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Variation in Activity of Lipase Immobilized on Chitosan and Alginate Nanoparticles by Changing Concentration of the Preparatory Reagents

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Abstract

Chitosan and alginate nanoparticles were prepared by ionic gelation method. The concentrations of each of the preparatory reagents (for chitosan: acetic acid, chitosan, sodium sulphate, glutaraldehyde and for alginate: calcium chloride, alginate, glutaraldehyde) were altered to check their consequences. Lipase was chemically crosslinked to these nanoparticles. Enzymatic assay was carried out for every alteration separately. To visualize the changes in each reagent at every step a precise table is incorporated in the text. It can be clearly understood from the outcomes that optimum concentration of each preparatory reagent is mandatory for maximum activity as can be seen from the graphical representations. Minute permutations in certain reagents can cause major difference in the results. During the study, it was also observed that certain changes in concentration affect the structure and rigidity of the nanoparticles.

Keywords: *Immobilization, glutaraldehyde, microspheres, nanospheres, ionic gelation*

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INTRODUCTION

Nanotechnology is the fastest emerging science in last few decades. It is an interdisciplinary science related to physics, chemistry, engineering science, material science, pharmaceutical science and biological science [1]. Nanotechnology combined with biotechnology gives rise to nanobiotechnology. One of the branches of nanobiotechnology is 'nanobiocatalyst science-NBC', which deals with the immobilization of enzymes on nanoparticles. Integration of biocatalyst in nanomaterials possessing properties required for stability and better activity of enzymes is the main aim of NBC science. Various methods to prepare suitable nanoparticles, different techniques for immobilizing enzymes on these nanoparticles and assay of enzyme activity are carried out in this field [2].

Nanomaterials are multifunctional; they are formed of polymeric materials, solid lipid nanoparticles, semiconductor materials, metal oxides and metals [3]. Different forms of nanomaterials are used as support such as nanoparticles, nanorods, nanotubes, nanomatrices, nanofilms, nanocrystals, nanocomposites, nanogels, nanowires, etc. Nanoparticles are considered as appropriate carriers owing to their ability to incorporate and release enzymes. Their higher surface area to volume ratio, minimum diffusional resistance and highly effective enzyme loading, increase their demand in the field of immobilization [4]. Polymeric nanoparticles are gaining a higher position in various fields such as environmental technology, biosensing, electronics, pollution control, medicine and biotechnology. These can be natural polymers as well as synthetic. Several techniques have been employed to prepare polymeric nanoparticles [5, 6].

In order to get the maximum functionality of nanoparticles it is mandatory to establish procedures and test the optimum requirements of the preparatory reagents. Various researchers have carried out such type of optimization studies [7, 8]. Along with the reagents, the method of preparation also has visible effects on the prepared nanoparticles [9, 10]. This paper deals with the preparation of two biopolymeric nanoparticles—chitosan

and alginate [11]. Literature survey show similar type of studies, such as effect of gel composition on polyacrylamide gels [12] and effect of polyelectrolyte carriers on polyacrylamide gels [13]. Chitosan is a natural biopolymer with reactive amino and hydroxyl groups and is one of the best support materials for immobilization [14]. It proves to be more useful when fabricated into nanosized spheres. It is a cationic polysaccharide that can easily crosslink with counter polyanions such as tripolyphosphate (TPP), sodium sulphate, etc. [15]. Alginate is a mucoadhesive, biocompatible, readily available anionic polysaccharide [16, 17]. It can easily form gels in the presence of divalent cations under mild conditions. Alginate gels are formed by exchange of sodium ions from guluronic acid (G) blocks with the divalent cations [18]. Most frequently used cation is calcium forming the strongest gels [19]. Combinations of both these biopolymers have proved to be extremely beneficial [20].

