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Research Article

PHYSICAL ADSORPTION OF LIPASE ONTO MESOPOROUS SILICA

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A B S T R A C T

Lipases are versatile enzymes that catalyze the hydrolysis of the ester bonds of lipids. For this study commercial enzyme Lipolase 100L was studied as a source of lipase. Two silica preparations, varying in mesh size ($Silica_{70-230}$ and $Silica_{230-400}$), were used for immobilisation of Lipolase 100L (1:10 = lipase: tris buffer). It was observed that activity assay gave the highest values when treated with 2% of guteraldehyde. 9 mM *p*- NPP gave the best results as suitable substrate. The enthalpy of activation for silica immobilized lipase decreased with an increase in temperature whereas K_m and V_{max} was analysed on the Lineweaver Burk Plot, the values were observed for S_{70-230} lipolase (K_m = 66.67 mM ,V_{max} = 3.34 U/mg/min., k_{cat} =35.22 s^{-1} and specificity constant =10.56 $s^{-1}Mm^{-1}$), $S_{230-400}$ lipolase (K_m = 52.64 mM , of V_{max} is 2.32 U/mg/min., k_{cat} =27.80 s^{-1} and specificity constant =12.00 $s^{-1}Mm^{-1}$) and pure lipolase enzyme (K_m value as 62.5mM , V_{max} 2.1875 U/mg/min , k_{cat} =33.02 s^{-1} and specificity constant =15.09 $s^{-1}Mm^{-1}$).The thermostability of silica immobilized showed that the half-life ($t_{1/2}$) of immobilized lipase was approximately 30 minutes at 55^0C. Among chelating and denaturing agents like SDS, EDTA, mercaptoethanol and PEG affected the activity by lowering its value too. The lipase immobilizes silica is reusable upto 5 cycles during which it retained it activity upto 50%.

INTRODUCTION

A lipase is an enzyme that hydrolyzes the fats like triglycerides into the fatty acid and glycerol molecules. Among biocatalysts microbial lipases have capability to catalyze a variety of reactions in aqueous and non-aqueous media (Rifaat *et al.,* 2010). Lipases are found in the plants, animals and microbes. However, the microbial lipases find immense industrial applications. Lipases are one of the most important groups of biotechnologically relevant enzymes used for application in manufacture of a variety of foods, fine chemicals, detergents, wastewater treatment, cosmetics, paper and pulp, pharmaceuticals and leather industry (Bose, 2014). The reason for the vast biotechnological potential of microbial lipases includes that they are; stable in organic solvents, do not require cofactors, possess a broad substrate specificity (Chandrasekaran and Bhartiya, 2009, Sharma *et al*., 2016, Sood *et al*., 2016) and also exhibit a high enantio-selectivity (Holt and Hanefeld, 2009).

Enzyme stability is important in a variety of commercial and industrial applications due to harsh conditions than in the laboratory assays. These drawbacks of the free enzymes are overcome through immobilization technique (Sharma *et al*., 2014, Sharma *et al*., 2016, Sood *et al*., 2016).

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their use even under harsh environmental conditions of pH, organic solvents and temperature There are many techniques used for immobilization of the enzymes onto supports which include simple adsorption, covalent attachment, entrapment, crosslinking, surface modification, etc (Tran and Balkus, 2011). The immobilization process can be affected by selection of the optimum support material because its nature, reactive group and interaction with enzymes can affect the enzyme activity. This technique makes use of enzymes in industries more attractive because it offers certain processing advantages over free enzyme that include ease of separation from the reactant and product, improved stability, and continuous operation. The porous nature of the hydrogel and particulate nature of silica or celite allow the solvent and reactants as well as the product(s) to diffuse freely; this enables the substrate to interact with the enzyme easily (Harun *et al.,* 2004). Generally silica has been used as a support due to its thermal, mechanical and chemical stability, biocompatibility, inertness, high hydrophobicity and microbial attacks resistant (Hartmann *et al.,* 2013).

METHODOLOGY

Chemicals and Source of Lipolase 100 L

All the chemicals were of analytic grade and were used as received.The enzyme used in the study named Lipolase 100 L is a commercial lipase enzyme obtained from a strain of bacteria namely *Thermomyces langinosus* and supplied by

Novoenzyme A/S (Bagsvaerd, Denmark) as a gift for use in the present study.

