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# Novel Approach of Alkaline Protease Mediated Biodegradation Analysis of Mustard Oil Driven Emulsified Bovine Serum Albumin Nanospheres for Controlled Release of Entrapped *Pennisetum glaucum* (Pearl Millet) Amylase

**Kirti Rani*, Shreya Goyal and Chanchal Chauhan**

Amity Institute of Biotechnology, Amity University Uttar Pradesh, Noida, Sec-125, Noida-201303 (UP), India

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**Address for Correspondence**

Amity Institute of Biotechnology, Amity University Uttar Pradesh, Noida,, Sec-125, Noida-201303 (UP), India.

**E-mail:** krsharma@amity.edu

## ABSTRACT

Amylase is known for starch saccharification and catalyses the starch hydrolysis producing glucose and limit dextrin which has wide range of applications in fermentation, textile, leather, detergents, food and paper industries. In the present work, amylase was extracted from *Pennisetum glaucum* (pearl millet) was entrapped in bovine serum albumin (BSA) nanoparticles in which emulsification was done by mustard oil and covalently coupled by using well known cross linking agents, glutaraldehyde. Bovine serum albumin is considered the most biodegradable, biocompatible and non-toxic biological matrix material for the preparation of enzyme bound nanospheres to enhance thermal stability, storage stability and suitability of bound enzymes as compared to free enzymes toward persisting ambient conditions. Formation of nanoparticles was confirmed by Dynamic Light scattering (DLS) and determination of exact size was observed by Scanning Electron Microscopy (SEM). Thermal stability of entrapped *Pennisetum glaucum* amylase at 70ºC and storage stability at 4ºC was enhanced for 3 hours 30 minutes and 12 months respectively as compared to free enzyme which was observed for 50 minutes and 1 day only. Studied optimal temperature of entrapped amylase was found to be 70ºC and it was significantly higher as compared to free enzyme (40ºC) which increased the industrial efficacy and catalytic viability of amylase after entrapment. Their biodegradation was carried out by using different units of alkaline protease (10U, 15U, 20U, 25U, 30U, 35U, 40U, 45U, 50U) to study sustained and controlled release of entrapped *Pennisetum glaucum*.

Characterization of kinetic parameters were also performed for *Pennisetum glaucum* extracted amylase and entrapped amylase for their comparative study for their optimal pH, incubation time, substrate concentration, $CaCl_2$ concentration and temperature for maximal enzyme activity which was tested by dinitrosalicylic acid method.

**Keywords**: Pearl millet, *Pennisetum glaucum*, Amylase, Bovine serum albumin, Mustard oil, Nanospheres, Glutaraldehyde, Encapsulated, Emulsified, Dynamic light scattering, Scanning electron microscopy.

## INTRODUCTION

Amylases (EC 3.2.1.1) are very popular ubiquitous enzymes produced by plants, animals and microbes, where they play a dominant role in carbohydrate metabolism.[1] The increased industrial use of biocatalysts is coined to be emphasized by the increasing demand of commercially enzymes worldwide approximately to US $ 3 billion. Globally, 45% of the enzymes produced are being used in the food industry and the rest % is shared by detergent (34.4%), textile (11 %), leather (2.8%), paper and pulp (1.2%) and other industries including their diagnostics and therapeutics implications (5.6%).[2] Yet, despite of industrial applications of enzymes, their uses has been limited due to several factors broadly categorised into two categories e.g. high cost of the biological catalysts and their labile nature.[3,4] Immobilized enzymes have several advantages over free enzymes such as more convenient handling of the enzyme, facile separation from the product, minimizing or eliminating protein contamination of the product and efficient recovery and reuse of the biocatalyst.[5] Immobilization of enzymes is done by entrapment, covalent Binding, cross-linking and adsorption. Advanced nanotechnology inspired researchers to immobilize the industrial important enzymes in chemically modified nanopreparation which have aroused innovative industrial interest for using supporting nanomaterials to provide high surface area/volume ratio, low mass transfer limitation and excellent particle mobility in enzyme catalysed reactions. Hence, various cross-linked enzyme aggregates, microwave-assisted immobilization, click chemistry technology, mesoporous supports and most recently nanoparticle-based immobilization of enzymes.[6] Enzymes immobilized into nanoparticles are showed to have broader operational range of pH and temperature as well as having higher thermal stability than the native enzymes.[7] Nanoparticles are well known, these days, for offering a wide variety of biomedical applications including cancer diagnosis, radiotherapy, drug and gene delivery, sensors and biosensors , environment remediation and water purification etc.[8,9] Researchers are still working to improve current technologies to develop new advanced applications that favour immobilization of industrial important enzymes into desired nanoparticles to make them more feasible and convenient in the point of their handling and resistivity.[10,11] Amylases have significance biotechnological approach which has already encased the class of industrial enzymes which has been grabbed approximately 25-30% of the world enzyme

