e-ISSN:2347-7857
p-ISSN:2347-7849

Research & Reviews: Journal of Pharmaceutics and Nanotechnology

Synthesis of an Antioxidant n-propyl Gallate by Magnetic Iron Nanoparticles-bound Lipolase

Surabhi Mehra[1], Ashok Kumar[2], Shamsher S Kanwar[1]*

[1]Department of Biotechnology, Himachal Pradesh University, Shimla, 171 005, India

[2]Lab of Enzyme Molecular Engineering, State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China

Review Article

Received date: 01/03/2016
Accepted date: 29/07/2016
Published date: 03/08/2016

***For Correspondence**

Shamsher S Kanwar, Department of Biotechnology, Himachal Pradesh University, Shimla, 171 005, India

E-mail: kanwarss2000@yahoo.com

Keywords: Magnetite iron nano-particles, Immobilization, Lipolase 100L, Enzyme stability, Esterification, n-propyl gallate.

ABSTRACT

In the present study, iron based magnetic nano-particles (NPs) were synthesized by a chemical co-precipitation method. The NPs were modified with tetraethoxysilane (TEOS) to prepare silane-coated nano-particles for covalent immobilization of *Thermomyces lanuginosus* derived Lipolase 100L. Lipolase/ protein bound to NPs retained about ~85% of its initial activity and reached saturation at 6.1 mg/mg NPs with binding efficiency of 97.4%. The nano-biocatalyst was capable of working at a broader temperature (40˚C to 70˚C) range. It exhibited enhanced activity in the presence of alkanes *i.e*. *n*-heptane, *n*-hexane and *n*-nonane. Lipolase bound to NPs retained ~99% of its initial hydrolytic activity at 55˚C after 10th cycle, which indicated the enormous stability attained by nano-biocatalyst. The nano-biocatalyst successfully catalyzed the conversion of *n*-propanol and gallic acid to *n*-propyl gallate. At equimolar (500 mM) concentartion of *n*-propanol and gallic acid, the maximum synthesis *n*-propyl gallate in 14 h at 55˚C with a conversion of ~81.4% was achieved by the use of NPs-bound Lipolase 100L.

INTRODUCTION

A variety of nano-carriers, nano-particles, nano-fibers, nano-tubes *etc.* have displayed greater competence to mimic nano-scale environment for the enzyme as compared to micro- and macro-environments provided by traditional support carriers that have been commonly employed for enzyme immobilization. The porous structured beads (silica, alginate *etc.*), membranes and fibers (>100 µm) have some inherent drawbacks that include lower stability, enzyme leaching and poor dispersibility. Among various types of nano-particles; the iron based magnetic nano-particles have been used for enzyme immobilization due to their non-toxicity and biocompatibility. Magnetic behavior of iron nano-particles results in the simple and speedy recovery of nano-biocatalyst from reaction mixture and reusability of biocatalyst. Surface modification of magnetic NPs is an important issue that needs consideration prior to immobilization of enzyme onto NPs. Functionalization of NPs with silane is a prerequisite for covalent attachment of enzyme onto modified nano-particles, which prevents enzyme leaching and results in enhanced stability and longer shelf life of enzyme. Monomeric stabilizers, inorganic materials like silica [1,2], gold [3] or gadolinium (III) [4,5] and polymer stabilizers like dextran, carboxymethylated dextran, carboxydextran, starch, arabinogalactan, glycosaminoglycan, sulfonated styrene-divinylbenzene, polyethylene glycol, polyvinyl alcohol, poloxamers, and polyoxamines [6,7] for carrying out surface modification and functionalization of nano-carriers have been reported. Lipases (EC 3.1.1.1) occupy a place of prominence among biocatalysts in a rapidly growing biotechnological and pharmaceutical market owing to their novel and multiple applications in synthetic chemistry.

