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Solid state fermentation for the production of lipolytic fungal enzymes

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Abstract - The production of lypolitic moulds in solid state fermentation was studied. *Aspergillus oryzae* and *Rhizopus oryzae* were grown on rice hulls using different media. Tween 80 and olive oil were employed as main carbon sources; the whole solid cultures were lyophilised and employed in heptane for catalysing the formation of various geranyl esters with molar conversions ranging from 40 to 95%, under optimised conditions, starting from 50 mM geraniol and equimolar amount of the acid.

Key words: solid state fermentation, rice hulls, *Rhizopus oryzae, Aspergillus oryzae*, esterification, flavours.

INTRODUCTION

Solid-state fermentation (SSF) is a process occurring in the near-absence of free water employing an inert and/or natural solid support for the growth (Pandey *et al*., 1999). SSF may have considerable advantages over traditional liquid fermentations, such as easy aeration, low energy requirements, non sterile conditions, higher product yields and easier product recovery (Ooijkaas *et al*., 2000). A lot of published information concerning SSF regards the production of microbial metabolites and industrial enzymes; the interest has been mainly devoted to extracellular enzymes which are easily recovered and do not undergo major proteolytic degradation (Pandey *et al*., 2000).

Many different fungi have been grown on natural or synthetic impregnated supports, such as vermiculite (Silman *et al*., 1993), clay granules (Desgranges *et al*., 1993), amberlite (Gutierrez-Rojas *et al*., 1995), polyurethane foam (Hongzhang *et al.*, 2006; John *et al.*, 2007) or polystyrene (Zhu *et al*., 1994; Gautam *et al.*, 2002). SSF can be carried out also on a variety of agricultural residues such as wheat straw, bagasse, corn cobs, rice hulls (Pandey *et al*., 2000), babassu cakes (Gutarra *et al*., 2007) and melon wastes (Alkan *et al.*, 2007). Rice hulls are cheap waste-products and have been employed for the growth of *Colletotrichum truncatum* for use as mycoherbicide (Silman *et al*., 1991).

Aspergillus oryzae and *Rhizopus oryzae* are among the microorganisms traditionally studied in SSF, such as the Koji process or grown on polyurethane foam for the production of extracellular lipases (Cbristen *et al*., 1995), proteases (Qzawa *et al*., 1996) and amylases (Murado *et al*., 1997). We have previously observed that strains of *A. oryzae* and *R. oryzae* produced extracellular and mycelium bound carboxylesterases with relevant activities and their dry mycelium efficiently and selectively catalysed the esterification between free acetic acid and primary alcohols in organic solvent (Molinari *et al*., 2000; Gandolfi *et al*., 2001). Acetic esters, such as geranyl acetate, are important flavour and fragrance components in the food and cosmetic industry (Longo and Sanroman, 2006).

In this work we have studied the growth and lypolitic activity of *A. oryzae* MIM in SSF employing rice hulls as support, since the mycelial morphology of these moulds seemed to be well suited for growing on solid supports. The carboxylesterase activity of the resulting biocatalyst has been exploited for the acylation of geraniol in organic solvent with acetic, butyric and hexanoic acid. The procedure developed with *A. oryzae* MIM was also applied to four other microbial strains, belonging to the species *R. oryzae* (namely *R. oryzae* CBS 112.07, *R. oryzae* CBS 260.28, *R. oryzae* CBS 328.47, *R. oryzae* CBS 391.34).

MATERIALS AND METHODS

Microorganisms. Five microorganisms were used: *Aspergillus oryzae* MIM (Microbiologia Industriale Milano) and *Rhizopus oryzae* CBS 112.07^T (Centraalbureau voor Schimmelcultures, Baarn, the Netherlands), *R. oryzae* CBS 260.28 formerly type of *Rhizopus liquefaciens*, *R. oryzae* CBS 328.47 formerly type of *Rhizopus delemar* and *R. oryzae* CBS 391.34 formerly type of *Rhizopus javanicus*. They were routinely maintained on a solid medium (malt extract 8 g/l, agar 15 g/l, pH 5.5). Rice hulls were gently furnished by AIRI (Associazione Italiana Risiere Italiane).

