STABILITY ANALYSIS OF IMMOBILIZED α-AMYLASE IN GUM ODINA-ALGINATE BEADS FOR INDUSTRIAL APPLICATION

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CERTIFICATE OF APPROVAL

This is to certify that the thesis entitled **"Stability Analysis of Immobilized α-Amylase in Gum-Odina Alginate Beads for Industrial Application"** submitted to Jadavpur University, Kolkata for the partial fulfilment of the Master Degree in Pharmacy, is a faithful record of bona fide and original research work carried out by **Mousumi Tudu** under my supervision and guidance.

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DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I, Mousumi Tudu, a student of Master of Pharmacy, 2nd year, bearing Exam Roll No: M4PHA19013, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-32, declare that my thesis work titled – **"Stability Analysis of α-Amylase in Gum Odina-Alginate Beads for Industrial Application."**, is original and presented in accordance with academic rules and ethical conduct and no part of this project work has been submitted for any other degree of mine. All the information and works are true to the best of my sense and knowledge.

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Place: Jadavpur, Kolkata
Date: 29-05-2019

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Mousumi Tudu

DEDICATED TO MY

BELOVED PARENTS

AND

MY RESPECTED

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Symbols and abbreviations

α	Alpha
α_1	Alpha one (interconnectivity parameter of sample 1)
α_2	alpha two(interconnectivity parameter of sample 2)
α_3	alpha three(interconnectivity parameter of sample 3)
λ	Wave length in SAXS analysis
q	Wave vector in SAXS analysis

Chapter 1

INTRODUCTION

Chapter 1

1. Introduction

1.1 Enzyme

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1.1 ENZYME

Enzymes are generally proteinous macromolecule that speeds up the rate of biochemical reaction. An enzyme acts as catalyst for specific biochemical reaction, converting a specific set of reactants (called substrates) into specific products. They are high molecular weight compounds made up principally of chains of amino acids linked together by peptide bonds. Enzymes can be denatured and precipitated with salts, solvents and other reagents (Elmarzugi et al., 2014). They have molecular weights ranging from 10,000 to 2,000,000 (Homaei et al., 2013). Many enzymes require the presence of other compounds, called as cofactors before their catalytic activity can be exerted. This entire active complex is referred to as holoenzyme; i.e., apoenzyme (protein portion) plus the cofactor (coenzyme, prosthetic group or metal ion activator) is called the holoenzyme (Elmarzugi et al., 2014).

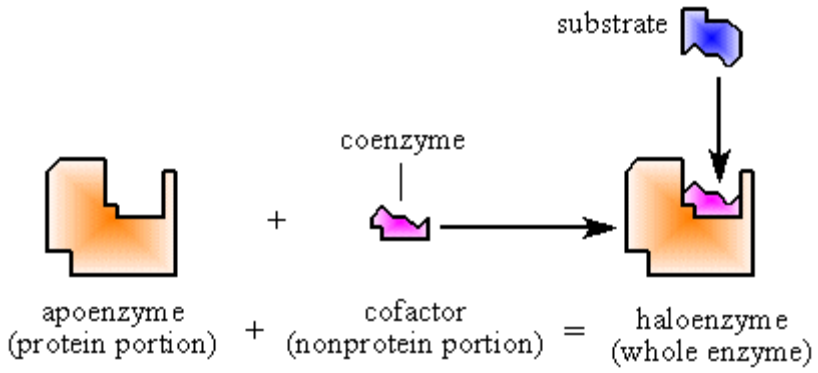

Fig. 1: Holoenzyme - apoenzyme plus cofactor (Source : http://www2.nau.edu/~fpm/bio205/chapter5.2.html . Last accessed on 27.05.2019)

The cofactor may be:

1. Coenzyme: A non-protein organic substance which is dialyzable, thermostable and loosely attached to the protein part (Homaei et al., 2013).

2. Prosthetic group: An organic substance which is dialyzable and thermostable which is firmly attached to the protein or apoenzyme portion (Homaei et al., 2013).

