Global Journal of Science Frontier Research: G
Bio-Tech & Genetics
Volume 14 Issue 1 Version 1.0 Year 2014
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN:2249-4626 & Print ISSN:0975-5896

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GJSFR-G Classification : *FOR Code: 100303*

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I. Introduction

Nanotechnology has been introduced into several aspects of the food science, including encapsulations and delivery systems, which protect and deliver functional ingredients. Bioactive ingredients such as nutrients, phytochemicals, nutraceuticals, drugs and enzymes may be incorporated into nanoparticles to maximize delivery efficiency and increase desirable benefits (Arnedo et al, 2002). Since nanoparticles are submicron and sub-cellular in size, they have versatile advantages such as increased surface area and reactivity, increased gastric residence time and permeability, and improved solubility in both aqueous and organic phases. Brownian motion can provide enough energy to keep exceptionally small particles agitated and hence precipitation is less likely to happen with nanoparticle suspension. Therefore, suspensions of nanoparticles are easier to stabilise because precipitation is less likely Bovine serum albumin (BSA) was chosen as the material for the particle matrix. BSA has great potential as a nanocarrier in food and pharmaceutical applications. BSA is non-toxic and degradable in vivo, so the nanoparticles generated by using it are easily adaptable to the human body (Jahanshahi et al,2008, Langer et al,2003, Desai et al,1996).The addition of bovine serum albumin (BSA) enhances stabilization both by protection against protease and by stabilization in the low concentration-regime protein.

Many chemical transformation processes used in various industries have inherent drawbacks from a commercial and environmental point of view. In particular, a greater awareness of conservation issues has forced industries to consider alternative, cleaner method. With this regard, the use of enzymes as industrial catalyst is becoming the best option, and enzymes are gradually replacing chemical catalysts in many areas of industry (Karthick Raja Namasivayam and Nirmala,2011).The major classes of enzyme offering immediate application are the hydrolytic enzyme (which account approximately 75% of the industrial enzymes produced (Prasanna ,2005).Through the use of hydrolytic enzymes many different natural and agricultural polymers can be processed and up graded for eventual human or animal consumption, or for further bioconversion in to value added products The ability to commercially produce sugars from starch using amylolytic enzymes is an example of such processes (Ivo safari,1989).

Due to the increasing demand for enzymes in various industries, there is enormous interest in searching for enzymes suitable for application, and their cost effective production techniques (Pandey et al, 1999). α -Amylases are starch degrading enzymes. They are widely distributed in microbial, plant and animal kingdoms. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes. Initially the term amylase was used originally to designate enzymes capable of hydrolyzing a-1, 4-glucosidic bonds of amylose, amylopectin, glycogen and their degradation products (Sodhi et al, 2005, Vishwanathan and Surlikar, 2001). In the present study, nano formulation of alpha amylase with BSA nanoparticles and the improved enzyme activity of nano encapsulated enzyme was studied.

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II. Materials and Methods

a) Preparation of BSA nanoparticles

Simple coacervation technique was implemented for preparation of BSA nanoparticles (Muller et al, 1996). Anhydrous ethyl alcohol was added to 150 ml BSA (5 mg/l in 10 mM Tris/HCl contained 0.02% sodium azide) till the solution became turbid then 150 μl of 25% glutaraldehyde was added for cross linking. The reaction was continued at room temperature (24°C). Ethanolamine was added to block the non-reacted aldehyde functional group. Also Tween-20 was added at a final concentration of 0.01% (v/v) to stabilize the preparation. The suspension is then ultrasonicated for 30 mins. Large aggregates were eliminated by centrifuge (50,000 g, 30 min, 4°C). The pellet is then lyophilized to form fine powder.

i. *Characterization of nanoparticles*

The morphologies of the BSA nanoparticles were observed by scanning electron microscopy (SEM) The sample was sputtered with palladium gold for 30s under Polaron machine (BAL-TEC, Model SCDOOS, Switzerland). Afterwards, SEM was performed with a Carl Zeiss supra 55 (Germany) Field emission scanning electron microscope with the upper detector at 15 kV. The magnification was set at 65,000.

b) Optimization of the nanoparticles preparation

i. *Determination of optimal pH*

The optimization of BSA nanoparticles synthesis was performed over a pH range between 6 and 10. For the analysis, the pH value of the suspension was automatically adjusted by the titration unit by addition of 0.1N hydrochloric acid or 0.1N sodium hydroxide solution, respectively. At 5 predefined pH values between 6 and 10, the yield percentage of the nanoparticles was measured and the particle size was determined by SEM.

c) Determination of optimal acetone to Albumin ratio

The optimization of the BSA nanoparticles preparation based on the acetone concentration used for coacervation process. Ethanol concentration used for the desolvation influences the yield and the particle size of the nanoparticles. Five different ethanol to album in ratios were preselected using earlier works on BSA nanoparticles synthesis. The different ratios include 2:1, 2. 5:1, 3:1, 4:1 and 5:1.optimal ethanol concentration was determined based on the particle size studied using SEM and yield percentage.

d) Determination of Crosslinking Time

The crosslinking of the acetone coacervates takes place after the addition of glutaraldehye. The crosslinking time influences the particle size and yield percentage of the nanoparticles. The cross linking time at a range from 6h to 16h was predetermined and the optimal crosslinking time for the maximum synthesis was determined.

e) Encapsulation of α-Amylase with BSA Nanoparticle

Alpha amylase (analytical grade) was obtained from SRL laboratories as powder form with 60 mu/g enzyme activity and the nano encapsulation with BSA nanoparticles was carried out by simple coacervation method that followed in free BSA nanoparticles synthesis. Analytical grade ethanol was added at a rate of 2ml for every 5 mins to 20 ml of 1% BSA by intermittent method (prepared in deionized water & the pH 9 was adjusted with 0.2M NaoH) and stirrer continuously in magnetic stirrer at 500 rpm till the solution became just turbid. Then 100 μl of 25% glutaraldehyde was added for cross linking. The reaction was continued at room temperature (24°C) for 3 hours. Ethanolamine (100μl) was added to block the non-reacted aldehyde functional group. 10mg of α-amylase was added to the medium and stirrer continuously for 1 hour. Also Tween-20 was added at a final concentration of 0.01% (v/v) to stabilize the preparation. The suspension was then ultracentrifuged (20,000g, 30minutes) for two cycles. The pellet was then lyophilized or keep it in hot air oven for 20 minutes at 55˚C to form fine powder of encapsulated α-amylase enzyme(Enz-A).Morphology of the encapsulated enzyme was characterized by scanning electron microscopy (SEM).

f) Enzyme assay

The activity of Enz-A was assayed using a reaction mixture comprising of 1ml encapsulated enzyme,1ml of 1% (w/v) soluble starch solution in 0.05 m citrate buffer solution (pH 4.5).The reducing sugars liberated were estimated by the 3, 5 Dinitrosalicylic acid (DNS) method. The reaction mixture was incubated at 60˚C for 20 minutes and the reaction was terminated by adding 2ml of DNS in the reaction tube and then immersing the tube in boiling water bath (60˚C) for 15 minutes. The absorbance was measured at 540 nm with spectrophotometer. The Enz-A activity was defined as by the following formula

$$\text{Amylase activity (IU/ml/min)} = \frac{\text{Amount of sugar released x 1000}}{\text{Molecular weight of maltose x Time of incubation}}$$

The enzymatic assay result was compared with the free and the immobilised enzyme assay data obtained from the literature to get the efficiency rate of the encapsulated enzyme (Enz-A).

g) Evaluation of improved enzyme activity of encapsulated enzyme.

i. *Effect of pH*

1ml of the free, immobilized and encapsulated enzyme was incubated with buffer with pH of 3, 4, 5, 6,7,8,9 and 10 respectively at 32C for 10 minutes and the enzyme activity was determined.

ii. *Effect of Temperature*

1ml of the free, immobilized and encapsulated α-amylase enzyme was incubated with different temperature ranged from 30°C, 40 °C, 50 °C, 60 °C, 70°C, 80°C, 90°C and 100°C respectively for 10 minutes. The enzyme assay was carried out as described earlier.

iii. *Effect of substrate concentration*

1ml of the free, immobilized and encapsulated enzyme was incubated with different concentration of starch solution (1 to 10mg/ml), incubated at 32°C for 10 minutes and the enzyme activity was determined.

h) Kinetic parameters of encapsulated enzyme

0.5 ml aliquot of starch solution(100- 1000 mg/ml) was mixed with equal volume of free, imkobilized and encapsulated enzyme ,incubated 32°C for 10 minutes and 1ml of GOD-POD reagent was added.