EXPERIMENTAL DESIGN

Materials

Chitosan flakes were procured from Sigma Aldrich, USA. Acetic acid and sodium hydroxide, sodium alginate, calcium chloride, glutaraldehyde were purchased from Acros, India. Folin's reagent and BSA were obtained from Qualigen's Fine Chemicals, India. Lipase was acquainted from Hi-Media Laboratories, India. Gum Arabic and phenolphthalein indicator were bought from Acros, India. All reagents used were of AnalaR grade and utilized without any further purification.

Methods

Preparation of Chitosan Nanoparticles

Enzyme loaded chitosan nanoparticles were prepared by desolvation technique [21, 22]. About 0.5 ml sodium-sulphate aqueous solution was added drop wise to 9.5 ml of chitosan solution (dissolved in aqueous acetic acid) containing 1% of Tween 80 during sonication which was maintained for 5 min. Lipase was added to chitosan solution. Then the suspension was agitated for 2 h with magnetic stirrer (500 rpm). The obtained particles were collected by centrifugation (10,000 rpm, 20 min) and washed with 0.02 mol/l potassium phosphate buffer (pH 7.5) until neutrality. Glutaraldehyde was used as a crosslinking reagent. The nanoparticles were suspended in 10 ml buffer solution after the last washing. Chitosan nanoparticles were prepared by changing concentrations of one of its preparatory reagents each time during its preparation as shown in Tables [1–4]. The effects of this were observed on lipase activity.

Table 1: *Change in Acetic Acid Concentration while Preparing Chitosan Nanoparticles.*

Acetic acid (%)	Chitosan (%)	Sodium sulphate (%)	Glutaraldehyde (%)
0.5	0.25	2.5	5
1.0			
1.5			
2.0			

Table 2: *Change in Chitosan Concentration while Preparing Chitosan Nanoparticles*.

Acetic acid (%)	Chitosan (%)	Sodium sulphate (%)	Glutaraldehyde (%)
1	0.15	2.5	5
	0.20		
	0.25		
	0.30		

Table 3: *Change in Sodium Sulphate Concentration While Preparing Chitosan Nanoparticles.*

Acetic acid (%)	Chitosan (%)	Sodium sulphate (%)	Glutaraldehyde (%)
1	0.25	2.0	5
		2.5	
		3 .0	
		3.5	

Table 4: *Change in Glutaraldehyde Concentration While Preparing Chitosan Nanoparticles.*

Acetic acid (%)	Chitosan (%)	Sodium sulphate (%)	Glutaraldehyde (%)
1	0.25	2.5	0
			5
			10
			15

Preparation of Alginate Nanoparticles

Alginate nanoparticles were prepared by ionotropic gelation method [23, 24]. Alginate powder was added to 10 ml distilled water to

make 1% alginate solution. Lipase was added to alginate solution. A surfactant Tween 80 was added to this solution in order to stabilize the nanoparticles. Calcium chloride (5%) was added intermittently i.e., 0.5 ml at every 2 min interval under sonication. It was then kept on a magnetic stirrer for 30 min at 1,000 rpm. After that, the solution was centrifuged at 10,000 rpm for 35 min. Nanoparticles after centrifugation were collected and glutaraldehyde was added for crosslinking. Nanoparticles were then washed with 0.2 M phosphate buffer and stored at 6 ºC. One by one, the concentrations of each of the preparatory reagents were changed during the preparation of alginate nanoparticles as shown in Tables [5–7]. The effects of these change in concentrations of each of these substances was studied on lipase activity.

Table 5: *Change in Alginate Concentration While Preparing Alginate Nanoparticles.*

Alginate (%)	Calcium chloride (%)	Glutaraldehyde (%)
0.5	5	5
1.0		
1.5		
2 .0		

Table 6: *Change in Calcium Chloride Concentration While Preparing Alginate Nanoparticles.*

Alginate (%)	Calcium chloride (%)	Glutaraldehyde (%)
1	1	5
	3	
	5	
	7	

Table 7: *Change in Glutaraldehyde Concentration While Preparing Alginate Nanoparticles.*

Alginate (%)	Calcium chloride (%)	Glutaraldehyde (%)
1	5	0
		5
		10
		15

Procedure for Immobilization

Lipase was immobilized on chitosan and alginate nanoparticles by crosslinking method by using glutaraldehyde as a crosslinker as described in the preparation procedures of nanoparticles.

