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ENZYMATIC SYNTHESIS OF METHYL MYRISTATE USING THERMOCOL-IMMOBILIZED LIPASE

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ABSTRACT

Fatty acid esters are now being produced commercially with immobilized commercial lipases in non aqueous solvents. In this study waste thermocol a commercial lipase (Steapsin) was immobilized on waste thermocol (polystyrene used as packing material for chemicals) by adsorption with a binding efficiency of 28 % (0.16 IU/mg matrix). Its efficacy for esterification was checked by Gas liquid chromatography for the synthesis of methyl myristate. The thermocol–bound lipase was used to synthesize methyl myristate from myristic acid and methanol in dimethyl sulphoxide. The optimization of the reaction conditions such as biocatalyst loading, effect of reactant concentration, reaction time, temperature, molecular sieve and scale up was studied. The immobilized lipase (20 mg/ml) was used to perform an esterification in DMSO that resulted in synthesis of approximately 88 mM of methyl myristate at 45°C, under continuous shaking at (120 rpm) after 16 h when myristic acid and methanol was used in an equimolar ratio. The addition of molecular sieve (3Å x 1.5 mm) has deleterious effect. The bound lipase resulted in 48.8 mM ester in the 6th cycle of repetitive use of matrix. When the reaction volume was scaled up to 50 mL, the ester syntheisized was 31 mM under optimized conditions.

INTRODUCTION

Enicostema axillare (E.littorale) Lam Raynal (synonym) Lipases of microbial origin are the most versatile enzymes and are known to bring about a range of versatile reactions, which include hydrolysis, inter-esterification, esterification, alcoholysis, acidolysis and aminolysis. Lipases constitute the most important group of biocatalysts for biotechnological applications. Novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers, biodiesel, the production of enantiopure pharmaceuticals agro-chemicals, and flavour compounds Jaeger and Eggert, 2002; Kanwar *et al.,* 2008. Lipases have emerged as one of the leading biocatalysts with proven potential for contributing to the multibillion dollar underexploited lipid technology bio-industry and have been used *in situ* lipid metabolism and *ex situ* multifaceted industrial applications (Joseph *et al.,* 2008). In the last few years, there has been an increasing interest in the use of enzymes for the biosynthesis of molecules in organic media (Kanwar *et al.,* 2005; Gargouri *et al.,* 2002; Castillo *et al.,* 2003). Many lipase-catalyzed esterification or condensation reactions have been developed by employing a variety of lipases of microbial origin (Acros *et al.,* 1998; Watanabe *et al.,* 2000; Kuwabara *et al.,* 2003; Sonwalkar *et al.,* 2003; Kanwar *et al.,* 2006). Such reactions were possible in organic solvents. Thermocol used in the present study as a support/ matrix for the immobilization of the lipase is used as a packing material for various chemicals and machines or their parts. Thermocol is chemically polystyrene/ an aromatic polymer. It is a waste material in the labs and industries. By chemical treatment we used it as a matrix for immobilization of a commercial enzyme Steapsin (lipase). The use of industrial enzymes allows the technologists to develop processes that more closely approach the gentle, efficient process in nature. Commercial preparations used for the desizing of denim and other cotton fabrics contain both alpha amylase and lipase enzymes. Alkaliphilic lipases are stable in detergents containing protease and activated bleach systems. Lipase decomposes fatty stains into more hydrophilic substances that are easier to remove than similar non-hydrolyzed stains (Fuji *et al.,* 1986). Methyl myristate is used as flavouring essence and the everyday use essence, and it is also used in organic synthesis.

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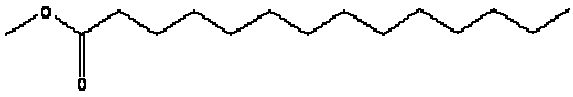


Fig. 1 Graphic structure of Methyl myristate

MATERIALS AND METHODS

Chemicals

Steapsin was purchased from RFCL ltd., New Delhi (India). Polystyrene was obtained as waste packing materials from Department of Biotechnology, Himachal Pradesh University, and Shimla, India. Methyl alcohol and myristic acid were procured from Merck, Mumbai (India). *p*-NP palmitate from Alfa-Aesar, Heysham, Lancs., England. Gum acacia and Tris buffer were obtained from SRL Ltd., Mumbai, India. Methyl myristate was used as an internal standard for GC and all other chemicals required were purchased from Merck, Mumbai (India).