Lipase assay

Lipase activity was assayed in the culture broth by the method of Winkler and Stuckmann, 1979 by measuring the micromoles of *p*-nitrophenol released from *p*-nitrophenyl palmitate. One unit (U) of lipase activity was defined as amount of enzyme required to release one micromole of *p*NP from the substrate (*p*NPP) per minute by one mL or one gram of the immobilized enzyme (including the weight of the matrix) prepared under standard assay conditions. Protein estimation was done by the method of (Bradford, 1976).

Immobilization of lipase on silica matrix

In a glass beaker, 10 g of silica was taken to which 50 mL of Tris buffer (0.05 M, pH 8.5) was poured and incubated for 24 hours at 8˚C to check the swelling capacity. After a day of incubation 2 g of silica was taken to which 5 mL of lipase enzyme (1:10 = Lipolase:tris buffer) was loaded and incubated for 24 hours at 8˚C. Further, 20 mL of Gluteraldehyde (2 %) was added as a cross linker before incubating for 24 hours at 8˚C. The immobilized protein in matrices was determined by subtracting unbound protein in the supernatant from the total protein used for immobilization as well as increased/decreased total activity was calculated by adding total activity of supernatant and matrix in comparison to total enzyme units in 5 mL of purified lipase incubated earlier. The matrix that gave higher activity and bound protein was used for further studies.

Effect of different percentage of Gluteraldehyde on the bound lipase activity

In this study different percentage concentration of Gluteraldehyde (1, 2, 3, and 4 %) was checked to find the maximum binding capacity of lipolase on the given matrices for a particular percentage of Gluteraldehyde.

Effect of different p- nitrophenyl acyl esters (substrates) on silica-immobilized lipase

To study the substrate specificity of the silica-immobilized lipase, different chromogenic substrates namely *p*NPA, *p*NPB, *p*NPL, *p*NPP and *p*NPS (9 mM each) were used. Each of the above chromogens was prepared by taking 0.04 g in 10mL of *iso*-propanol. The reaction was performed using 40 mg of silica-immobilized lipase and Tris buffer (0.05 M) of pH 8.5 at 55ºC for 10 min.

Effect of reaction temperature on silica-immobilized lipase

To study the effect of reaction temperature, enzyme activity was assayed at selected each of the reaction temperatures (35, 45, 55, 65, and 75ºC) with 9 mM substrate (*p*NPP). The reaction was performed using 40 mg of silica-immobilized lipase and Tris buffer (0.05 M) of pH 8.5 for 10 min.

Kinetic study of silica-immobilized lipase

The K_m, $V_{max,}$ K_{cat} and specificity constant of the immobilized lipase(s) was determined by measuring the reaction velocities at the different concentration of the *p*NPP *i.e* 2-20 mM, the reciprocal of the reaction velocity was plotted against the reciprocal of the substrate concentration to determine the K_m and V_{max} value by Lineweaver-Burke plot (Lineweaver and Burke, 1934). The reaction was performed using 40 mg of immobilized enzyme and Tris buffer (0.05 M) of pH 8.5 at 55ºC for 10 min. Activation energy (Ea) was determined from Arrhenius plot (Ghori *et al.,* 2011, Sharma *et al*., 2016).

Thermostability of silica-immobilized lipase

To examine the effect of temperature on stability of the immobilized enzyme, enzyme immobilized silica was kept separately in test tubes for different intervals (0, 10, 20 and 30 minutes) at 55^{o}C. The activity measured immediately before incubation was defined as 100% of hydrolytic activity. The reaction was performed using 40 mg of silica-immobilized enzyme, *p*NPP and Tris buffer (0.05 M) of pH 8.5 at 55ºC.

Effect of denaturing/ chelating agents on silica-immobilized lipase

To study the effect of denaturing/ chelating agents on lipase, each of the selected compounds (SDS, EDTA, DTT, mercaptoethanol and PEG; 1 % (v/v) each) were included separately in reaction mixture. The reaction was performed using 40 mg of silica-immobilized enzyme and Tris buffer (0.05 M) of pH 8.5 at 55ºC for 10 min.

Effect of organic solvents on silica-immobilized lipase

The silica-immobilized lipase was assayed in the presence of different 1% (v/v) organic solvents *viz* methanol, ethanol, propane-1-ol, *iso*-propanol, butane-1-ol, butane-2-ol, pentyl alcohol, hexane-1-ol, octanol, decanol, phenol, *p*-xylene, acetonitrile, DMSO, *n*-pentane, *n*-hexane, *n*-heptane, *n*-octane and *n*-nonane. The reaction was performed using 40 mg of silica-immobilized lipase and Tris buffer (0.05 M) of pH 8.5 at 55ºC for 10 min.