market.[12,13] These are ubiquitous enzymes which are produced by plants, animals and microbes where they have prominent role in carbohydrate metabolism [14]. Amylases saccharifiy starch, glycogen and oligosaccharides in a random manner, liberating reducing sugars as glucose, maltose and limit dextrins which have potential industrial applications.[15-17] Recently, amylase was immobilized onto silica, alumina, chitin, tannin sepharose, ionic binding on Amberlite IR-120, Dowex 50W, DEAE-Cellulose DE-52 by either physical adsorption or entrapment in calcium alginate beads.[18,19] It was also found to be entrapped within a semi-permeable polymeric membrane and gel matrix too.[20] In our present study, entrapment of *Pennisetum glaucum* amylase was achieved into bovine serum albumin nanospheres by chemical modification done by butanol, emulsification done by mustard oil and enzyme was loaded by using glutaraldehyde as cross-linking agent into nanospheres in which the bound active gradient was dispersed in a hydrophilic or non-polymer layer aqueous medium.[21,23,24] Hence, this immobilization approach was coined reasonably safe, simple, cheap and offering good mechanical strength, high porosity for substrate/ product diffusion used for immobilization of enzymes.[25] Bovine serum albumin was chosen biocompatible, biodegradable, non-toxic and echo-friendly matrix in which emulsified entrapment or encapsulation of amylase was done where sustained release of bound enzyme form prepared nanospheres was achieved successfully by alkaline protease.[21,24-27] For the determination of size and characterization of prepared enzyme loaded nanospheres was done by Dynamic light Scattering (DLS) and Scanning electron Microscope was used.[28,29] Their biodegradation of mustard oil driven emulsified bovine serum albumin nanospheres was done with alkaline protease (10U, 15U, 20U, 25U, 30U, 35U, 40U, 45U, 50U) for controlled release of entrapped *Pennisetum glaucum* amylase. Successful sustained release of enzyme from emulsified chemically modified bovine serum albumin is widened the application of immobilized amylase by increased sustained and controlled usability.[30,31] Kinetic parameters of free and immobilized enzymes was carried out to study the effect of pH (1.5-11.5), effect of temperature (5°C-100°C), effect of incubation time (20mins- 4 hours), effect of substrate concentration (0.50%-1.5%) and effect of $CaCl_2$ (1%-10%) was studied for their activity by dinitrosalicyclic acid method (DNS method).[24,23]

## MATERIALS AND METHODS

### Extraction amylase from *Pennisetum glaucum* seeds

Cotyledons of 3-day old seedlings of germinating seeds of *Pennisetum glaucum* were homogenized in 0.05M sodium phosphate buffer (pH 7.0) in the ratio of 6ml: 1grams of seeds. It was centrifuged for 15min at 4°C at 3000rpm. The supernatant was collected for crude enzyme and stored at 4°C.[10,22,23]

### Amylase assay by DNS method

Amylase activity was estimated by determining the amount of reducing sugars released from starch. 0.5 ml of the enzyme extracts were added to 1 ml of the starch solution and the mixture incubated at 37ºC for 20 min. After incubation, 2 ml of dinitrosalicylic acid (DNS) was added and the reaction mixture was boiled at 100ºC for 5 minutes. The amount of reducing sugars in the final mixture was determined spectrophotometrically at 570 nm. One unit of enzymatic activity is defined as the amount of enzyme that produces 1 µmol of maltose per minute.[32, 34]

### Preparation of *Pennisetum glaucum* amylase loaded mustard oil driven emulsified bovine serum albumin nanospheres

Mustard oil bath was prepared by adding 25% glutaraldehyde and 2.6ml of n-butanol into 50ml of almond oil in glass beaker. 50U *Pennisetum glaucum* amylase was mixed with 8ml of bovine serum albumin. This solution filled in a 10 guage syringe and was dispersed in the prepared mustard oil bath. It was continuously stirred overnight on the magnetic stirrer. Next day, it was centrifuged at 5000rpm at 4°C for 20mins and the supernatant was discarded. The pellet was washed with chilled diethyl ether and acetone. The pellet was re-dispersed into acetone in bath sonicator for 12mins.[11] Enzyme assay of supernatant was performed by DNS method to know % of entrapment of enzyme in mustard oil driven bovine serum albumin nanospheres.

### % of entrapment

% of entrapment of enzyme was calculated by determining the residual enzyme activity from reaction mixture in which enzyme entrapment was done into bovine serum albumin nanospheres. Amylase assay was performed by using DNS.[31,32]

% of encapsulated enzyme = Specific activity of encapsulated / Specific activity of free enzyme X 100.