Experimental

Activation of polystyrene (thermocol)

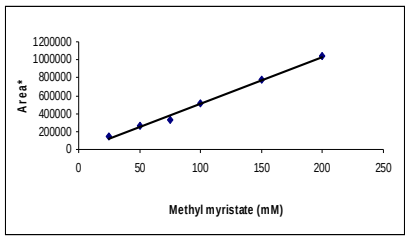
Waste thermocol from lab was collected, weighed and washed thrice with water. Two grams thermocol was kept into 20 ml of acetone inside a 50 ml screw capped glass tube for 24 h. The thermocol was sedimented by centrifugation at 10,000 g for 10 min in excess of acetone and the later was discarded by inversion. The brittle thermocol was crushed in pestle-mortar to prepare crude powder. The powder/ pellets of thermocol were washed with Tris buffer (pH 8.0: 0.5 mM) thrice and the material was dried thereof in oven set at 60°C to achieve a constant weight.

Immobilization

The crude thermocol (2 g) was exposed to Steapsin (lipase) prepared in Tris buffer (0.05 M, pH 8.0; 10 ml) overnight. The pellets were separated by centrifuging at 10,000 g for 10 min. The matrix/ thermocol pellets-associated/ bound lipase activity (Winkler and Stuckmann, 1979) and the bound protein content (Lowry *et al.,* 1951) were calculated by subtracting the unbound protein/ lipase activity from the total protein/ lipase used during the immobilization onto thermocol beads. The immobilization of commercial enzyme on waste polystyrene matrix shows 27.8 % binding and lipase activity of 0.16 IU/mg when assayed using 20 mg of bound lipase (weight of matrix included) and 7 µg proteins per ml, specific activity of free enzyme was 71.4 U/ml and bound lipase is 32.8 U/mg. The efficiency of this immobilized matrix was checked for the synthesis of methyl myristate.

Preparation of standard profile of methyl myristate

A reference curve (Fig. 2) was plotted between molar concentration of methyl myristate and the area under the peak (retention time 1.0-1.3 min).



* arbitrary units

Fig. 2 Standard profile of methyl myristate (25-200mM) in DMSO

A sample size of 2 µl was used for GLC analysis. The GLC (Michro-9100, Netel chromatographs, was programmed for oven temperature 250 ºC, injector 260 ºC and FID temperature 270 ºC. The assay of methyl myristate was performed on 10% SE 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 methyl myristate

After the completion of esterification reaction at specified time intervals the reaction mixture was withdrawn (2 µl) and subjected to analysis of methyl myristate by esterification.

Effect of biocatalyst load

The immobilized matrix was washed twice, in 1 ml of DMSO (solvent) at room temperature. Thereafter the matrix was recovered by decantation of DMSO and used to catalyze the esterification of methyl myristate. The effect of bound lipase concentration on ester formation was evaluated by enhancing the concentration of bound lipase (5, 10, 15, 20, 25 and 30 mg) in the reaction mixture comprising 100: 100 (mM) methyl alcohol and myristic acid at 45 ºC.

Effect of relative proportion of reactant on methyl myristate synthesis

The effect of concentration and relative molar ratio of methyl alcohol and myristic acid on the synthesis of methyl myristate was determined by keeping the concentration of one of the reactants (methyl alcohol or myristic acid) at 100 mM and varying the concentration of second reactant (25-100mM) in a reaction volume of 1ml in DMSO. The esterification was carried out using matrix bound lipase (20 mg) at 45° in 10 ml plastic capped glass vial for 24 hour under continuous shaking. The methyl myristate formed in each of the combinations of the reactants was determined by GLC analysis.

Effect of reaction time for synthesis of methyl myristate

The reaction mixture 1ml contained 10 mg of bound lipase 100 mM final concentration of ethyl alcohol and myristic acid in DMSO in 10 ml plastic capped glass vial. The reaction mixture was incubated at 55ºC in an incubator under shaking conditions (120 rpm) up to 24 h. The reaction mixture was sampled (2 µl) in duplicate at an interval of 2 h and subjected to analysis by GLC for the formation of methyl myristate.

Effect of reaction temperature

Temperature for the esterification reaction was studied at 35, 45, 55, 65 and 75ºC for 16 hour in DMSO using 20 mg bound lipase. The methyl myristate formed in each case was determined by GLC analysis.

Effect of addition of molecular sieve on synthesis of methyl myristate

A molecular sieve was selected to study its effect on synthesis of methyl myristate by bound lipase. To the above reaction mixture prepared in DMSO varying amount (25-500 mg) of molecular sieve was added. The esterification was carried out in duplicate by adding 20 mg of bound lipase at 45 ºC with continuous shaking for 16 h. Methyl myristate synthesized in each case was determined by GLC.

