International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Functionalization of Metal and Metal Oxide Nanoparticles for Enzyme Immobilization

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Abstract
The enzymes lipase and urease were immobilized on inorganic nanoparticles by the crosslinking method using glutaraldehyde. The nanoparticles were purchased commercially and used without any further modifications. Lipase activity was measured by titrimetric method whereas Urease activity was calculated using Nessler's reagent method, in free as well as immobilized state. Activity as a function of pH, temperature, storage stability, reusability and K_m was analyzed and their results were compared. The optimum pH range of free lipase, 7.2 to 7.4 showed an increase in the range from 7 to 8 when immobilized. Similarly, in thermal regulation optimum temperature range for free lipase was increased on immobilization. The immobilized lipase showed better storage stability as compared to free lipase, which was maximum for lipase on zinc oxide nanoparticles. A similar trend was observed in the activities of immobilized urease. The results clearly depict the beneficial effects of immobilization. Further, it revealed zinc oxide nanoparticles to be the best support material, followed by silver nanoparticles and lastly aluminium oxide nanoparticles. Kinetic studies were performed to confirm these outcomes.

Keywords: Enzyme immobilization; inorganic nanoparticles; optimization; reusability; enzyme kinetics.

1. Introduction

Immobilization of enzymes has been carried out on various supports of different shapes and sizes. Amongst all, nanoparticles have been the best support materials owing to their large surface area to volume ratio as they provide minimum diffusional limitation and highly effective enzyme loading. Nanoparticles as supports are generally of two types: polymeric NPs and inorganic NPs. Polymeric nanoparticles include calcium alginate [1], chitosan [2], agar, k-carrageenan, agarose, etc. Inorganic nanoparticles have the tendency to increase the enzyme stability [3]. Literature studies show that different metal and metal oxide nanoparticles have been employed for immobilizing enzymes. These supports are ZnO, TiO_2, Fe_3O_4, NiO, SnO_2, Silica, Ag, C, Au, Pt and $CaCO_3$. Khan et al. [4] concluded in their article that metal oxide nanoparticles such as iron oxide, zinc oxide and titanium dioxide serve as excellent support materials for enzyme immobilization. Murugappan G and Sreeram KJ successfully demonstrated that copper oxide nanoparticles can act as a dual-functional support for co-immobilization of multiple enzymes [5]. Hybrid nanocomposites have also been used as supports *viz*. iron oxide and carrageenan composites [6], silica and chitosan nanocomposites [7], ZnO and Polyvinyl alcohol hybrid nanofilm [8], N_2 and diamond nanowires [9], iron oxide and chitosan hybrid [10], silica and alginate nanogel [11], platinum and alginate composites [12], chitin and alginate fibers [13], etc. In recent years, single enzyme nanoparticles are being fabricated to modulate and stabilize the immobilized enzymes [14].

Present study deals with the immobilization of lipase and urease on zinc oxide, aluminium oxide and silver nanoparticles. These NPs have innumerable properties and characteristics [15]. Zinc oxide is a semiconductor, generally insoluble in water and alcohol but can dissolve in acids such as dilute hydrochloric acid. It is used in many fields such as paints and cosmetics industries, plastic and rubber manufacturing, electronics and pharmaceuticals [16, 17]. Recently, zinc oxide nanoparticles have been employed in enzyme immobilization techniques owing to its large surface area and better thermal stability [18]. Khalid et al. [19], concluded in their study that zinc oxide nanoparticles provide a superior microenvironment that enhances the stability and catalytic activity of immobilized enzyme tyrosine hydroxylase. Zinc oxide is mostly used in the form of nanorods to immobilize enzymes. Another metal oxide used here is aluminium oxide, however, not much work has been done on this metal oxide support. It is thermally stable, white coloured compound, often referred to as alumina and has properties like high stability, high hardness, transparency and high insulation [20]. Owing to these properties, it is used as an insulator and forming protective coverings. Alumina is suitable as a support material for immobilizing enzymes due to its nanoporous membrane [21, 22]. It has low cost, can be easily prepared and do not chemically interact with the enzyme [23]. The third inorganic support is a metallic nanoparticle, AgNP. Colloidal silver has many properties, which have increased its use in science and technology [24, 25]. It has sterical properties, thermal and electrical conductivity, antimicrobial properties, etc. [26]. Silver NPs can be incorporated into biosensor materials, composite fibers, cryogenic superconducting materials, cosmetic products, medical imaging, computer circuits, drug delivery and support for enzyme immobilization [27, 28, 29, 30]. Lipase and urease have been immobilized on these nanoparticles by the method of cross-linking using glutaraldehyde as a cross-linker. Enzymatic assay of these enzymes were carried out in free as well as immobilized states. Optimum pH and Temperature studies were carried out and it was found that these ranges improve in immobilized enzymes when compared to the free ones. Reusability and storage ability were then analysed in both the enzymes with and without support. Finally enzyme kinetics were measured using Michaelis Menten equation to conclude that immobilization proves to be beneficial for enhancing the catalytic abilities of these biocatalysts.

