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Comparative study on Enhanced Activity of Bovine Serum Albumin Nanodriblet Ensnared Textile Industrial Important Enzymes through Biocompatibility.

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ABSTRACT

In this Present work, bovine serum albumin (BSA) nanodriblets were prepared using a modified desolatory method. BSA nanodriblets were fabricated at the targeted magnitude of 100nm. The mean diameters of the prepared BSA nanodriblet were 382.9, 355.9, 289.3, 105.6, 132.1nm with respect to optimisation factors of framework. The surface area-to-volume-ratios of the prepared BSA nanodriblet were 0.006, 0.008, 0.010, 0.024 and 0.020.The magnitude and the surface-area-to-volume-ratio of the BSA nanodriblet were controlled by adjusting BSA concentration, pH, and NaCl content, which affected the coagulation of the BSA molecules. The surface-area-to-volume-ratio is a more useful parameter than the mean diameter of driblet for comparing effectiveness of nanodriblet. After the successful BSA nanodriblet preparation, the Textile industrially important enzymes such as Pectinase and Protease were Ensnared to facilitate its improved activity were compared like temperature resistant,pH survivability, overcome the effect of inhibitors observed through the serious of results that are obtained from various comparative framework and enzyme assays which gives rise to much greater extent in the wide field of industrial applications and significant role on cost effective to get the desired products in much conservative way using BSA nanodriblet Ensnared enzymes.

Keywords: BSA nanodriblet, Pectinase, Protease, Ensnared, SEM

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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 (Rhaese, von briesen, rubsamen-waigmann, kreuter & Langer, 2003). 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 nanodriblet suspension. Therefore, suspensions of nanodriblet 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 (Arnedo.A, 2002) 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 (Jahashahi, Najafpour, & Rahimnejad, 2008,).The addition of bovine serum albumin (BSA) enhances stabilization both by protection against protease and by stabilization in the low concentration-regime protein.

The desolatory process has been successfully used to prepare nanoparticles using food-grade polymeric materials such as BSA. Desolatory is a thermodynamically driven self-assembly process for polymeric materials. The addition of desolatory agents such as ethanol or acetone separates and coacervates the polymeric molecules in the aqueous phase (Soppimath, Aminabhavi, Kulkarni, & Rudzinski, 2001b).Recently, desolvation process has been successful to prepare nanoparticles, but these methods indicated unreliable reproducibility is important. In the preparation of BSA nanoparticles, pH and salt affect the aggregation of protein molecules. The pH and salt are important factors in size controlling. In the preparation of protein nanoparticle using modern desolatory method (Ji Yeon Jun, Feb 2011), facilitated by two distinct steps to get more sensitive reproducibility of BSA nanoparticle through continuous and intermittent method.

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 methods (Rao et al 1998). 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 (Smith 1996).

The major classes of enzyme offering immediate application are the hydrolytic enzymes (Kirk et al 2002), which account approximately 75% of the industrial enzymes produced (Lowe, 2002). 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 (Fogarty and Kelly 1990.

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 (Burhan et al 2003).

Pectinases have been used in conjunction with amylases, lipases, cellulases and hemicellulases to remove sizing agents from cotton in a safe and ecofriendly manner, replacing toxic caustic soda used for the purpose earlier. Bioscouring is a novel process for removal of noncellulosic impurities from the fiber with specific enzymes. Pectinases have been used for this purpose without any negative side effect on cellulose degradation.

Protease is of commercial value and various industrial applications. They are widely used as detergent, in food, pharmaceutical and leather tanning industries.The vast variety of proteases, with their specificity of their action and application has attracted worldwide attention to exploit their physiological as well as biotechnological applications.It has been considered as eco-friendly because the appropriate producers of these enzymes for commercial exploitation are non-toxic and non- pathogenic that are designated a safe(Saraswathy et al.)

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MATERIALS AND METHODS

Reagent preparation

- 50 mM Sodium Acetate Buffer, pH 4.5 at 30°C (Prepare 50 ml in deionized water using Sodium Acetate, Trihydrate, Adjust to pH 4.5 at 30°C with 1 M HCl.)
- 1.0% (w/v) Xylan Substrate Solution (Xylan) (Prepare 5 ml in Reagent A.
- 0.05% (w/v) Bovine Serum Albumin (Enz Diluent) (Prepare 25 ml in Reagent A.
- Ensnared Protease Enzyme (QEZ-2) Solution (Immediately before use, prepare a solution containing 5 - 10 units/ml of Protease in cold Reagent C.)
- 16 mM Copper Sulphate, 1.3 M Sodium Sulphate,226 mM Sodium Carbonate, 190 mM Sodium Bicarbonate and 43 mM Sodium Potassium Tartrate Solution (Copper Solution)(Prepare 1 litre in deionized water)
- 40 mM Molybdic Acid, 19 mM Arsenic Acid and 756 mM Sulphuric Acid Solution (Ars-Mol)(Prepare 1 litre in deionized water).
- 1 mg/ml Xylose Standard Solution (Prepare 10 ml in deionized water)

Iodine solutions

- 1g of iodine and 3 g of potassium iodide in 100ml of water.

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Optimization of the BSA nanodriblet's Preparation:

S.no	Framework	Factor1	Factor2	Factor3	Factor4	Factor5
1.	pH	6	7	8	9	10
2.	BSA in (%)	5	4	3	2	1
3.	Acetone volume(in ml)	20	19	24	34	29
4.	Crosslinking incubation time(in hrs)	16	13	10	4	7
5.	RPM	250	350	450	550	650
6.	Mean size of the Driblet (in nm)	382.9	355.9	289.3	105.6	132.1

Table 1: Escalation of preparation condition for BSA nanodriblet at theTarget size of 100nm

Determination of optimal pH

- The optimization of BSA nanodriblet's synthesis was performed over a pH range between 6 to 10 with the preferred components as shown in table no.1.
- 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.
- The effect of pH range over the target size (100nm) of the BSA nanodriblet preparation was analysed and the Driblet size was determined by SEM.

Determination of optimal Acetone to Albumin ratio

- The optimization of the BSA nanodriblet's preparation based on the acetone concentration used for desolvation process.
- The Acetone concentration used for the desolvation influences the yield and the Driblet size of the nanodriblets.
- Different acetone: albumin ratios were optimized as mentioned in table no.1 to get the desired target size (100nm) of the BSA nanodriblet's synthesis.
- Optimal Acetone proportion (in ml) was determined based on the driblet size of the BSA nanodriblet's was measured using SEM.

Determination of Crosslinking Time

- The crosslinking of the acetone coacervates takes place after the addition of glutaraldehye (25%).
- The crosslinking time influences the driblet size and yield percentage of the nanodriblet's.
- The cross linking time at a range from 4hours to 16hours was optimized as shown in table no.1 and the optimal crosslinking time for the desired target size of the driblet was determined by using SEM.

Determination of optimal BSA concentration

- The optimal BSA concentration for the preparation of BSA nanodriblet at a target size of 100nm was optimised with the rest of the Framework as shown in the table no.1
- The effect of BSA concentration on driblet target size was measured by using SEM.