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Culture conditions. Submerged fermentations (SMF) were carried out in 750 ml Erlenmeyer flasks containing 100 ml of medium and incubated at 28 °C for 48 h on a reciprocal shaker (100 spm). The moulds were grown on media containing a basal medium (BM: Difco yeast extract 1 g/l, K_2HPO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 0.2 g/l, pH 5.8) added with a nitrogen source (peptone, polypeptone, $NaNO_3$ or $(NH_4)_2SO_4$ 5 g/l) and a carbon source (olive oil or Tween 80, 5-20 g/l). The flasks were inoculated with 10 ml of spore suspension (2 x 10^6 spores/ml).

Solid state fermentations were performed in 20 cm Petri dishes. Rice hulls were dried in an air oven at 60 °C for 48 h, homogenised and sieved to obtain fractions between 1.0 and 2.0 mm particle size; 10 g of the obtained rice hulls were impregnated with 13.5 ml of a solution containing: Difco yeast extract 1 g/l, K_2HPO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 0.2 g/l, pH 5.8, added with a nitrogen source (polypeptone 5 g/l). Carbon sources (olive oil or Tween 80) were separately added at 5-20 g/l after sterilisation; the plates were finally inoculated with 1.5 ml of a spore suspension (2 x 10^6 spores/ml) and incubated at 28 °C for 48 h; the resulting microbial cultures were lyophilised. The lyophilised cultures were assayed for esterase activity and employed as biocatalyst in esterification trials.

Esterification reactions. Geraniol esterification was performed at 50 °C under magnetic stirring in 10 ml screw capped test tubes by suspending lyophilised cultures from SSF in *n*-heptane and then adding geraniol (50 mM) and an equimolar amount of the free acids. Control reactions with lyophilised cells coming from SMF were carried out in the same conditions (see Results and Discussion). All the conversion values shown are averages for three different measurements (standard deviation, $\sigma < 5\%$).

Esterase activity assays. Esterase activity was assayed using α-naphtylacetate, α-naphtylbutyrate and α-naphtylcaprylate as substrate, measuring the absorbance (wavelength = 560 nm) relative to the cromophores originated by reaction of the hydrolysed substrate with Fast Garnet GBS salt (Molinari *et al*., 2000; Gilham and Lehner, 2005). The mycelia resulting from the SMF growth and the lyophilised cultures obtained with SSF were both suspended in distilled water and homogenised at 4 ºC to obtain a suspension suited for the test.

The solutions of naphtyl derivatives were prepared by adding the substrates (0.42 mM) in 4 ml of ethanol to 96 ml of buffer solution (0.1 M Tris-HCl, pH 7.0) containing arabic gum (10 mg) and dioctyl sulfosuccinate sodium salt (AOT, 200 mg). The reaction mixtures were obtained by adding 1.5 ml of the substrate solution to 100 mg of lyophilised biocatalyst in 0.1 M Tris-HCl, pH 7. The enzymatic activity was expressed as unit (U), defined as the amount of biocatalyst which catalysed the transformation of 1 μmole of substrate in 1 minute at 45 ºC.

Analytical methods. Biomass in submerged and solid state cultures was determined by ergosterol content measurements (Desgranges *et al*., 1991). 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 x 2000 mm) was packed with Carbowax 1540 (10% on Chromosorb 80-100 mesh). The injector temperature was 200 ºC. Samples (0.25 ml) were taken at intervals; cells were separated by centrifugation (15000 rpm, 5 min) and to the organic phase was added an equal volume of an internal standard solution (1-octanol) in *n*-heptane.