2. Experimental

2.1 Materials

Inorganic nanoparticles viz., Al_2O_3 NP and ZnO NP were purchased from Sigma Aldrich, USA. Folin's reagent and BSA were obtained from Qualigen's Fine Chemicals, India. Lipase and Urease were obtained from Hi-Media Laboratories, India. Urea and Nessler's reagent (NR) were possessed from Qualigen's Fine Chemicals, India. SDS, silver nitrate solution, hydrazine hydrate and sodium citrate were bought from Merck, India. Gum Arabic and phenolphthalein indicator were bought from Acros, India. All reagents used were of AnalaR grade and utilized without any further purification.

2.2 Preparation of silver nanoparticles

Silver nanoparticles were prepared by the chemical reduction method. 6mM silver nitrate solution was used as a metal salt precursor, to which 8% sodium dodecyl sulphate was added. After that Hydrazine hydrate solution (12 mM) and Citrate of sodium solution (20 mM) were added as reducing agents. Citrate of sodium was also used as stabilizing agent at room temperature. The transparent colourless solution was converted to the characteristic pale yellow and pale red colour. The colouration indicated the formation of silver nanoparticles, which were then purified by centrifugation. To remove excess silver ions, the particles were washed with distilled water.

2.3 Characterization/particle size measurement

The prepared silver nanoparticles were characterised by the Dynamic Light Scattering method. DLS measurements were performed to measure the average size of silver nanoparticles. Each measurement system was repeated 5 times and averaged to get mean particle size.

2.3 Protein estimation and enzyme assay

The protein content of soluble and immobilized lipase was estimated by the method of Lowry et al., using BSA as standard. The amount of protein immobilized on various supports was determined by subtracting the proteins in washings from the total protein introduce in the nanoparticles.

The enzymatic activities of free and immobilized lipase were measured by titration of the fatty acid (oleic acid, linoleic acid), which came from the hydrolysis of olive oil. This was carried out at 37ºC for 30 min on a rotary shaker. The reaction was stopped by the addition of 10 mL of ethanol–acetone solution (1:1). The liberated fatty acid in the medium was determined by titration with 50 mM NaOH solution using phenolphthalein as indicator. One unit of lipase activity (U) was defined as the amount of enzyme that hydrolyzed olive oil liberating 1 mol fatty acid per minute under the assay condition. Soluble or immobilized urease was incubated with 1 ml urea (0.5 M) solution. The amount of ammonia liberated in 30 minute was determined using Nessler's reagent. The reaction was stopped by adding 0.66 N H_2SO_4 (1 ml) at constant temperature (55ºC) and pH 7. The absorbance was measured at 405 nm using Spectrophotometer.

2.4 Procedure for immobilization

The three inorganic nanoparticles were added to 5% glutaraldehyde solution each. Lipase and urease were also added to this solution separately. The solution was kept on a rotary shaker for 30 min for the crosslinking process to occur. After 4 hours the particles were separated by centrifugation and were washed several times to remove excess glutaraldehyde and unbound enzyme.

2.5 Properties for immobilized enzymes

Immobilized lipase and urease were screened for pH, temperature, storage stability and reusability. The effect of pH on the activity of enzyme (soluble as well as immobilized) was investigated in pH range, 4–9. Phosphate buffer and acetate buffer were used in the experiment. The relative enzyme activity was determined at each pH by the method described for enzyme assay above. The thermal stability of both the free and immobilized enzymes was determined by measuring the enzymatic activity by incubating the solution for 30 min at temperatures ranging from 10ºC to 80ºC keeping the other factors same. The relative enzyme activity was determined by the method described for enzyme assay above. For storage stability studies, the immobilized enzymes were kept at room temperature. Free and immobilized enzymes were stored for 1 month and their activity was analyzed after every 5 days at optimum pH (7) and temperature (55ºC). Similarly, reusability studies were also performed by using the same repeatedly, where the nanoparticles were reused after every 30 min.