Determination of optimal agitation rate

- The variation in agitation rate implies in the size of the BSA nanodriblet .
- The desired rate of agitation speed was optimised to get the target size of the BSA nanodriblet as mentioned in the table no.1

Preparation of BSA nanodriblet's by Intermittent Method

- Analytical grade acetone was added at a rate of 2ml for every 5 mins to 20 ml of 1% BSA (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 turbid (Hoang Hai Nguyen, 2010).
- The turbid solution was stirred continuously for 30 mins without acetone addition.
- Then 100 µl of 25% glutaraldehyde was added for cross linking. The reaction was continued at room temperature (24°C) for 12 hours.
- Ethanolamine (100µl) 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 was ultracentrifuged (20,000g, 30minutes) for two cycles.
- The suspension was then ultrasonicated for 10 minutes.
- The pellet is then lyophilized or keep it in hot air oven for 20 mins at 55˚C to form fine powder.

Characterization of BSA nanodriblet's

- The morphology of the BSA nanodriblet's was observed by scanning electron microscopy (SEM) (Zhang Z, 2001), with the magnification set at 30, 000, 40,000, 60,000 and 100,000X respectively.

Surface-area-to-volume-ratio of BSA nanodriblet's

- The surface-area-to-volume ratio can be calculated using size distribution data of driblets under that all driblets are spheres.
- Thus, the surface area is 4 πr^2, whereas the volume is (4/3) $\pi r3$. So the surface-area-to-volume ratio of a sphere is (4 πr^2) / [(4/3) $\pi r3^{1.}$

Ensnared of Pectinase Enzyme & Protease Enzyme with BSA nanodriblet

- Analytical grade acetone 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.
- Then add 10mg of Pectinase enzyme 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 mins at 55˚C to form fine powder of ensnared Pectinase enzyme(QEZ-1).
- Repeat the same steps mentioned above for the encapsulation of Protease Enzyme with the BSA to get the desired BSA nanodriblet ensnared Protease enzyme (QEZ-2).

Characterization of BSA nanodriblet's with QEZ-1& QEZ-2

- The morphology of the BSA nanodriblet Ensnared enzymes was observed by scanning electron microscopy (SEM), with the magnification was set at 60,000.

Enzymatic Assay for Ensnared (QEZ-1)

- The activity of QEZ-1 was assayed using a reaction mixture comprising of 1ml ensnared 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(Mandels M, 1976).
- 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 waterbath (60˚C) for 15 minutes.
- The absorbance was measured at 540 nm with spectrophotometer.
- The QEZ-1 activity was defined as by the following standard formula

$$\text{Enzyme activity (IU/ml/min)} = \frac{\text{Amount of sugar released x 1000}}{\text{Molecular weight of maltose x Time of incubationunder the assay conditions.}}$$

- 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 ensnared enzyme (QEZ-1).

Kinetic Analysis for Ensnared (QEZ-1)

- 1% starch solution was prepared by dissolving 1g in 100ml of double distilled water.
- Eleven different test tubes were taken, blank solution, 0.1ml of starch .0.2ml, 0.3ml... to 1ml were pipetted out in their respective test tubes.
- It was all made upto 1ml with distilled water and 0.5ml of QEZ-1 was added and incubated for 5mins at room temperature.
- Then 1ml of GOD-POD reagent was added to it and made upto final volume of 3ml with distilled water in all test tubes.
- After the colour development the OD was taken by using spectrophotometer at 540nm.

Assessment of bettered enzyme activity of ensnared enzyme(QEZ-1).

Effect of pH

1ml of Ensnared Pectinase enzyme (QEZ-1) was incubated with different buffer of different pH ranged from 3,4,5, 6,7,8,9 and 10 respectively at 32˚C for 10 minutes. The enzyme assay was carried out as described below.

- Use buffers, and starch solutions of differing pH to prepare of pH profile of enzyme activity.
- Take starch solutions (1g/L) of differing pH's and add 0.1 ml of each to an assay tube.
- Add Ensnared enzyme (QEZ-1) stock to 1/10 dilution to corresponding pH buffer.

- Add 0.1 ml diluted Ensnared enzyme to 0.1 ml starch solution. Start timing immediately for three minutes.
- Add 2 ml of iodine reagent. Measure A540. Be sure to run a control set of three starch tubes with 0.1 mL of buffer instead of enzyme solution) for each new pH.“control” Pectinase
- The effect of pH on assay was compared with the free and the immobilised enzyme assay to get the efficiency rate of the ensnared enzyme (QEZ-1).

Effect of Temperature

- 1ml of ensnared Pectinase 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 below.
- Equilibrate 6 tubes containing 0.1 mL of starch solution at each assay temperature (on ice, room temperature, and in 40, 50, 60, 70, 80°C).
- For each temperature prepare 3 tubes containing 0.1 mL of ensnared enzyme solution and 3 tubes containing 0.1 mL of buffer pH 7.0.
- Quickly add the contents of one starch tube to each of 3 enzyme/buffer tubes (keeping the solutions at the desired assay temperature). Time the reaction for 5-10 minutes.
- Stop the reaction by adding acidic iodine solution (2.0 ml). Cool at room temperature for 5 minutes and measure the A540.
- The effect of temperature on assay was compared with the free and the immobilised enzyme assay to get the efficiency rate of the ensnared enzyme (QEZ-1).

Effect of Substrate

- Perform the assay using solutions with differing initial starch concentrations (1 to 10mg/ml). For each concentration, make 3 control tubes “C”.
- For each concentration, make 3 assay tubes “A”. And three control tubes "C."
- Add 0.1 mL of the corresponding starch solution to each of the 6 tubes labeled for that dilution. Vortex to mix.
- Add 0.1 ml of buffer to the "C" tubes. Mix.
- Add 0.1 ml of ensnared Pectinase to the "A" tubes. Mix immediately starts timing the reaction.
- Exactly 5 minutes after adding enzyme to the "A" tubes quickly add 2 ml of iodine solution to the tubes labeled "A" and "C". Vortex to mix.
- The "A" tubes should be less blue than the “C” tubes, which contained no enzyme.
- Cool each tube on ice (5 minutes) before measuring absorbances.
- Zero the spectrophotometer with water. Measure the absorbance (A540) of each of the tubes and record the values.
- The effect of substrate on assay was compared with the free and the immobilised enzyme assay to get the efficiency rate of the ensnared enzyme (QEZ-1).

Effect of Incubation Time

- Prepare to measure the activity at incubations of 2hrs to 16 hrs with 2 hrs regular intervals.
- Prepare eight sample tubes with 0.1 ml of unknown, and three tubes with 0.1 ml buffer at pH 7.0. Incubate at 50°C for minutes.
- Incubate 6 ml of starch solution at 50°C for 5 minutes.
- Add 0.1 ml of starch solution to the QEZ-1 tubes and control tubes.
- After 5 minutes stop one of the enzymatic reactions and stop one of the control tubes by adding with 2 ml of iodine solution. Allow the tubes to cool to room temperature measure absorbance at 540 nm.
- Stop the second set after 20 minutes and the third set after 40 minutes.
- Estimate the activity in the unknown.

- The effect of incubation time on assay was compared with the free and the immobilised enzyme assay to get the efficiency rate of the ensnared enzyme (QEZ-1).