### Characterization of amylase loaded bovine serum albumin nanospheres

Prepared enzyme loaded bovine serum albumin nanoparticles were confirmed by Dynamic Light Scattering Technique. The size and morphologies of the prepared nanospheres were further studied by scanning electron microscopy.[11,28]

### Alkaline mediated biodegradation of prepared bovine serum albumin nanospheres for controlled release of entrapped *Pennisetum glaucum* amylase

Entrapped *Pennisetum glaucum* Amylase was incubated at 37°C with different concentrations of alkaline Protease (10U, 15U, 20U, 25U, 30U, 35U, 40U, 45U, 50U). Alkaline protease was already reported excellent enzyme to combat high alkaline reaction conditions and has suitability for biodegradation.[34,38] The enzyme assay of entrapped enzyme was performed for consecutive twelve days by DNS method.[11,24,27,34]

### Study of kinetic properties

The free enzyme and immobilized enzyme are characterized for their different kinetic properties i.e. effect of pH, effect of temperature, effect of incubation time, effect of $CaCl_2$ concentration and effect of substrate concentration.[11,24] The effect of pH on activity of free and immobilized enzymes was studied by performing enzyme assay at different pH using acetate and phosphate buffer by varying pH from 1.5 to 11.5. The effect of time on the activity of free and immobilized enzyme was studied by performing the enzyme assay at different time (20 minutes to 4 hours). Optimal substrate concentration for maximal enzyme activity for free and immobilized enzymes which were estimated by incubating the reaction mixture at different concentrations of starch solution (0.50%-1.50%). The effect of $CaCl_2$ on activity for free and entrapped enzymes was studied by performing the enzyme assay at different concentrations (1%-10%). Optimal temperature for free and encapsulated enzyme for maximal activity was studied by incubating the reaction mixture for 15minutes at different1 temperature (5°C – 100°C). These kinetic properties of enzyme were determined by performing DNS method.[33-37]

## RESULT AND DISCUSSION

### Percentage of entrapment

Entrapment of *Pennisetum glaucum* extracted amylase in to mustard oil driven chemically modified bovine serum albumin nanospheres was observed to be 74% which was found to be comparable with previous studies done.[11,12,31,32,36]

### Characterization of *Pennisetum glaucum* amylase loaded bovine serum albumin nanospheres

The prepared enzyme loaded bovine serum albumin nanospheres was confirmed by using Dynamic Light Spectroscopy that average particle size were in range of 1 to 10nm with a observed sharp peak in between (DLS) (Fig 1).[11,28] The nanospheres was found to be spherical in shape and fluroresence also observed under Scanning Electron Microscope (SEM) (Fig. 2) which confirmed the formation of diene adduct that leads of exposure of tryptophan residue during the encapsulation by using glutaraldehyde which acts as free acid and has much stronger fluroresence as compared to phenylalanine and tyrosin.[12] Observed size for prepared amylase loaded bovine serum albumin nanospheres under Scanning Electron Microscope was found to be in the range of 76.3nm to 111.5nm which were pretty lesser than the previous study (Fig. 2).[11,40]

### Studied kinetic properties

The free and encapsulated enzyme were characterized for their different kinetic properties i.e. effect of pH, effect of temperature, effect of incubation time, effect of $CaCl_2$ concentration and effect of substrate concentration. Optimal pH was found to be similar which was 11.5 for encapsulated amylase and free enzyme whose results were slightly comparable to previous studies (Fig 3).[11,24] Optimal incubation time for free enzyme was found to 50 minutes and 30 minutes for encapsulated enzyme whose results were also comparable with previous observations (Fig. 6).[11,24,33] Optimal substrate concentrations of free enzyme and encapsulated enzyme was found to be 1.5% for both by varying starch concentration from 1%-1.5% whose results were also pretty comparable to previous finding (Fig. 7).[11,24,33,35,36] Optimal $CaCl_2$ concentration for maximal amylase activity for free and bound enzyme was found to be 5% and 4% respectively whose results were similar to previous results (Fig. 8).[11,24,33-35] Optimal temperature for maximal activity for free enzyme and bound enzyme was observed $40^oC$ and $70^oC$ respectively whose results were also pretty comparable to previous results (Fig. 4).[11,33-36] It was also found that after encapsulation, thermal stability (Fig. 5) and storage stability (Fig. 9) were enhanced for bound enzyme (at 70ºC for 2 hours 30 minutes and 12 months respectively) as compared to free enzyme (at 70ºC for 50 minutes and one day only respectively) which were also sharply comparable to previous observations (Table 1).[34-36,39,40]