Lipase Assay

The enzymatic activities of immobilized lipase were measured by titration of the fatty acid (oleic acid), which came from the hydrolysis of olive oil. About 100 ml of olive oil emulsion was prepared by mixing of olive oil (50 ml), bile salts (2%) and gum Arabic solution (50 ml, 7% w/v). The assay mixture consisted of emulsion (5 ml), phosphate buffer (2 ml, 100 mM, pH 7.4) and free enzyme (1 ml, 6.47 mg/ml) or immobilized lipase (50 mg in 1 ml buffer). Oil hydrolysis was carried out at 37 °C for 30 min on a rotary shaker (Biotechnics, India). 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 indicator. One unit of lipase activity (U) is defined as the amount of enzyme that hydrolyzed olive oil liberating 1 M fatty acid per minute under the assay condition.

RESULTS AND DISCUSSION

Chitosan Nanoparticles

Chitosan is a linear polyamine with many free amine groups that are readily available for ionic crosslinking with multivalent ions [25]. Weak basic groups present in chitosan are responsible for the positive charge of this hydrophilic polymer. In ionic gelation method, electrostatic interactions occur between the amine groups of chitosan and the polyanion. Ionic gelation of chitosan occurs due to complexation between the opposite charges that results in precipitation of chitosan [26]. Chitosan nanoparticles with variations in proportions of their preparatory reagents were successfully prepared. While assaying enzyme activities differences in the activities as well as in nanoparticle structures were observed due to variations in proportions [27]. Apart from the proportions of these reagents, physiochemical properties, the molecular weight and degree of acetylation of chitosan

also has effect on chitosan gel formation [28–30]. Following effects were observed:

Effect of Change in Acetic Acid Concentration

Due to increase in acetic acid concentration, the size of the nanoparticles decreased. This can be due to decrease in solution viscosity. The spherical shape of nanoparticles was lost as the gel was not viscous enough to maintain spherical shape. This also affected the enzyme loading capacity of the support. On assaying the lipase activity following results was obtained:

The enzyme showed 30% activity at low acetic acid concentration. The reason could be that chitosan might not have dissolved properly which resulted in poor formation of nanoparticles. When 1% acetic acid was used lipase showed maximum activity. This gradually decreased as the concentration was further increased as shown in Figure 1. This could be due to increased acid content, which might have interfered with the enzyme activity.

Effect of Change in Chitosan Concentration

Increase in chitosan concentration increases the solution viscosity. The size of nanoparticles increased due to high viscosity. The shape became less spherical as the concentration gradually increased. The smoothness of the particles disappeared gradually. Increase in chitosan concentration resulted in stiff nanoparticles formation. The enzyme activity was analysed by changing the chitosan concentration. At 0.15% chitosan, lipase showed 63% activity that was increased to almost 68% at 0.2% chitosan concentration as shown in Figure 2. It became maximum at 0.25% chitosan concentration. However, at 0.3% chitosan concentration the activity of lipase decreased upto 80%. This decrease in activity could be due to high increase in viscosity.

Effect of Change in Sodium Sulphate Concentration

Increase in sodium sulphate concentration resulted in increased size of chitosan nanoparticles. The reason here is that greater availability of sodium sulphate tends to higher chances of bond formation. Thus, the size increases which results in higher accumulation of chitosan. This has a contradictory effect on the efficiency of enzyme. Lipase shows 30% activity at 2% sodium sulphate, which becomes 100% at 2.5% sodium sulphate as shown in Figure 3. However, after this the activity decreases upto 40% at 3% sodium sulphate and 30% at 3.5% sodium sulphate. This implies that on increasing the concentration of sodium sulphate reaction becomes faster but enzyme efficiency decreases.