Besides bacteria, yeast and fungi are also good sources for lipase production. The lipase hydrolyzes preferentially long chain of fatty acids [8,9] besides other reactions like esterification, amidation, alcoholysis and aminolysis. However, to achieve biocatalysis at industrial scale, it is essential to employ a stable and cheaper biocatalyst, which is non-toxic, stable, easily separable/ recoverable and could be used repetitively in aqueous or organic media. The Lipolase 100L sourced from a thermophilic fungus *Thermomyces langinosus* was chosen for immobilization on to iron magnetic NPs because of its inherent thermostability (up to

60°C), alkalophilic nature (up to pH 9) and its known potential as an industrial biocatalyst [10]. The *n*-popyl gallate (E310), a propyl ester of gallic acid is a well-known antioxidant used in foods containing oils and fats, food packaging materials, non-food items like hair products, cosmetics, lubricants *etc.* Propyl gallate is considered a safe antioxidant to protect oil & fat-containing foods from rancidity and spoilage by preventing the formation of peroxides. The biological effects of *n*-propyl gallate include antimicrobial activity, ultraviolet (UV) radiation protection, chemoprotection, antimutagenesis, antitumorigenesis, antiteratogenesis and anticarcinogenesis (Safety assessment sheet of propyl gallate, 2007). In the present study, Fe_3O_4/ γFe_2O_3 nano-particles were prepared, characterized for enzymatic activity and subsequently employed to achieve esterification of *n*-propanol and gallic acid to synthesize *n*-propyl gallate in an organic medium.

EXPERIMENTAL

Materials and methods

The chemicals that included $FeCl_3.6H_2O$, NaCl, K_2HPO_4 and KCl were purchased from S.D. Fine-Chem. Ltd., Hyderabad, India; tetraethoxy silane (TEOS), $FeCl_2.4H_2O$, Bovine Serum Albumin (BSA), *p*-nitrophenyl palmitate (*p*-NPP) and *p*-nitrophenol (*p*-NP) were purchased from Alfa Aesar, Manchaster, England. NaH_2PO_4 and methanol were purchased from HIMEDIA Laboratory Ltd., Mumbai, India. Ammonia solution, ethanol, *n*-hexane, *n*-heptane, *n*-nonane, cyclohexane, DMSO, *n*-propanol, *iso*-propanol, *iso*-butanol, and Tris buffer were purchased from Merck Darmstadt, Germany. All the chemicals used in the present study were of high analytical grade. A commercial Lipolase 100L of *Thermomyces lanuginosus* was supplied as a gift by Novozymes A/S (Bagsvaerd, Denmark). The Lipolase 100L contained 8,202 U/ml lipase activity and 505.6 mg protein/ml representing a specific enzyme activity of 16.2 U/mg protein.

Synthesis of iron magnetic nano-particles

Co-precipitation [11] a widely used method for synthesis of magnetic nano-particles (Fe_3O_4 or γFe_2O_3) is based on stoichiometric molar ratio of ferrous and ferric salts in the mixture and may be represented by following chemical reaction;

$$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + H_2O$$

At a molar ratio of 2:1 (Fe^{3+}/Fe^{2+}), pH 8 to 14 and in the presence of non-oxidizing environment, the precipitate of Fe_3O_4 was obtained [12]. However, Fe_3O_4 (magnetite) form is unstable as it is readily oxidized to γFe_2O_3 (maghemite) form in the presence of oxygen.

$$Fe_3O_4 + 2H^+ \rightarrow \gamma Fe_2O_3 + Fe^{2+} + H_2O$$

The aqueous solutions of ferric (III) chloride hexahydrate ($FeCl_3.6H_2O$) (0.6M) and ferrous (II) chloride tetrahydrate ($FeCl_2.4H_2O$) (0.3M) were taken in molar ratio 2:1. Aqueous ammonia was drop-wise added to the mixture with continuous stirring and passage of N_2 in the reaction cocktail. The reaction was performed at a pH 10-12. Thereafter, the solution was stirred at 40˚C for 30 min, 150 rpm followed by heating at 80˚C for another 30 min under shaking. The magnetic NPs were retained followed by extensive washing with distilled water. The NPs were completely dried by freeze drying.

Surface modification of magnetic NPs

In brief, the NPs (Fe_3O_4 or γ Fe_2O_3) were set in the form of a suspension by dropping 1.5 g of NPs in 150 ml of ethanol. The suspension was ultrasonicated (35% amplitude for 20 min at room temperature). Deoxygenated water (1 ml) and 750 µl of TEOS were added to above NPs suspension. The suspension was stirred for 12 h at 37˚C and NPs were collected by using a magnet, The NPs were ethanol washed to remove traces of unbound silane. These NPs dried in a rotary-evaporator were stored under N_2 to get finer particles with reduced particle size [13].