Reusability of silica-immobilized lipase

The silica-immobilized enzyme was used for 5 repeated cycles and the reaction was performed using 40 mg of silica-immobilized lipase and Tris buffer (0.05 M) of pH 8.5 at 55ºC for 10 min. The silica bound biocatalyst was recovered after each cycle and was used to catalyze a fresh reaction.

RESULTS

Immobilization of lipase on silica

Two silica preparations, varying in mesh size ($Silica_{70-230}$ and $Silica_{230-400}$), were used for immobilisation of S1, S2 Lipolase 100L (1:10 = lipase: tris buffer). In a glass beaker 10 g of silica was taken to which 50 mL of tris buffer (0.05 M, pH 8.5) was poured and incubated for 24 hours at 8˚C to check the swelling capacity. 2 g of swell silica was taken to which 5 mL of lipase enzyme (1:10 = Lipolase: tris buffer) was loaded and incubated for 24 hours at 8˚C. Gluteraldehyde (2 %) was loaded as a cross linker and incubated for 24 hours at 8˚C before washing it with Tris buffer (Fig. 1).

Fig 1 Silica-bound lipase cross linked with glutaraldehyde.

Effect of different percentage of Gluteraldehyde on silica-immobilized lipase

To examine the effect of different percentage of Gluteraldehyde (0%, 1%, 2%, 3% and 4%), 40 mg of immobilized silica was taken (equivalent to 40 µL of enzyme put in activity assay) along with 9 mM of *p*NPP and Tris buffer (0.05 M) of pH 8.5 at 55ºC for 10 min, lipase showed maximum activity at the use of 2% of Gluteraldehyde ($S_{70\text{-}230}$ = 73.0 U/mg and $S_{230\text{-}400}$ = 52.6 U/mg).

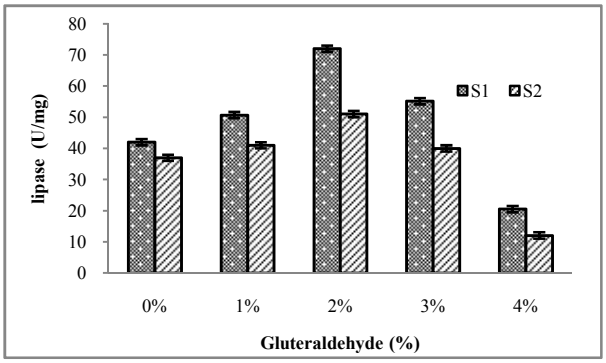

Fig 2 Effect of different percentage of Gluteraldehyde on silica-immobilized lipase

Effect of different p- nitrophenyl acyl esters (substrates) on silica-immobilized lipase

To examine the effect of selected *p*-nitrophenyl acyl esters varying in their C-chain length *i.e. p*-nitrophenyl acetate, *p*-nitrophenyl benzoate, *p*-nitrophenyl larute, *p*- nitrophenyl palmitate and *p*-nitrophenyl stearate, each of these chromogens were separately included in the reaction mixture containing 40 mg of immobilized silica (equivalent to 40 µL of enzyme put in activity assay along with Tris buffer (0.05 M) of pH 8.5 at 55ºC for 10 min. Both the silica-immobilized lipase preperation showed maximum activity towards *p*- nitrophenyl palmitate ($S_{70\text{-}230}$ = 72.0 U/mg and $S_{230\text{-}400}$ = 52.1 U/mg) (Fig. 3).

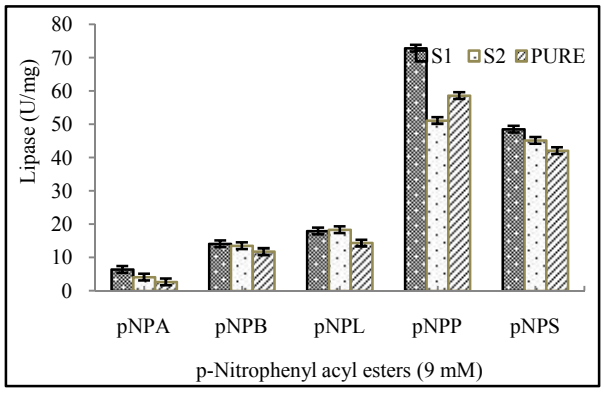

Fig. 3 Effect of different *p*-nitrophenyl acyl esters (9 mM) on silica-immobilized lipase