Effect of Inhibitors

- A rapid and simple procedure (Safari I, 1990) for the detection the presence of a required inhibitor.
- It was based on the ensnared Pectinase hydrolysis of a thin layer of starch lying on a glass plate.
- The starch was suspended in 100ml of water and the suspension was boiled in a water bath for 15mins.
- The hot starch solution(3ml) were poured on the glass plates(8x8cm) to cover the plates evenly.
- The plates was incubated at room temperature for overnight, a thin layer of firmly attached starch was formed on the plates.
- The solutions to be tested solutions of various chemicals (1.water-control, 2.silvernitrate, 3.coppersulphate, 4.leadnitrate, 5.EDTA, 6.sodium sulphate) were mixed, in the wells of a microtitre plate with buffered ensnared Pectinase enzyme (QEZ-1),Free and immobilised enzyme in the ratio ranging from 1:1 to 3:1.
- After 10-30mins incubation, 10-40µl of the mixture was pipetted onto the surface of the starch plate to form a small drop.
- The plates were incubated at room temperature for 20-30 mins after the application of the last mixture drop.
- The plate was then thoroughly rinsed with running water.
- The plate was then overlaid with an iodine solution.
- The plates was again rinsed with running tap water and evaluated.

Enzymatic Assay for Ensnared Protease Enzyme (QEZ-2)

- The enzymatic assay was done by using the following reagent into suitable reaction tubes.
- The reagent B (xylan) 1.9ml was added in the respective test tubes and the rest of the tubes with 0.02, 0.05, 0.07 and 0.1ml reagent G respectively.
- Mix it well by swirling and equilibrate to 30˚C in boiling water bath.
- Then 0.1 ml of reagent D (QEZ-2) was added in the tubes 1 and 2 then the reagent C was added to made up to 2ml into the rest of the tubes and mix it well by swirling and equilibrate to 30˚C for exactly 10mins.
- Then add 2 ml of reagent E to all the tubes and mix it well.
- Transfer the tubes to a boiling water bath and incubate for 10mins.
- Remove the tubes from allow to the boiling water bath and cool to room temperature.
- Then add 2ml of reagent F to all the test tubes, vortex the tubes until foaming stops and any precipitate present was dissolved.
- Centrifuge the solution present in the tubes to get the clarified solution.
- Transfer the solutions to cuvettes, to obtain the absorbance at 540nm using spectrophotometer.

Kinetic Analysis for Ensnared (QEZ-2)

- 1% xylan solution was prepared by dissolving 1g in 50 mM Sodium Acetate Buffer, pH 4.5 at 30°C.
- Eleven different test tubes were taken, blank solution, 0.1ml.0.2ml, 0.3ml... to 1ml of xylan were pipetted out in their respective test tubes.
- It was all made upto 1ml with buffer solution and 0.5ml of QEZ-2 was added and incubated for 10mins at 60ºC.
- Then 1ml of DNS reagent was added to it and made upto final volume of 3ml with buffer solution in all test tubes.

- After the colour development the OD was taken by using spectrophotometer at 540nm.

Assessment of bettered enzyme activity of ensnared enzyme(QEZ-2).

Effect of pH

- The optimum pH value for the reaction was determined by varying pH values.
- 0.1 M of sodium acetate buffer between 3 to 6.5 while all other Frameworks were kept constant.
- After incubation at 37°C for 30 min, Ensnared Protease (QEZ-2) activity was determined.
- The effect of pH was done with the free enzyme and the immobilised enzyme to get the efficiency rate of the ensnared enzyme (QEZ-2).

Effect of temperature

- The reaction tubes were incubated for 30 min at different temperatures between 30°C and 100°C while all other Frameworks were kept constant.
- Temperature stability was assessed by preincubating enzyme(QEZ-2) solutions for 2 h at 30, 40, 50, 60 and 70°C prior to addition of substrate and the determination of activity at 37°C.

Effect of substrate

- The optimum substrate concentration for the reaction was determined by varying substrate concentration.
- The effect of substrate concentration, xylan ranging from 0 to 20mg/ml (in 0.1 M sodium acetate buffer) was added to the reaction medium, while all other frameworks were kept constant, process the enzyme assay.

Effect of Reaction Time

- The effect of reaction time on enzyme activity was investigated by incubating the reaction media at 37°C for 20 to 60 min while keeping all other Frameworks constant and processes the enzyme assay.

Effect of Metal ions

- The mixture of 1 ml xylan substrate, 0.5 ml dialyzed enzyme(QEZ-2) and 1 ml different metal ions (Mn^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, K^{2+}, Mg^{2+}) were incubated at 37°C for 30 min.
- After incubation, enzyme activities were determined.
- Ions were added in the assay buffer at a level of 1 mM.
- The percent activity was determined based on the reference sample, which did not contain any metal ion.

RESULTS AND DISCUSSION

Optimization of the BSA nanodriblet's Preparation

The escalation of preparation condition for BSA nanodriblet at the target size of 100nm plans are shown in table.1.and the Scanning electron micrographs results of those plans are shown in Figure.1 to 5. The strategy to control size was to adjust BSA concentration, pH, ionic strength, crosslinking reaction rate and agitation speed in the desolvating conditions. BSA nanodriblet's formed in different sizes at the above conditions since the desolvation process was influenced by the electrostatic attraction and repulsion among BSA molecules (Mohsen Jahanshahi, 2008).