2.6 Enzyme activity and kinetic parameters

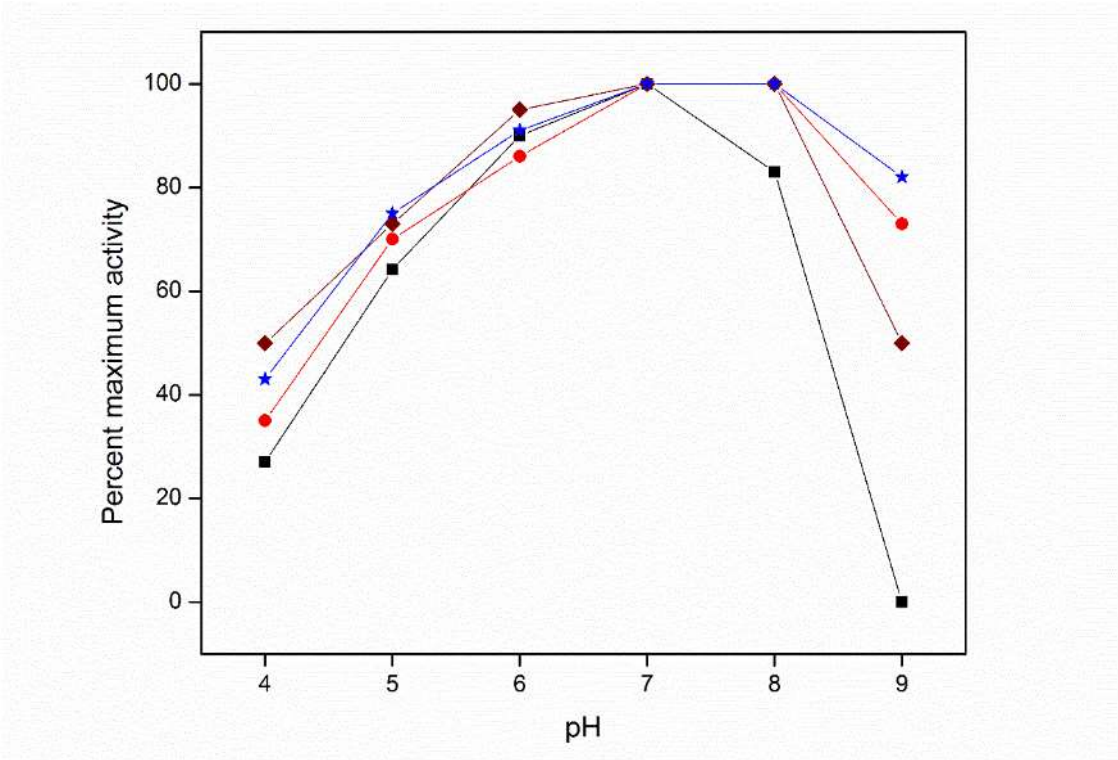
The effect of substrate concentration on free and immobilized enzyme activity was investigated by varying the substrate concentration at optimum pH and temperature for soluble and immobilized enzyme. A plot was made for free enzymes and was compared with plots made for enzymes immobilized on inorganic supports, silver nanoparticles, zinc oxide nanoparticles and aluminium oxide nanoparticles. The Michaelis-Menten constant K_m and V_{max} values for both free and immobilized enzymes were determined by Lineweaver–Burk plot.

3. Results and Discussion

The results showing the activities of lipase and urease immobilized on various nanoparticles have been described below as graphs and table along with detailed explanation.

3.1 Effect of pH

The relative activity of free enzyme at different pH was compared with that of enzyme immobilized on the three inorganic nanoparticles. The effect of pH on the activity of free and immobilized lipase is shown in the Figure 1(a). The reaction was carried out in pH range 4-9 at 55°C. The free enzyme showed maximum activity at pH 7. When lipase was immobilized on inorganic nanoparticles, its optimal pH range increased. Rise in optimum pH range was also observed by Khoobi et al. [31]. Lipase on aluminium oxide nanoparticles showed maximum activity at pH 7 to 8. At pH 6, it showed almost 85% activity and at pH 9 almost 75% of activity. Lipase immobilized on zinc oxide nanoparticles showed maximum activity at pH 7 to 8. It showed almost 95% activity at pH 6. When on silver nanoparticles showed maximum activity at pH 7 to 8. It showed 80 % activity at pH 9. Stability of lipase in a broad range of pH increased due to immobilization. Urease was immobilized on the inorganic nanoparticles and screened for activity at pH range 4 – 9. Free urease showed maximum activity at pH 7, which was decreased to 65% at pH 8 as can be seen in Figure 1(b). At pH 9, the activity of free urease was 58%. Urease on Al_2O_3 NP showed maximum activity at pH range 7-8. 75% activity was seen at pH 6 and 79% of activity at pH 9. Optimum pH range of 6-8 was earlier observed when urease was immobilized on carbon nanotubes [32]. In case of urease on ZnO NP, maximum activity was observed at pH 7 to 8. At pH 6, 82% activity was observed. Almost 90% activity retention was visible at pH 9. Urease on Ag NP showed maximum activity at pH 7-8. Moreover, showed 89% activity at pH 6 whereas, 85% activity retention at pH 9. The optimal pH range for all the three supports was similar, but the activity retention was different. ZnO NP provided best results for stability of urease at varying pH.