### Alkaline mediated biodegradation Study

Biodegradation of mustard oil driven emulsified bovine serum albumin nanospheres of encapsulated *Pennisetum glaucum* amylase was performed by incubating 2 mg of encapsulated amylase loaded nanosphere with alkaline protease (10U, 15U, 20U, 25U, 30U, 35U, 40U, 45U, 50U) overnight at 4°C. The study was carried out for consecutive 14 days and 45U of alkaline protease was found to achieve controlled and sustained release of encapsulated *Pennisetum glaucum* amylase form almond oil driven emulsified bovine serum albumin nanoparticles (Fig. 10). First 4 days, the release of the enzyme was negligible while it increased over the next 2 days. From $6^{th}$ day onwards, the enzyme activity was stable and not much change was observed

from day 6 till day 12th and then increased till day 14th. Enzymatic functionality was observed even after 2 weeks of protease incubation owing to highly stable nanoparticle formation whose results are found to be comparable with previous findings.[11,34,36,38-40]

## CONCLUSION

In our study, *Pennisetum glaucum* (Pearl millet) amylase was entrapped into mustard oil driven emulsified bovine serum albumin nanospheres via glutaraldehyde coupling with 74% of % of retention activity. The Characterization of prepared amylase loaded nanoparticles was performed by Dynamic Light Spectroscopy (DLS) showed the presence of nanoparticles and the exact size was confirmed by observing under the Scanning Electron Microscope (SEM). The observed size of prepared nanoparticles was found to be 56.2nm to 84.3nm and prepared enzyme loaded bovine serum albumin nanopsheres were spherical in shape as well fluroresence was also observed due to diene adduct formation by having tryptophan residue after the glutaraldehyde coupling. The result of biodegradation study were performed for consecutive 14 days which showed that 45U of alkaline protease was found efficient in sustained and more sustained release of bound enzyme from almond oil driven emulsified bovine serum albumin nanoparticles. It was observed that in the initial 7 days, the release of amylase was negligible. From the 11th day onwards, there was an increase in amylase activity and at 13th day the release of bound enzyme was noticed at its highest. The bound amylase was found to have increase storage stability for 12 months when stored at 4°C and excellent reproducibility and thermal stable up to 70ºC for 3 hours 30 minutes which was remarkably higher as compared to free enzyme with storage stability for one day only and having thermal stability at 70ºC for 50 minutes only. Hence, the spectrum of encapsulated amylase in alkaline protease mediated biodegradation of mustard oil driven emulsified bovine serum albumin form of nanospheres is coined significant scientific approach due to which storage stability, thermal stability and reusability of industrial important amylase are increased which, in turn, whose application may be expanded to automatic dishwashing detergents as well as textile desizing, pharmaceutical, paper industries and also may be used as bio-robotic tool for drug targeted delivery system to combat myriad of lethal epidemics and diseases as most advanced bio-clinical appliances for betterment of mankind too.

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**Table 1.** Kinetic Parameters of free and mustard oil driven emulsified bovine serum albumin nanospheres of encapsulated *Pennisetum glaucum* amylase

| Kinetic Parameters | Free amylase | Encapsulated amylase |
|---|---|---|
| Optimal pH | 11.5 | 11.5 |
| Temperature Optima | 40°C | 70°C |
| Thermal Stability at 70°C | Up to 50 minutes | Up to 2 hours 30 minutes |
| Optimal time of incubation | 50 minutes | 30 minutes |
| Optimal Substrate concentration | 1.5% | 1.5% |
| Optimal $CaCl_2$ concentration | 5% | 4% |
| Storage stability at 4ºC | Up to 1 day | Up to 12 months |

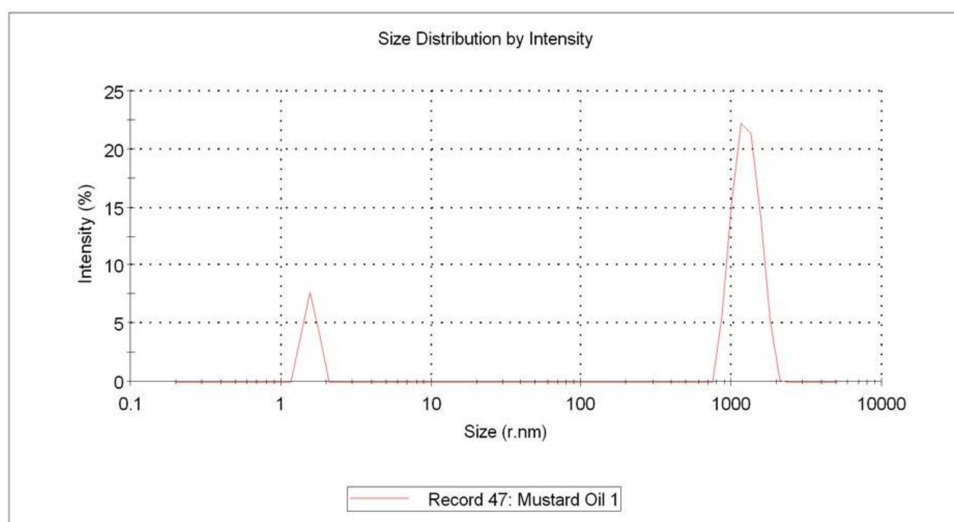


**Figure 1.** DLS of Mustard oil driven emulsified bovine serum albumin nanoparticles of encapsulated *Pennisetum glaucum* (Pearl millet) amylase

