Comparison of cutinase bioencapsulation in sol-gel and PVA versus lyophilized form on biosynthesis of Ethyl Caproate in organic solvent

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Introduction

Ethyl caproate belong to the group of flavors compounds that can contribute for the aroma of pineapple flavor, in the preparation of food; tobacco, liquor flavor, among others. An enzymatic process, as an alternative route to chemical synthesis of this and many other fruit flavor compounds, has technological and economical interesting due to use of mild reaction conditions and the synthesis of flavors with high quality and purity. The high purity of these flavors allows their labeling as *natural* by food regulatory agencies and strongly contributes for better public acceptance as ingredients for food industry in relation to those synthesized by chemical processes (S. Hari-Krishna et al., 2002).

Cutinase (E.C. 3.1.1.3.) belongs to the class of the serine esterases. In comparison with lipases, cutinases display very little interfacial activation, being active on both soluble and emulsified triglycerides. As small carboxylic ester hydrolases, the sub-family of cutinases consists of 20 members based on amino acid sequence similarity which display hydrolytic activity on cutin polymers and efficiently hydrolyze soluble esters and emulsified triacylglycerols. In recent years, the esterolytic activity of cutinase has been largely exploited and several applications in different industrial field have been presented (Carvalho et al., 1999; Egmond et al., 2000).

In order to use cutinase economically and efficiently in non-conventional reaction medium various enzyme preparations have been tested: as a free form, immobilized on solid supports, encapsulated in reversed micelles and entrapped in hydrogel gel matrices (Carvalho et al., 1999). Encapsulation in a sol –gel matrix has proved to be a sound strategy to improve cutinase activity and stability in organic solvent media (Vidinha et al., 2006).

Entrapment of enzymes in lens-shaped hydrogels mainly based on polyvinyl alcohol (PAVL) has also been shown to provide a successful solution for reactions of synthesis. It is a cheap technique and hydrogel beads can be easily separated from reaction media and can be used in stirred reactor (Gröger et al., 2001).

In this work was carried out comparison of esterolytic activity of cutinase encapsulated in sol-gel and PVAL hydrogel versus lyophilized enzyme preparation in the esterification of caproic acid with ethanol using isooctane as organic solvent. The possibility of reutilization of immobilized enzyme in more than a single reaction with a high conversion yield of ethyl caproate contributes to lowering the cost of the biotechnological process.

Material and methods

The *F. solani pisi* cutinase wild-type was produced by recombinant *S. cerevisiae* SU50 strain and characterization was done as previously described (Calado *et al*., 2002a and 2002b). Tetramethoxysilane (TMOS) and sodium dioctyl sulfosuccinate (AOT) were all purchased from Sigma-Aldrich. Isooctane and PEG 600 were supplied by FLUKA (Germany). Lentikats® liquid was purchased from Genialab, Germany. Caproic acid (Sigma-Aldrich, Germany), and ethanol 95% (AGA, Portugal) were also purchased.

All other reagents used were either laboratory or analytical grade.

Methods for monitoring substrates and ester concentrations

The concentration of ethanol, caproic acid, and ethyl caproate was determined by a Hewllet-Packard model 5890 gas chromatography (GC) equipped with a flame ionization detector (FID). The WCOT Fused Silica coating CP Chirasil- Dex CB column, 25m x 0.25mm, DF=0.25 (Varian Inc.) was used. 20 mM n-decane (Merck, Germany) was used as the internal standard inside of reaction media. Oven temperature was held at 50ºC for 4 min before being elevated to 160ºC for 1.67 min at 15ºC/min; injector temperature was set at 200 ºC; detector temperature was set at 250 ºC; carrier gas was nitrogen.