III. Result and Discussion

The strategy to control size was to adjust BSA concentration, pH, ionic strength, crosslinking reaction rate and agitation speed in the desolvating conditions. BSA nanoparticles formed in different sizes at the above conditions since the desolvation process was influenced by the electrostatic attraction and repulsion among BSA molecules (Fig 1 to 8 a).

a) Determination of optimal pH

Optimisation of pH condition for BSA nanoparticle at the target size of 100nm plans are shown in table 1.Size control of BSA nanoparticles was conducted by modifying desolvation methods. The particle size was controlled by adjusting pH which affects the coagulation of the BSA molecules. The isoelectric point (pI) of BSA is about 4.9. When pH shifts toward the pI, the enhanced protein–protein interactions increase coagulation among BSA molecules, larger BSA particles was formed. On the other hand, the enhanced protein–solvent (or water) interactions decrease coagulation when pH was far from the pI. pH 9 provides a highly electrostatic repulsive condition for the BSA molecules and concomitantly, coagulations by protein–protein interactions are limited, as a result in table 1,2, fine BSA particles with required target mean size was formed.

b) Determination of optimal Acetone to Albumin ratio

Optimization of volume consumptions of acetone for the BSA nanoparticle preparation at target size of 100nm.The amount of acetone for the 100 nm targeted BSA nanoparticle preparation was 35 ml. Consumption of acetone was inversely proportional to the targeted size.

c) Determination of Crosslinking Time

Crosslinking incubation time on BSA nanoparticle preparation was optimised as in table 1. It was Influences the cross linking processes; as a result different mean size of BSA nanoparticles was formed. The target size of 100nm was achieved for 3hrs incubation from source 4)

d) Determination of optimal BSA concentration

The effect of BSA concentration on BSA nanoparticle size was conducted as the concentration of BSA in creased the particle size was also increased. The target particle mean size of (115.6nm) was achieved at the concentration of 10mg/ml.

e) Determination of optimal agitation rate

Agitation speed that in terfere with the target size of the BSA nanoparticle, mean size of particle (115.6 nm) close to the target size was obtained at agitation speed of 500 rpm.

f) Surface-area-to-volume-ratio of BSA nanoparticles

The greater surface-area-to-volume-ratio of BSA nanoparticles particle than rest of the sources was found in source 4 parameter. Surface area is a material property of particles that is often a determining factor in bioavailability, dissolution rate, adsorption, catalyst activity, and toxicity (Oberdorster et al, 1996).The surface area is a more useful and accurate metric than concentration or dosage, which is traditionally believed to be the most important .The concept of total surface area can be used to describe the surface area in a sample. One such is the surface-area-to-volume-ratio, and is more useful than the concentration or the mean diameter of particles. The surface-area-to-volume ratio is the amount of surface area per unit volume of particles. The surface-area-to-volume ratio can be calculated using size distribution data of particles under that all particles are spheres. Table 2 shows the surface-area-to volume-ratios of BSA nanoparticles of target size 100 nm. The particles for the 100 nm plan obtained the largest surface area in source 4, whereas those from sources 1, 2, 3, & 5 had a relatively small surface area. In any case, the surface-area-to-volume-ratio can be used to represent a major physical property of the particles instead of diameter-related values Furthermore; the surface-area-to volume-ratio is useful for comparative studies of nano-scaled materials.

g) Encapsulation of α-Amylase Enzyme with BSA Nanoparticle.

The encapsulation of α-Amylase Enzyme with BSA Nanoparticle was done based on hydrophobic ion pairing (HIP) complexation. And the Scanning electron micrographs of BSA nanoparticle encapsulated enzyme

(Enz-A) at the targeted size of 100nm by Intermittent Method was shown in figure 9.

h) Kinetic Analysis for Encapsulated (Enz-A)

Kinetic parameters of Enz-A with the free and immobilized α-amylase were measured. For these forms of α-amylase enzyme activity, Michaelis-Menten type kinetic behavior was observed. The Km and V max values as determined from the figure 10 found to be 3.2 mg/ml and $1.89x10^{-3}$ μ mole/ml. s (for Enz-A), 3.8 mg/ml and $1.46x10^{-3}$ μ mole/ml. s (for free enzyme, 5.9mg/ml and $1.58x10^{-3}$ μ mole/ml. s. Therefore, for Enz-A form of α-amylase Km value was decreased and the V max was increased as compared to free and immobilised form. The Km value shows the affinity of enzyme for its substrate. Lower the Km value more is the affinity of enzyme for its substrate.

i) Effect of pH

Effect of pH on activity of Enz-A, free and immobilized α-amylase was given in fig 11.Optimum pH values were 8, 7 and 6 for Enz-A, Free and immobilized α-amylase respectively. This shift in optimum pH could be resulted from the change in acidic and basic amino acid side chain ionization in the microenvironment around the active site. The pH is one of the major parameters capable of shifting enzyme activities in reaction mixture, encapsulation and Immobilization usually results in shift of optimum pH due to conformational changes in enzymes

j) Effect of Temperature

The activity of enzyme is also strongly dependent on temperature. The activity of Enz-A, free, Immobilised α-amylase increased with temperature and maximum activity was observed at 80°C,50°C,60°C as shown in fig.12.The optimum temperature of α-amylase was shifted to 60°C after immobilization in calcium alginate beads and 80°C for Enz-A. As was evident from the fig 12, α-amylase after encapsulation possessed temperature /resistance than the free enzyme and Immobilised enzyme

k) Effect of substrate

The activity of Enz-A, free, Immobilised α-amylase enzyme based on substrate concentration was varied with respect to Km value as shown in fig.13.. The increased activity facilitated by Enz-A within low substrate concentration than the Free and immobilised α-amylase enzymes was observed. The Km value was furnished with low numbers represents strong activity even in low concentration of substrate was observed The optimum activity with respect to substrate concentration for Enz-A, free and Immobilised α-amylase was 4,5 and 8 mg/ml

l) Effect of Incubation Time

The enzymatic activity of Enz-A, free, Immobilised α-amylase enzyme was varied with respect to time of incubation. The retained activity facilitated by Enz-A with an incubation time of 14 hrs. followed by immobilised and free α-amylase enzymes at a rate of 12 and 10 respectively The present study clearly revealed the biocompatible bovine serum albumin nanoparticles encapsulated alpha amylase would suggest the possible utilization of enzyme preparation with improved activity.

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Table 1 : Optimization of the parameters for the BSA nanoparticles at the target size of 100nm

S.no	*Parameters*	*Source 1*	*Source 2*	*Source 3*	*Source 4*	*Source 5*
1.	Ph	6	7	8	9	10
2.	BSA in (%)	5	4	3	2	1
3.	Acetone volume(in ml)	21	20	25	35	30
4.	Crosslinking incubation time(in hrs)	15	12	9	3	6
5.	RPM	200	300	400	500	600
6.	Mean size of the particle (in nm)	379.8	352.9	286.4	115.6	130.1

Table 2 : Mean size and surface-area-to-volume-ratio of BSA nanoparticles at the targeted size: 100nm

Target size (nm)	Method	Mean size (nm)	Surface-area-to-volume ratio
100	Intermittent Method	115.06	$6.62x10^{10}$
	Continuous Method	137.06	$1.58x10^{11}$

