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IMMOBILIZATION OF LIPASE ON WASTE THERMOCOL AND ITS HYDROLYTIC PROPERTIES

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ABSTRACT

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). The effects of various physico-chemical parameters such as buffer pH, reaction temperature, surfactants, chelating agents, salt ions etc. on the hydrolytic activity of the bound lipase were studied consecutively. The bound lipase was also evaluated for its hydrolytic specificity toward various p-nitrophenyl esters of various C-chain lengths. The bound lipase showed optimal hydrolytic toward p-nitrophenol palmitate at a pH of 8.5 and a temperature of 45 $^{\circ}$C. The hydrolytic activity of thermocol-bound lipase was inhibited in the presence of salt-ions that included Li^{+}, Co^{2+}, Al^{3+}, Ca^{2+}, Hg^{2+} and Mn^{2+} ions at 5 mM.

1. INTRODUCTION

Lipases are a class of enzymes which catalyze the hydrolysis of long-chain triglycerides. They constitute a most important group of biocatalysts for biotechnological applications. Novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers, biodiesel, production of enantiopure pharmaceuticals, agro-chemicals and flavour/ fragrance compounds.[1,2] 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.[3] In the last few years, there has been an increasing interest in the use of enzymes for the biosynthesis of molecules in organic media.[4,5,6] Many lipase-catalyzed esterification or condensation reactions have been developed by employing a variety of lipases of microbial origin.[7,8,9,10,11] 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 de-sizing of denim and other cotton fabrics contain both alpha amylase and lipase enzymes. 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. 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.[12]

2. MATERIALS AND METHODS

2.1 Chemicals

Steapsin was purchased from RFCL Ltd., New Delhi (India). Polystyrene was obtained as waste packing materials from Department of Biotechnology, Himachal Pradesh University, Shimla, India. Various acyl esters such as p-nitrophenyl formate (p-NPF), p-nitrophenyl acetate (p-NPA), p-nitrophenyl caprylate (p-NPC), p-nitrophenyl laurate (p-NPL), p-nitrophenyl benzoate (p-NPB) and p-nitrophenyl palmitate (p-NPP) from Alfa-Aesar,

Heysham, Lancs., England. Gum acacia and Tris base were obtained from SRL Ltd., Mumbai, India. All the chemicals were of analytic grade and were used as received.

2.2 Optimization of reaction conditions for free and bound biocatalyst

2.2.1 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 in 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 sieved through 0.1 mm (diameter) mesh were washed with Tris buffer (pH 8.0: 0.5 mM) thrice and the material was dried thereof in oven at 60 $^{\circ}$C to achieve a constant weight.

2.2.2 Immobilization

The crude thermocol (2 g) was exposed to Steapsin (lipase) solution 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 and the bound protein content were calculated by subtracting the unbound protein/ lipase activity from the total protein/ lipase used during the immobilization onto thermocol beads. The protein was assayed by a standard method.[13]

2.2.3 Assay of lipase activity

The lipase activity was assayed by a colorimetric method[14] using p-NPP. The reaction mixture contained 80 µl of p-NPP stock-solution (20 mM p-NPP prepared in isopropanol), 20 mg (weight of polystyrene included) of test sample (lipase) and Tris buffer (0.05 M, pH 8.5) to make final volume to 3 ml. The reaction mixture was incubated at 45 $^{\circ}$C for 20 min in a water bath. The reaction was stopped by keeping the reaction mixture in -20 $^{\circ}$C for 7 min. The absorbance of p-nitrophenol released was measured at A_{410}. The unknown concentration of p-nitrophenol released was determined from a reference curve of p-nitrophenol (2-50 µg/ml final concentrations in 0.05 M Tris buffer, pH 8.5). Each of the assays was performed in triplicate, unless otherwise stated and mean values were recorded. One unit (U) of lipase activity was defined as micromole (s) of p-nitrophenol released per minute by hydrolysis of p-NPP by one ml soluble enzyme or 1 g thermocol-bound enzyme (weight of matrix included) at 45 $^{\circ}$C under assay conditions. Specific activity was expressed as µ mole (s) of the p-nitrophenol released per min by 1 mg of protein.