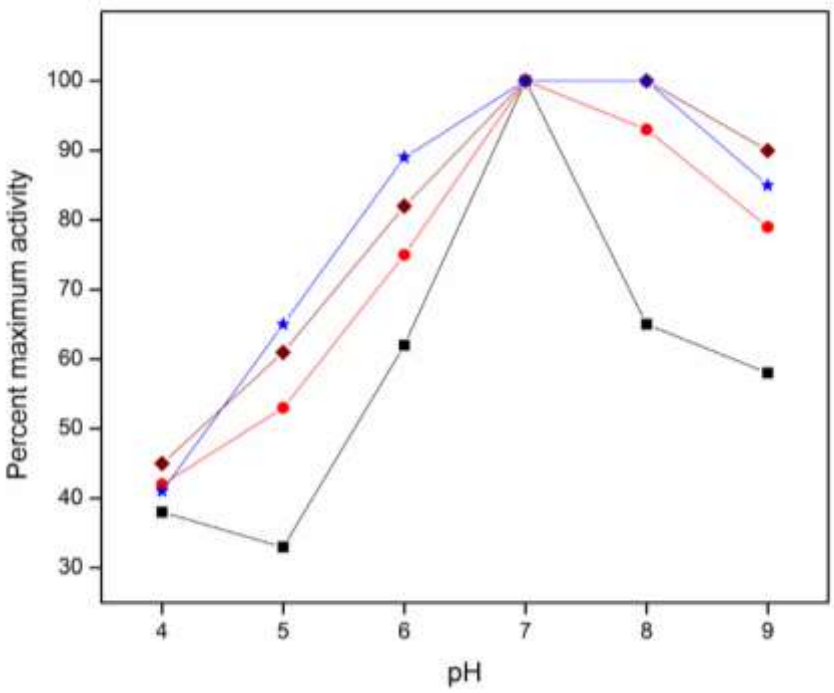


Figure 1: Plot of Percent Maximum Activity (%) vs. pH Profile of Free (■) and Immobilized (a) Lipase and Urease on Al_2O_3 NP (●), ZnO NP (◆) and Ag NP (★).

3.3 Thermal stability

The stability of free and immobilized lipase was studied in the temperature range of 15°C–80°C as shown in Figure 2(a). Free lipase showed maximum activity at 40°C. Lipase immobilized on Al_2O_3 NP showed maximum activity in the temperature range of 40 to 55°C. At 30°C immobilized lipase showed 76% activity which was 50% for free lipase. At 70°C, immobilized lipase showed 46% activity, which was 30% for the free lipase. When lipase was immobilized on ZnO NP, it showed maximum activity in the range of 55-70°C. The optimum temperature range shifted towards higher side due to immobilization. It showed 79% activity at 40°C and 70% activity at 80°C. Free lipase was able to show only 20% activity at 80°C. Lipase immobilized on Ag NP also showed its optimal temperature range to be 55-70°C. It showed 75% activity at 40°C and 60% activity at 80°C. Similar enhancement in activity was observed when lipase was immobilized on other nanoparticles [33].

The thermal stability of free and immobilized urease was studied in the temperature range of 30°C–80°C and is shown in Figure 2(b). Free urease showed maximum activity at 55°C, which is considered as optimum temperature for urease. Change in optimum temperature was observed when urease was immobilized on inorganic nanoparticles. Urease immobilized on Al_2O_3 NP showed maximum activity in the range of 55°C to 65°C, this was considered as the optimum range for immobilized urease. It showed 48 % activity at 80°C, which was 30% for free urease. When urease was immobilized on ZnO NP, it showed the best results in thermal stability. Optimum range became 55-70°C. At 45°C, it showed 88% activity, which was 60% for free urease. At 30°C, the activity of immobilized urease was 47% which was 25% for free urease. Similarly, at 80°C, the activity of immobilized urease was 58%. When urease was immobilized on Ag NP, the optimum temperature range was 60 to 70°C. There was a shift towards right side. Here, urease showed 97% activity at 55°C and 50% activity at 80°C. Similar enhancement in thermal stability was observed in the activity of glucose isomerase immobilized on silica and chitosan hybrid

particles [34] and of penicillin G acylase immobilized on magnetic $Ni_{0.7}Co_{0.3}Fe_2O_4$@SiO_2-CHO nanocomposites [35].