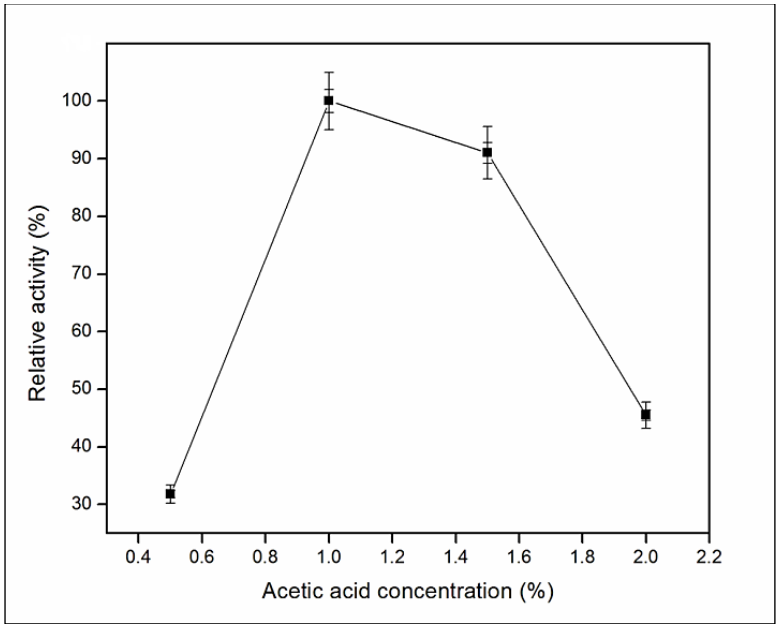

Fig. 1: *Effect of Acetic Acid Concentration on Lipase Immobilized on Chitosan Nanoparticles.*

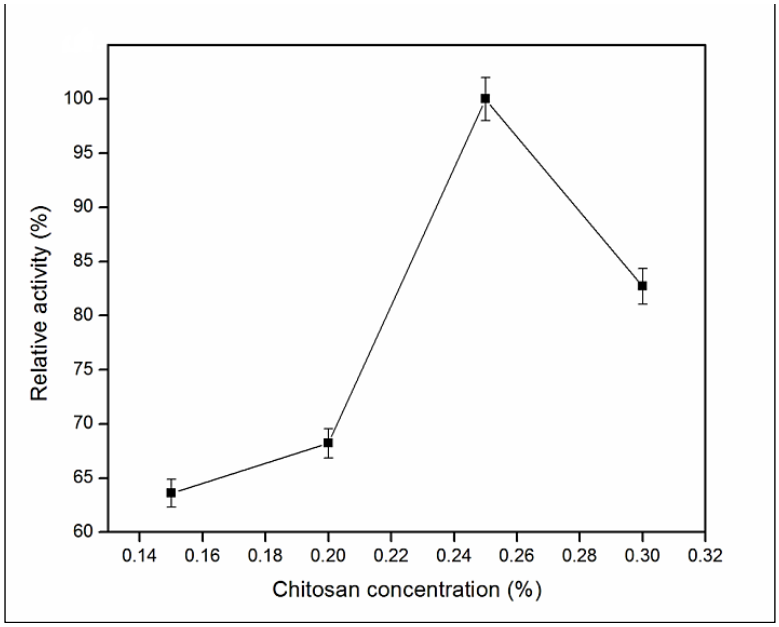

Fig. 2: *Effect of Chitosan Concentration on Lipase Immobilized on Chitosan Nanoparticles.*