Binding of lipolase on silane-coated NPs

A commercial Lipolase 100L (Novozymes, Denmark) was covalently immobilized on to silane-coated modified magnetic NPs. For lipase immobilization [14], 30 mg of modified NPs were suspended in sodium phosphate buffered saline (PBS, pH 7.4) and the suspension was sonicated for 5 min at room temperature. Lipase-PBS solution (5 ml of certain concentration) was added to it in the presence of N_2 and suspension was kept under shaking for 24 h at 37˚C for efficient immobilization of lipase onto magnetic NPs. Lipase-immobilized NPs were separated by a magnet and supernatant was collected. These NPs were given several PBS washing and were finally dispersed into PBS for further use.

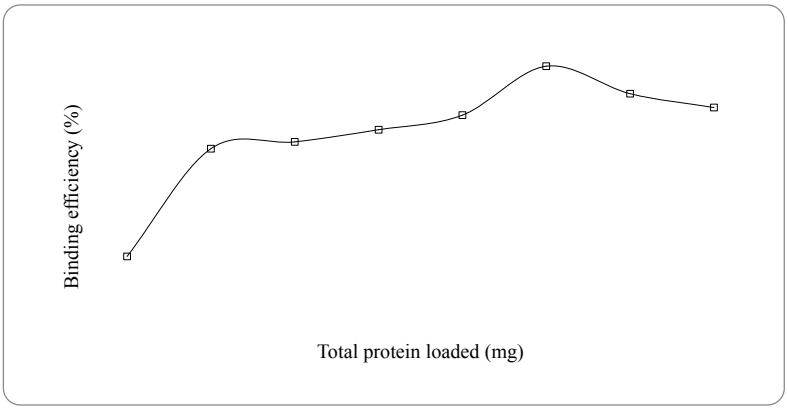
Lipase activity assay

Lipase activity of supernatant and immobilized biocatalyst was measured by a previously reported method [15] using *p*-NPP as a substrate. The absorbance of *p*-nitrophenol released during the reaction was measured at 410 nm. One unit (U) of Lipolase activity was defined as µmol(s) of *p*-nitrophenol released per minute by hydrolysis of *p*-NPP by 1 ml of soluble enzyme or 1 mg of immobilized biocatalyst (weight of NPs included) at 45°C under standard assay conditions.

Loading capacity of NPs for lipolase and determination of protein binding efficiency

To investigate the total loading capacity and protein binding efficiency of magnetic NPs, various dilutions of Lipolase in PBS

(pH 7.4) were prepared **(Figure 1).** The 5 ml of each of the Lipolase-PBS stock (dilution) was loaded onto 5 ml of NPs suspension (30 mg NPs in 5 ml PBS) and at 37°C, overnight with gentle stirring. Thereafter, supernatant from each of the sample(s) was recovered and used to determine protein binding on NPs, which was calculated by subtracting the total protein used for binding from that of total protein recovered in the supernatant [16].



The appropriately diluted Lipolase-PBS stock mixed with 5 ml of NPs suspension (30 mg NPs in 5 ml PBS pH 7.4) was kept at 37°C overnight under gentle stirring. The supernatant from each of the preparations was recovered and assayed to determine protein binding on NPs, which was calculated by subtracting the amount of protein recovered in the supernatant from the total protein used for binding on to NPs.

Figure 1. Amount of Lipolase immobilized onto magnetic iron nano-particles.

Characterization of synthesized and NPs-bound lipolase

The size and morphology of synthesized and NPs-bound Lipolase were observed by Scanning Electron Microscope (SEM) with an accelerating voltage of 15 kV.

Thermo-tolerance of NPs-bound lipolase

To determine the maximum activity of NP-Lipolase at different temperatures, NPs-bound Lipolase was assayed at temperature range 40-70˚C in separate reaction mixtures after 10 min incubation under shaking. The obtained enzyme activities were recorded thereafter.

Effect of different solvents on NPs-bound lipolase

Ideally, biocatalyst must be able to work in different organic solvents in order to carry out various synthetic reactions effectively. NPs-bound Lipolase was pre-exposed to different pure solvents *i.e. n*-heptane, *n*-nonane, cyclohexane, DMSO, *iso*-propanol and *iso*-butanol at 45˚C for 10 min. The NPs-bound biocatalyst was washed with 1X PBS (pH 7.4) and checked for lipase activity.