3. Metal-ion-activator: These include K+ , Fe++, Fe+++, Cu++, Co++, Zn++, Mn++, Mg++, Ca++ , and Mo+++ (Elmarzugi et al., 2014).

Based on structure, enzyme can be divided in two groups: monomeric, having only one peptide chain and multimeric, having more than one peptide chains. In some adverse conditions (e.g. pH, temperature, in presence of some chemicals), monomeric enzyme can be deactivated by changing tertiary structure and multimeric enzyme can be deactivated by dissociation to subunits or due to disorder in the assembly of their structures (Singh and Kumar, 2011). Thus, stability of enzyme molecule is an important factor. There are several methods by which enzyme molecule can be stabilized. Immobilization by entrapment method is one of them. The main aim of immobilization is to obtain stable and reusable enzymes with resistance to different environmental factors (Guzik et al., 2014). In enzymology, enzyme immobilization is a process where the enzyme is immobilized onto a stationary phase prior to the reaction. This immobilization protocol simplifies the separation of the enzymes from the products, once the reaction is done (Swarnalatha et al.2013).

1.2 IMMOBILIZED ENZYME: Immobilization of enzymes is one of the important applications of biotechnology. Enzymes have an enormous potential as catalysts in chemical processes in a wide range of industries (Munjal et al., 2002). Many methods exist for the immobilization of enzymes, but usually, one of four methods is used such as entrapment, physical adsorption, co-polymerization, and covalent attachment. Encapsulation technology is designed to entrap materials within a semi-permeable polymeric membrane and/or a gel matrix (Baysal et al.2005).

1.3 IMMOBILIZATION BY ENTRAPMENT METHOD: In this method enzymes are physically entrapped inside a porous matrix. Bonds involved in stabilizing the enzyme to the matrix may be covalent or non-covalent. The matrix used will be a water soluble polymer. The form and nature of matrix varies with different enzymes. Pore size of matrix is adjusted to prevent the loss of enzyme. Pore size of the matrix can be adjusted with the concentration of the polymer used. Agar-agar and carrageenan have comparatively large pore sizes. The greatest

disadvantage of this method is that there is a possibility of leakage of low molecular weight enzymes from the matrix (Sharma et al., 2014).

Examples of commonly used matrices for entrapment are:

(1) Polyacrylamide gels
(2) Cellulose triacetate
(3) Agar
(4) Gelatin
(5) Carrageenan
(6) Alginate

Advantages of entrapment:

(a) Fast method of immobilization,
(b) Cheap (low cost matrixes available),
(c) Easy to practice at small scale,
(d) Mild conditions are required,
(e) Less chance of conformational changes in enzyme,
(f) Can be used for sensing application.

Disadvantages of entrapment:

(a) Leakage of enzyme,
(b) Pore diffusion limitation,
(c) Chance of microbial contamination,
(d) Not much success in industrial process (Kumar et al., 2006)

1.4 α-Amylase: α-Amylase is an enzyme that hydrolyses alpha bonds of large, α-linked polysaccharides, such as starch and glycogen, yielding glucose and maltose.It is the major form of amylase found in humans and other mammals. It is also present in seeds containing starch as a food reserve, and is secreted by many fungi *Aspergillus oryzae*, *Aspergillus niger etc.*

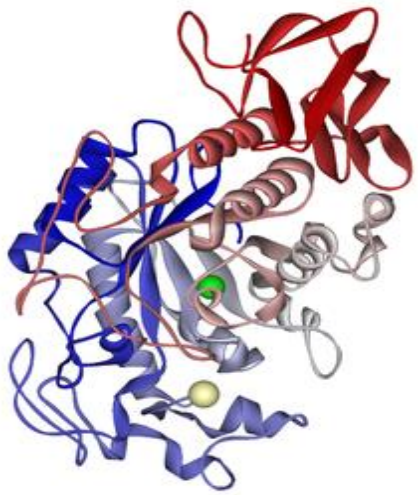

Fig. 2: 3D structure of α-Amylase

α-Amylase can be produced by different species of microorganisms, but for commercial applications α-amylase is mainly derived from the genus *Bacillus.* α-Amylases produced from *Bacillus licheniformis, Bacillus stearothermophilus,* and *Bacillus amyloliquefaciens* find potential application in a number of industrial processes such as in food, fermentation, textiles and paper industries α-Amylase is used in ethanol production to break starches in grains into fermentable sugars (Elmarzugi et al., 2014).