RESULTS AND DISCUSSION

Aspergillus oryzae MIM was preliminarily grown on submerged cultures employing media containing different nitrogen sources (peptone, polypeptone, $NaNO_3$ or $(NH_4)_2SO_4$) and 0.5% olive oil as main carbon source. The use of 0.5% polypeptone as nitrogen source gave the highest biomass yields; polypeptone was therefore used as nitrogen source in all the following trials.

It has been previously shown that olive oil and Tween 80 are among the carbon sources suited for producing mycelium of *A. oryzae* MIM with high lipolytic activity (Molinari *et al*., 2000). Experiments were therefore performed using solid media containing olive oil and Tween 80 as main carbon sources using rice hulls as support; submerged fermentations were also carried out as comparison. The resulting growth values are reported in Table 1.

TABLE 1 - Growth of *Aspergillus oryzae* MIM on solid-state media (SSF) and submerged cultures (SMF). Growth is expressed as grams of dry mycelium per litre of culture (SMF) or as mg of dry mycelium per gram of inert support.

Carbon source	Concentration (%)	Growth	
		SSF	SMF
Olive oil	0.5	43	4.1
	1.0	52	5.3
	2.0	60	5.8
Tween 80	0.5	18	2.5
	1.0	24	3.2
	2.0	21	3.7

The activity of the SMF mycelia and the whole SSF cultures was tested using α-naphtyl acetate, butyrate and caprylate as current substrates (see figures 1A and 1B).

The highest degree of activity was observed in most of the cases with naphtylbutyrate, while the highest activity towards acetate ester was obtained with mycelia grown using 0.5% Tween 80. This approach indicates that the enzyme(s) expressed under these conditions can be considered as "true" esterases (Bornscheuer, 2002).

The whole lyophilised SSF cultures and the lyophilised mycelium from SMF were used as biocatalyst for the synthesis of different geranyl esters (acetate, butyrate and hexanoate). Figure 2A reports the acylation of geraniol (50mM) with equimolar amounts of acetic, butyric and hexanoic acid catalysed by 300 g/l of lyophilised SSF culture (corresponding to a concentration of dry mycelium ranging from 5 to 20 g/l depending on the growth medium) suspended in heptane at 50 ºC. Biotransformations performed with 15 mg/ml of free lyophilised mycelium are also reported for comparison (see figure 2B).