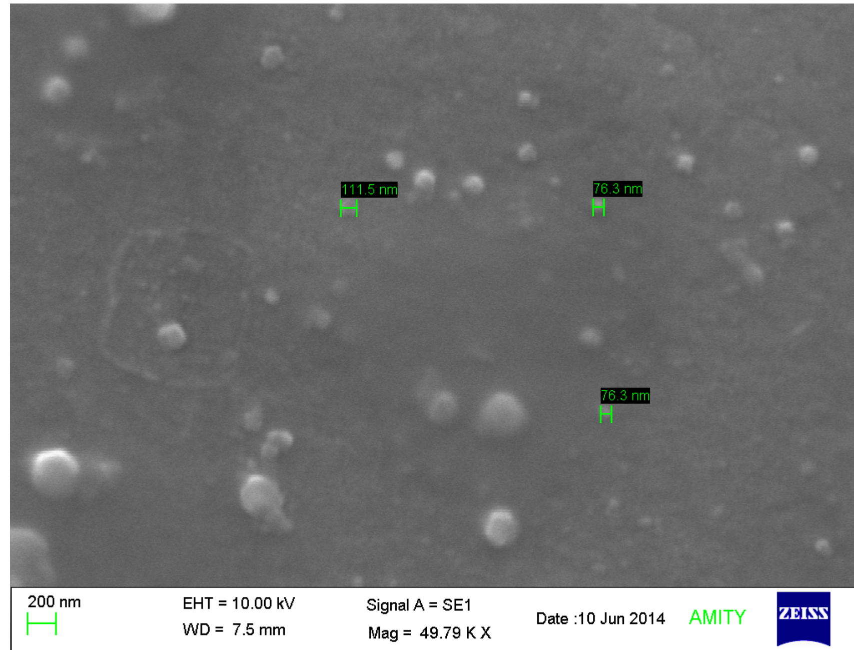


**Figure 2.** SEM of mustard oil driven emulsified bovine serum albumin nanospheres of encapsulated *Pennisetum glaucum* (Pearl millet) amylase

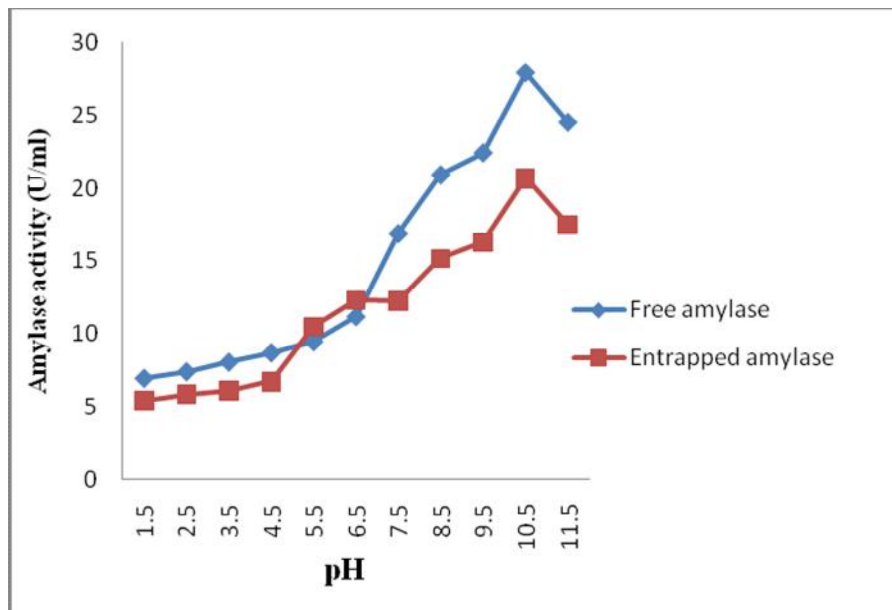


**Figure 3.** Effect of pH on free amylase and mustard oil driven emulsified bovine serum albumin nanospheres of encapsulated *Pennisetum glaucum* amylase