Reusability of NPs-bound lipolase

Reusability and recovery become very important parameters when we use immobilized enzymes. Magnetic NPs have turned out as an excellent lipase carriers resulting in stability of enzyme molecules. Reusability of the biocatalyst was examined by recycling NPs-bound Lipolase up to 10 cycles of hydrolysis of *p*-NPP conducted at 45˚C for 10 min, each. After each cycle, NPs-bound Lipolase was separated using magnet and was given thrice PBS (pH 7.4) washes to prepare it for fresh cycle of enzymatic assay with *p*-NPP.

Enzymatic esterification of n-propanol and gallic acid

Esterification reaction involving the synthesis of propyl gallate in solvent-mediated system was carried out by varying the molar ratios of *n*-propanol/ gallic acid (100-500 mM) catalyzed by 50 µl homogeneous suspension of NPs-bound Lipolase. Final reaction volume was made to 2 ml by DMSO taken in Teflon-capped glass-vial (5 ml capacity). The reaction was performed at 55°C for 14 h along with respective controls (with no enzyme). Samples were analyzed by High performance liquid chromatography (HPLC) for presence of *n*-propyl gallate.

HPLC analysis

A C18 column (250 × 4.6 mm, 5 m; Intersil, GL Sciences Inc) was employed to determine the amount of *n*-propyl gallate synthesized in the reaction cocktail by the action of NPs-bound Lipolase. The sample size was 20 µl and all assays were done in duplicate and mean values were presented. An isocratic mobile phase comprising formic acid (0.1%, v/v) and acetonitrile (80%, v/v) taken in ratio 50: 50 was used at a flow rate of 1 ml/min. A reference curve was calibrated with *n*-propyl gallate (0.1 to 0.5 mM).

RESULT

The results of the various experiments are presented in the following sections

e-ISSN:2347-7857
p-ISSN:2347-7849

Loading capacity of lipolase and determination of protein binding efficiency on magnetite nano-particles

Different dilutions of Lipolase (conc. <5%, ~ 41.5 mg/ml) with PBS (1X, pH 7.4) were prepared to determine the loading capacity and protein binding efficiency of Lipolase onto magnetite nano-particles. The loading of enzyme on to NPs reached a saturated amount of about 6.1 mg Lipolase per milligram magnetite nanoparticles with 97.4% binding efficiency **(Figure 1).** However, it was found that with any further increase in the loaded protein, % binding of Lipolase onto nano-particles thereby decreased.

Characterization of lipolase bound NPs

The size and morphology of the synthesized nano-particles and NPs bound Lipolase were observed using SEM **(Figure 2).** The average size of distinct particles was found to be in the range of 30-90 nm. The SEM images of NPs-bound Lipolase revealed that the resulting nano-biocatalyst comprises of agglomerated structures of NPs-Lipolase.

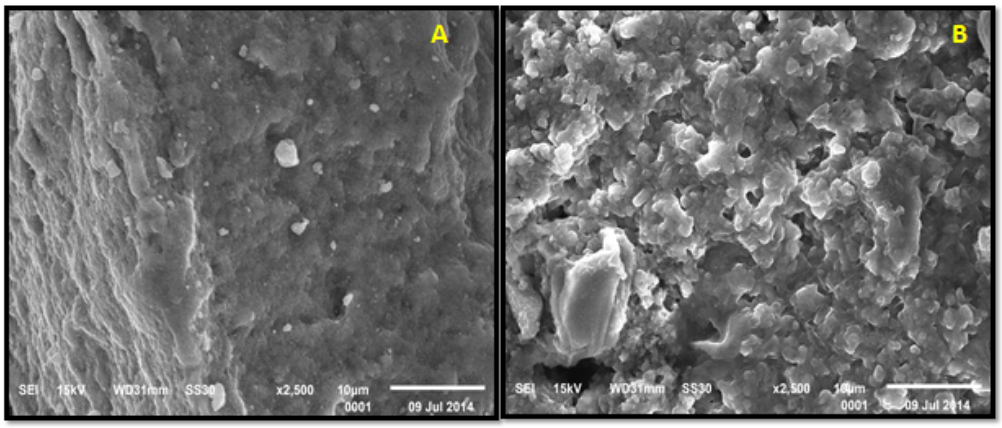
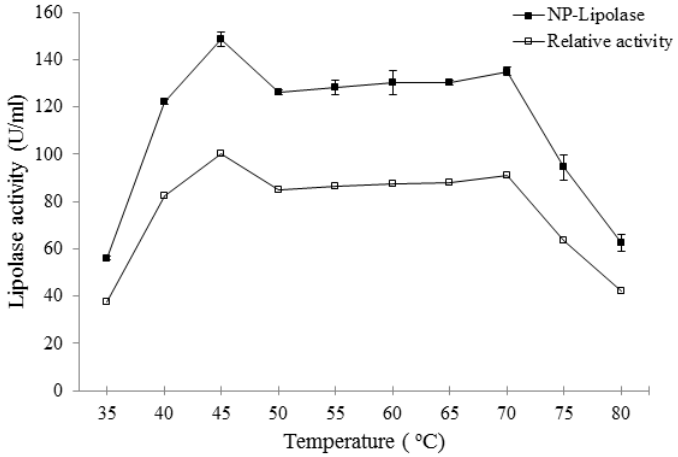