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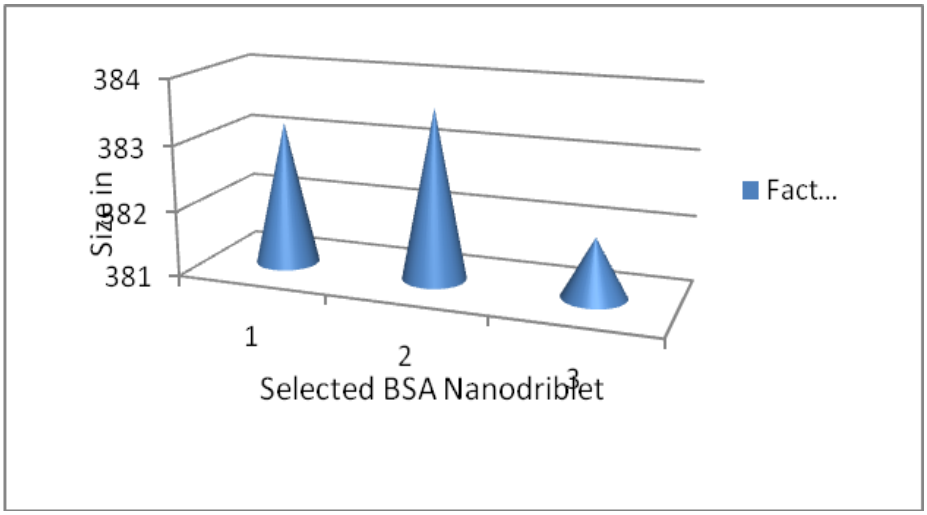
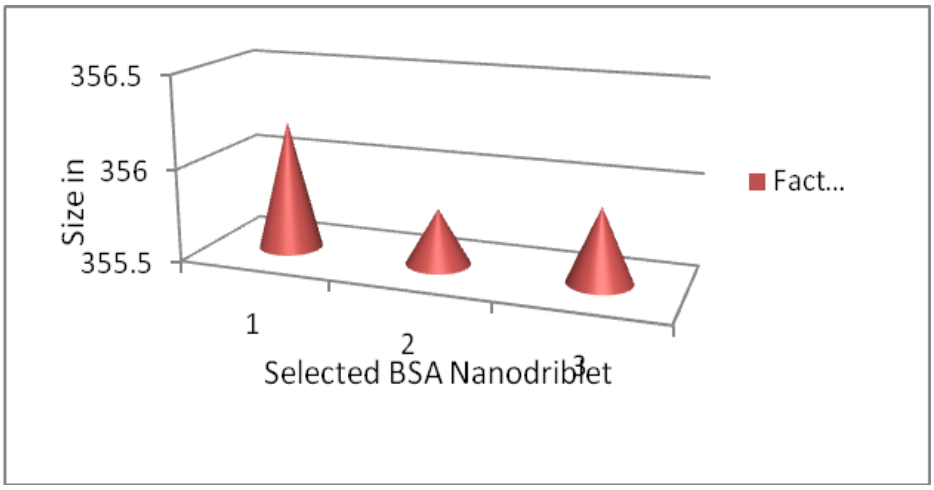
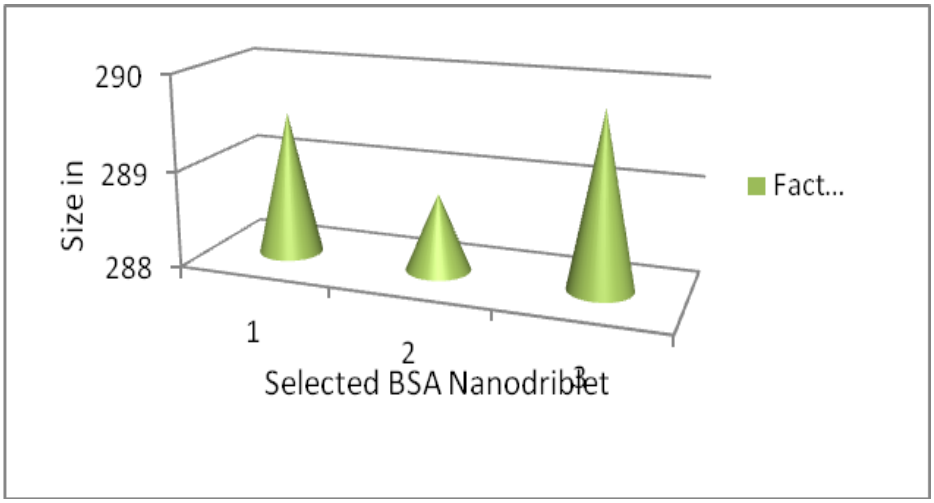


Graph 1: The escalation of size of BSA nanodriblet's by Factor1 Framework



Graph 2: The escalation of size of BSA nanodriblet's by Factor2 Framework



Graph3: The escalation of size of BSA nanodriblet's by Factor3 Framework

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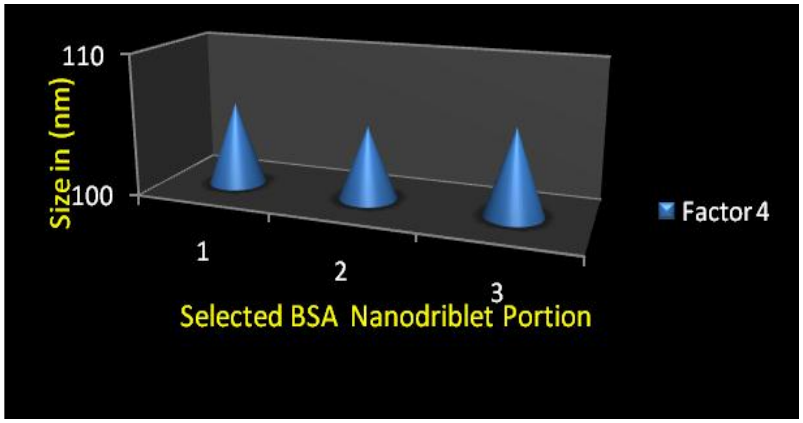
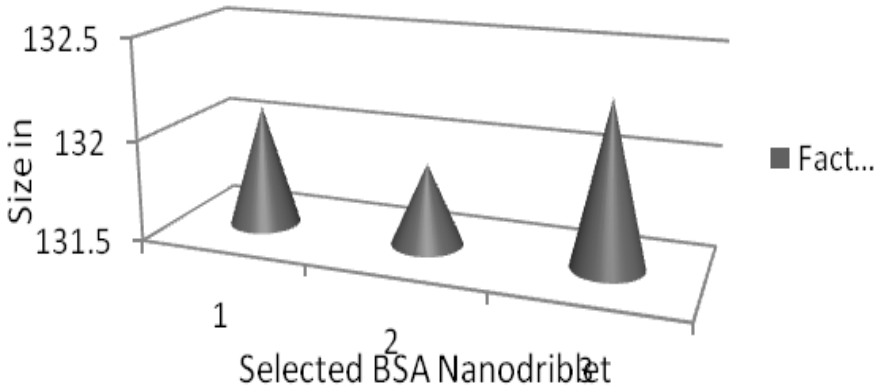


Graph 4: The escalation of size of BSA nanodriblet's by Factor4 Framework



Graph 5: The escalation of size of BSA nanodriblet's by Factor5 Framework

Determination of optimal pH:

The escalation of pH condition for BSA nanodriblet at the target size of 100nm plans are shown in table.1.Size control of BSA nanodriblet's was conducted by modifying desolvation methods (Ko & Gunasekaran, 2006). The driblet 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 driblets 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.2, fine BSA driblets with required target mean size was formed.

Determination of optimal Acetone to Albumin ratio:

The Optimization of volume consumptions of acetone for the BSA nanodriblet preparation at target size of 100nm are listed in table.1.The amount of acetone for the 100 nm targeted BSA nanodriblet preparation was 34 ml. Consumption of acetone was inversely proportional to the targeted size as shown in table 2 .

S.no	Factor no.	Mean size (in nm)
1	1	382.9
2	2	355.9
3	3	289.3
4	4	105.6
5	5	132.1

Table 2: Mean size of BSA nanodriblet's by different sources of Framework

Determination of Crosslinking Time

The crosslinking incubation time on BSA nanodriblet preparation was optimised as in table.1. It was Influences the crosslinking processes; as a result different mean size of BSA nanodriblet's was formed. The target size of 100nm was achieved for 4hrs incubation from Factor4) as shown in table.2.

Determination of optimal BSA concentration

The effect of BSA concentration on BSA nanodriblet size was conducted and shown in table.1. As the concentration of BSA increased, the driblet size was also increased. The target Driblet mean size of (105.6nm) was achieved at the concentration of 10mg/ml.

Determination of optimal agitation rate

As a result in table.2 , the agitation speed that interfere with the target size of the BSA nanodriblet , mean size of Driblet (105.6 nm) close to the target size was obtained at agitation speed of 550 rpm.