Sol-gel immobilization

The solution containing 100 µL TMOS (2.32 mol dm^{-3}) and 40 µL HCl (1.37 mmol dm^{-3}) was mixed in a Transsonic T 460 sonication water bath for 10 min until the hydrolysis reaction was complete (sol-solution). In a typical immobilization reaction 75 µL of cutinase *Fusarium solani pisi* SU50 (2 mg/mL, 150 U/mg) was suspended in 85 µL of 50 mmol dm^{-3} phosphate buffer, pH 7, and then added into the sol-solution. To obtain micro-particles and minimize diffusion limitations, 300 µL of the sol-gel solution with enzyme was immediately added to 6 mL of 150 mmol dm^{-3} AOT/isoctane solution, before gelation. The resulting mixture was under vortex for 2 min and washed twice with 4 times with isooctane to take out AOT before start reaction of esterification. The immobilized cutinase obtained were suspended in isooctane and used in reaction esterification.

Immobilization in PVAL particles

500 µl of a cutinase solution (61.2 mg ml^{-1}) were added to 10 ml of Lentikat® liquid and the whole extruded into 100 ml of PEG 600 under magnetic stirring, to yield half-spherical like beads. After a 2-hour incubation period at room temperature, the beads were harvested, washed once with 50 mmol dm^{-3} phosphate buffer, pH 7 buffer, and either stored in isooctane or immediately used for bioconversion runs. After swelling 4.5g PVAL particles with cutinase concentration of 6.8 mg/g PVAL was obtained.

Enzymatic esterification reaction

Unless otherwise stated a typical esterification reaction for ethyl ester synthesis was carried out in 7 ml of working volume inside of 10 ml capped flasks with stopper (EPDM stoppers, black, SIGMA-ALDRICH, Germany). Ethanol, caproic acid and n-decane were mixed thoroughly in isooctane which consist the reaction medium before the addition of enzyme (lyophilized form, immobilized on sol-gel and immobilized on PVAL particles). Zero point of the reaction was withdrawn before enzyme addition. The enzymatic ester synthesis occurred in an incubator at 30ºC under 300 rpm magnetic stirring. Samples were withdrawn periodically using a needle, thus preventing tampering with the integrity of the rubber cap, and then dried before ester and substrates analysis by GC.

The esterification conversion or reaction yield was calculated according to the molar ration between ethyl ester and respective limiting substrate alcohol or acid.

Results and Discussion

The lyophilized cutinase preparation shows potential for synthesis of short alkyl esters displaying roughly 91% conversion yield after 8h (Figure 1).

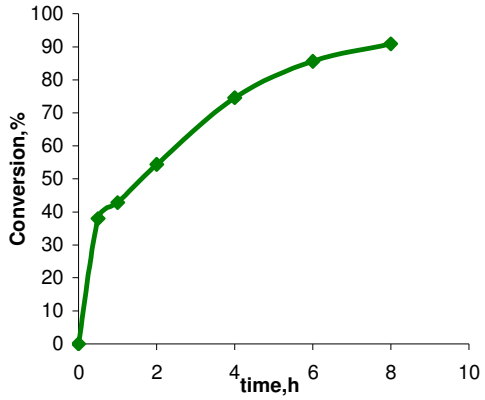

Fig. 1: Synthesis of Ethyl caproate by lyophilized cutinase *F.s. pisi* SU50 alcohol/acid molar ratio=2 ([ethanol]=0.2M), 300 rpm, t=30^{0}C, 1.4mg/ml enzyme, 170U/mg

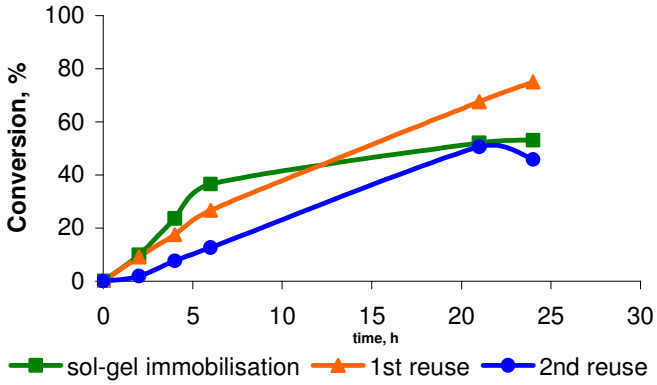

Fig. 2: Synthesis of Ethyl caproate by sol-gel encapsulated cutinase *F.s.pisi* SU50, 300 rpm, T=30^{0}C, alcohol/acid molar ratio=2 [ethanol]=0.2M