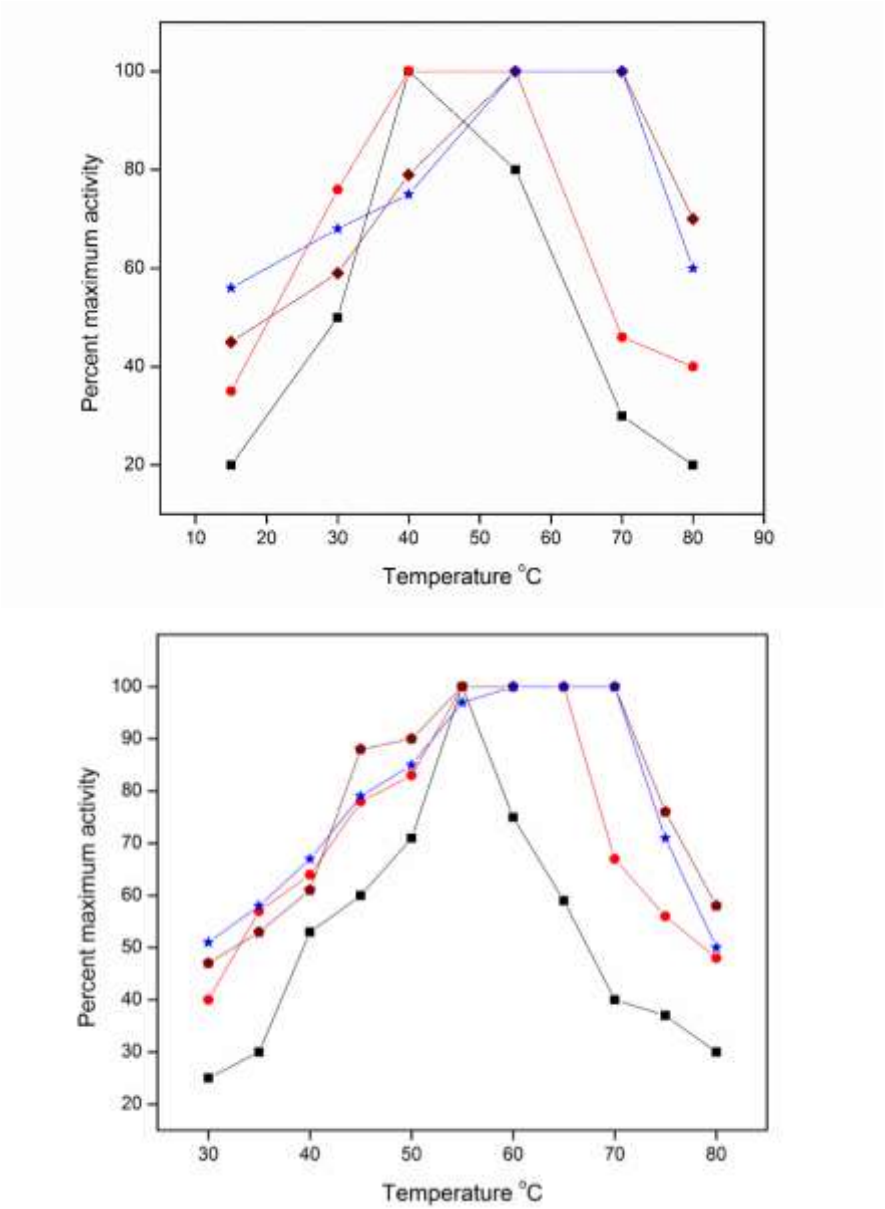


Figure 2: Plot of Percent Maximum Activity (%) vs. Thermal Stability of Free (■) and Immobilized (a) Lipase (b) Urease on on Al_2O_3 NP (●), ZnO NP (◆) and Ag NP (★).