1.5 Industrial demand of α-amylase: α-Amylase is one of the most largely consumed enzymes and their sale is estimated to be approximately USD 225 million throughout the world. Amylases, which are produced by a wide range of organisms, constitute an approximate 25% of the entire global enzyme market (Swarnalatha et al., 2013). Detergents (37%), textiles (12%), starch (11%), baking (8%) and animal feed (6%) are the main industries, where about 75% of industrially produced enzymes are used. Amylases are among the most important enzymes

having great significance for biotechnology, constituting a class of industrial enzymes having approximately 25-30% of the world enzyme market (Elmarzugi et al.).

1.6 Alginate: Alginic acid, also called algin or alginate, is a polysaccharide distributed widely in the cell walls of brown algae. It forms a viscous gum by binding with water. Alginic acid is linear copolymer of u-l guluronic acid and p-o manuronic acid that is linked by α-(1-4) glycosidic bond (Chan et al, 2002, Sevens et al.2006). It is also a significant component of the biofilms produced by the bacterium *Pseudomonas aeruginosa*. Its colour ranges from white to yellowish-brown. It is sold in filamentous, granular or powdered form. Alginate absorbs water quickly, which makes it useful as an additive in dehydrated products such as slimming aids, and in the manufacture of paper and textiles. It is also used for waterproofing and fireproofing fabrics, in the food industry as a thickening agent for drinks, ice cream and cosmetics, and as a gelling agent for jellies. Alginate is used as an ingredient in various pharmaceutical preparations, such as Gaviscon, in which it combines with bicarbonate to inhibit reflux. It is used as a material for microencapsulation. It is used in different types of medical products including skin wound dressings to promote healing and can be removed with less pain than conventional dressings (Gombotz and Wee, 2012).

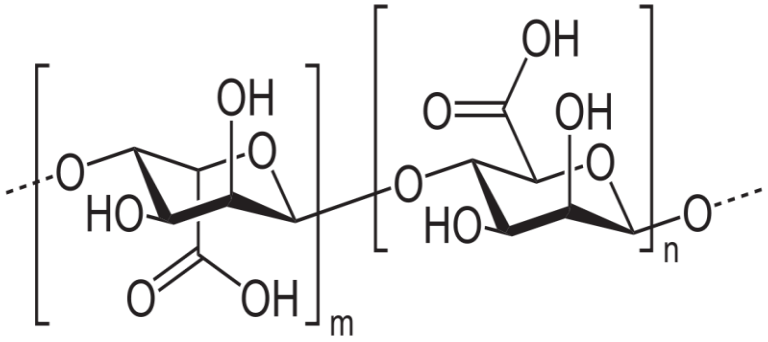

Fig. 3: Alginic acid molecular structure

Fig.4: Brown algae

Fig. 5: Alginate powdered form

3. Gum odina: Gum odina is the solid exudates of bark obtained from the tree *Odina wodier*, Roxb, family Anacardiaceae. It is a mixture of various polysaccharides and chemically is a negatively charged polyelectrolyte. The structure of gum odina was reported earlier which is a polymer of D-galactose, L-arabinose and two uronic acids (D-galactouronic acid and aldobiuronic acid). It is used as binder for tablet preparation (Mukherjee et al., 2006) and as emulsifying agent (Jena et al., 2018). Gum odina is a potential prebiotic which not only provides nutrition but also improves gut ecology (Mitra et al., 2016).

Fig 6.: Collected Gum odina

Chapter 2

LITERATURE REVIEW

MacGREGOR, 1978, investigated physical properties and action pattern of αamylase-I from malted barley on amylose. The enzyme had a molecular weight of 52,000, an isoelectric point of pH 5.1, an activation energy of 9.7 kcal/mol between 20 and 50º C, an optimum temperature of 55º C, and an optimum pH of 5.5.

