by comparison of the activity with the type of Tween employed, but all the tested strains gave mycelium with good carboxylesterase activity. These results on the whole indicate that microbial cells can be also used to perform the resolution of racemic mixtures of chiral secondary alcohols when this is difficult to achieve with commercial enzymes.

It can be concluded that the production of efficient and selective mycelium-bound carboxylesterases is not strain-specific among the species *Rhizopus oryzae*. Although a limited num-

ber of *Rhizopus oryzae* was tested, the results obtained show that strains able to carry out selective direct acylations of primary and secondary alcohols in organic solvents can be often found within this species, provided that suited cultural media are employed for their growth.

Acknowledgements

This work was supported by the C.N.R. Target Project on Biotechnology (Nos. 97.01019. PF 115.08601).

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