Effect of salt ions on ester synthesis

Effects of salt ions were studied by pre-incubating metal ions at a concentration of 5 mM in 1 ml DMSO. Each of the salt ions (50 µl) was mixed with reaction mixture.

Reusability of bound lipase on esterification of methyl myristate

The formation of methyl myristate from myristic acid and ethyl alcohol (100 mM: 100mM) with bound lipase was assayed for 6 cycles of 16 h each. After each cycle of esterification, the bound lipase was washed twice for 5 min each in DMSO (1 ml) at room temperature. Thereafter, DMSO was decanted and matrix was reused for fresh cycle of ester synthesis under similar conditions.

Bioprocess development at the 50 ml level for the methyl myristate

Under optimized conditions, the 2 ml reaction volume was scaled up to a 50 ml reaction volume. The esterification was performed in a 250 ml capped flask at 55°C for 16 h under shaking and the ester synthesized was assayed by GC.

RESULTS

Effect of biocatalyst load

The esterification reaction with Methyl alcohol: Myristic acid (100 mM: 100 mM) in DMSO was performed (Fig. 3). The formation of ester remained more or less the same with an increase in concentration of matrix bound lipase under continuous shaking condition after 16 h at 45˚C. In the subsequent esterification reactions, 20 mg of hydrogel bound lipase was used for bio-catalysis.

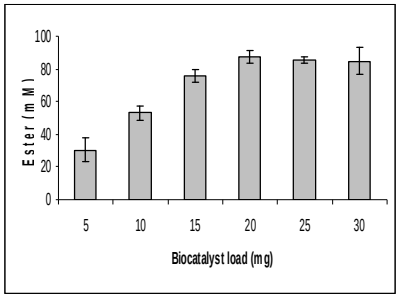


Fig. 3 Effect of biocatalyst load on ester synthesis

Effect of relative proportions of reactant on synthesis of methyl myristate

The formation of ester was highest when myristic acid and alcohol was used as 100:100 mM in DMSO. Under continuous shaking condition (120rpm) after 20 h at 45ºC (Fig.4). In the subsequent reactions, same concentration of reactant was employed. Amount of methyl myristate was estimated from a standard profile of pure methyl myristate.

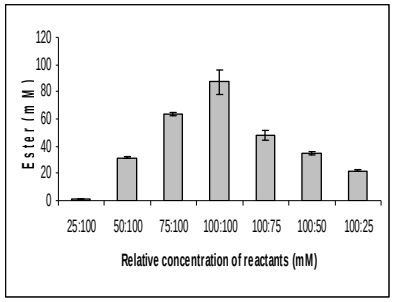


Fig. 4 Effect of relative proportion myristic acid and methanol

Effect of reaction time for synthesis of methyl myristate

The effect of reaction time on synthesis of methyl myristate using immobilized lipase was studied at a temperature of 45˚C in DMSO under shaking condition up to 20 h. The synthesis of the ester was time dependent and a maximum amount of methyl myristate (87.8 mM) was produced after 16 h of reaction (Fig.5). Thus in subsequent reaction a reaction time of 16 h was considered optimum to perform synthesis of methyl myristate using bound lipase.

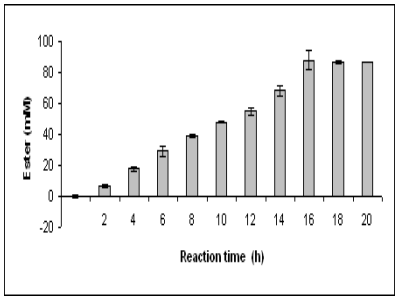


Fig. 5 Effect of reaction time on ester synthesis

Effect of reaction temperature for ester synthesis

Maximum synthesis (87.8 mM) of methyl myristate was obtained at 45˚C after 16 h (Fig. 6). At 70˚C, there was a marked decrease (41.3 mM) in the ester synthesis, which might be on account of denaturation of the lipase. At 75ºC there was no ester synthesis.

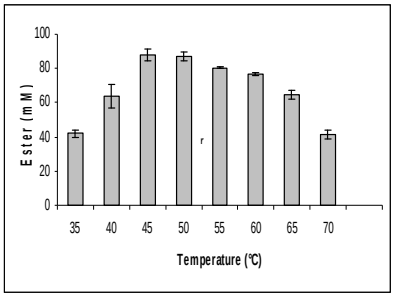


Fig. 6 Effect of reaction temperature on ester synthesis

Effect of addition of molecular sieve on esterification

When the effect of molecular sieve was studied by adding a molecular sieve (25 to 250 mg per reaction volume), a gradual decline (85.9 to 67.6 mM) in the amount of ester formed was noticed (Fig. 7). Thus addition of a molecular sieve had a deleterious effect on the esterification reaction at this present study.