Amiel et al., 1982, studied the effect of immobilization on collagen turnover in connective tissue. Immobilization of the knee joint for 9 weeks results in a reduction of the mechanical properties in the lateral collateral ligament. Specifically, ligament stiffness is reduced in this tissue. No statistical change in collagen mass was detected for the medial collateral ligament (MCL) or patellar tendon. An increase in collagen turnover (synthesis and degradation) was, however, found in the immobilized medial collateral ligament and patellar tendon. It is thus proposed that stiffness reduction is due to a change in the ligament substance itself, rather than a result of tissue atrophy.

Spagna et al., 1995, showed immobilization of pectinlyase (PL, EC 4.2.2.3) derived from Aspeqillus niger and the supports that permitted an effective immobilization with good activity levels and sufficient stability under the operational conditions were found to be the acrylic resin XAD 7 activated with trichlorotriazine and, in particular, bentonite activated with glutaraldehyde.

GE et al., 1997, suggested immobilization of glucose isomerase and its application in continuous production of high fructose syrup. the activity of immobilized glucose isomerase were optimized, with the enzyme concentration of 308 IU/mL, enzyme-to-matrix ratio of 924 IU/g wet carrier, and hexamethylene bis(trimethyl ammonium iodine) concentration of 15 mg/mL giving the maximum catalytic activity (2238 IU/g dry gel) of the immobilized bilayer glucose isomerase, retaining 68.5% of the initially added activity.

Xing et al., 2000, suggested a series of zeolite immobilized a-chymotrypsin and thermolysin with micro porous Y zeolites (HY, NH4Y, NaY) and mesoporous dealuminized DAY zeolites (HDAY, HNH4DAY) as matrices have been prepared to catalyse peptide bond formation in organic solvents. The immobilization effect of micro porous Y zeolite was better than that of mesoporous DAY zeolite, suggesting that micro porous Y zeolite can form more powerful hydrogen bonds with enzyme molecules since there are more hydroxyl groups on the Y zeolite than on the DAY zeolite. In addition, the influences of some reaction conditions Chapter 2 Literature Review 25 such as reaction time and water content of the solvent on the enzymatic peptide synthesis were also studied and optimized.

Betigeri and Neau, 2002, showed immobilize lipase (triacylglycerol ester hydrolase, E.C. 3.1.1.3) from Candida rugosa using various polymers in the form of beads; to evaluate enzyme loading, leaching, and activity; and to characterize the beads. Entrapment efficiency, however, was the same for different chitosan levels as well as different alginate levels (43%– 50%). Activity in alginate was low at 240733 and 220726, compared to 1110751 and 1150711 units/ml in chitosan, for fresh and freeze-dried beads, respectively.

Mulhbacher et al., 2002, studied the modification high amylose starch for immobilization of uricase for therapeutic application. The N-ethyl-5-phenylisoxazolium-3'- sulphonate (Woodward reagent K) gave a high binding but totally inhibited the enzyme activity. Best results were obtained with CM-HASCL-35 using 1-ethyl-3-(3- dimethylaminopropyl) carbodi-imide as a coupling agent. The immobilized enzyme retained 88%of its initial limit rate [Vmax (app) =16 EU/mg for immobilized uricase versus Vmax=18 EU/mg for the free enzyme], with an apparent decrease of affinity for urate substrate [Km (app) =0.17 mM versus Km=0.03 mM for the free enzyme]. The coupling yield was 60% and the modified uricase was found more resistant to proteolysis than the free enzyme. The immobilized uricase retained 25% of its initial activity after 60 min in pancreatic proteolysis medium (pancreatin), whereas the free enzyme retained only 5% of its initial activity. The best immobilization yield was obtained with the polymeric

support based on CM-HASCL-35 (53%), which gave better results than commercial supports based on agarose.

