P-021 **Entrapped lipase hydrogel used in ester synthesis**

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INTRODUCTION

Immobilized enzymes are used in many commercialized products for higher yields. The lipases (*E.C. 3.1.1.3*) that constitute a most versatile group of enzymes have been used for performing esterification and trans-esterification reactions in organic solvents. In the last few years, there has been an increasing interest in the use of enzymes for the biosynthesis of molecules in organic media [Gargouri *et.al*., 2002, Castillo *et. al.*, 2003 and Kanwar et al., 2006]. Esters of short chain fatty acids and alcohols are known as flavor and fragrance compounds that are used in food, beverage, cosmetic and pharmaceutical industries. Geranyl acetate occurs naturally and is used in perfumes/ fragrances, as a flavoring agent in non-alcoholic beverages, ice cream, candy, baked goods, acquers, paints, formaldehyde, synthetic resins and waxes, celluloid and camphor; for masking unpleasant odors and to perfume shoe polish. In the present study a synthetic *poly*(MAc-*co*-DMA-*cl*-DMAm) hydrogel bound lipase of *Bacillus coagulans* MTCC-6375 has been employed in organic media to synthesize geranyl acetate and a few other short-chain esters by transesterification.

MATERIALS AND METHODS

Microorganism The *Bacillus coagulans* MTCC-6375 isolate was obtained from Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla (India).

Synthesis of hydrogel The hydrogel *poly* (MAc-*co*-DMA-*cl*-MBAm) was prepared by co-polymerizing methacrylic acid (23.6 mM) and dodecyl methacrylate (6.85 mM) in acetone (4 ml), in the presence of a cross-linker *N*, *N*-methylene bisacrylamide (376 mg) and benzyol peroxide (30 mg) as an initiator.[5] The reactants were vigorously stirred an[1]d transferred inside an airtight glass vial under vacuum. The vial was sealed and heated at 80°C for 30 minutes in a water bath. Insoluble product (polymer) was separated from the reaction mixture by filtration to remove unreacted soluble constituents. Successive washings with water, geranyl alcohol and acetone in that order separated the unreacted compounds trapped in the body of the polymer. The polymer was dried completely in an air-oven at 40°C for 24 h to obtain a xerogel.

Immobilization Purified lipase (8 ml; 35.6 U; 0.52 mg protein/ml) was incubated with 2 g of pre-swollen *poly* (AAc-*co*-HPMA-*cl*-MBAm) hydrogel for 18 h at 4°C. The matrix was pre-swollen in Tris buffer (0.05 M, pH 8.5) for 16 h at 8°C. The lipase-bound matrix was filtered through Whattman No.1 filter paper. The bound lipase activity as well as percent protein binding was determined (by estimating unbound protein in the filtrate). Total unbound protein in filtrate was deducted from total protein incubated with the matrix.

Ester synthesis employing immobilized lipase A reference curve was calibrated between molar concentration (mM) of vinyl acetate and the corresponding area under the peak (retention time 0.48 min). A sample size of 2 µl was used for GLC analysis. The GLC (Michro-9100, Netel Chromatographs, Thane, India) was programmed for oven temperature 100°C, FID temperature 160°C and injector temperature 160°C. The assay of vinyl acetate was performed on 15% SE30 Chromo WHP column (2 meter X 1.8 inch) using N_2 as a carrier gas (flow rate 30 ml/min).

Determination of amount of geranyl acetate synthesized After the completion of esterification at specified time interval, the reaction mixture was withdrawn (2-5 µl) and subjected to analysis of residual vinyl acetate. The amount of residual vinyl acetate was subtracted from the original amount of vinyl acetate to determine the amount of vinyl acetate consumed. The amount of ester produced in the transesterification reaction was considered to be equivalent to the amount of vinyl acetate consumed in the transesterification reaction.

RESULTS AND DISCUSSIONS

Kinetics of synthesis of geranyl acetate The effect of reaction time on synthesis of geranyl acetate using immobilized lipase was studied at a temperature of 55˚C in *n*-heptane under shaking condition up to 21 h. The synthesis of the ester was time dependent and a maximum amount of geranyl acetate (64.5 mM) was produced after 15 h of reaction when geranyl alcohol and vinyl acetate were used at 100 mM each in *n*-heptane. Thus in subsequent reaction a reaction time of 21 h at 55˚C was considered optimum to perform synthesis of geranyl acetate using hydrogel-bound lipase.

Effect of temperature on synthesis of geranyl acetate The effect of change in the reaction temperature on the synthesis of geranyl acetate by immobilized lipase was also studied. Maximum synthesis (64.6 mM) of geranyl acetate was obtained at 55˚C after 21 h . At 65˚C, there was a marked decrease (55.2 mM) in the ester synthesis, which might be on account of denaturation of the lipase. At 75°C there was a further decline (50.1 mM) in the amount of ester produced.