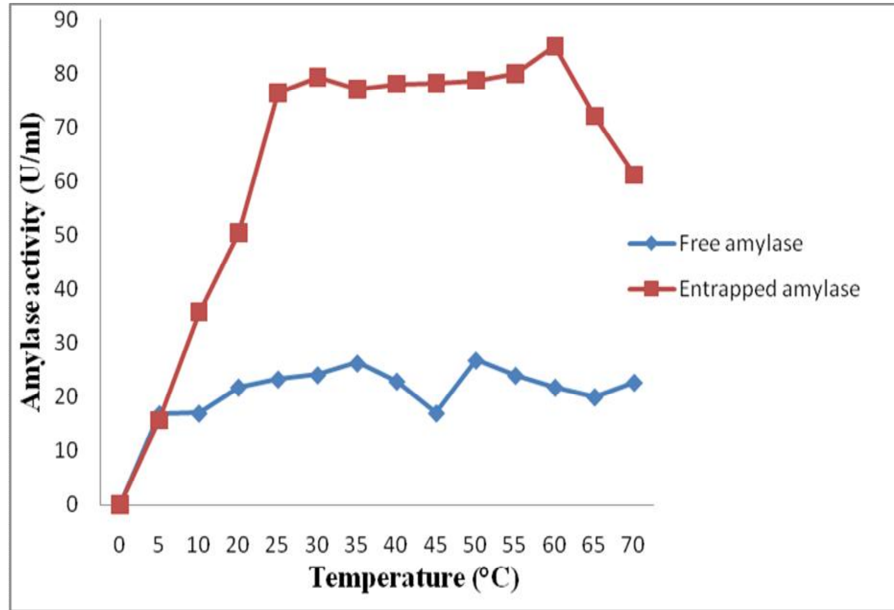


**Figure 4.** Effect of temperature on free amylase and mustard oil driven emulsified bovine serum albumin nanospheres of encapsulated *Pennisetum glaucum* amylase

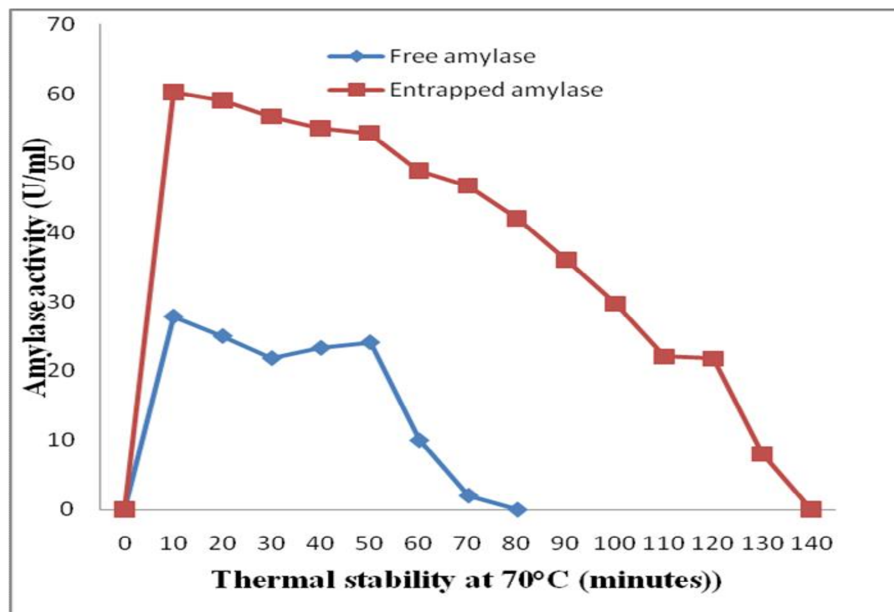


**Figure 5.** Study of thermal stability at 70°C of free amylase and mustard oil driven emulsified bovine serum albumin nanospheres of encapsulated *Pennisetum glaucum* amylase