Figure 2. Surface morphology of (A) naked Fe_3O_4/ γFe_2O_3 nano-particles; (B) Lipolase bound to NPs [Magnification *X 8,500*], respectively.

Thermo-tolerance of NPs-bound lipolase

The effect of temperature and thermos-tolerance on the NP-immobilized Lipolase was evaluated **(Figure 3).** The results showed that maximum NP-Lipolase activity was obtained at temperature 45˚C (148 U/ml). However, it was found that NPs-bound lipase was active even at temperature of 70˚C (135 U/ml) and thus the NPs-bound biocatalyst was capable of working at broader temperature range *i.e.* 40 to 70˚C.



The NPs-bound Lipolase was incubated at 40 to 70˚C in separate reaction mixtures for 10 min under shaking and the residual lipase activity was assayed thereafter.

Figure 3. Thermo-tolerance of NPs-bound Lipolase.

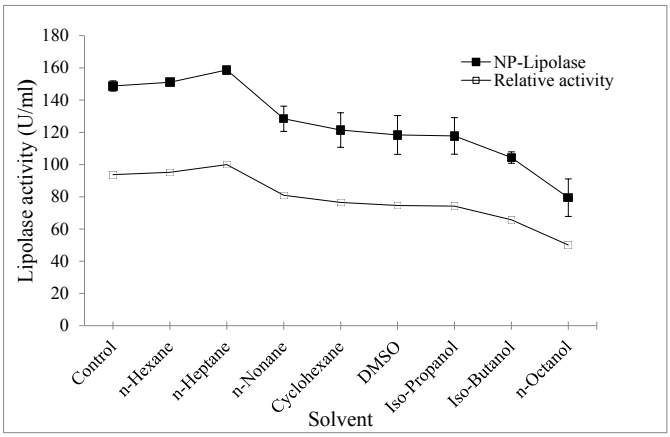
Effect of different solvents on the activity of NPs-bound lipolase

NPs-bound Lipolase showed greater activity in the presence of alkanes as compared to alcohols when used as solvents. The highest activity of the NPs-bound biocatalyst was found after exposure to *n*-heptane (158.7 U/ml) followed by *n*-hexane (151.1 U/ml), *n*-nonane (128.5 U/ml) and cyclohexane (121.4 U/ml). It was observed that with an increasing C-chain length of alcohols, the hydrolytic activity of NPs-bound biocatalyst decreased **(Figure 4).**

Reusability of NPs-bound biocatalyst

The hydrolytic activity of NPs-bound Lipolase more or less (~99% of original Lipolase activity) was retained up to 10 cycles of repetitive hydrolysis **(Figure 5).** Moreover, the NPs-bound biocatalyst was easy to wash and retained by magnetic attraction.

e-ISSN:2347-7857
p-ISSN:2347-7849



NPs-bound Lipolase was pre-exposed to each of the pure solvents at 45˚C for 10 min. The biocatalyst washed with PBS (pH 7.4) after each use was assayed for residual lipase activity.

Figure 4. Effect of various organic solvents on hydrolytic activity of NPs-bound Lipolase.