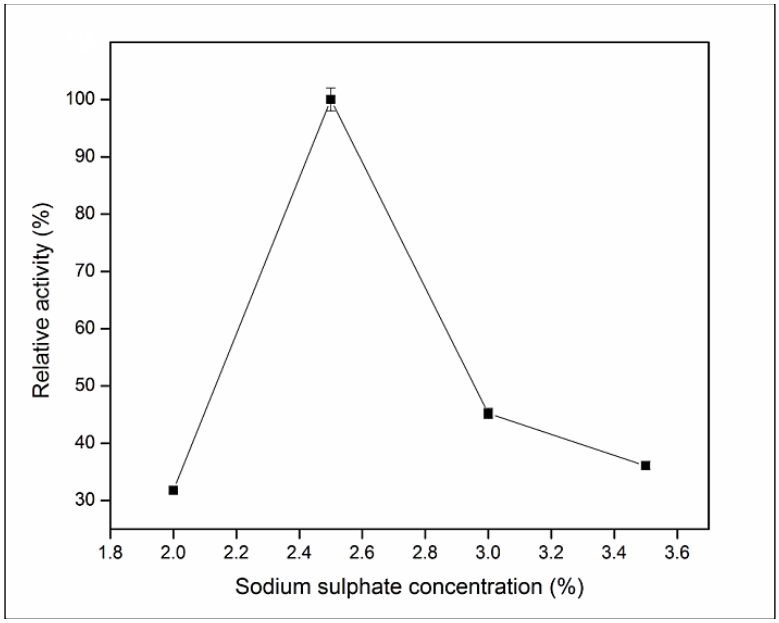

Fig. 3: *Effect of Sodium Sulphate Concentration on Lipase Immobilized on Chitosan Nanoparticles*.

Effect of Change in Glutaraldehyde Concentration

Glutaraldehyde is a crosslinking reagent. The amine groups of chitosan react with the aldehyde group of glutaraldehyde to form imine group. It is responsible for stable formation of polymeric nanoparticles but higher amount could prove to be deleterious. Higher amount of glutaraldehyde altered the spherical geometry of nanoparticles. It decreased the surface smoothness. Higher amount of glutaraldehyde decreases the water content of the chitosan nanoparticles making them shapeless and dark colored. The effect of glutaraldehyde concentration on enzyme activity has been studied. Lipase showed lower activity in the absence of glutarldehyde. At 5% glutaraldehyde it showed maximum activity. This decreased upto 80% at 10% glutaraldehyde. A further decrease in activity was noticed at 15% glutaraldehyde (Figure 4).

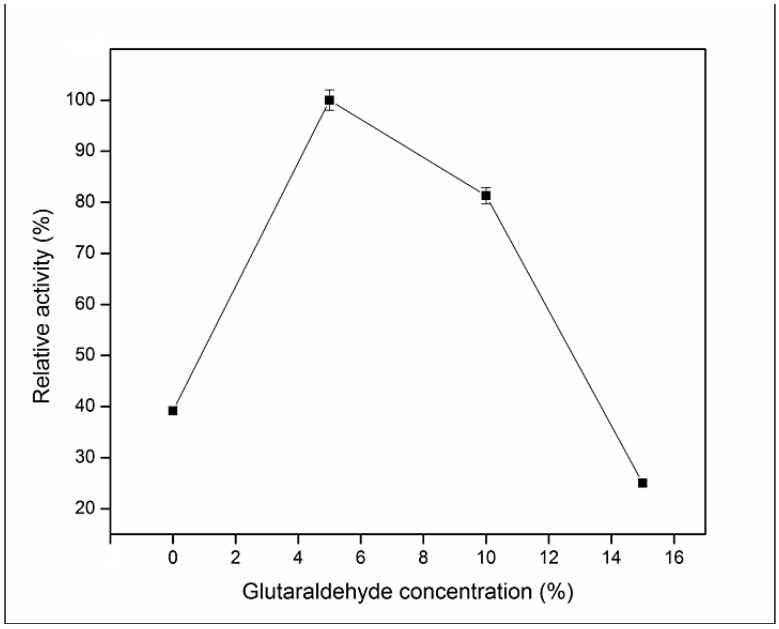

Fig. 4: *Effect of Glutaraldehyde Concentration on Lipase Immobilized on Chitosan Nanoparticles.*