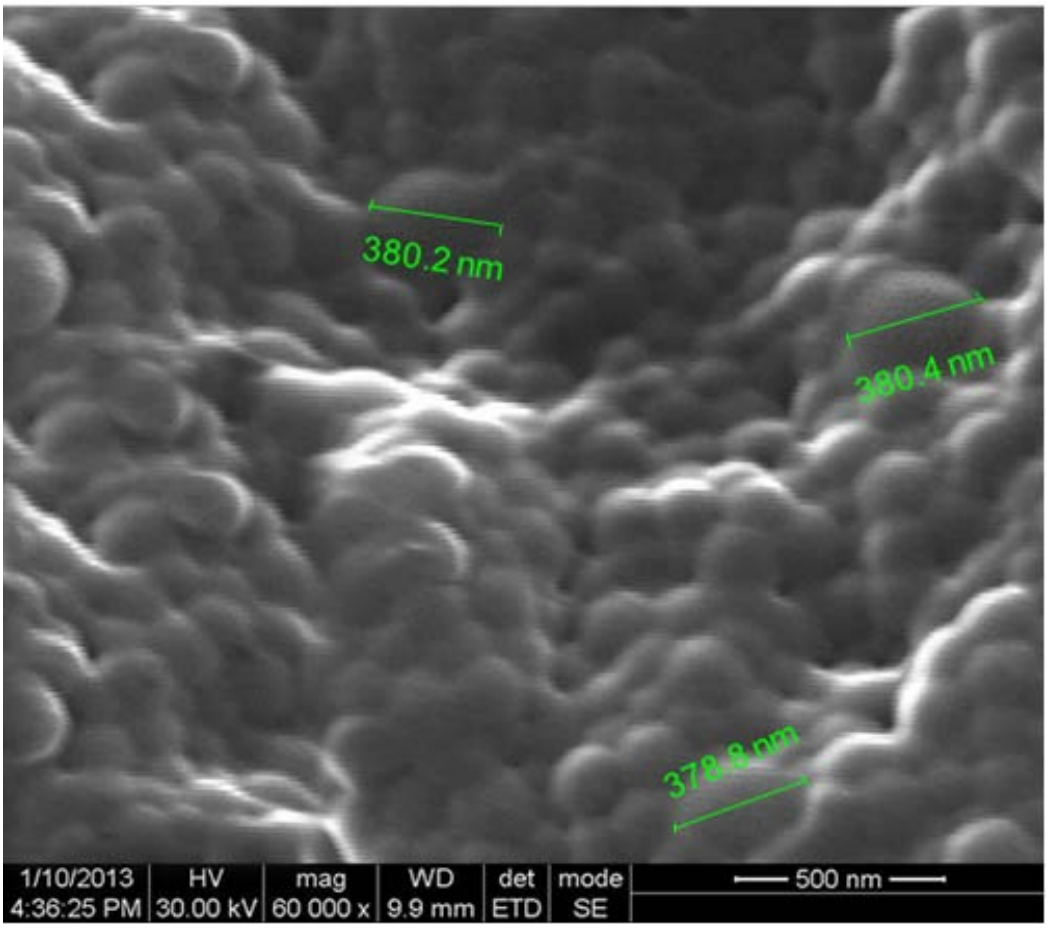
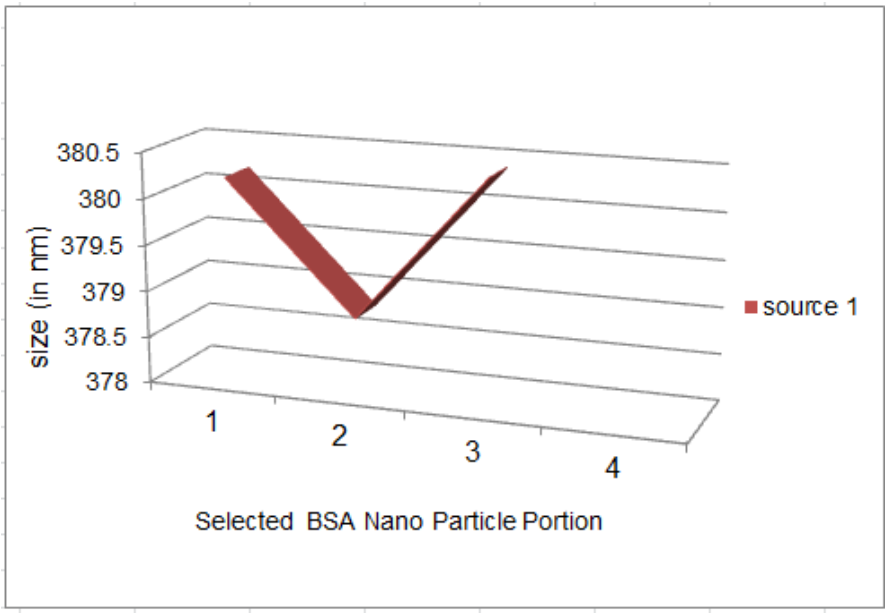


Figure 1 : Scanning electron micrographs of BSA nanoparticles at the targeted size of 100nm by Source 1 parameter



Graph 1 a : The optimisation of size of BSA nanoparticles by source 1 parameter

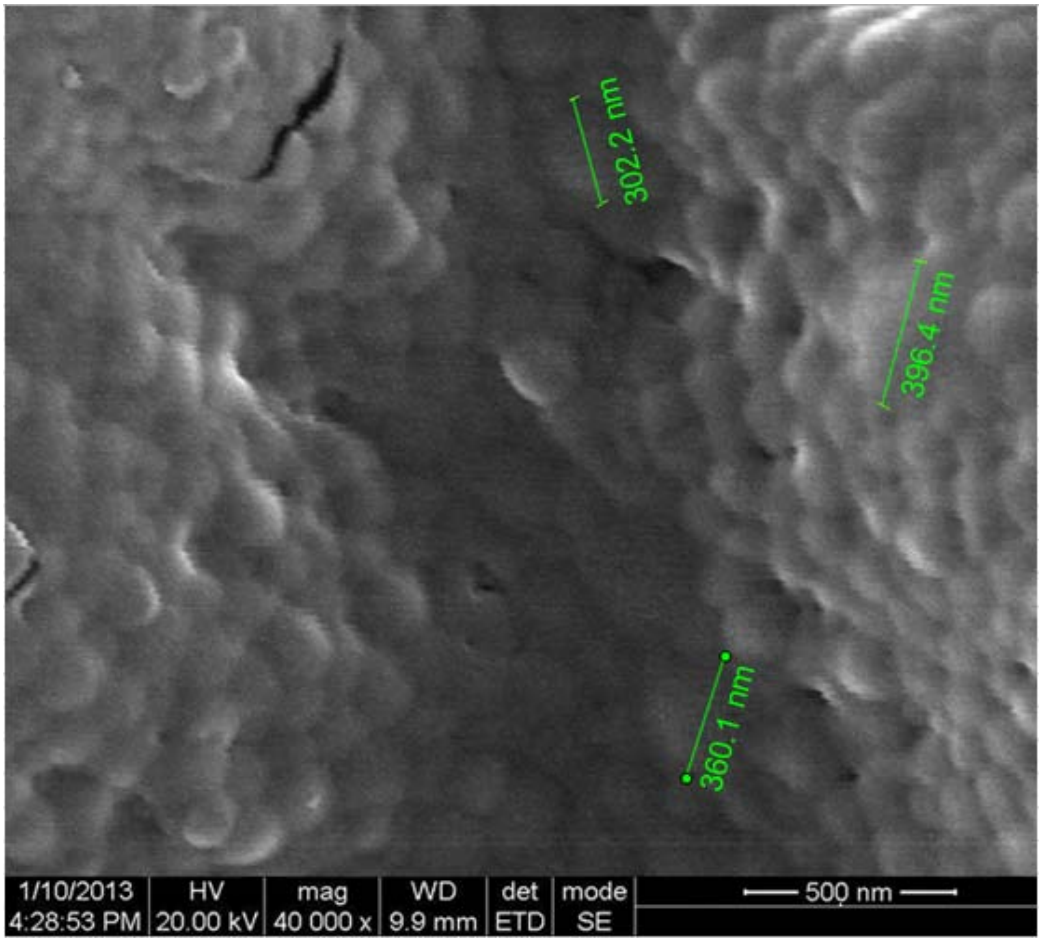
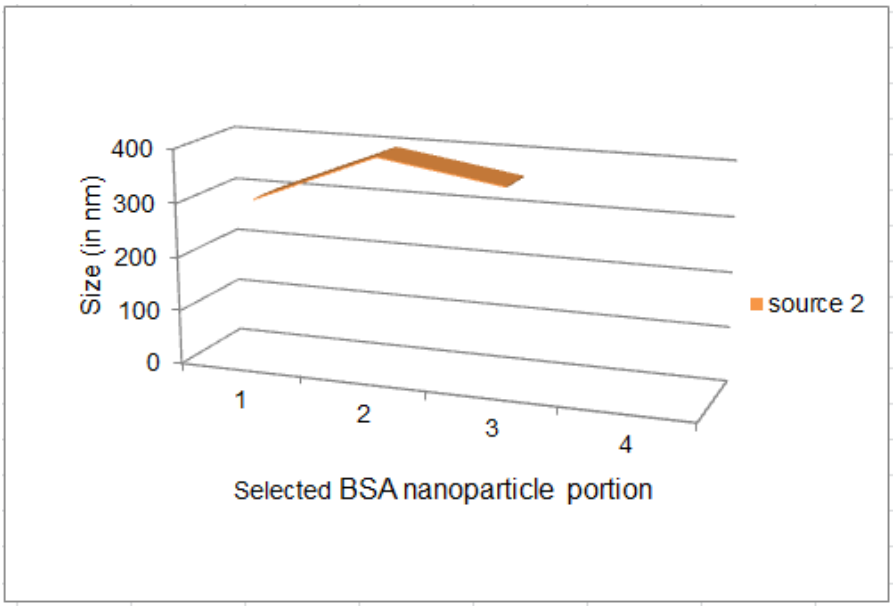