Effect of reaction temperature on silica-immobilized lipase

To examine the effect of reaction temperature on the hydrolytic activity of lipase, 40 mg of immobilized silica was taken (equivalent to 40 µL of enzyme put in activity assay) along with 9 mM of *p*NPP and Tris buffer (0.05 M) of pH 8.5 for 10 min was incubated separately in the reaction mixture(s) at 35, 45, 55, 65 and 75, kept under shaking (130 rpm). For immobilized lipase, the maximum activity ($S_{70\text{-}230}$= 72.9 U/mg and $S_{230\text{-}400}$= 52.0 U/mg) was recorded at 55±1°C (Fig. 4.6). A further increase in temperature resulted in a gradual decrease in the lipase activity (Fig.4).

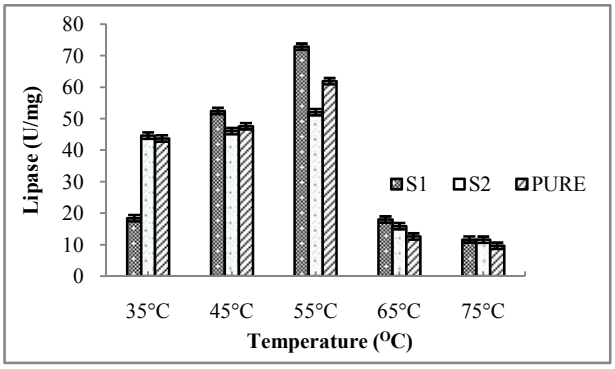

Fig 4 Effect of reaction temperature on silica-immobilized enzyme

Kinetic study of silica-immobilized lipase

The rate of reaction V_{max} and K_m using the best colorimetric substrate *p*NPP were studied by; 40 mg of immobilized silica was taken (equivalent to 40 µL of enzyme put in activity assay) along with 9 mM of *p*NPP and Tris buffer (0.05 M) of pH 8.5 at 55ºC for 10 min 1-20 mM of *p*NPP concentration (Fig.8) in 0.05 M Tris-buffer (pH 8.5) under shaking at 55± 1°C. A Lineweaver-Burk plot was calibrated to determine K_m and V_{max} values of immobilized lipase which is in (Fig. 5). Energy of activation (E_a) for lipase was found to be 2.75 $KJmol^{-1}$.

Table 1 Kinetic study analysis of silica-immobilized lipase

Biocatalysts used	V_{max} (U/mg/min)	K_m (mM)	K_{cat} (s^{-1})	Specificity constant ($s^{-1}mM^{-1}$)
$S_{70\text{-}230}$ lipolase	66.67	3.34	35.22	10.56
$S_{230\text{-}400}$lipolase	52.63	2.31	27.80	12.00
Pure lipolase	62.50	2.18	33.02	15.09

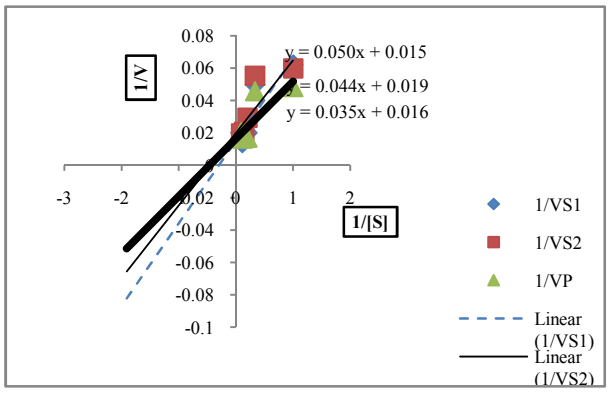

Fig 5 Lineweaver Burk Plot of silica bound lipase using

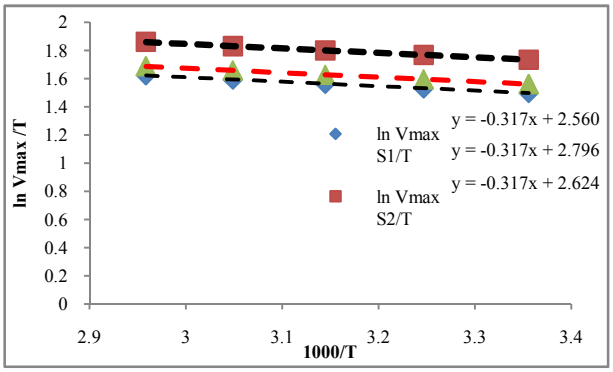

Fig 6 Arrhenius plot for determination of energy of activation of silica-immobilized lipase