3.4 Reusability

One of the main aims of immobilizing enzymes is to render multiple uses of the enzyme to avoid unnecessary wastage. Reusability studies were done by using the immobilized enzyme after every 30 min interval. The enzyme was reused 10 times as shown in Figure 3(a). Lipase immobilized on Al_2O_3 NP showed maximum activity for 4 cycles, after which it showed 91% activity on 5th cycle of reuse. During 10th cycle, it was 50% active. When lipase was immobilized on ZnO NP, it showed 100% activity even when used for 5 times. At the 6th cycle of reuse, it showed 88% activity. 54% activity was retained after 10 times reuse of the enzyme. Lipase on Ag NP showed 100% activity for first four uses after which it lost 2% activity, i.e. 98% activity retention was observed when it was reused for the 5th time. 90% activity

was seen at the 6th cycle of reuse and 53% activity was observed after 10 times reuse. Figure 3(b) shows the reusability of free and immobilized urease. Free urease does not show reusability hence its activity is completely lost after 1st cycle. Urease immobilized on Al_2O_3 NP was 100% active until 3 cycles of reuse. It showed 93% activity when used for the 4th time. 34% of activity retention was observed after 10th cycle of reuse. When urease was immobilized on ZnO NP, it showed 100% activity upto 4 cycles of reuse. On reusing for the 5th time it lost 10% of its activity. It retained 46% activity after 10 times of reuse. Urease on Ag NP was 100% active upto 4 cycles. It showed 87% activity retention when reused for the 5th time and 40% activity retention when used for the 10th time.

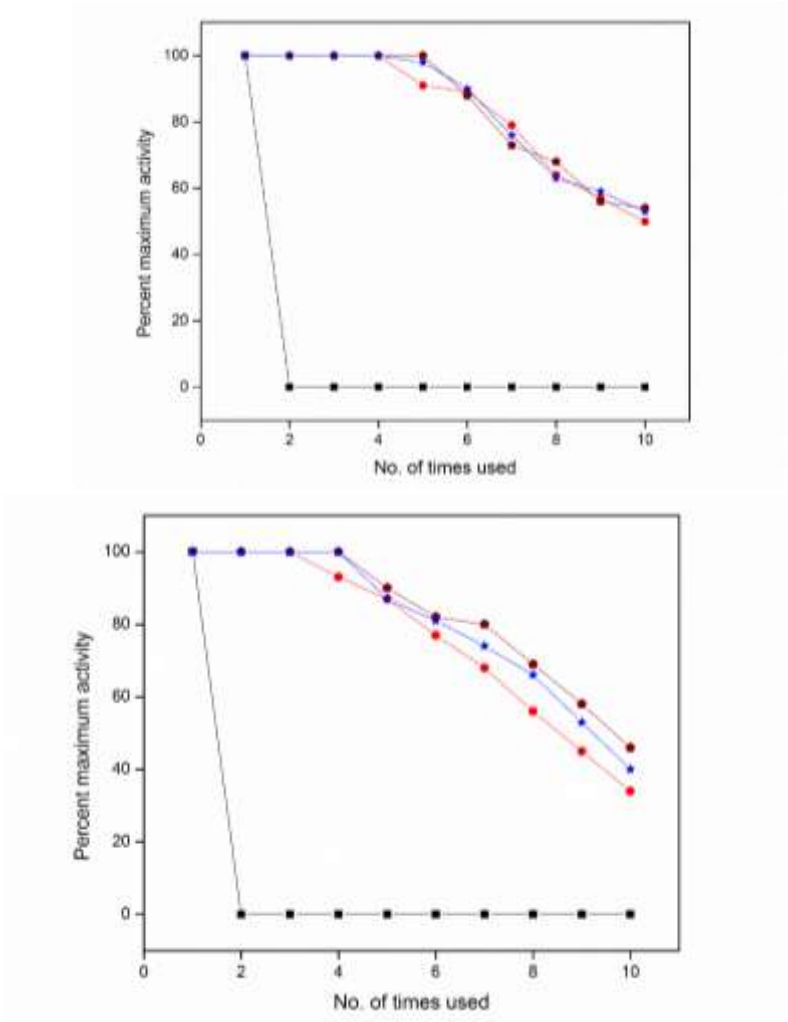


Figure 3: Plot of Percent Maximum Activity (%) vs. Number of Times Used for Reusability Studies of Free (■) and Immobilized (a) Lipase (b) Urease on Al_2O_3 NP (●), ZnO NP (◆) and Ag NP (★).

3.5 Storage stability

Free and immobilized lipase were stored for 45 days to check their storage stability. The results are graphically shown in Figure 4(a). Free lipase showed 100% activity on the first day of storage after which it decreased to 90% on the fifth day. Free lipase was active for 35 days when it showed 25% activity. After this, it lost its complete activity on 40th day. Lipase immobilized on Al_2O_3 NP showed 100% activity for 15 days. It lost 10% of its activity on 20th day. After 45 days, it showed 30% activity retention. Lipase