Surface-area-to-volume-ratio of BSA nanodriblet's

The data from the graph.7 , the greater surface-area-to-volume-ratio of BSA nanodriblet's driblet than rest of the sources was found in Factor4 Framework.Surface area is a material property of Driblets that is often a determining factor in bioavailability, dissolution rate, adsorption, catalyst activity, and toxicity(Aguzzi,Cerezo,Viseras & Caramella,2007). The surface area is a more useful and accurate metric than concentration or dosage, which is traditionally believed to be the most important (Oberdorstre, ferin, & Lehnert, 1994). The concept of total surface area can be used to describe the surface area in a sample(Desai, M. P,1996). One such is the surface-area-to-volume-ratio, and is more useful than the concentration or the mean diameter of Driblets. The surface-area-to-volume ratio is the amount of surface area per unit volume of driblets. The surface-area-to-volume ratio can be calculated using size distribution data of driblets under that all driblets are spheres. Graph.7, shows the surface-area-to volume-ratios of BSA nanodriblet's of target size 100 nm. The Driblets for the 100 nm plan obtained the largest surface area in Factor4, 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 driblets instead of diameter-related values (Phyu,warne,& Lim,2005). Furthermore, the surface-area-to volume-ratio is useful for comparative studies of nano-scaled materials.

Ensnared of Pectinase enzyme with BSA nanodriblet

The Ensnared of Pectinase enzyme with BSA nanodriblet was done and the Scanning electron micrographs of BSA nanodriblet Ensnared enzyme (QEZ-1) at the targeted size of 100nm by Intermittent Method was shown in figure .

Kinetic Analysis for Ensnared (QEZ-1)

Kinetic Frameworks of QEZ-1 with the free and immobilized Pectinase were measured. For these forms of Pectinase enzyme activity, Michaelis-Menten type kinetic behavior was observed. The Km and Vmax values as determined from the graph.8 found to be 3.9 mg/ml and $0.686x10^{-3}$ µmole/ml.s (for QEZ-1) ,3.5 mg/ml and $0.648x10^{-3}$ µmole/ml.s (for free enzyme), 3.9mg/ml and $0.622x10^{-3}$ µmole/ml.s as shown in Table.3.Therefore, for QEZ-1 form of Pectinase Km value was decreased and the Vmax 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.

Enzyme	Km(mg/ml)	Vmax(µmole/ml.s)
QEZ-1	3.1	$0.686x10^{-3}$
Free Enzyme	3.5	$0.648 x10^{-3}$
Immobilised Enzyme	3.9	$0.622 x10^{-3}$

Table 3: KineticFrameworks of QEZ-1, Free and Immobilized Pectinase

Effect of pH

. The effect of pH on activity of QEZ-1, free and immobilized Pectinase was given in graph.9. Optimum pH values were 8, 7 and 6 for QEZ-1, Free and immobilized Pectinase 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 frameworks capable of shifting enzyme activities in reaction mixture, encapsulation and Immobilization usually results in shift of optimum pH due to conformational changes in enzymes.

Effect of Temperature

The activity of enzyme is also strongly dependent on temperature. The activity of QEZ-1, free, Immobilised Pectinase increased with temperature and maximum activity was observed at 80ºC,50ºC,60ºC as shown in Graph 10.The optimum temperature of Pectinase was shifted to 60°C after immobilization in calcium alginate beads and 80ºC for QEZ-1. As was evident from the graph 10 ,Pectinase after ensnared possessed temperature resistance than the free enzyme and Immobilised enzyme.

Effect of substrate

The activity of QEZ-1, free, Immobilised Pectinase enzyme based on substrate concentration was varied with respect to Km value as shown in graph 11. The increased activity facilitated by QEZ-1 within low substrate concentration than the Free and immobilised Pectinase enzymes was observed. The Km value was furnished with low numbers represents strong activity even in low concentration of substrate was observed as shown in graph 11. The optimum activity with respect to substrate concentration for QEZ-1, free and Immobilised Pectinase was 4,5 and 8 mg/ml as shown in graph 11.

Effect of Incubation Time

The effect of incubation was observed as shown in graph 12.The enzymatic activity of QEZ-1, free, Immobilised Pectinase enzyme was varied with respect to time of incubation. The retained activity facilitated by QEZ-1 with an incubation time of 14 hrs. Followed by immobilised and free Pectinase enzymes at a rate of 12 and 10 respectively was observed as shown in graph 12.

Effect of Inhibitors

The effect of inhibitors are shown in figure 7,that the starch layer was hydrolysed for the control QEZ 1-A, Free and immobilised Pectinase on the plate (row 1).The ability of various compounds to inhibit enzyme activity like on plate (row 2) was completely inhibited by silver nitrate and in row 3 ,the immobilised and free enzymes are inhibited whereas QEZ-A in row 3 column C facilitated the starch hydrolysis. In row 4 the lead nitrate inhibited the free enzyme activity on column A rest of the column B and C furnished the hydrolysis of starch in mild way. Then the EDTA and sodium sulphate compounds not affected the enzymatic activity in their respective columns of the plate as shown in figure 7.

Ensnared of Protease Enzyme with BSA nanodriblet

The *Ensnared* of Protease Enzyme with BSA nanodriblet was done and the Scanning electron micrographs of BSA nanodriblet Ensnared enzyme (QEZ-2) at the targeted size of 100nm by Intermittent Method was shown in figure 8.

Kinetic Analysis for Ensnared (QEZ-2)

Kinetic Frameworks of QEZ-2 with the free and immobilized Protease were measured. For these forms of Protease Enzyme activity, Michaelis-Menten type kinetic behavior was observed. The Km and Vmax values as determined from the Graph 13 found to be 3.4 mg/ml and 0.696 µmole/ml.s (for QEZ-2), 3.0 mg/ml and 0.658 µmole/ml.s (for free enzyme), 3.1mg/ml and 0.632 µmole/ml.s respectively as shown in Table 4. Therefore, for QEZ-2 form of Km value was decreased and the Vmax 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.

Enzyme	Km(mg/ml)	Vmax(µmole/ml.s)
QEZ-2	3.4	0.696
Free Enzyme	3.0	0.658
Immobilised Enzyme	3.1	0.632

Table 4: Kinetic Frameworks of QEZ-2, Free and Immobilized Protease Enzyme

Effect of pH

The effect of pH on activity of QEZ-2, free and immobilized Pectinase was given in Graph 14. Optimum pH values were 6, 5 and 5.5 for QEZ-2, Free and immobilized Protease respectively. This shift in optimum pH could be resulted from the change in acidic and basic amino acid side chain ionization in the micro environment around the active site. The pH is one of the major Frameworks capable of shifting enzyme activities in reaction mixture, encapsulation and Immobilization usually results in shift of optimum pH due to conformational changes in enzymes.

Effect of Temperature

The activity of enzyme is also strongly dependent on temperature. The activity of QEZ-2, free, Immobilised Protease Enzyme increased with temperature and maximum activity was observed at 80ºC,60ºC,70ºC as shown in Graph 15.The optimum temperature of Protease was shifted to 70°C after immobilization in barium alginate beads and 80ºC for QEZ-2. As was evident from the graph 15, Protease Enzyme after encapsulation possessed temperature resistance than the free enzyme and immobilised enzyme.