Alginate Nanoparticles

Alginate nanoparticles are prepared by the reaction taking place between polymeric anion and metallic cation. The size of the metallic cation is smaller than that of anion. Hence, calcium ions tend to diffuse between the chains of alginate polymer to bond with the unoccupied ares of the polymer [31]. When calcium ions were drop-wise added to alginate solution, an instantaneous membrane was formed around the droplet. There should be proper selection of suitable cation. Calcium has proved to be comparatively better than other cations such as barium and strontium [32]. Optimization of alginate nanoparticles before lipase immobilization was also studied by various researchers [33]. Due to change in concentrations of its preparatory reagents, variations in lipase activity were observed as described below:

Effect of Change in Alginate Concentration

On increasing the concentration of alginate in the solution rigidity of the nanospheres increased. More unoccupied sites were available to the calcium ions hence more binding took place. This resulted in increased stiffness of the nanoparticles. The size of the spheres decreased due to decrease in the membrane thickness. When enzyme activity was assayed it was found that at lower concentration of alginate in the solution, lipase showed 80% activity. Nevertheless, when alginate concentration increased, lipase reached its maximum activity. On further increasing the concentration, decrease in lipase activity was observed. This could be due to decrease in membrane thickness which leads to enzyme leakage therefore decreasing its activity (Figure 5).

Effect of Change in Calcium Chloride Concentration

On increasing the concentration of calcium chloride membrane thickness increased. This happened due to large amount of influx of calcium ions inside the droplets. As a result of large amount of influx, a concentration gradient was developed. Inhomogeneity decreased as the concentration of calcium ions increased. On performing the assay of lipase, following results were obtained:

Lipase showed almost 70% activity at lower calcium chloride concentration. The activity gradually increased on increasing the concentration. When the concentration of calcium chloride was 5%, lipase showed 100% activity. This decreased on further increase in the concentration. This could be due to increase in membrane thickness, which did not allow the substrate to bind the enzyme completely (Figure 6).

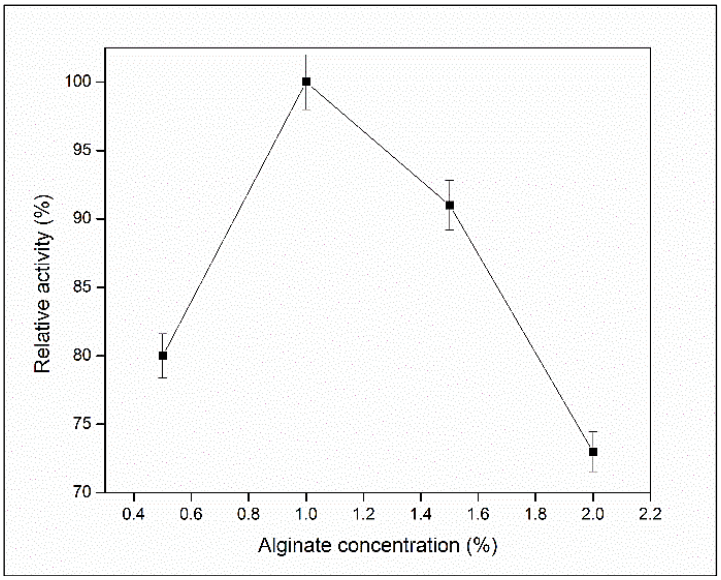

Fig. 5: *Effect of Alginate Concentration on Lipase Immobilized on Alginate Nanoparticles*.