immobilized on ZnO NP was completely active until 20 days of storage. On the 25th day of storage, it lost 10% of its activity. It showed 42% activity retention on the last day, i.e. 45th day, of storage. Lipase on Ag NP showed 100% activity until 15 days of storage. It showed 97% activity on the 20th day. On the 25th day, the activity became 83% and on the 45th day, it was 40%. Lipase also shows enhancement in storage stability when immobilized on other nanoparticles [36, 37]. The storage stability studies of free and immobilized urease was done for 45 days. Free urease showed 100% activity on the initial day and lost about 10% of its activity until the 5th day of storage as shown in Figure 4(b). On the 10th day it lost 40% of its activity, it was 60% active. On the 40th day it showed 15% of activity retention after which it completely lost its activity on the 45th day. Urease immobilized on Al_2O_3 NP showed 100% activity upto 10 days of storage. It lost 5% of its activity when assayed on 15th day. On 45th day of storage it retained 60% of its original activity. Urease immobilized on ZnO NP showed 100% activity for 20 days. On 25th day, the activity was 91% and on the 45th day, it was 67%. Urease on Ag NP showed 100% activity up to 15 days. When assayed on the 20th day, it showed 93% of its original activity. This activity became 63% on the 45th day of storage. Amylase also showed increased storage stability when immobilized on magnetic nanoparticles [16].

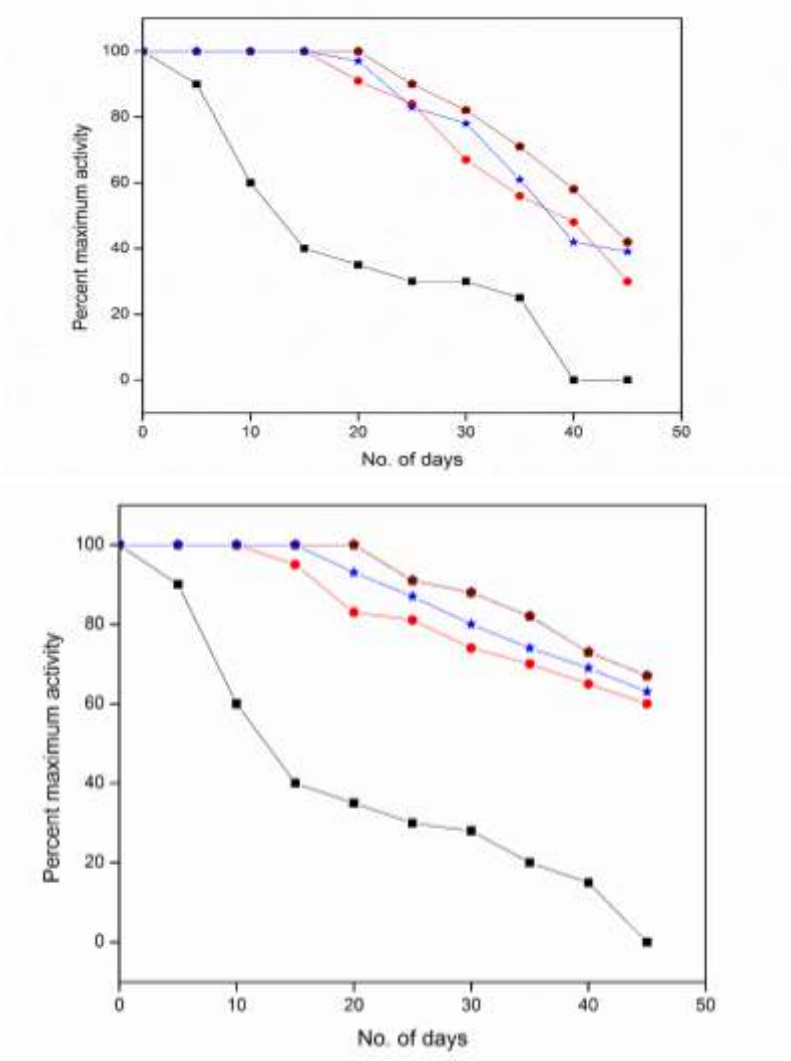


Figure 4: Plot of Percent Maximum Activity (%) vs. Storage Stability of Free (■) and Immobilized (a) Lipase (b) Urease on on Al_2O_3 NP (●), ZnO NP (◆) and Ag NP (★).

3.6 Effect of substrate concentration/ Enzyme kinetics

The effect of substrate concentration on free and immobilized lipase activity is shown in Figure 5(a). It is expressed as Lineweaver Burk plot or double reciprocal plot. K_m and V_{max} values of free and immobilized lipase were evaluated from the reciprocal plots of enzyme activity versus substrate concentration. K_m is the Michaelis constant, it is considered equal to the substrate concentration at half the maximum rate. V_{max} is the maximum rate of enzyme activity at which total enzyme concentration is present as enzyme-substrate complex. It can be observed from the graph that lipase on ZnO NP has the highest Km followed by Ag NP and Al_2O_3 NP. Kinetic parameters Km and Vmax of free and immobilized urease are shown in Figure 5(b). It is expressed as Lineweaver Burk plot or double reciprocal plot. Km and Vmax values of free and immobilized urease were evaluated from the reciprocal plots of enzyme activity versus substrate concentration. It can be observed from the data that urease on ZnO NP has the maximum Km followed by Ag NP and Al_2O_3 NP.