However, for industrial applications, the use of an immobilized biocatalyst is, in general, more advantageous, if the right encapsulation strategy to obtain an economical enzyme immobilization form is identified. Key issues to be addressed are the maintenance of catalytic activity upon immobilization, and the recycling capability of the immobilized biocatalyst. The effect of sol-gel immobilization on the synthesis of ethyl caproate on the course of reaction is shown in Figure 2.

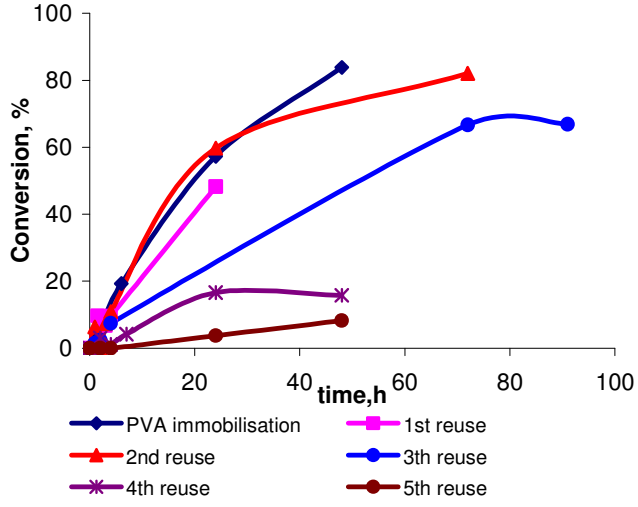

Fig. 3: Synthesis of Ethyl caproate by PVAL encapsulated cutinase *F.s.pisi* SU50, 300 rpm, T=30^{0}C, alcohol/acid molar ratio=2 ([ethanol]=0.2M)

Fig. 4: PVAL particles of immobilized cutinase

Ester conversions of 75% were achieved by cutinase sol-gel preparation after 24 and 120h for first and second biocatalyst reutilizations (table 1).

Conversion (%)	24h	32h	120h
Cutinase Sol-gel.	53	ND	ND
1st reuse	75	ND	ND
2nd reuse	46	66	76

Table 1: **Reuse of cutinase encapsulated in Sol-gel preparations**

In the case of PVAL-cutinase preparation (Figure 3) the time course of conversion proceeded at a lower speed when matched with the lyophilized enzyme, but after 48 h the conversion yield (82 – 84%) roughly achieved the result with the lyophilized enzyme (91%). This result could be ascribed to partition/diffusion limitations due to the greater diameter of PVAL particles, about 3-5 mm (Figure 4) which is related with similar observation effect reported by Gröger et *al*. (2001). However, with this size the PVAL beads are easy separated from the reaction mixture. The conversion yield was not significantly altered in the first two reutilization cycles but a marked decrease occurred afterwards, to about 16% in the fourth cycle and to only 9% in the fifth cycle. This result could be due to partial enzyme leakage from the PVAL particles.

With these experiments was noticed that the rate of reactions of for both immobilized cutinase preparations are smaller than with the lyophilized enzyme preparation probably due to diffusion limitations.

Conclusions

Cutinase encapsulation in sol-gel and PVAL hydrogel matrices is promising strategy to efficiently use immobilized enzyme preparations in esterification reactions in organic media. The conversion yields of lyophilized and immobilized enzymes were roughly similar upon immobilization, although the enzyme reaction rate was lower in the immobilized forms. Diffusion limitation could be reason for this behavior. The encapsulation methods led to promising results when the immobilized biocatalysts were challenged in consecutive batch runs especially with sol-gel preparation.

References

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