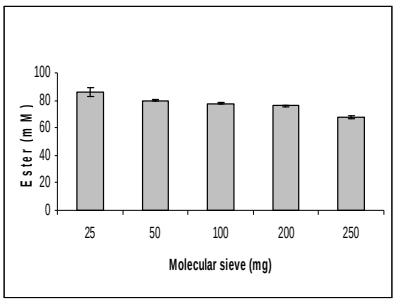


Fig. 7 Effect of molecular sieve on ester synthesis

Effect of metal ions on ester synthesis

Most of the metal ions had inhibitory effect on ester synthesis. The maximum inhibition was done by Li^+ (Fig.8).

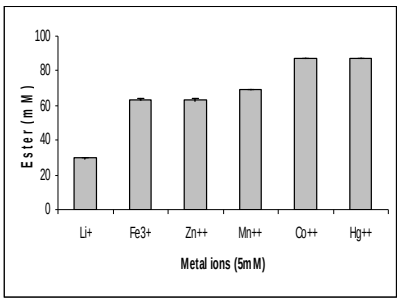


Fig. 8 Effect of metal ions on ester synthesis

Reusability of immobilized enzyme for ester synthesis

The bound lipase when repetitively used to perform esterification at 45°C under optimized conditions resulted in 48.8 mM methyl myristate after 6th cycle of esterification (Fig.9). In each cycle esterification was performed for 16 h.

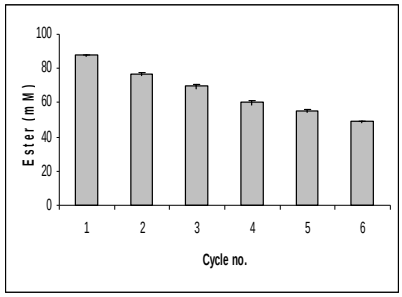


Fig. 9: Reusability of bound lipase

Bioprocess development at the 50 ml level for methyl myristate synthesis

Under optimized conditions, a 2 ml reaction volume was scaled up to the 50 ml Teflon-capped flask. When the reaction volume was increased from 2 to 50 ml the reaction time increased from 16 to 30 h and the conversion was 30.9 mM (Fig. 10).

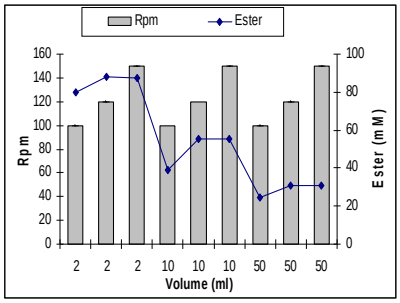


Fig. 10 Bioprocess development at the 50 ml level for the methyl myristate.

DISCUSSION

Lipases also referred as triacylglycerol hydrolases, are an important group of biotechnologically relevant enzymes that find immense applications in the food, dairy, detergent and pharmaceutical industries. Hydrolytic enzymes are widely used in organic synthesis as eco-friendly catalysts that possess broad substrate specificities, display high stereoselectivity, work under mild reaction conditions are commercially available and do not require the use of cofactors (Reetz, 2002). Lipases are successfully being used in organic chemical processing, detergent formulations, bio-surfactant synthesis, the oleo-chemical, dairy, agrochemical industries, paper manufacture, nutrition, cosmetics and pharmaceutical processing. This is mainly a result of the huge achievements made in the cloning and expression of enzymes from microorganisms, as well as of an increasing demand for these biocatalysts with novel and specific properties such as specificity, stability, pH, and temperature (Bornscheuer *et al.*, 2002; Menoncin *et al.*, 2008). However, smaller amounts of lipases are used in oleo-chemical transformations. Lipases can play an important role in the processing of g-linolenic acid, a polyunsaturated fatty acid (PUFA); astaxanthine, a food colorant; methyl ketones, flavor molecules characteristic of blue cheese; 4-hydroxydecanoic acid used as a precursor of g-decalactone, a fruit flavor; dicarboxylic acids for use as polymers; and inter-esterification of

cheaper glycerides to more valuable forms (Undurraga *et al.,* 2001). The ester of myristic acid appears to have flavoring properties as well cosmetic and therapeutic applications.