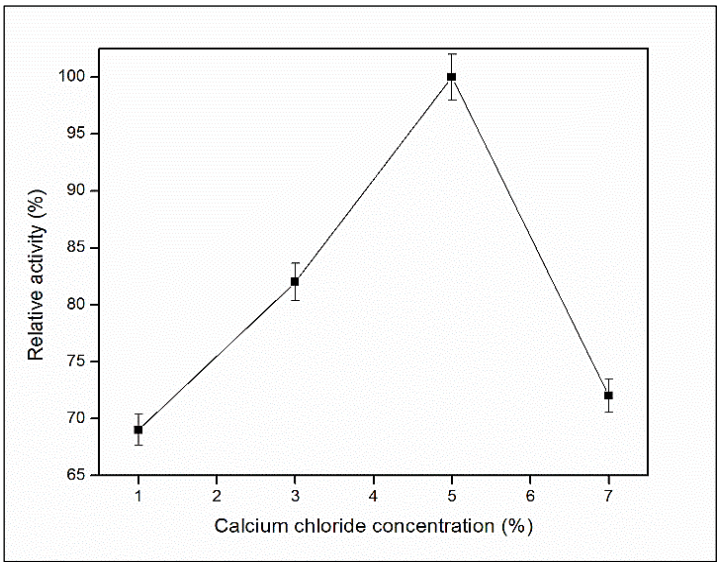

Fig. 6: *Effect of Calcium Chloride Concentration on Lipase Immobilized on Alginate Nanoparticles.*

Effect of Change in Glutaraldehyde Concentration

Glutaraldehyde plays an important role in preparation of nanoparticles. It consists of aldehyde group which reacts with the cis-oriented vicinal hydroxyl groups at the C-2 and C-3 positions of alginate to form acetal-linked alginate networks. Generally, in the absence of glutaraldehyde the absorbency of alginate nanoparticles is less due to tightly packed junctions between alginate and calcium ions. This absorbency can be increased by chemically crosslinking with glutaraldehyde, which results in creation of linkers of adjustable lengths that in turn form crosslink junctions allowing more liquid to be absorbed. The nanoparticles which were prepared in the presence of high concentration of glutaraldehyde were found to be asymmetrical in shape.. Stiffness also increases in the particles due to increase in glutaraldehyde concentration. The color of the particles gradually darkens with gradual increase in its concentration. On examining the lipase activity, it was found that in the absence of glutaraldehyde the activity was low as compared to its presence. Lipase showed maximum activity at 5% glutaraldehyde. The activity gradually decreased on increase in glutaraldehyde concentration. This could be either due to stiffness in nanoparticles or due to their asymmetrical structure (Figure 7).

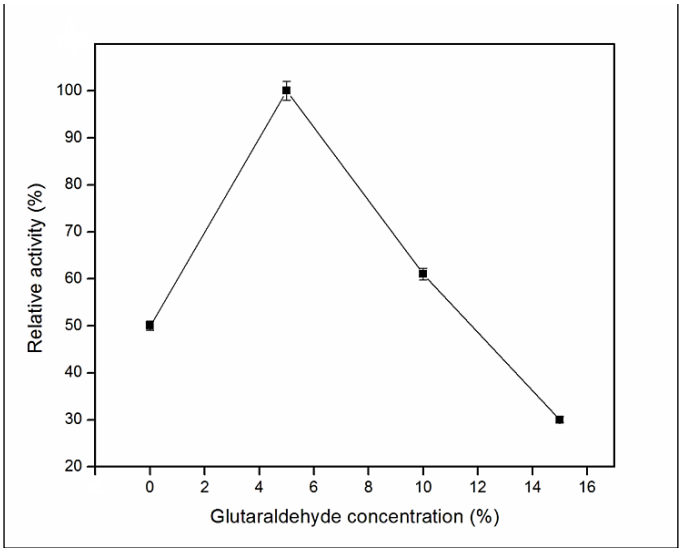

Fig. 7: *Effect of Glutaraldehyde Concentration on Lipase Immobilized on Alginate Nanoparticles.*

CONCLUSION

This work dealt with the preparation of chitosan and alginate nanoparticles. Different methods of preparing these nanoparticles are available. Various researchers differently optimize the concentrations of the substances used to prepare these nanoparticles. The main aim of this work was to optimize the concentration of the preparatory reagents required to prepare these nanoparticles. Successful preparation of nanoparticles is important. Another thing that was kept in mind was the enzyme activity. Hence, lipase assay was carried out at each and every step. The changes in concentrations of these reagents affected the stability and activity of enzymes. Finally, optimum conditions for preparation of alginate and chitosan nanoparticles were obtained.

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