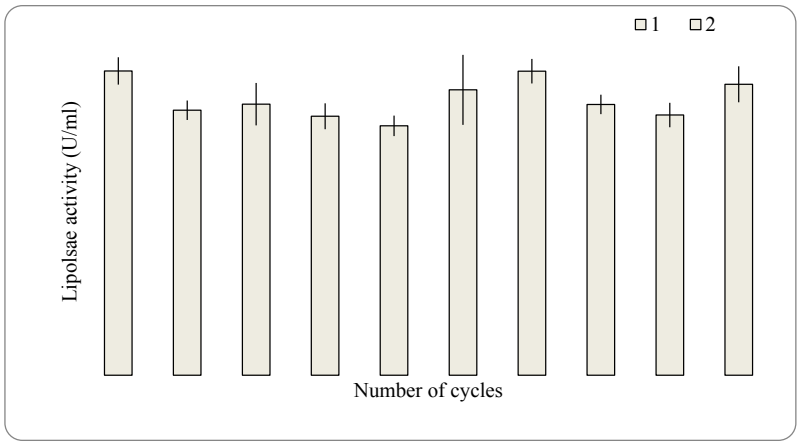


Figure 5. Reusability of NPs-bound Lipolase in continuous cycles of hydrolysis of *p*-nitrophenyl palmitate.

Synthesis of n-propyl gallate by using NP-bound lipolase

The ability of NPs-bound Lipolase to carry out esterification reaction was tested by HPLC analysis of the reaction products. The results **(Table 1)** showed the presence of *n*-propyl gallate in the reaction mixture. In order to determine optimum ratio of reactants to obtain the maximum yield of product, different concentration of reactants were prepared by varying the concentration of one reactant at a time and keeping the other constant, making the final volume of reaction mix to 2 ml with DMSO. At equimolar (500 mM) concentartion of *n*-propanol and gallic acid, the maximum synthesis *n*-propyl gallate with a conversion of ~81.4% was achieved. On the other hand, an excess of either *n*-propanol or gallic acid reduced the formation of *n*-propyl gallate in the reaction system. It seems that excess of acid or alcohol might be responsible for denauration of protein/ enzyme ledaing to a decline in the catalytic activity of the NP-bound Lipolase.

Table 1. Synthesis of *n*-propyl gallate at different molar concentrations of reactants.

Propanol: Gallic acid (mM: mM)	n-Propyl gallate (mM)
500: 100	57.37
500: 200	165.08
500: 300	265.65
500: 400	366.43
500: 500	406.98
400: 500	367.10
300: 500	201.69
200: 500	185.14
100: 500	76.96

The reaction mixture containing varying ratio of *n*-propanol and gallic acid in DMSO was incubated at 55˚C for 14 h in the presence of the NPs-bound biocatalyst.

DISCUSSION

Recent advances in nano-technology have led to the development of various nano-structured materials which for

immobilization of enzymes and biocatalysts. Surface chemistry [17] and different physiochemical properties like surface area to volume ratios, aquaphilicity, hydrophobicity, particle size-shape, topography and chemical reactivity coupled with inherent chemical, electrical and magnetic properties of the nano-particles make them suitable host for immobilization of some industrially important enzymes [18]. In the light beneficial properties of nano-particles, successful attempt was made to synthesize the magnetic nano-particles of iron that efficiently bound to commercial enzyme Lipolase, the NPs possessed remarkable stability and reusability [19,20]. In an another experiment with immobilization of *Candida rugosa* lipase on alkyl silane coated Fe_3O_4 nano-particles, amount of immobilized lipase was found to be about 0.20 mg lipase per mg of nanoparticles [14]. In the present study, the NPs-bound enzyme reached a saturated amount of about 6.06 mg Lipolase per milligram magnetite nano-particles with 97.4% binding efficiency, which was noticeably higher than previous reports. A decrease of protein % binding that was observed while increasing the protein loading is quite likely on account of the saturation of binding of lipase/ protein molecules on to surface of NPs. Further, it might also be because of obscuring of enzyme molecules placed beneath the top layer of enzyme molecules or stearic hindrance of substrate molecules to the enzyme catalytic site(s).