Owing of important applications of myristic acid derivatives in methyl myristate is used in the flavoring essence and the everyday use essence, and it is also used in the organic synthesis. Immobilization of commercially lipase was performed on easily available and waste thermocol matrix by simple physical adsorption in order to prevent mixing of product with enzyme and enhance reusability of biocatalyst. A variety of fatty acid esters are now being produced commercially using immobilized lipases in aqueous solvents that obviously require knowledge of optimal conditions to achieve hydrolysis in aqueous medium before the biocatalyst is exploited/ used in organic synthesis in water restricted medium or organic solvents (Pandey *et al.,* 1999; Verma *et al.,* 2008).

Immobilizations of lipase often improves stability of enzyme under reaction condition, enhance enzyme activity, make the repeated use of enzyme feasible, permit use of enzyme for diverse applications and thus lowers the production cost too (Guncheva *et al.,* 2009; Liu *et al.,* 2009). The obtained experimental data established that lipase was efficiently immobilized on thermocol matrix. In the present study, a commercial lipase (Steapsin) immobilized on waste thermocol showed a 27.9% binding efficiency.

The thermocol-bound lipase was optimally active at pH 8.5 and temperature over a wide range 25- 70°C. Most bacterial lipases act best at alkaline pH in nature and are thus promising catalysts for many industrial processes (Lee and Parkin 2001). In another study, *Bacillus cereus* MTCC 8372, lipase immobilized on a poly (methacrylic acid-co-dodecyl methacrylate-cl-N, N-methylene bisacrylamide) hydrogel optimally active at pH 8.5 and temperature 55°C (Kanwar *et al.,* 2004). Generally temperature above ambient promotes liquefaction of reactants and also tend make substrate more diffusible and hence easily acceptable to enzymes (Ahmed *et al.,* 2009). Above 60°C there is decrease in activity of bound enzyme, which might be on account of denaturation of lipase. Heat is likely to promote protein unfolding also thus leads to loss of enzyme activity. Often immobilized enzyme preparation is found to be more stable than soluble enzyme as seen in the present study using thermocol. The hydrogel-bound lipase of *P. aeruginosa* MTCC 4713 was highly hydrolytic towards longer carbon chain esters, *p*-NPP (Kanwar *et al.,* 2007).

Lipase the serine hydrolase and have high stability in organic solvent (s) system that keep the reactants dissolved and do not react with enzyme matrix or any other reactant and also did not evaporate at temperature of catalysis are very important in achieving efficient esterification. Esterification is generally water limited reaction, because the equilibrium catalyzed by the lipase is often in favor of hydrolysis (Halling *et al.,* 1984). Traditional extractions from plant material and direct biosynthesis by fermentation are two methods for flavour and fragrance production (Yadav *et al.,* 2003). However, the natural esters extracted from plant material often one either too scarce or too expensive for commercial use. For industrial purpose, flavors are usually produced by chemical synthesis that does not considered as natural products. Such esters may be considered as natural when produced by lipase-mediated synthesis (Abbas and Comeau, 2003) now a day, the synthesis of flavor compounds by biotechnological processes plays increasing role in food industry. Hence in present study methyl myristate was synthesized that may be used as a natural flavoring essence.

In the present study, efficacy of thermocol bound commercial lipase (Steapsin) to catalyze esterification of methanol and myristic acid in to methyl myristate in organic solvent DMSO was achieved efficiently under optimized conditions. Esterification of thermocol-bound immobilized alkaline lipase was enhanced when equimolar concentration of hydrophobic reactants were taken *i.e*. 100 mM: 100 mM (myristic acid: methyl alcohol) optimized for methyl myristate production in reaction mixture of 2 ml volume in 16 h at 45 °C. Further increased in temperature reduces the product and there were no synthesis at 75°C. It might be possible that reaction mixture might interfere with the diffusion of reactants or products at catalytic site of thermocol-bound enzyme at higher temperature. Enzyme concentration is known to influence esterification reaction. To establish the optimal amount of immobilized lipase, different quantities of immobilized lipase were used to study reaction kinetics of ester synthesis. An increase in biocatalyst concentration (20-30 mg/ml reaction volume) resulted in decrease in apparent enzyme activity in production of methyl myristate.

The esterification 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, addition of molecular sieves (25-300 mg) decreased the yield (87.5 to 67.5 mM). When, thermocol-bound lipase exposed to salt ions did not show any improvement in ester yield; and thus most of the tested metal ions had inhibitory effect on ester synthesis. Thus exposure to salt-ions had inhibitory effect on ester synthesis. Moreover, immobilized lipase had retained ~48.8% of native catalytic activity up to 6^{th} cycle in repeated batch reactions. Further, lipase gave ~31 mM yield in scaled up batch reaction of 50 ml. Thus, thermocol-bound lipase showed efficient esterification potential in d organic solvent/water free systems.

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