2.2.4 Effect of reaction temperature on free and bound lipase activity

The effect of temperature on the commercial lipase was studied by separately pre-incubating enzyme (0.2 ml) in an Eppendorf tube at the selected temperature (25 to 85 °C) in a water-bath for 20 min. Thereafter 5 µl free and 20 mg bound enzyme was sampled and assayed for residual lipase activity.

2.2.5 Effect of pH on free and bound lipase activity

Tris buffer (0.05 M) was adjusted separately to pH ranging 5.0 to 10.5. To the reaction mixture containing p-NPP and Tris buffer at each of the above pH, 5 µl free or 20 mg bound enzyme was added separately, and the reaction was carried out at 45 °C for 20 min. The heat inactivated lipase control was also included with each of the test sample.

2.2.6 Thermo-stability of free and bound lipase

The sensitivity of lipase to heat was evaluated by incubating free (0.2 ml) and thermocol-bound (20 mg) biocatalyst separately taken in Eppendorf tube at 55 °C in a water-bath for a period of 30 h. At intervals of 6 h, 5 µl free and 20 mg bound enzyme was sampled and assayed for residual lipase activity.

2.2.7 Substrate specificity of free and bound lipase

Either free lipase or thermocol-bound lipase was separately incubated with each of the p-nitrophenyl esters (*i.e.* p-NPF, p-NPA, p-NPC, p-NPL, p-NPB and p-NPP) for 20 min at 45 °C and the lipase activity was assayed thereafter.

2.2.8 Effect of salt ions on free and bound lipase

To evaluate the study of various metal ions on lipase activity, various salts (5 mM) were taken separately along with either free lipase or thermocol-bound lipase in the reaction mixture and the lipase activity was assayed after 20 min incubation at 45 °C.

2.2.9 Effect of chelating agent EDTA on hydrolytic activity of free and bound lipase

The effect of EDTA (50-200 mM in Tris buffer, pH 8.5) on lipase activity was evaluated. The mixture containing EDTA (50 µl) and either free lipase (50 µl) or thermocol-bound lipase (20 mg) was pre-incubated for 30 min at 37 °C. The p-NPP was added thereafter to initiate its hydrolysis at 45 °C by the EDTA-treated lipase free or thermocol-bound lipase. The reaction was stopped after 20 min and the residual lipase activity was assayed in each case at 45 °C thereof.

3. RESULTS

3.1 Optimization of assay condition for free and bound lipase activity

3.1.1 Immobilization

The immobilization of commercial enzyme on waste polystyrene matrix showed 28% binding and lipase activity of 0.16 U/mg when assayed using 20 mg of bound lipase (weight of matrix included). The corresponding protein content was 7 µg/mg (matrix), specific activity of free enzyme was 71.4 U/mg and thermocol-bound lipase was 32.8 U/mg protein matrix.

3.1.2 Effect of reaction temperature on free and bound lipase activity

The free as well as the thermocol-bound lipase preparations possessed optimal hydrolytic activities at 45 $^{\circ}$C and beyond this temperature the hydrolytic activities of both preparations decreases gradually up to 65 $^{\circ}$C. The enzyme activity was completely inhibited at 85 $^{\circ}$C (Table 1) both for free and thermocol-bound enzyme. Thus 45 $^{\circ}$C was chosen as optimum temperature to assay hydrolytic activity of free or thermocol-bound lipase in subsequent reactions.

3.1.3 Effect of pH on free and bound lipase activity

At pH 5.0, 7% relative activity was recorded for free enzyme, and 18% for thermocol-bound lipase, respectively. The maximum hydrolytic activity for both lipase preparations was found at pH 8.5 (100%). The relative activity at pH 10.5 was 60% for bound lipase as compared to 49% for free enzyme (Table 2). The thermocol-bound lipase was optimally active at pH 8.5 over a wide range of temperature (25-70 $^{\circ}$C).