Thermostability of silica-immobilized lipase at 55°C

The thermostability of silica-immobilized lipase was studied by when 40 mg of immobilized silica was taken (equivalent to 40 µL of enzyme put in activity assay) along with 9 mM of *p*NPP and Tris buffer (0.05 M) of pH 8.5 at 55ºC for 3 h. After 30 minutes, 50% decrease in the activity of purified along with immobilized lipase was recorded (S_{70-230} having 31.4 U/mg, $S_{230-400}$ having 32.7 U/mg and of pure enzyme was 32.7 U/mg) and thus the half-life ($t_{1/2}$) of immobilized lipase was approximately 30 minutes at 55^0C.

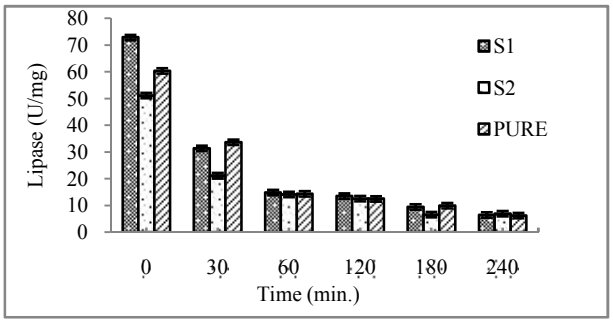

Fig 7 Thermostability of silica-immobilized lipase

Effect of denaturing/ chelating agents on silica-immobilized lipase

To study the effect of PEG, DTT, EDTA and Mercaptoethanol each on lipase activity, these were pre-incubated separately at 1% (w/v) concentration with the enzyme (n=3) at 55± 1°C for 10 min (Fig. 8). The residual lipase activity was assayed thereafter and relative activity in each case was calculated. The mercaptoethanol gave an activity result of silica bound lipase (S_{70-230} = 51.103 U/mg, $S_{230-400}$ = 51.07 U/mg).

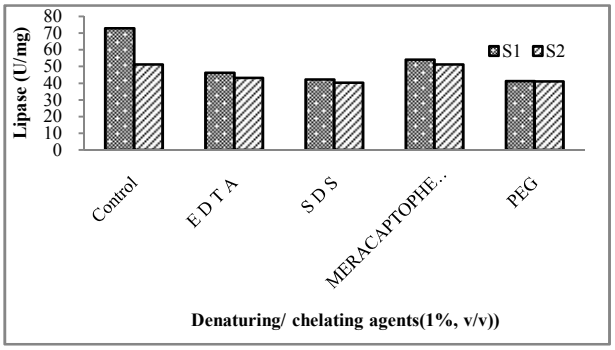

Fig. 8 Effect of denaturating and chelating agents on silica-immobilized lipase

Effect of organic solvents on silica-immobilized lipase

To study the effect of organic solvents, methanol, ethanol, propane-1-ol, butane-1-ol, butane-2-ol, pentyl alcohol, hexane-1-ol, octanol, decanol, phenol, p-xylene, acetonitrile, DMSO, pentane, hexane, heptane, octane, nonane and iso-propane, on silica-immobilized lipase, each of the selected organic solvents was pre-incubated with silica bound lipase (40 mg) at 55± 1°C for 10 min. Lipase activity was assayed at 55± 1°C and all showed toxicity towards silica bound lipase. Decanol, DMSO, *n*-nonane and *n*-octane strongly inhibited the activity of immobilized lipase. (Table 2).

Reusability of silica-immobilized lipase

The immobilized enzyme was used for 5 repetitive cycles with ~50% loss in activity after 4th cycle (Fig.9).