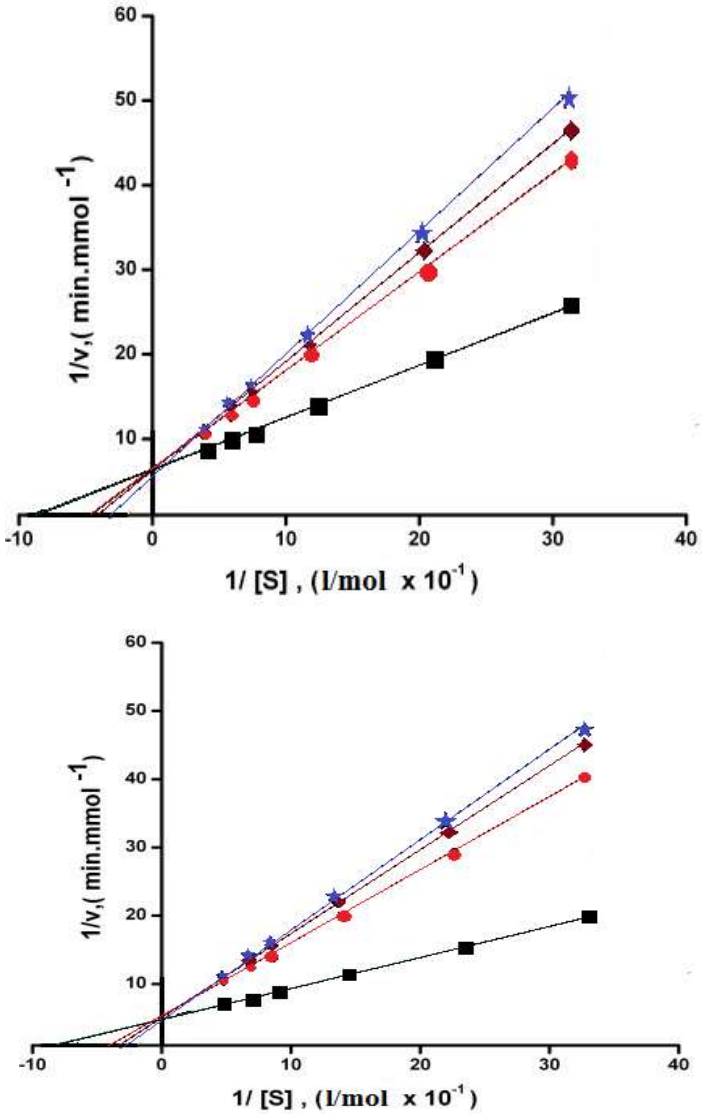


Figure 5: Double Reciprocal Plot of Velocity V (m Moles of Fatty Acid Produced/min/mg vs. Concentration of Substrate for Free (■) and Immobilized (a) Lipase (b) Urease on Al_2O_3 NP (●), ZnO NP (◆) and Ag NP (★)

4. Conclusion

Immobilization of enzymes on inorganic nanoparticles is comparatively better. Inorganic nanoparticles are stable, cheap and easy to prepare. These provide large surface area to volume ratio and do not interfere with the catalysis reactions of enzymes. Amongst the three nanoparticles used here, ZnO NP proved to be the best ones for both these enzyme. The pH stability and thermal stability results showed that it has a tendency to increase the optimum range in both the cases. Enzymes lipase as well as urease immobilized on ZnO nanoparticles showed better reusability and storage stability. After this, on comparing AgNP and Al_2O_3 NP, it was found out that Ag NPs were better support for these enzymes. The kinetic results showed that zinc oxide nanoparticles are the best ones followed by silver nanoparticles and then aluminium oxide nanoparticles.

Declaration of generative: AI and AI-assisted technologies in the writing process. While preparing this work, the authors utilised the ChatGPT tool/ service to rephrase several statements in the manuscript. After using this tool/ service, the authors reviewed and edited the content as needed and took full responsibility for the publication content.

Funding sources: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement: The authors acknowledge the Department of Zoology, P. T. Sarvajanik College of Science, Surat, 395001, Gujarat, India, for providing instrument facilities.

Data availability: Data will be made available on request.

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