3.1.4 Thermo-stability of free and bound lipase

In comparison to free enzyme the thermocol-bound enzyme showed greater residual hydrolytic activity (48%) than the free enzyme (9.8%) after 30 h of incubation (Table 3).

3.1.5 Substrate specificity of free and thermocol-bound lipase

The free as well as thermocol-bound enzyme exhibited highest affinity towards the p-NPP followed by p-NPC (Table 4). Both free and bound biocatalysts were highly specific toward p-NPP but showed variable specificities towards other esters. The hydrolytic activity of free lipase for hydrolysis of p-NPL was approximately 37% lower than p-NPP. Hydrolytic activity for p-NPF and p-NPB for matrix bound lipase was 31% lower than for p-NPP.

3.1.6 Effect of salt ions on hydrolytic activity of free and bound lipase

In present study, hydrolytic activity of thermocol-bound lipase was inhibited in the presence of salt-ions that included Li^{+}, Co^{2+}, Al^{3+}, Ca^{2+}, Hg^{2+} and Mn^{2+} ions in that order (Table 5).

3.1.7 Effect of chelating agent EDTA on free and bound lipase

It was observed that as the concentration of EDTA increased in the reaction cocktail, hydrolytic activities of both free as well as thermocol-bound lipase decreased drastically

(Table 6). The free enzyme retained a residual activity of 15% only, and for bound lipase the residual activity was 12%.

Table 1: Effect of reaction temperature on the hydrolytic activity of free and thermocol-bound lipase

	Free lipase		Bound lipase	
Temperature	Activity	Relative	Activity	Relative
(ºC)	(U/ml) ± SD*	activity (%)	(U/mg) ± SD*	activity (%)
25	0.34 ± 0.01	67	0.10 ± 0.01	60
30	0.37 ± 0.00	72	0.12 ± 0.01	73
35	0.39 ± 0.00	76	0.13 ± 0.00	79
40	0.45 ± 0.01	88	0.13 ± 0.00	79
45	0.51 ± 0.02	100	0.16 ± 0.01	100
50	0.50 ± 0.01	98	0.15 ± 0.00	91
55	0.47 ± 0.03	92	0.13 ± 0.00	79
60	0.44 ± 0.02	86	0.10 ± 0.00	60
65	0.38 ± 0.01	74	0.10 ± 0.00	60
70	0.20 ± 0.00	39	0.08 ± 0.01	48
75	0.11 ± 0.01	21	0.05 ± 0.02	30
80	0.03 ± 0.00	5.8	0.02 ± 0.01	12

SD* is standard deviation.

Table 2: Effect of pH on the hydrolytic activity of free and thermocol-bound lipase

	Free Lipase		Bound lipase	
pH	Activity (U/ml ± SD*)	Relative activity (%)	Activity (U/mg ± SD*)	Relative activity (%)
5.0	0.04 ± 0.00	7	0.03 ± 0.00	18
5.5	0.09 ± 0.00	17	0.04 ± 0.01	24
6.0	0.10 ± 0.01	19	0.06 ± 0.04	36
6.5	0.22 ± 0.02	43	0.07 ± 0.01	42
7.0	0.31 ± 0.01	60	0.09 ± 0.01	56
7.5	0.34 ± 0.01	67	0.11 ± 0.01	68
8.0	0.48 ± 0.00	94	0.12 ± 0.02	73
8.5	0.51 ± 0.00	100	0.16 ± 0.01	100
9.0	0.49 ± 0.01	96	0.13 ± 0.01	79
9.5	0.42 ± 0.00	82	0.12 ± 0.01	73
10.0	0.29 ± 0.00	56	0.11 ± 0.00	67
10.5	0.25 ± 0.01	49	0.10 ± 0.01	60

SD* is standard deviation.