A *Mucor javanicus* lipase, which was effectively immobilized on silica nanoparticles [21] showed optimized pH at 9.0 with working range 8 to 10 as compared to free enzyme optimized at 8.0 pH. Immobilized lipase onto the EDA-GA or EDA-NCS activated nano-particles at 60°C retained 62% or 68% to the initial activity after 120 min incubation, respectively. In an another study, [22] a bacterial lipase from *Arthrobacter* sp. (ABL: IIIM Jammu, India strain, MTCC No. 5125) immobilized on non-magnetic (Type A) and magnetic (Type B) supports derived from copolymerization of 3-aminopropyltriethoxysilane and TEOS showed enhanced stability at pH 5-9 retaining 65-95% activity after 48 h incubation as compared to free enzyme, which was not stable under these conditions [23]. The non-magnetic and magnetic sol-gel composites retained 60-70% activity after 1 h incubation at 70˚C. However, in the current work, NPs-bound Lipolase was capable working in much broader pH and temperature range, with pH 6.5 to 9.5 with good activity at extremes pH as well and showed temperature tolerance in the range 40˚C to 70˚C. Moreover, NPs-bound Lipolase retained almost 80-85% of its initial activity even after 28 h of incubation at 70˚C much higher than previous reports [14,22,24]. Reusability and recovery of immobilized enzyme are amongst greatly desired and important parameters that have to be considered in order to ensure efficient and effective immobilization. The immobilized lipase on alkyl trimethoxysilane-coated Fe_3O_4 nano-particles (C18-Fe_3O_4) retained 65% of its initial activity after 7 cycles [14]. The decrease of activity was attributed to possible denaturation or leaching (detachment) of lipase molecules from nano-particles. In case of porcine pancreas lipase covalently immobilized on the surface of silica-coated modified magnetite nanoparticles, immobilized lipase retained 63.5% of its initial activity after 6 cycles [24]. However, NP-Lipolase showed enormous stability and repetitive use. It was active and retained 99% of its activity even after 10th cycle. This confirmed that the lipase bound to silane-coated NPs are highly stable and overcomes enzyme-leaching problem reported by earlier workers.

DMSO is miscible with water and has the property to dissolve the enzyme, which invariably results in the inactivation of protein of interest [25]. The tolerance of NPs-bound Lipolase in different solvents suggested its likely use in organic synthesis, enantioselectivity and associated applications. The NPs-bound Lipolase showed enhanced activity in case of alkanes and DMSO. It showed the highest activity towards *p*-NPP (C16 acyl group) among various tested in the presence of *n*-heptane. When the NPs-bound Lipolase was employed for the synthesis of an ester namely '*n*-propyl gallate' the maximum synthesis of *n*-propyl gallate in DMSO with a conversion of ~81.4% could be achieved. However, an excess of either *n*-propanol or gallic acid reduced the formation of *n*-propyl gallate in the reaction system possiblly because of denauration of protein/ enzyme ledaing to a decline in the catalytic activity of the iron magnetic NPs-bound Lipolase.

CONCLUSION

Magnetite/maghemite nanoparticles (Fe_3O_4/ γFe_2O_3) synthesized by using $FeCl_3$ and $FeCl_2$ in a molar ratio 2:1 were modified with TEOS. A commercial lipase from *Thermomyces lanuginosus i.e.* 'Lipolase 100L' was used for immobilization. Nano-biocatalyst was highly stable in broader temperature range and showed increased activity in the presence of alkanes. NP-Lipolase was highly stable and enzyme-leaching problem as reported previously was resolved. NPs-bound Lipolase remained almost active over 10 cycles of hydrolysis and retained ~99% of its initial hydrolytic activity. *n*-Propyl gallate was effectively synthesized by NPs-bound Lipolase at 55˚C. An equimolar (500 mM) reaction mixture of *n*-propanol and gallic acid in DMSO gave the maximum yield of the product. The study showed remarkable stability and reusability of NPs-bound biocatalyst not only in aqueous and organic solvents but also employed successfully the NPs-bould Lipolase for the synthesis of *n*-propyl gallate with a conversion rate of 81.4% in a reasonably short period of time indicating suitability of NPs-bound Lipolase for achieving biocatalysis in an organic solvent system. *n*-Propyl gallate is an antioxidant commonly used in foods containing oils & fats, food packaging materials, non-food items like hair products, cosmetics, lubricants *etc.*