Figure 2 : Scanning electron micrographs of BSA nanoparticles at the targeted size of 100nm by Source 2 parameter



Graph 2 a : The optimisation of size of BSA nanoparticles by source 2 parameter

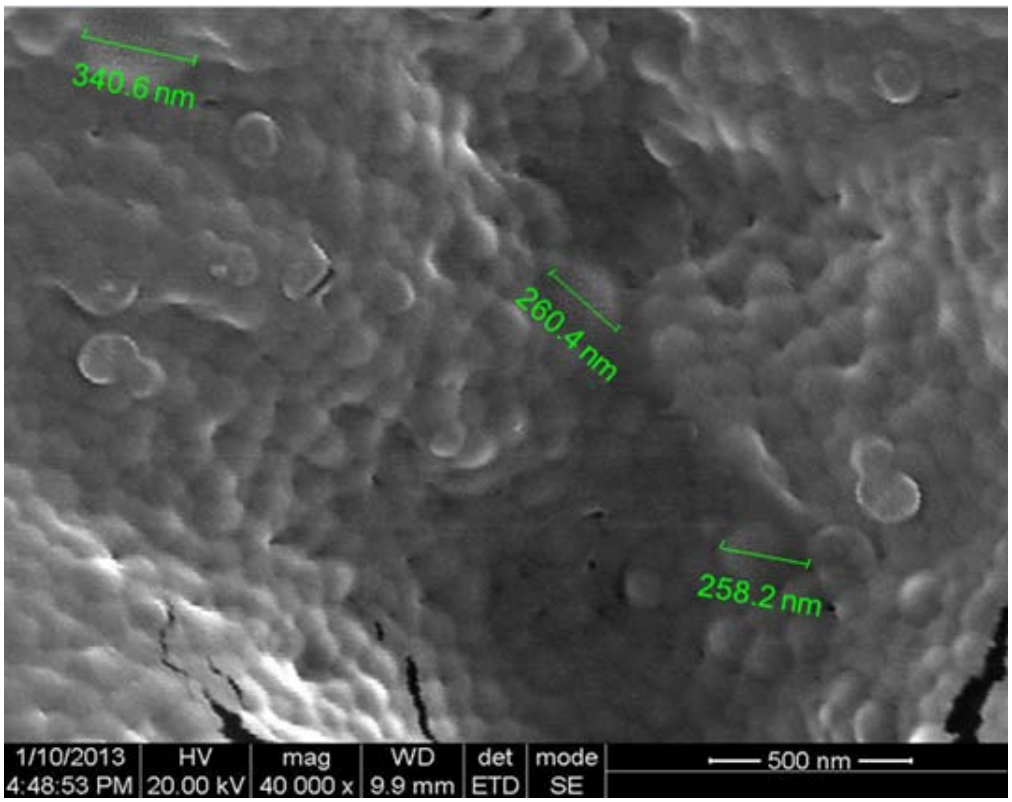


Figure 3 : Scanning electron micrographs of BSA nanoparticles at the targeted size of 100nm by Source 3 parameter

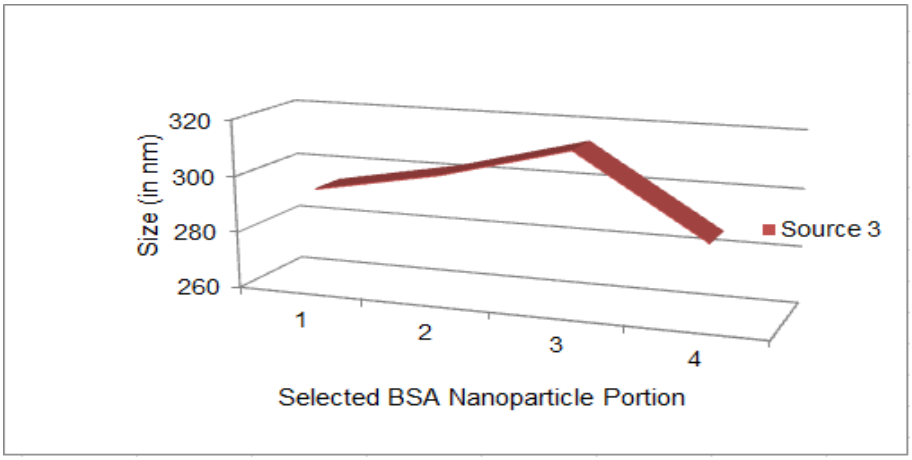


Figure 3 a : The optimisation of size of BSA nanoparticles by source 3 parameter

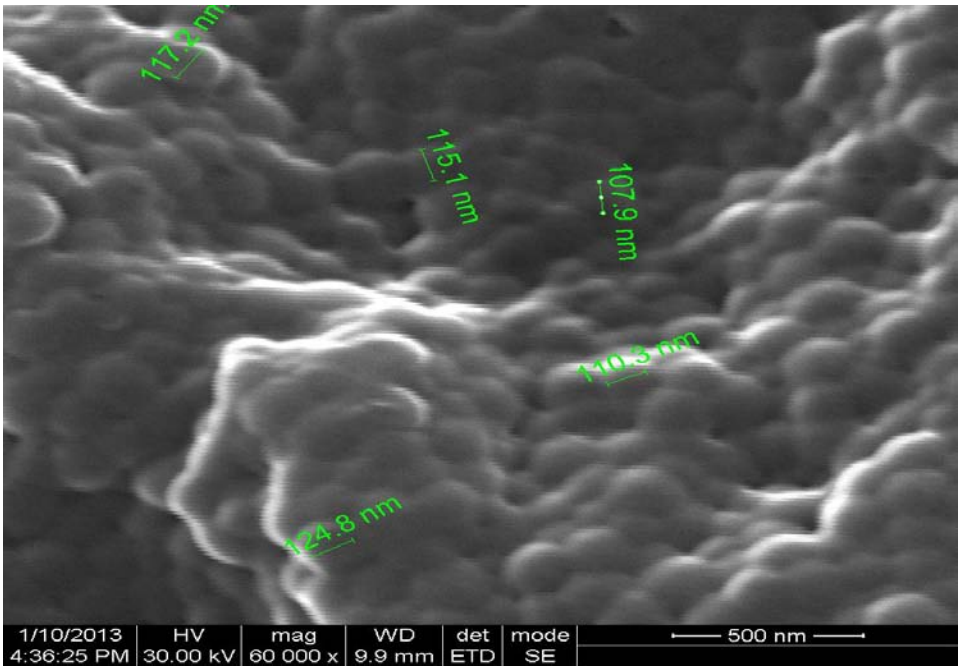


Figure 4 : Scanning electron micrographs of BSA nanoparticles at the targeted size of 100nm by Source 4 parameter