Table 3: Thermo stability of free and thermocol-bound lipase

Free lipase			Bound lipase	
Time period (h) at 55°C)	Activity(U/ml ± SD*)	Relative activity (%)	Activity (U/mg± SD*)	Relative activity (%)
Control	**0.51 ± 0.01**	**100**	**0.16** ± 0.01	**100**
1	0.39 ± 0.00	76	0.15 ± 0.01	94
6	0.15 ± 0.00	29	0.12 ± 0.01	75
12	0.09 ± 0.01	17	0.11 ± 0.01	68
18	0.07 ± 0.02	13	0.10 ± 0.02	62
24	0.06 ± 0.01	11	0.09 ± 0.03	56
30	0.05 ± 0.01	9	0.08 ± 0.04	48

SD* is standard deviation.

Table 4: Effect of salt ions on the hydrolytic activity of free and thermocol-bound lipase

Free lipase			Bound lipase	
Salt ions (5 mM)	Activity(U/ml ± SD*)	Relative activity (%)	Activity (U/mg ±SD*)	Relative activity (%)
Control	0.50 ± 0.02	100	0.16 ± 0.010	100
$LiCl_3$	0.10 ± 0.002	19	0.09 ± 0.01	56
NaCl	0.21 ± 0.003	41	0.10 ± 0.002	62
KCl	0.20 ± 0.007	39	0.10 ± 0.003	62
$FeCl_3$	0.32 ± 0.002	62	0.16 ± 0	100
$ZnSO_4$	0.40 ± 0.003	78	0.7 ± 0.01	43
$MnCl_2$	0.35 ± 0.03	68	0.12 ± 0.002	75
$CoCl_2$	0.21 ± 0.05	41	0.007 ± 0.003	4.3
$HgCl_2$	0.24 ± 0.01	47	0.008 ± 0.01	5.0
$CuSO_4$	0.35 ± 0.04	68	0.15 ±0.003	93

SD* is standard deviation.

Table 5: Substrate specificity of free and thermocol-bound lipase

Free lipase			Bound lipase	
pNP-substrate (20 mM)	Activity (U/ml ± SD*)	Relative activity (%)	Activity (U/mg ±SD*)	Relative activity (%)
Formate	0.25 ± 0.02	49	0.05 ± 0.006	31
Acetate	0.21 ± 0.07	41	0.06 ± 0.002	37
Caprylate	0.28 ± 0.03	54	0.08 ± 0.003	50
Laurate	0.19 ± 0.05	37	0.09 ± 0.007	56
Palmitate	0.51 ± 0.01	100	0.16 ± 0.002	100
Benzoate	0.25 ± 0.04	49	0.05 ± 0.003	31

SD* is standard deviation.

Table 6: Effect of chelating agent EDTA on the hydrolytic activity of free and thermocol-bound lipase

	Free lipase		Bound lipase	
EDTA (mM)	Activity (U/ml ± SD*)	Relative activity (%)	Activity (U/mg ±SD*)	Relative activity (%)
0	0.51 ± 0.01	100	0.16 ± 0.01	100
50	0.25 ± 0.03	50	0.07 ± 0.007	43
100	0.12 ± 0.06	23	0.05 ± 0.002	31
150	0.09 ± 0.02	18	0.03 ± 0.003	18
200	0.08 ± 0.01	15	0.01± 0.001	6

SD* is standard deviation.

4. 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.[15] 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.[16,17] 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.[18,19]

Immobilization of commercially lipase was performed on an easily available and waste thermocol matrix by simple physical adsorption in order to prevent mixing of product with enzyme and enhance reusability of biocatalyst. The thermocol-bound lipase was optimized for various physiochemical parameters like temperature, pH, substrate specificity, and salt-ions etc. to achieve hydrolysis in aqueous medium. 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.[20,21] The obtained experimental data established that lipase was efficiently immobilized onto thermocol matrix. Various features of reaction selectivity of lipases are modulated by exogenous factors[22] such as choice of substrate, pH, temperature, pH, salt-ions etc. Most bacterial lipases can generally act in a wide range of pH and temperature.[23] An extracellular lipase isolated from a *Bacillus sp.*[24] possessed an optimum activity at 50 °C, with half-life of 15 min at 75°C; and its stability was observed even in presence of oxidizing/reducing agents, surfactants, chelating agents and certain organic solvents. The present study also showed that the immobilization of lipase on thermocol matrix promoted its thermo-stability that was higher for the bound catalyst than free form.