Effect of substrate

The activity of QEZ-2, free, Immobilised Protease Enzyme based on substrate concentration was varied with respect to Km value as shown in graph 13. The increased activity facilitated by QEZ-2 within low substrate concentration than the Free and immobilised Protease Enzymes was observed. The Km value was furnished with low numbers represents strong activity even in low concentration of substrate was observed as shown in graph 16. The optimum activity with respect to substrate concentration for QEZ-2, free and Immobilised Protease was 12.5,10 and 15 mg/ml as shown in graph 16.

Effect of Reaction Time

The effect of reaction time was observed as shown in graph 17.The enzymatic activity of QEZ-2, free, Immobilised Protease Enzyme was varied with respect to time of incubation. The retained activity facilitated by QEZ-2 with an incubation time of 30mins followed by immobilised and free Protease Enzyme at a rate of 50 and 60 mins respectively was observed as shown in graph 17.

Effect of Metal ions

The effect of Metal ions was observed as shown in graph 18.The enzymatic activity of QEZ-2, free, Immobilised Protease Enzyme was varied with respect to addition of different metal ions. The activity was inhibited by K ions with almost all the enzymes types. Followed by mild effect by Mg to all QEZ-2, immobilised and free Protease Enzyme, with enhancement of these enzymes was also observed in Mn and Zn ions respectively as shown in graph 18.

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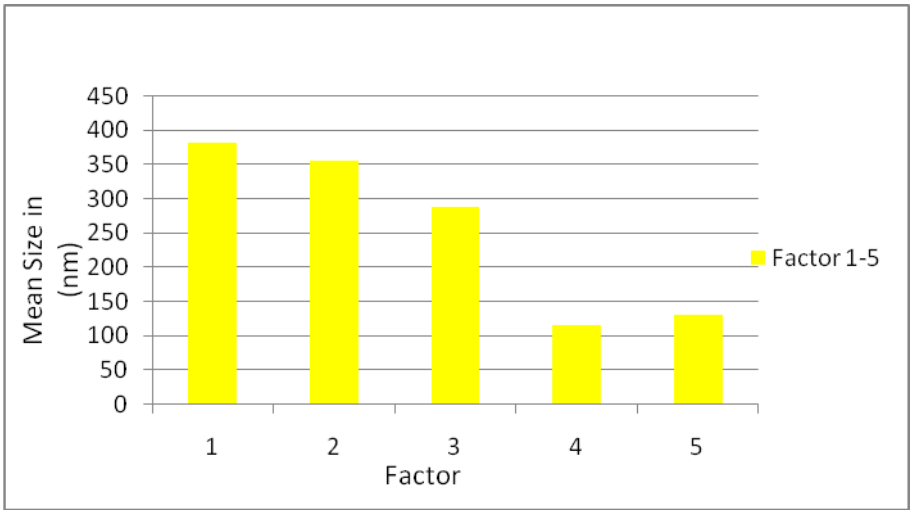
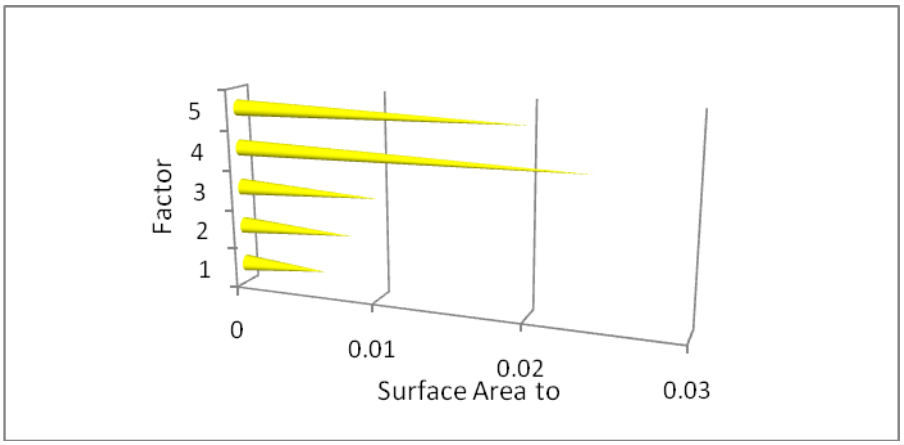
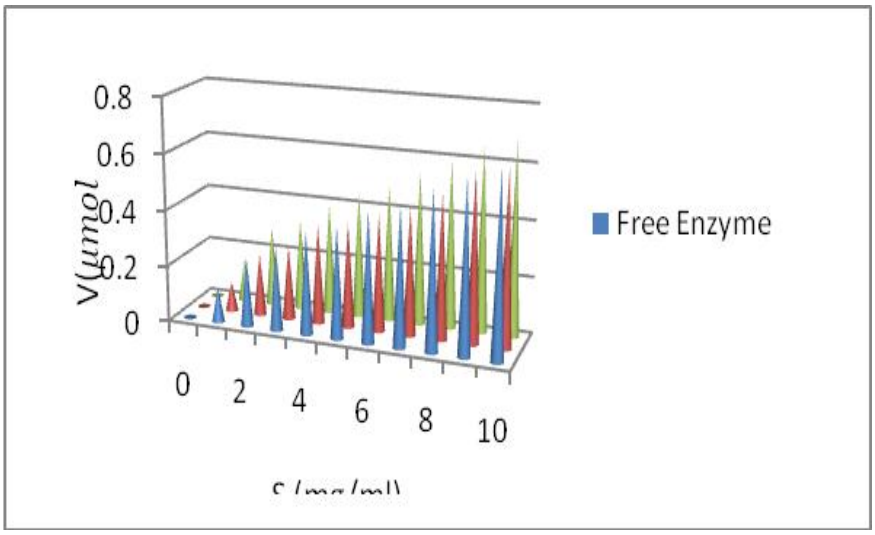


Graph 6: Mean size of BSA nanodriblet's by different sources of Framework



Graph 7: Surface-area-to-volume-ratio of BSA nanodriblet's from different sources at the targeted size of 100nm