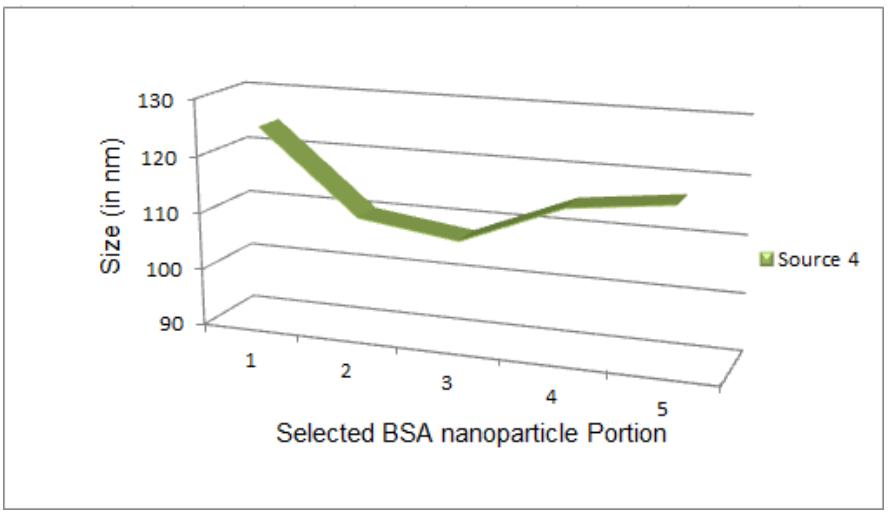


Figure 4 a : The optimisation of size of BSA nanoparticles by source 4 parameter

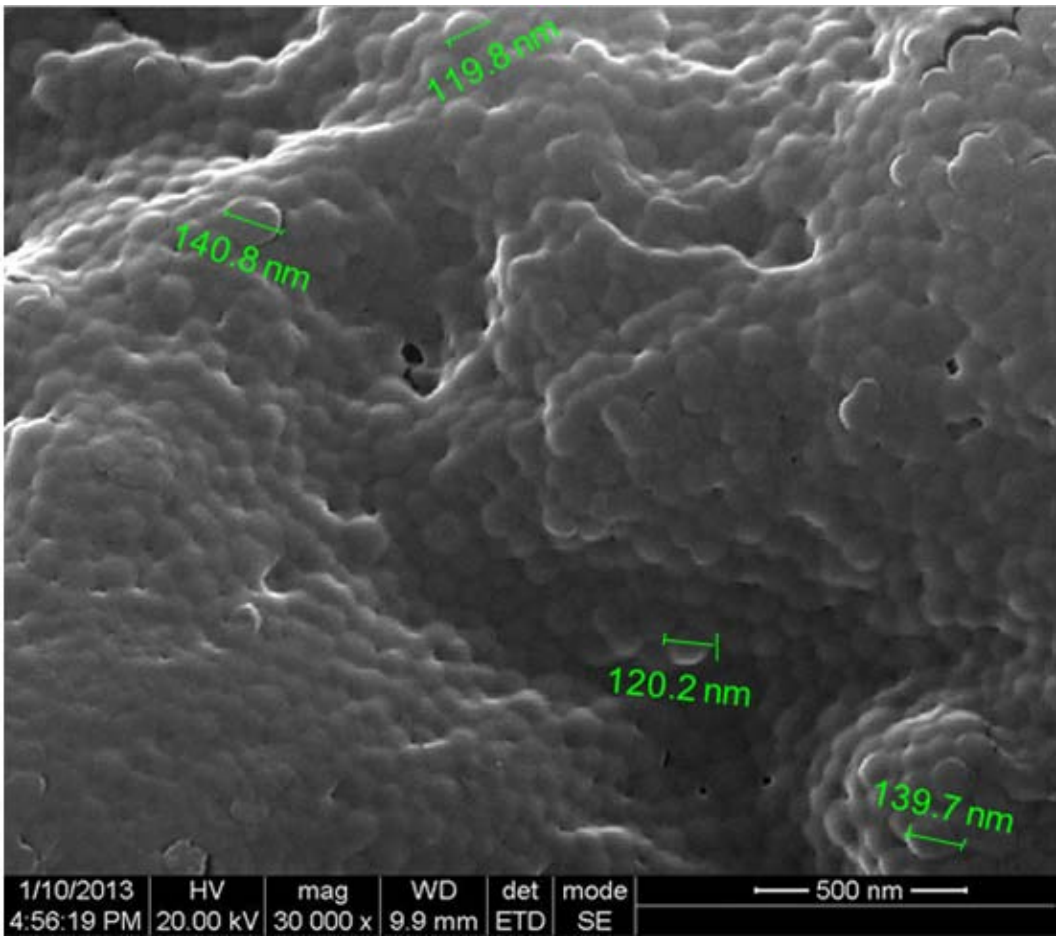


Figure 5 : Scanning electron micrographs of BSA nanoparticles at the targeted size of 100nm by Source 5 parameter