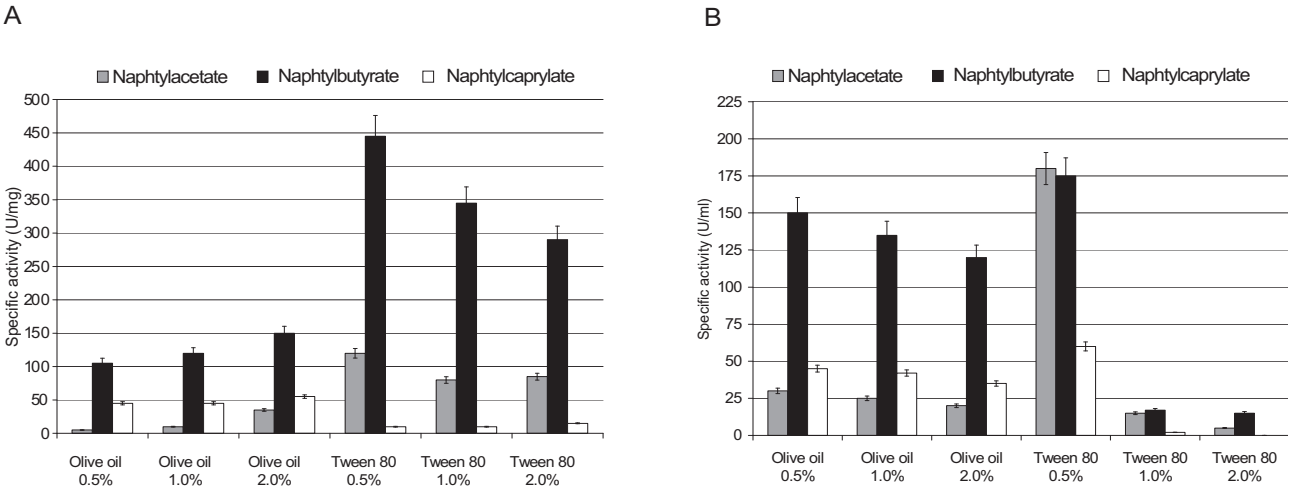

FIG. 1 - Hydrolysis of naphtyl-esters with *Aspergillus oryzae* MIM grown on solid-state media (A) and in submerged cultures (B). Specific activity is expressed as Units per ml of culture (SMF) or as Units per mg of biocatalyst (SSF).

Mycelium grown in SMF cultures with 0.5% Tween 80 catalysed complete esterification of geranyol with all the carboxylic acids tested, while low activity was found using other C-sources. Molar conversions higher than 70% were found in the case geranylbutyrate with lyophilised SSF-cultures of *A. oryzae* grown with olive oil or Tween 80 at various concentrations, while the production of geranylacetate and geranylhexanoate was strongly dependent on the carbon source employed; high yields of geranylhexanoate were obtained only using olive oil. Therefore it is likely that different enzymes are expressed depending on the type of fermentation employed.

The good results obtained with *A. oryzae* led us to investigate other four microbial strains, belonging to the species *R. oryza*e (namely *R. oryzae* CBS 112.07, *R. oryzae* CBS 260.28, *R. oryzae* CBS 328.47 and *R. oryzae* CBS 391.34). These strains had previously shown very effective in promoting the synthesis of esters in organic solvents (Gandolfi *et al*., 2001). The four strains were grown in SSF using rice hulls as solid support with the medium that afforded the best results with *A. oryzae* (i.e. 2% olive oil, polypeptone 5g/l). A fungal biomass of 51 (± 5.0) mg of mycelium per gram of solid support was found.

Figure 3A reports the acylation of geraniol (50 mM) with equimolar amounts of acetic, butyric and hexanoic acid catalysed by 300 g/l of lyophilised SSF culture (corresponding to about 15 mg/ml of mycelium) suspended in heptane at 50 ºC. Biotransformations performed with 15 mg/ml of free lyophilised mycelium are also reported for comparison (Fig. 3B).

Lyophilised mycelia of *R. oryzae* grown in SMF furnished higher rates and conversions for acetylation than when grown in SSF. The acylation with butyric acid occurred with similar results using SMF- or SSF-grown mycelium, while the latter was a better catalyst for geranylhexanoate formation, being able to yield more than 80% molar conversion after 24 hours. It is likely that different sets of enzymes are expressed under different growth conditions. It should be pointed out that *A. oryzae* MIM and *R. oryzae* strains produced also extra-cellular carboxylesterases in SMF, while SSF cultures are directly lyophilised without any downstream operation.