Graph 8: Kinetic analysis of QEZ-1 with free and immobilised Pectinase enzyme

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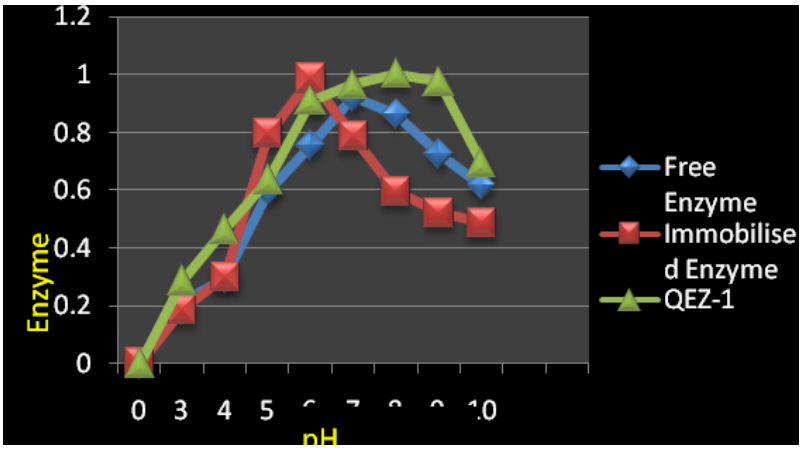
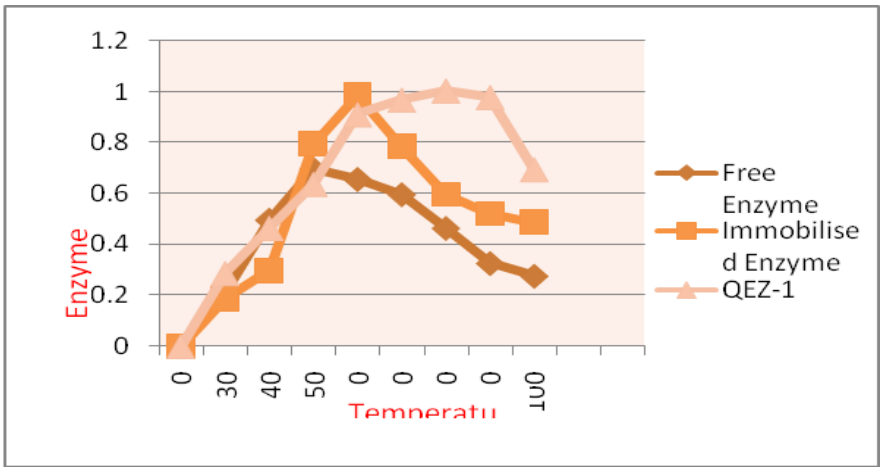
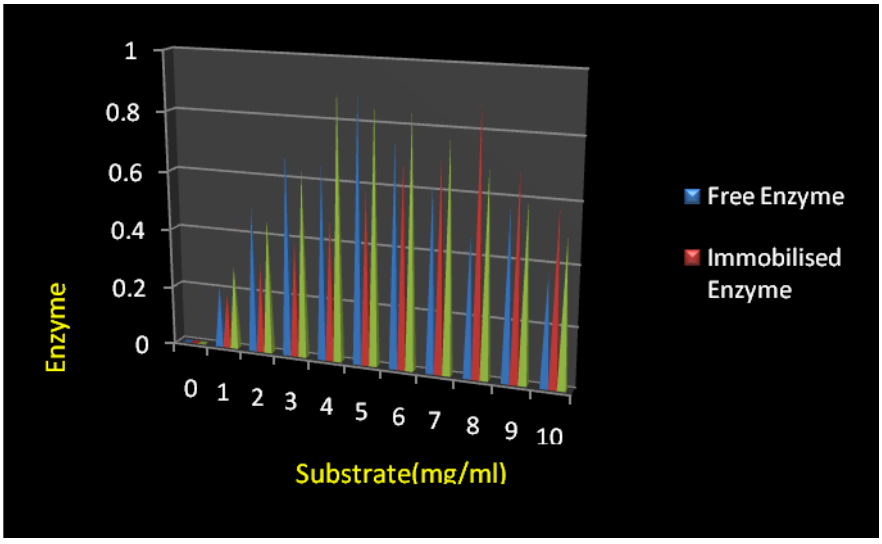


Graph 9: Effect of pH on activity of QEZ-1, Free and Immobilized Pectinase



Graph 10: Effect of temperature on activity of QEZ-1, Free and Immobilized Pectinase enzyme



Graph 11: Effect of substrate on activity of QEZ-1, Free and Immobilized Pectinase enzyme

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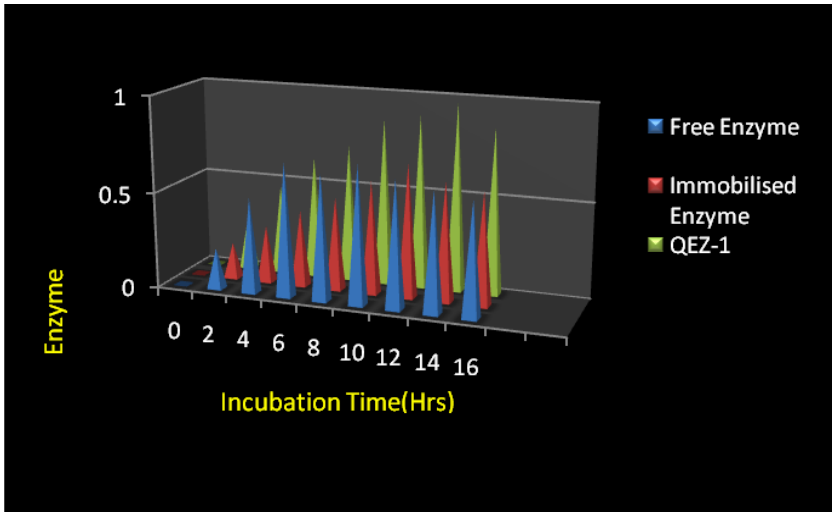
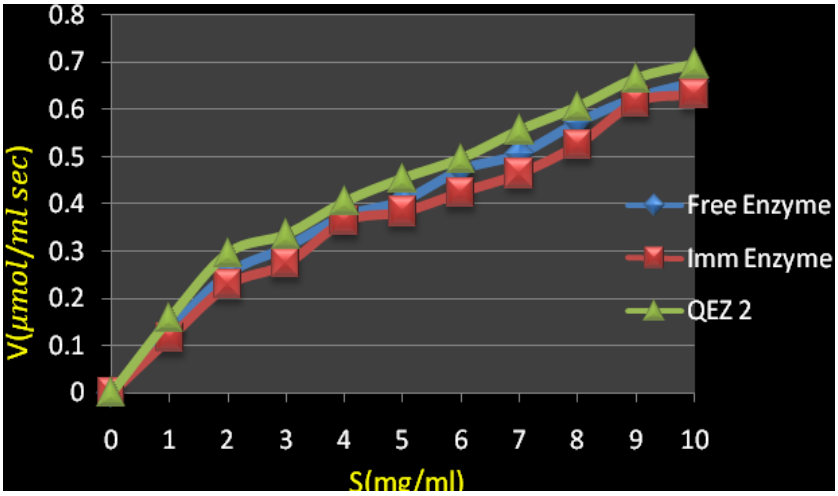
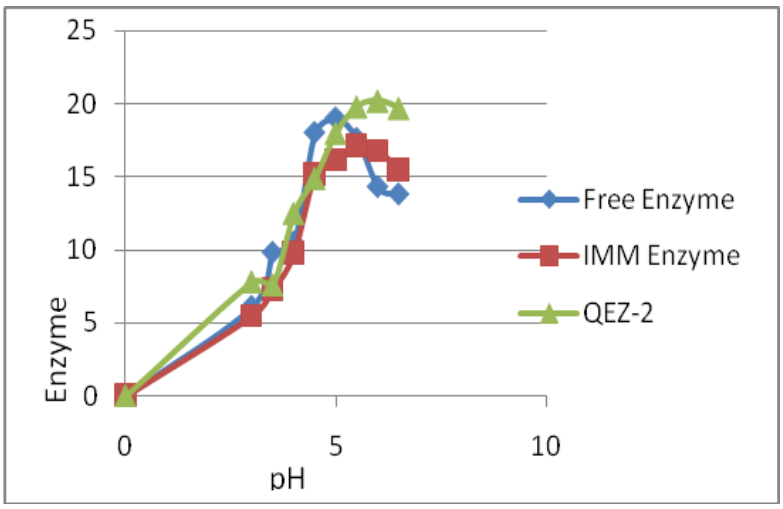