Table 2 Effect of organic solvents (2% v/v) on silica-immobilized lipase

Organic solvents	*logP* value	Activity (U/mg)	Relative activity (%)	Activity (U/mg)	Relative activity (%)
		Silica $_{230-400}$		Silica$_{70-230}$	
Control	-	72.90	100	51.19	100
Methanol	-0.69	12.22	16.73	17.84	34.98
Ethanol	-0.58	10.64	14.58	11.51	22.56
Propan-1-ol	0.28	58.99	80.80	55.39	108.6
Propan-2-ol	-0.19	36.68	50.24	23.39	45.13
Butan-1-ol	0.61	51.36	70.35	48.19	94.49
Butan-2-ol	0.68	44.60	61.09	29.63	58.09
Pentyl alcohol	1.20	25.46	34.87	42.01	82.37
Hexan-1-ol	1.80	13.20	18.08	25.61	50.21
Octanol	2.91	11.36	15.56	20.57	40.33
Decanol	-	4.46	6.10	1.87	3.66
Phenol	-	33.23	45.52	28.63	56.13
p-Xylene	3.1	48.91	66.99	46.76	91.68
Benzene	2.13	36.40	49.86	39.13	76.72
Acetonitrile	-0.33	27.19	37.24	32.94	64.58
DMSO	-1.22	25.03	34.28	30.50	59.80
Pentane	3.25	9.20	72.60	6.04	11.84
Hexane	3.76	30.21	41.38	23.30	45.68
Heptane	4.27	34.09	46.69	27.62	54.15
Octane	4.78	8.92	12.21	9.78	19.17
Nonane	5.29	3.02	4.13	4.17	8.17

The increased reusability, higher pH and storage stability of immobilized enzyme as compared to the free enzyme would be important for its sustained use and economic viability of biosynthetic processes.

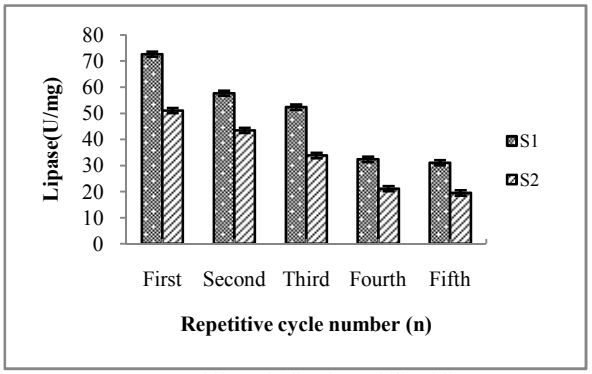

Fig 9 Reusability of silica-immobilized lipase

DISCUSSION

Lipases have drawn much attention for their potential use in bioprocess, mainly due to their availability and stability in both, aqueous and organic media (*Lanka and Lavanya Latha* 2015). The interest in microbial lipase production has increased in the last decades, because of its large potential in a wide range of industrial applications as additives in food processing (flavor modification), fine chemicals (synthesis of esters), detergents (hydrolysis of fats), waste water treatment (decomposition and removal of oil substances), diagnostics, cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fats in foods), leather (removal of lipids from animal skin) and medical assays (blood triglyceride assay). The lipase after immobilization onto silica was exposed to glutaraldehyde that acts as a cross-linking agent and is effective against dilution induced dissociation of enzyme (Zhang *et al.,* 2004). Lipase-immobilized by surface adsorption onto silica when pre-treated with 1% glutaraldehyde showed 89% binding of protein (Chandel *et al.,* 2015), as compared to 2% glutaraldehyde which showed

96% bnding in Silica$_{70-230}$ and 80% Silica$_{230-400}$ observed in this study.

When lipase activity was determined with the use of *p*NPP, *p*NPL and *p*NPB as substrates, a unit of activity (U*p*NP) was expressed as the quantity of enzyme preparation that released 1 mM of p-nitrophenol per 1 min in the reaction conditions. Standard assays were performed at 30°C, using *p*NPP as the substrate (Krakowiak *et al*., 2003). Whereas, in this study selected *p*-nitrophenyl acyl esters varying in their C-chain length *i.e. p*NPA, *p*NPB, *p*-NPP, *p*NPL and *p*NPS. Both the silica immobilized lipase preperation showed maximum activity towards *p*NPP (S_{70-230} = 72.0 U/mg and $S_{230-400}$ = 52.1 U/mg) when taken at a concentration of 9mM.

During the 1st, 2nd, 3rd, 4th, and 5th cycles of loading of silica with the enzyme, the protein-binding on the silica achieved 51.73 %, 48.27 %, 26.92 %, 10.73 %, and 4.29 %, respectively (Kumar *et al.,* 2013). The immobilized enzyme was used for 5 repeated cycles with ~50% loss in activity after 4th cycle when observed in this study.

Concluding remarks

In this work, the use of a simple approach to employ a cheap silica support to achieve stable binding of a commercial lipase by cyclic adsorption of lipase followed by glutaraldehyde cross-linking was successfully demonstrated. Thus, after immobilisation and optimisation of immobilisation paraneters it was observed that there was a significant increase in the activity of the pure enzyme Lipolase 100 L.

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Conflict of interest

The authors declare no conflict of interests publishing this article in this journal.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

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