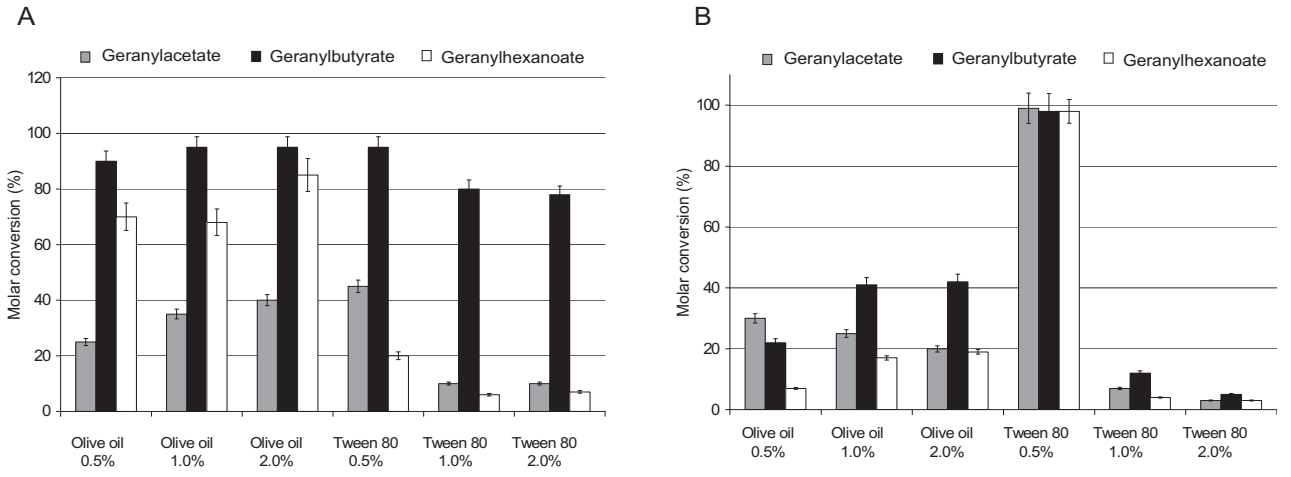

FIG. 2 - Formation of geranyl esters catalysed by *Aspergillus oryzae* MIM grown on solid-state media (A) and in submerged cultures (B). Molar conversions refer to 24 h of reaction.

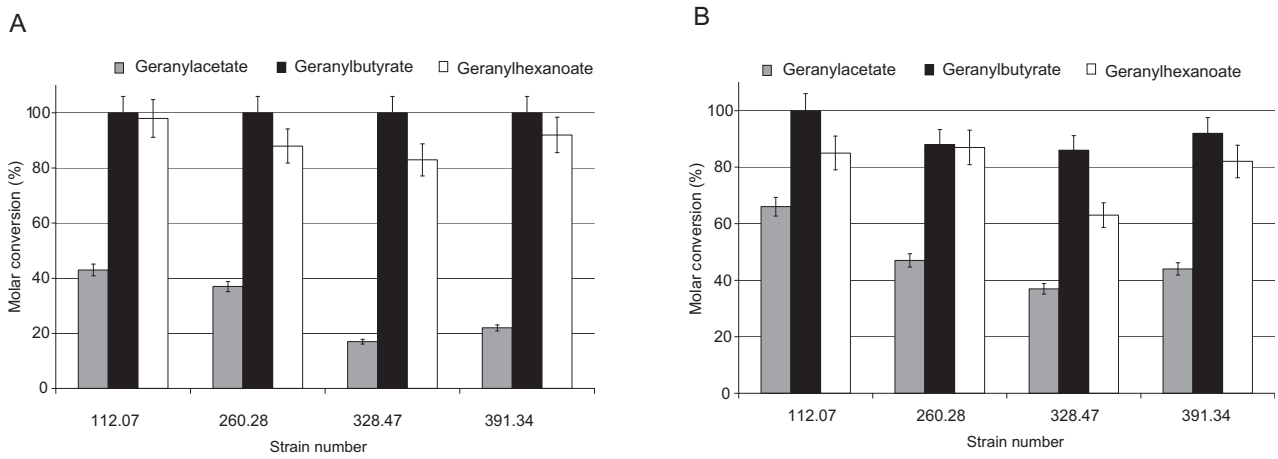

FIG. 3 - Formation of geranyl esters catalysed by *Rhizopus oryzae* strains grown on solid-state media (A) and in submerged cultures (B). Molar conversions refer to 24 h of reaction.

In conclusion the esterification in organic solvent catalysed by moulds grown in SSF was studied using *A. oryzae* and *R. oryzae* and compared with the performances of mycelium grown submerged cultures. The whole procedure is very simple and allowed for a stable and efficient catalyst employable in organic solvents for direct esterification of geraniol.

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