ACKNOWLEDGEMENT

The financial support from Department of Biotechnology, Ministry of Science and Technology, Government of India to Department of Biotechnology, Himachal Pradesh University, Shimla (India) is thankfully acknowledged. Moreover, the authors do not have any conflict of interest at their place of work.

e-ISSN:2347-7857
p-ISSN:2347-7849

REFERENCES

1. Tartaj P, et al. Langmuir. 2002; 18:4556-4458.
2. Zhang C, et al. Silica- and alkoxysilane-coated ultrasmall superparamagnetic iron oxide particles: a promising tool to label cells for magnetic resonance imaging. Langmuir. 2007; 23:1427-1434.
3. Chen M, et al. Gold-coated iron nanoparticles for biomedical applications. J Appl Phys. 2003; 93:7551-7553.
4. Xu HK, et al. J Mater Res. 1992; 77:12-716.
5. Morawski AM, et al. Targeted nanoparticles for quantitative imaging of sparse molecular epitopes with MRI. Magnet Resonan Med. 2004; 51:480-486.
6. Lacava LM, et al. Magnetic resonance of a dextran-coated magnetic fluid intravenously administered in mice. Biophys J. 2001; 80:2483-2486.
7. Zhang Y, et al. Surface modification of superparamagnetic magnetite nanoparticles and their intracellular uptake. Biomaterials. 2002; 23: 1553-1561.
8. Arpigny JL and Jaeger KE. Bacterial lipolytic enzymes: classification and properties. Biochem J. 1999; 343: 177-183.
9. Fojan P, et al. What distinguishes an esterase from a lipase: a novel structural approach. Biochimie 2000; 82: 1033-1041.
10. Fernandez-Lafuente R, J Mol Catal B Enzymatic. 2010; 62: 197-212.
11. Can K, et al. Immobilization of albumin on aminosilane modified superparamagnetic magnetite nanoparticles and its characterization. Colloids Surf B. 2009; 71: 154-159.
12. Jolivet JP, et al. Iron oxide chemistry. From molecular clusters to extended solid networks. Chem Commun 2004; 5: 481-487.
13. Gupta AK and Wells S. Surface-modified superparamagnetic nanoparticles for drug delivery: preparation, characterization, and cytotoxicity studies. IEEE Trans Nanobiosci. 2004; 3: 66-73.
14. Wang J, et al. Immobilization of lipases on alkyl silane modified magnetic nanoparticles: effect of alkyl chain length on enzyme activity. PLoS ONE. 2012; 7: e43478.
15. Winkler UK and Stuckmann M. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by Serratia marcescens. J Bacteriol 1979; 138: 663-670.
16. Lowry OH, et al. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.
17. Larsen G, et al. A Method for Making Inorganic and Hybrid (Organic/Inorganic) Fibers and Vesicles with Diameters in the Submicrometer and Micrometer Range via Sol−Gel Chemistry and Electrically Forced Liquid Jets. J Am Chem Soc 2003; 125: 1154-1155.
18. Kim K, et al. Nanobiocatalysis and its potential applications. Trends Biotechnol 2008; 26: 639-646.
19. Vaidya BK, et al. Immobilization of Candida rugosa lipase on poly (allyl glycidyl ether-co-ethylene glycol dimethacrylate) macroporous polymer particles. Bioresour Technol 2008; 99: 3623-3629.
20. Wu Y, et al. In situ preparation of magnetic Fe_3O_4-chitosan nanoparticles for lipase immobilization by cross-linking and oxidation in aqueous solution. Bioresour Technol 2009; 100: 3459-3464.
21. Kim MI, et al. J Mol Catal B Enzym. 2006; 39: 62–68.
22. Chaubey A, et al. Process Biochem. 2009; 44: 154-160.
23. Chaubey A, et al. Arthrobacter sp. lipase immobilization for improvement in stability and enantioselectivity. Appl Microbiol Biotechnol 2006; 273: 598-606.
24. Ranjbakhsh E, et al. Enhancement of stability and catalytic activity of immobilized lipase on silica-coated modified magnetite nanoparticles. Chem Eng J 2012; 179: 272-276.
25. Hun CJ, et al. A newly isolated organic solvent tolerant Bacillus sphaericus 205y producing organic solvent-stable lipase. J Biochem Eng 2003; 15: 147-151.