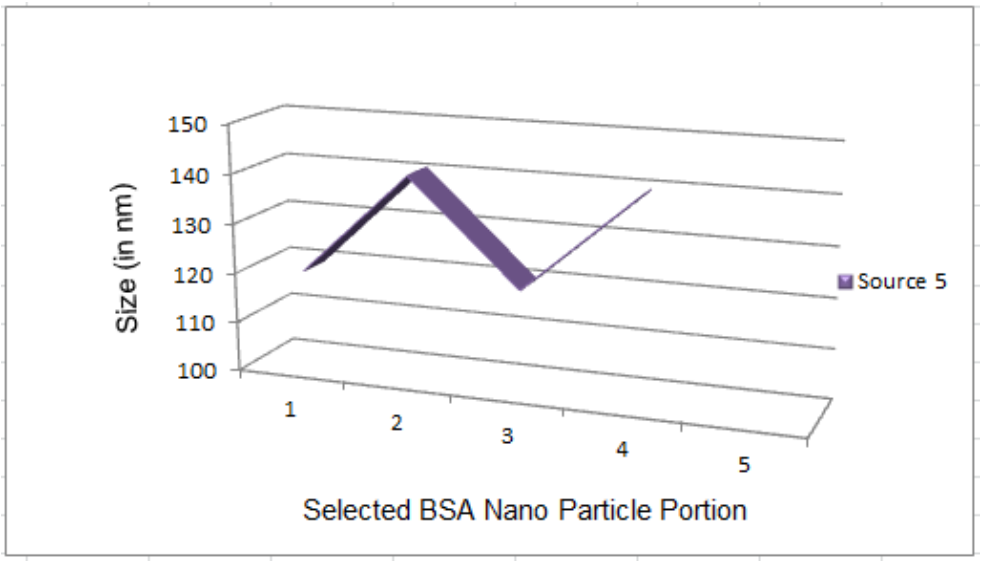


Figure 5 a : The optimisation of size of BSA nanoparticles by source 5 parameter

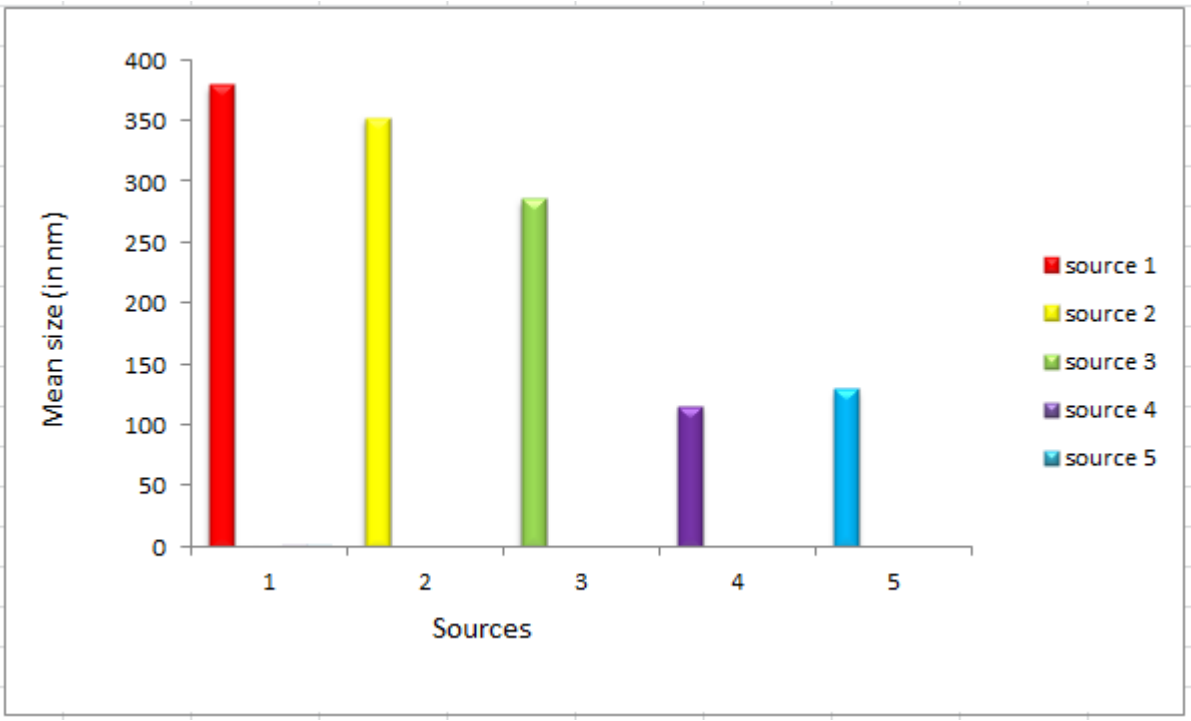


Figure 6 : Mean size of BSA nanoparticles by different sources of parameter

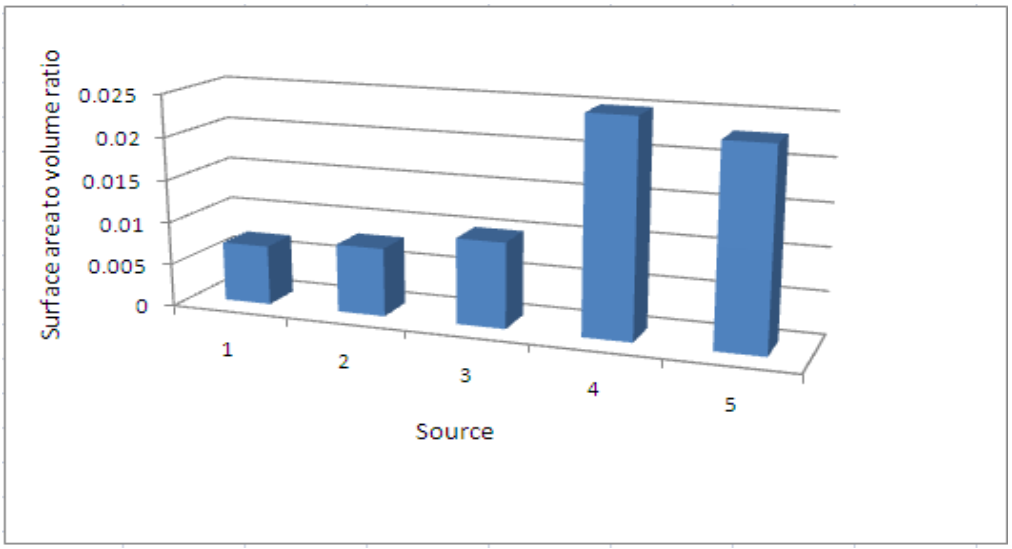


Figure 6 a : Surface-area-to-volume-ratio of BSA nanoparticles from different sources at the targeted size of 100nm

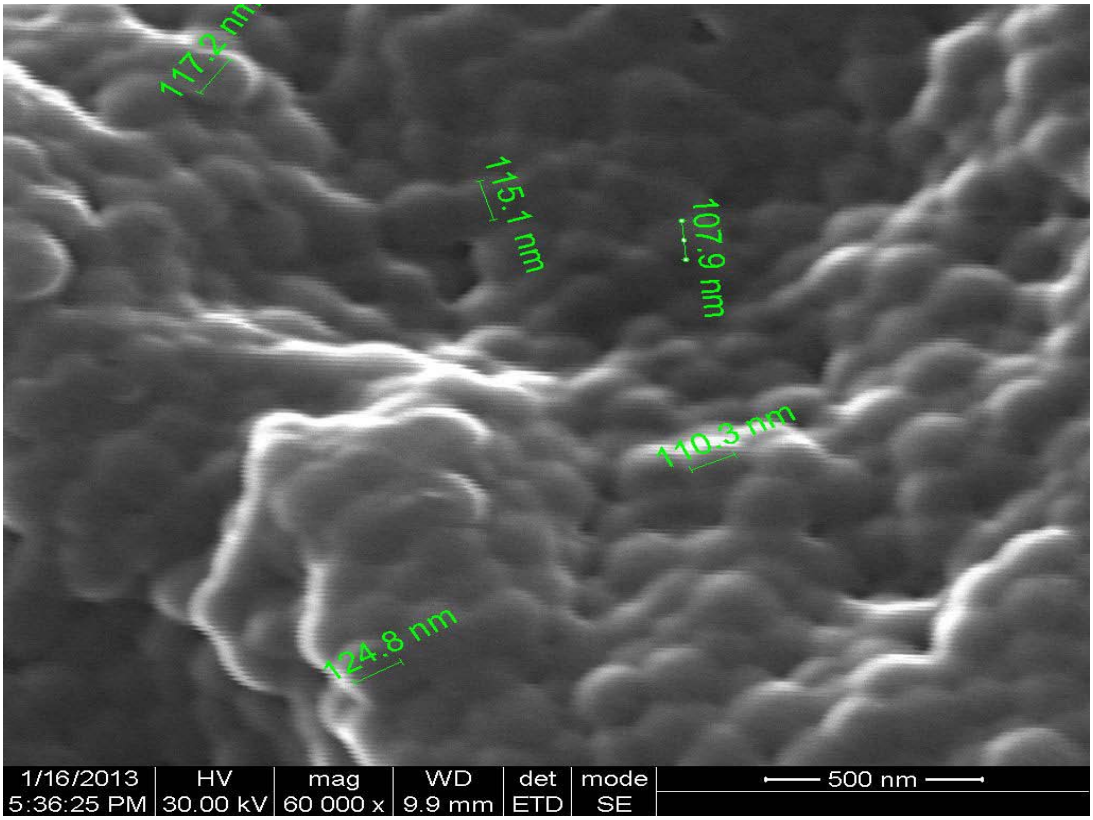


Figure 7 : Scanning electron micrographs of BSA nanoparticles at the targeted size of 100nm by Intermittent Method.

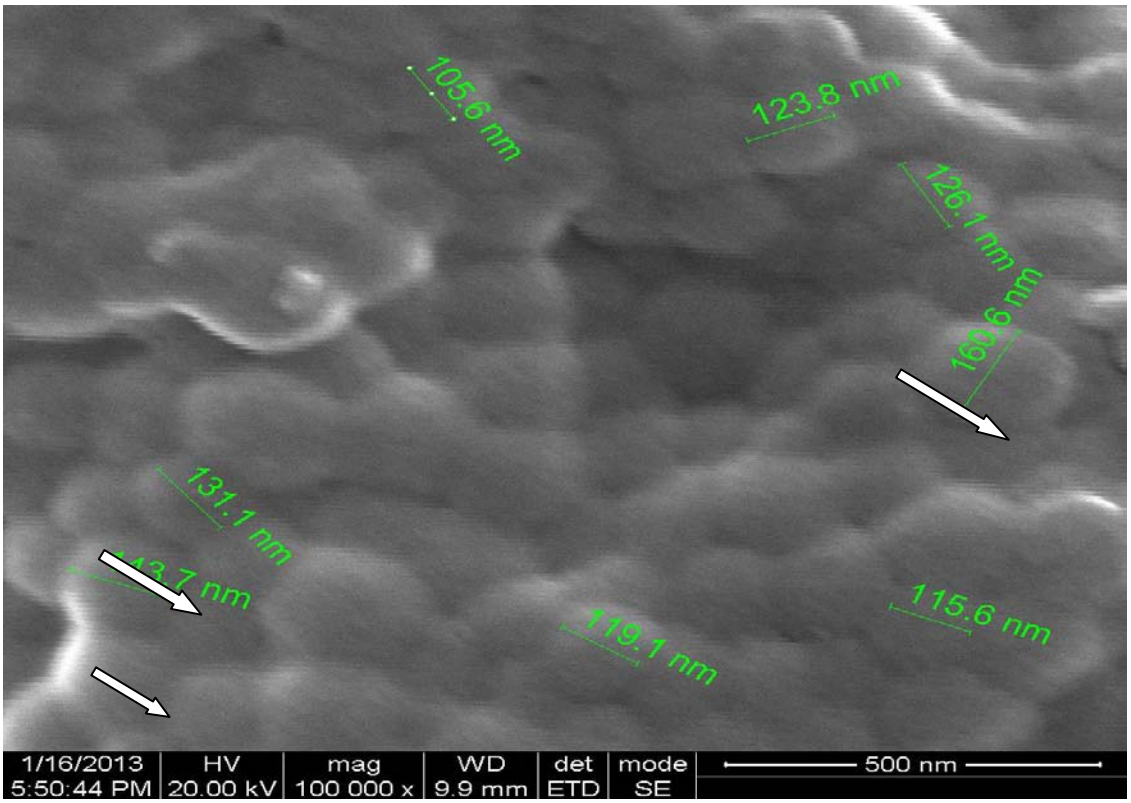


Figure 8 : Scanning electron micrograph of BSA nanoparticles at the targeted size of 100nm by Continuous Method