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.[25,26] 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. Early in a study, *Bacillus sp.* lipase was stable in pH range of 7.0-10.0 with optimum pH 8.0. Generally temperature above ambient promotes liquefaction of reactants and also tend make substrate more diffusible and hence easily acceptable to enzymes.[27] 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. When immobilization of lipase from *B. thermocatenulatus* was done on hydrophobic support, the bound lipase maintains 100% of its original activity at 65°C. The hydrogel-bound lipase of *P. aeruginosa* MTCC 4713 was highly hydrolytic towards longer carbon chain esters, p-NPP.[28] In another study, a lipase from psychrotrophic *Pseudomonas sp.* displayed highest activity towards carbon: 10 acyl groups of p-nitrophenol esters.[29] Immobilized lipase from *Bacillus coagulans* BTS-1 was found to be more hydrolytic towards medium carbon chain length esters. In present study, free and thermocol-bound lipase was also highly hydrolytic towards p-NPP.

Salt ions enhance the catalytic activity of some enzymes.[30] In present study, hydrolytic activity of thermocol-bound lipase was inhibited in the presence of salt-ions that included Li^{+}, $Co2^{+}$, $Al^{3,+}$ Ca^{2+}, $Hg2^{+}$ and Mn^{2+} ions. Structure elucidation of lipase has shown that catalytic site of most lipases resembled that of serine protease.[31] Presences of ionizable salts

of metals as well as non-metals are quite likely to cause structural changes at catalytic site of immobilized or free lipase. However exposure to Ca^{2+} ion to an extracellular lipase of *Pseudomonas fluorescence* 2D was reported to effect an ~360% increase in lipase activity, but presence of Hg^{+2} and Co^{+2} strongly inhibited lipase activity.[32] However Hg^{+2}, Al^{+3}, Mn^{+2} and Co^{+2} exerted drastic decline in lipase activity of *Rhizopus oryzae.*[33] Lipase from *Pseudomonas fluorescens* RB02-3 was resistant to most metal ions except Mn^{2+} and Fe^{3+}. Cd^{2+} had no effect on enzyme activity, while Zn^{2+}, Co^{2+}, Cu^{2+} and Ni^{2+} salts did not show remarkable inhibitory effect on lipase activity.[34] Several workers have reported that metal ions like Zn^{2+} and Cu^{2+} have inhibitory effects on *Pseudomonas* lipases.[35,36]

Unlike most other bacterial lipases, the *Bacillus coagulans* MTCC-6375 lipase is a metallo-enzyme as its activity was inhibited by EDTA as well as PMSF.[37] On other hand PMSF and EDTA did not affect lipase activity of *Mucor hiemalis f. hiemalis*[38] and lipase of non-microbial origin.[39] In an earlier study *Acinetobacter bayly* lipase was completely inhibited by EDTA, SDS and Triton X-100. The complete inhibition by EDTA implies the involvement of metal ions at its active site believed to be Ca^{2+} located at the substrate binding pocket and stabilizes the structure of lipase.[40] In the present study, the hydrolytic activities of both free as well as the thermocol bound-lipase were drastically antagonized by EDTA.

The study showed that a cheap, easily available waste material such as thermocol could be used successfully for creating a thermostable and alkaline-pH stable matrix-bound lipase to achieve efficient hydrolysis of the target substrate. However, the amenability of thermocol-bound lipase in organic synthesis is yet to be evaluated in experimental studies.

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