Graph 12: Effect of incubation time on activity of QEZ-1, Free and Immobilized Pectinase enzyme



Graph 13: Kinetic analysis of QEZ-2 with free and immobilised Protease Enzyme



Graph 14: Effect of pH on activity of QEZ-2, Free and Immobilized Protease Enzyme

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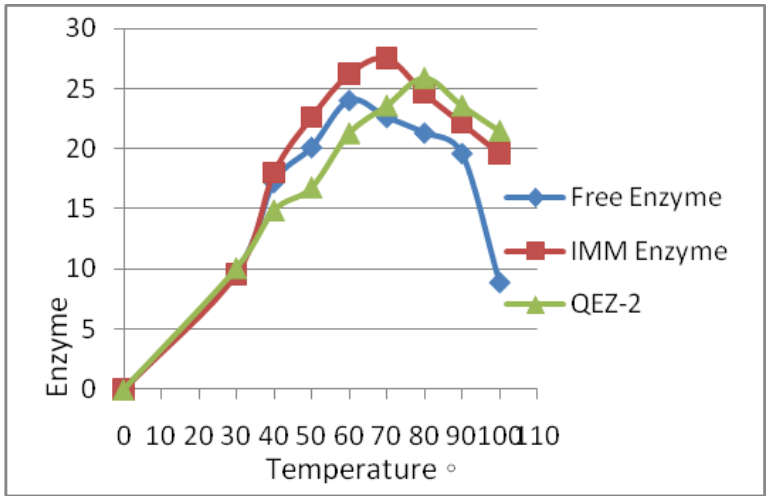
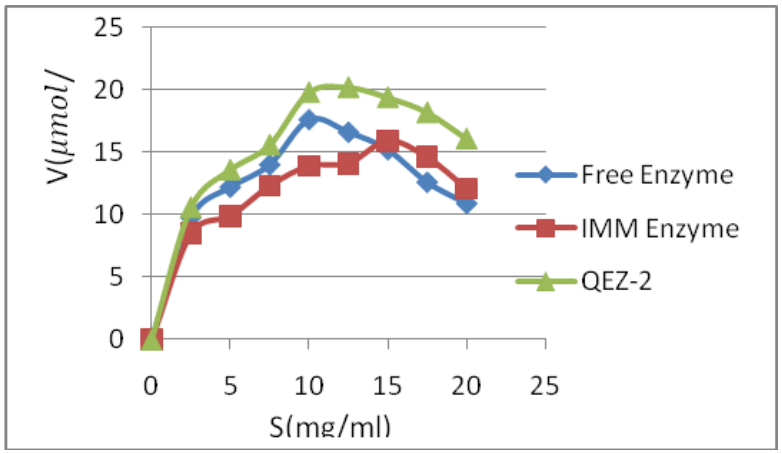
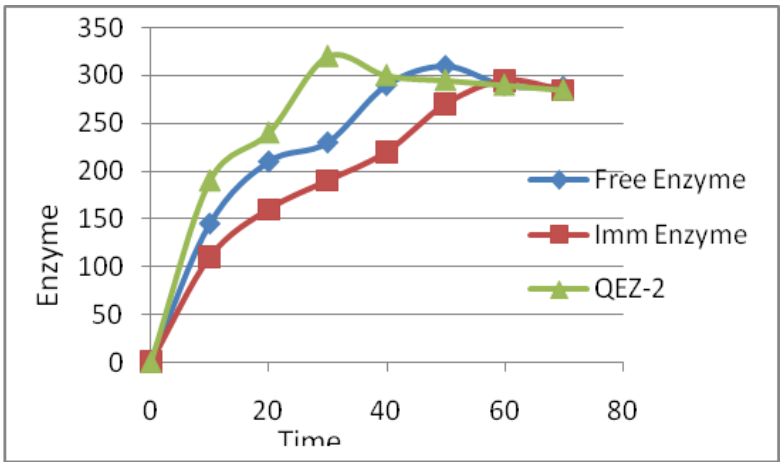


Graph 15: Effect of temperature on activity of QEZ-2, Free and Immobilized Protease Enzyme



Graph 16: Effect of substrate on activity of QEZ-2, Free and Immobilized Protease Enzyme



Graph 17: Effect of reaction time on activity of QEZ-2, Free and Immobilized Protease Enzyme

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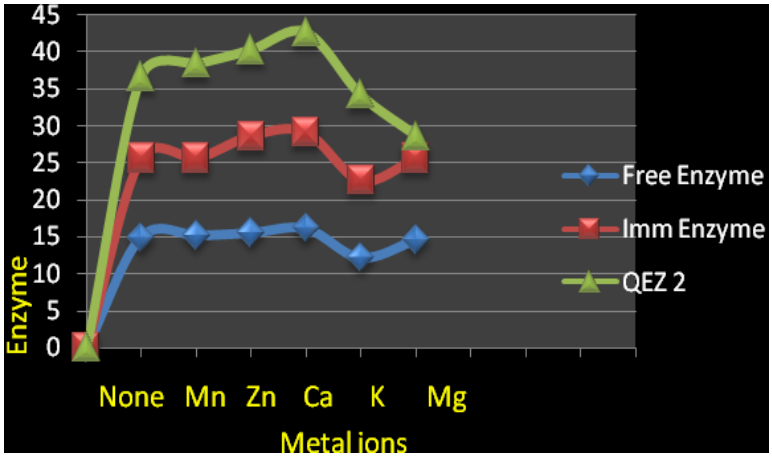

Graph 18: Effect of Metal ions on activity of QEZ-2, Free and Immobilized Protease Enzyme

CONCLUSION

The Escalation of preparation condition for BSA nanodriblet's at the target size of 100nm plans were successful in Factor4 Framework and the Scanning electron micrographs results of this (Factor4) plans are significantly fulfil the desired target size which exhibits the relative small BSA nanodriblet's facilitates the basic requirement that enhances by means of intermittent method of addition quite smaller and homogenous driblets were formed rather using continuous method of addition. From the data table the greater surface-area-to-volume-ratio of BSA nanodriblet's driblet than rest of the sources was found in Factor4 Framework.Surface area is a material property of driblets that is often a determining factor in bioavailability, dissolution rate, adsorption, catalyst activity, and toxicity(Aguzzi,Cerezo,Viseras & Caramella,2007). One such is the surface-area-to-volume-ratio, and is more useful than the concentration or the mean diameter of driblets. The surface-area-to-volume ratio is the amount of surface area per unit volume of driblets. The surface-area-to volume-ratios of BSA nanodriblet's of target size 100 nm.the Driblets for the 100 nm plan obtained the largest surface area in Factor4, 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 driblets instead of diameter-related values (Phyu,warne,& Lim,2005).After the successful BSA nanodriblet's preparation,the industrially important enzymes such as Pectinase ,Protease were ensnared to facilitate its improved activity like temperature resistant,pH survivability,overcome the effect of inhibitors observed through the serious of results that are obtained from various Frameworks and enzyme assays which gives rise to much greater extent in the wide field of industrial applications and significant role on cost effective to get the desired products in much conservative way using BSA nanodriblet ensnared enzymes.

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