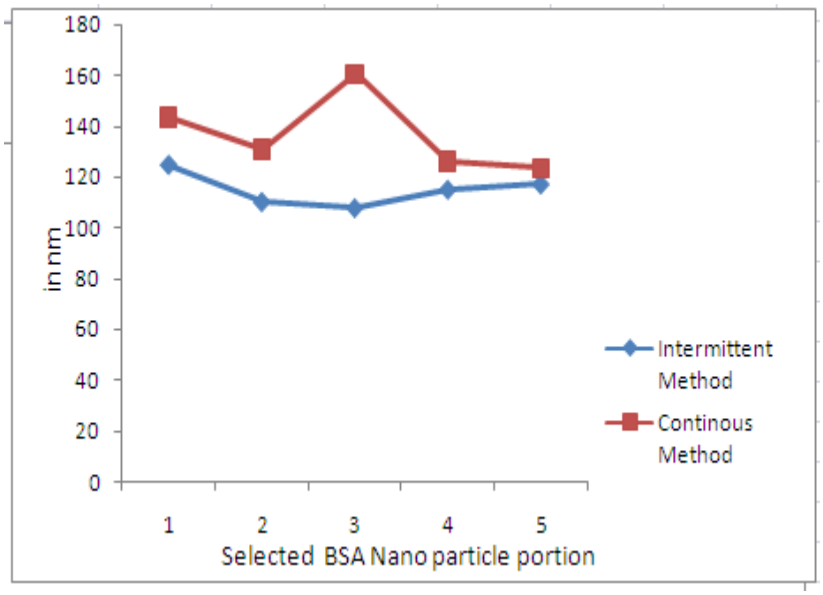


Figure 8 a : The Size of BSA Nanoparticles by the Intermittent and Continuous Method

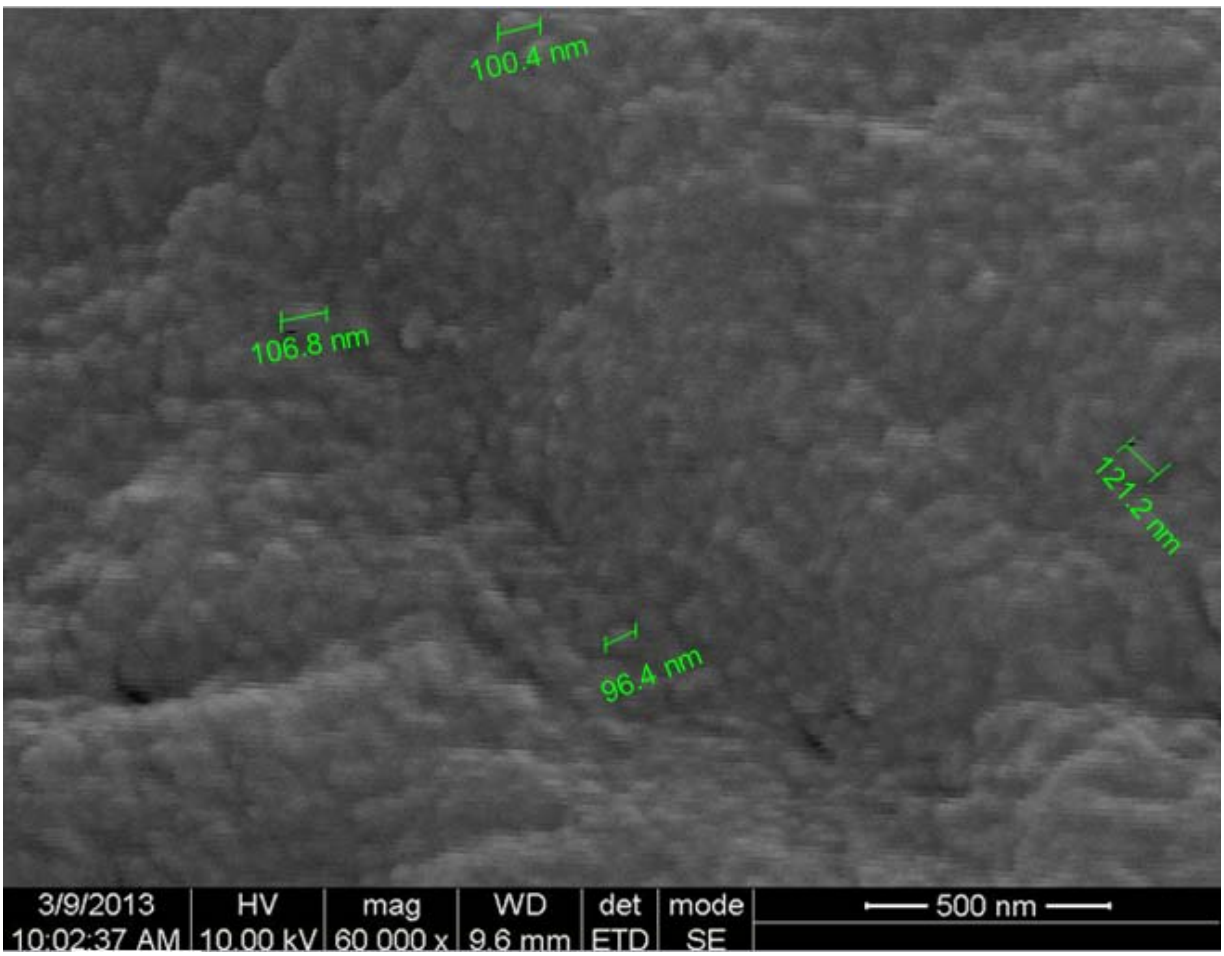


Figure 9 : Scanning electron micrographs of BSA nanoparticle encapsulated enzyme (Enz-A) at the targeted size of 100nm by Intermittent Method.