Effect of solvent (n-alkane) on the ester synthesis*:* Use of *n*-pentane, *n*-hexane, *n*-hexadecane or *n*-heptadecane reduced the amount of ester formed under similar conditions . The use of *n*-heptane, *n*-octane and *n*-nonane resulted in 63.7, 63.2 and 66.2 mM of geranyl acetate. Thus the maximum conversion of reactants into ester was recorded in *n*-nonane (66.2 mM) at 55˚C under shaking after 21 h.

Effect of addition of molecular sieve on transesterification*:* The transesterification reaction resulted in formation of water as a by-product of the reaction, and its removal using a molecular sieve might enhance the synthesis of ester by pushing the reaction equilibrium in the forward direction. However, when the effect of molecular sieve was studied by adding a molecular sieve (25 to 500 mg per reaction volume), a gradual decline (64.0 to 28.4 mM geranyl acetate) in the amount of ester formed was noticed . Thus addition of a molecular sieve had a deleterious effect on the transesterification reaction.

Lipase is a ubiquitous activity possessed by most of the organisms. Many bacteria, fungi and yeasts produce extra-cellular lipases. Newer diverse microbial sources of lipases have been reported in the recent years. In the present study an extra-cellular alkaline lipase of *Bacillus coagulans* MTCC-6375 was purified and immobilized on to a *poly* (MAc-*co*-DMA-*cl*-MBAm) hydrogel. In the esterification reaction, the hydrogel bound lipase of *B. coagulans* optimally produced geranyl acetate at 55^{o}C in 15 h. However, further increase in the reaction temperature to 65 or 75^{o}C decreased the esterification rate. The alteration in temperature of reaction mixture might interfere with the porosity, hydrophobic character and diffusion of the reactants and/ or products at the catalytic site of enzyme or hydrogel. At temperature more than 55^{o}C, there was no further increase in the amount of ester synthesized, which might be on account of denaturation of the lipase as well as alteration in the native structure of lipase. Recently, the hydrogel-bound lipase of *Bacillus coagulans* MTCC-6375 has been used for optimal synthesis of ethyl laurate at 65^{o}C in 21 h [Kanwar *et. al*., 2005].

The use of alkanes of C-chain length shorter and longer than *n*-heptane decreased the rate of esterification. When *n*-alkane with a shorter C-chain length was used as a solvent, a gradual decrease in rate of geranyl acetate synthesis was noticed. It was observed that as the log *P* value of an *n*-alkane increased corresponding to increase in the C-chain length of the alkanes (log *P* value for *n*-pentane, *n*-hexane, *n*-heptane, and *n*-nonane is 3, 3.5, 4 and 5, respectively), the hydrophobicity of the alkane *i.e.* solvent also increased in that order, and that appeared to be very important for modulation of catalytic activity of hydrogel-bound *B. coagulans* lipase. Thus it appeared that choice of an appropriate solvent system was critical for the synthesis of ester using hydrogel-immobilized lipase of *Bacillus coagulans*. A relatively higher hydrophobicity appeared to be an important property to be built into a synthetic hydrogel for increasing the catalytic activity of bound lipase of *B. coagulans*. Higher the log *P*, the more hydrophobic is the solvent. In a recent study using hydrogel-immobilized lipase of *P. aeruginosa* BTS-2, we have shown a similar effect that resulted in an enhanced synthesis of ethyl propionate in *n*-nonane.[39] However, previously reported lipase of *P. aeruginosa* was more efficient in synthesis of ester as indicated by approximately 94% conversion of reactants into ester. In another study, hydrogel-bound *B. coagulans* MTCC-6375 lipase could achieve 61% conversion of reactants into ethyl laurate.[36] This lower conversion into ester might be due to use of a higher carbon chain length fatty acid (lauric acid, C 12) than the one used previously (propionic acid, C 3). It appeared that *B. coagulans* MTCC-6375 lipase is inherently less efficient in performing esterification than the *Pseudomonas* lipase as in the [1]present study approximately 64-67 mM of geranyl acetate could be produced by manipulating various physical and kinetic parameters.

CONCLUSIONS

Foregoing discussion indicated that a systematic approach could be devised on the basis of solvent engineering to favor optimal synthesis of an ester of interest. . This study is among very few reports on the use of a synthetic hydrogel bound extra-cellular alkaline lipase of a moderately thermotolerant *B. coagulans* for the synthesis of geranyl acetate and a few-other alcohol-acetate esters, of short chain lengths in a water free organic solvent system. The efficacy of this lipase to catalyze interesterification and transesterification reactions in organic solvents/ water restricted/ controlled media are still required to be explored in future studies.

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