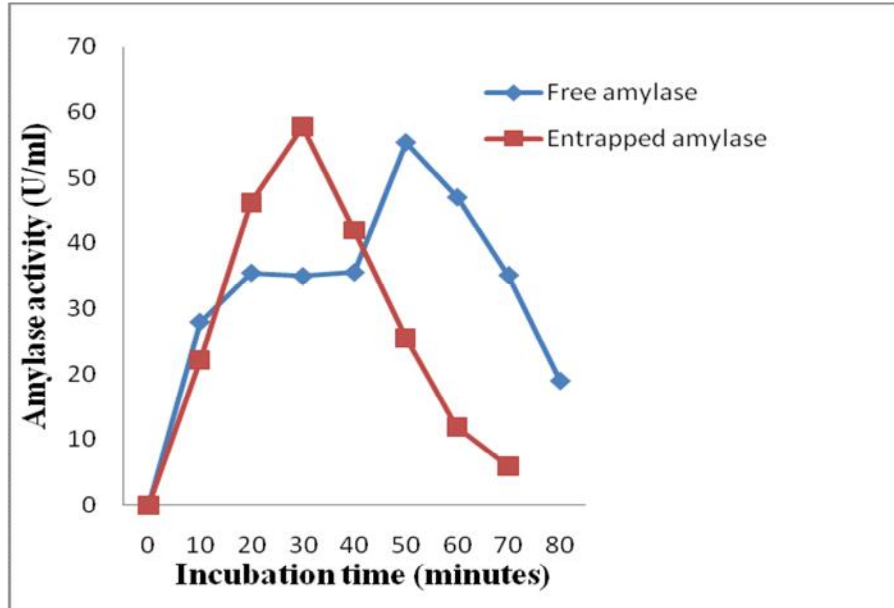


**Figure 6.** Effect of incubation time on activity of free amylase and mustard oil driven emulsified bovine serum albumin nanospheres of encapsulated *Pennisetum glaucum* amylase

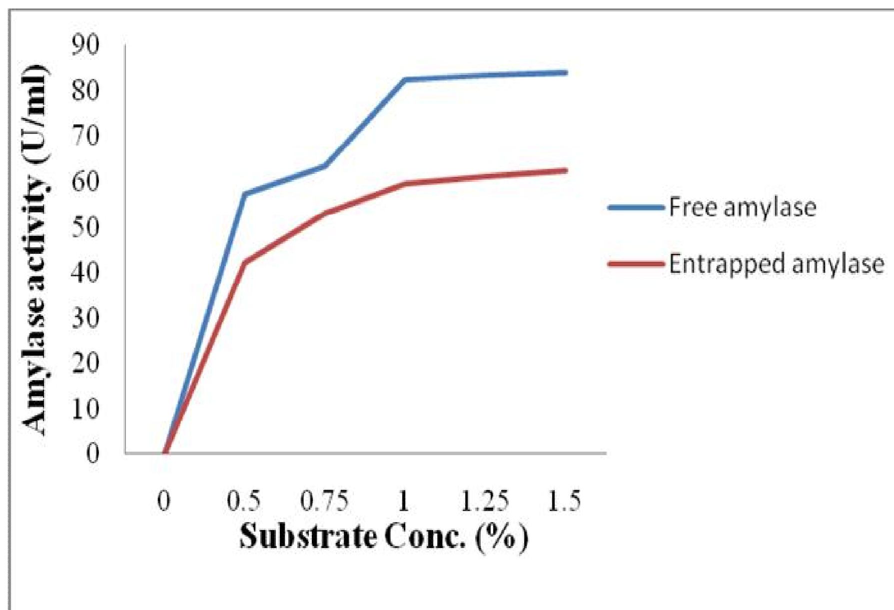


**Figure 7.** Effect of substrate concentration on activity of free amylase and mustard oil driven emulsified bovine serum albumin nanospheres of encapsulated *Pennisetum glaucum* amylase

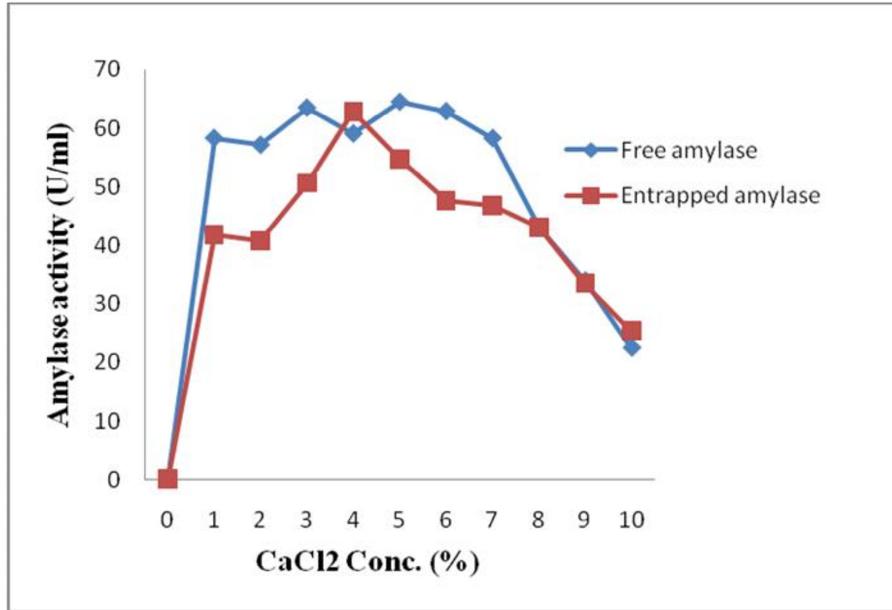


**Figure 8.** Effect of CaCl2 concentration on activity of free amylase and mustard oil driven emulsified bovine serum albumin nanospheres of encapsulated *Pennisetum glaucum* amylase