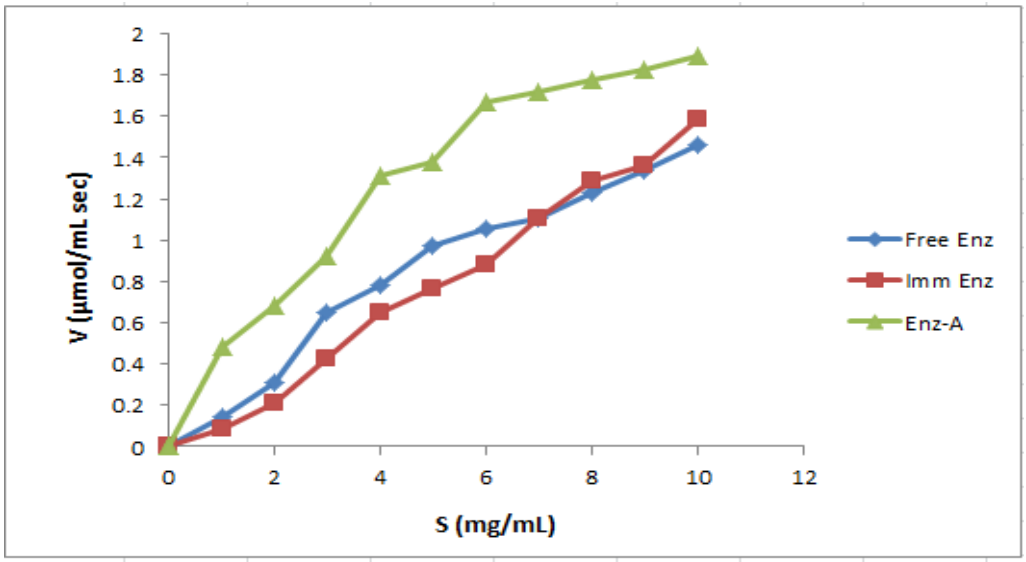


Figure 10 : Kinetic analysis of Enz-A with free and immobilised α-amylase enzyme

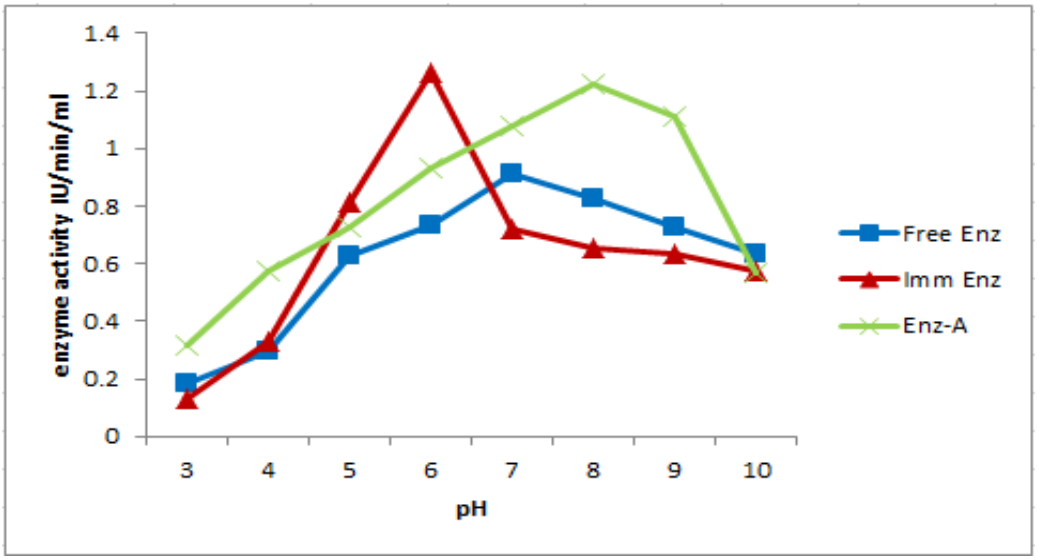


Figure 11 : Effect of pH on activity of Enz-A, Free and Immobilized α-amylase

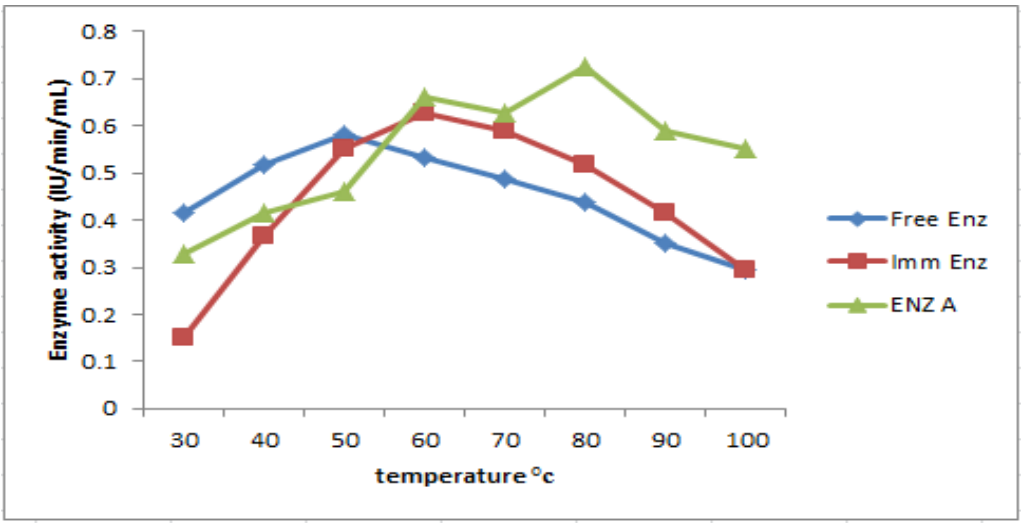


Figure 12 : Effect of temperature on activity of Enz-A, Free and Immobilized α- amylase enzyme

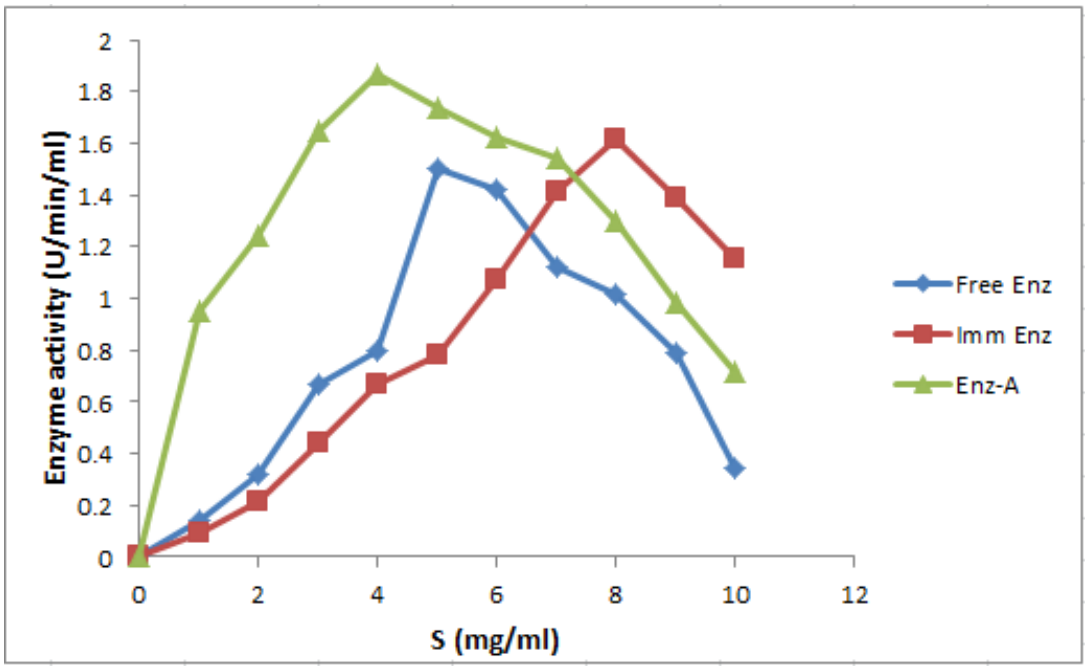


Figure 13 : Effect of substrate on activity of Enz-A, Free and Immobilized α- amylase enzyme

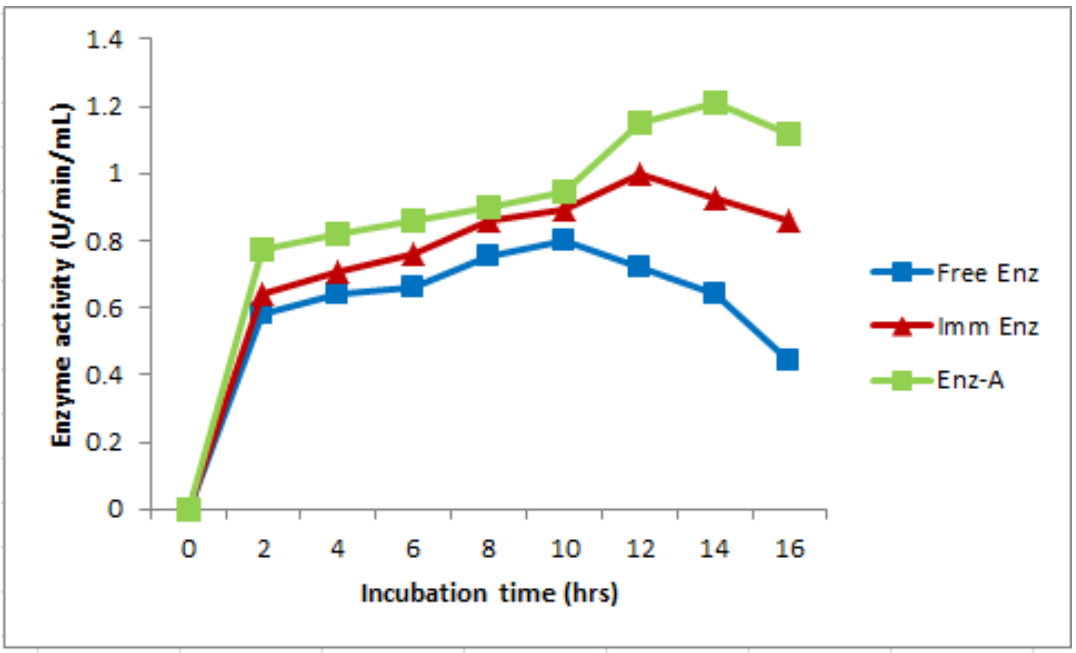


Figure 14 : Effect of incubation time on activity of Enz-A, Free and Immobilized α- Amylase enzyme

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