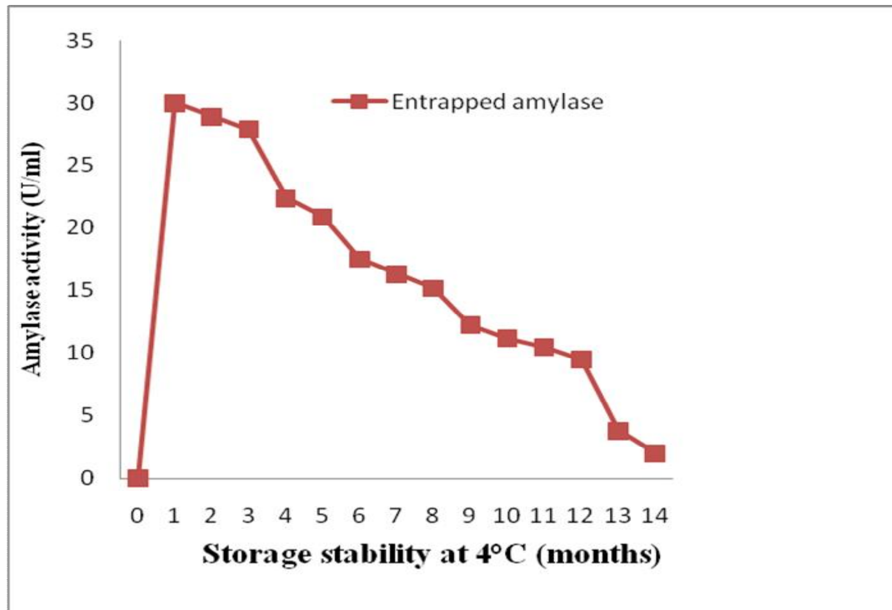


**Figure 9.** Study of storage stability at 4°C of mustard oil driven emulsified bovine serum albumin nanospheres of encapsulated *Pennisetum glaucum* amylase

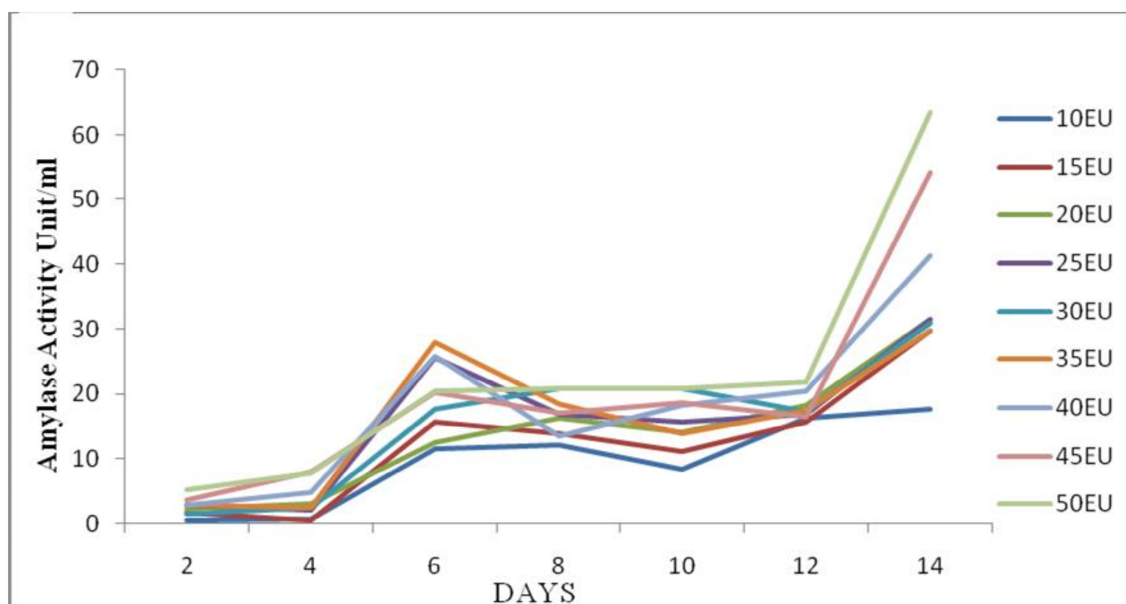


**Figure 10.** Biodegradation analysis of mustard oil driven emulsified bovine serum albumin nanospheres to study controlled release of encapsulated *Pennisetum glaucum* amylase with alkaline protease (10U, 15U, 20U, 25U, 30U, 35U, 40U, 45U, 50U)**EU-Enzyme Units*