Jawaheer et al., 2002, suggested pectin as a novel material for enzyme entrapment and stabilisation. This work relates to the development of an electrochemical enzyme-sensor array for the monitoring of fruit quality. The enzymes investigated are glucose oxidase, mutarotase, invertase and ascorbate oxidase. Sensors were constructed by screen-printing, with a metallised (rhodium) carbon working electrode, silver/silver chloride printed reference electrode and carbon counter.

Tsen et al., 2004, studied Fermentation of banana media by using n-carrageenan immobilized Lactobacillus acidophilus. Gel beads of diameters around 3.0 mm were prepared for the immobilized cells, ripe bananas were used for preparation of media, and both free and immobilized cells were employed to carry out the fermentation for 80 h. Cells leaked out from the gel beads and proliferated in the medium solution during the fermentation of immobilized cells. The final viable cell number reached 105 CFU/ml in the medium suspension, over 108 CFU/ (ml gel) in gel beads for the immobilized cell fermentation and around 106 CFU/ml for the free cell fermentation. Immobilized cells withstand the adverse conditions in banana media resulting in better fermentation efficiency compared to free cells.

Kumar et al., 2006, studied alginate as the affinity matrix for entrapment of αAmylase and subsequent precipitation of the beads with calcium chloride. Amylase was recoverable by addition of 0.5 M NaCl containing 0.2 M Ca2+. The enzyme was found to be homogenous (recovery of 76%) with a specific activity of 1764 U/mg. The pH and temperature optima shifted (on entrapment) from 5.5 to 6.0 and 54 to 60oC, respectively. The entrapped enzyme had higher thermal stability compared to the free enzyme. The midpoint of thermal inactivation for the enzyme increased by 6±1oC on entrapment. The entrapped enzyme had an Ea value of 51.7 compared to 40.9 kcal mol-1 for the free enzyme. The positive increase in Ea value as well as the

half-life of the entrapped enzyme is indicative of increased stability. The reusability of the beads was dependent on bead size.

Gomez et al., 2006, explained immobilization of soybean and horseradish peroxidases (SBP and HRP) were immobilized on glutaraldehyde-activated aminopropyl glass beads. The reaction with immobilized HRP was faster than with SBP at all the enzyme concentrations assayed. However, at enzyme concentrations lower than 0.028 mg/ml, 7% and 20% more phenol was removed with SBP than with HRP. This behaviour changed as the enzyme dose increased. Thus, the addition of 0.039 mg/ml of either immobilized SBP or HRP eliminated 77% of the initial phenol. Increasing the amount of hydrogen peroxide added to the reactor led to higher conversions and more than 95% of the initial phenol present in the solution was eliminated with immobilized SBP at an H_2O_2/phenol molar ratio of 1.5 for all the phenol concentrations studied. However, immobilized HRP was more susceptible to inactivation/inhibition effects and its phenol removal capacity was lower (less than 93%).

de Lathouder et al., 2007, investigated Carbon–ceramic composites for enzyme immobilization. The composites were used as a carrier for lactase from Aspergillus oryzae. ACM monoliths allow for a higher CNF loading, and thus for a higher enzyme loading. Lactase adsorption per gram of carbon increases by 15–20% compared to cordierite samples. The total enzyme adsorption capacity for CNF-coated ACM and cordierite monoliths was found to be 350 and 300 mg g-1 Carbon respectively. Oxidation treatment of the CNFs affected the surface chemistry, while the porosity was not affected. The growth catalyst could be removed completely from both the support and the CNFs by treatment in HCl or HNO3, without destroying the carbon structure.

Sankalia et al., 2007, designed reversed chitosan–alginate polyelectrolyte complex for stability improvement of alpha-amylase. A topographical characterization was carried out using scanning electron microscopy (SEM), and the entrapment was confirmed using Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC). Stability testing was carried

out according to the International Conference on Harmonization (ICH) guidelines for zones III and IV. Beads prepared using 2.5% w/v chitosan and 3% w/v sodium alginate with a hardening time of 60 mins had more than 90% entrapment and a T90 value greater than 48 mins. Moreover, the shelf-life of the enzyme-loaded beads was found to increase to 3.68 years, compared with 0.99 years for the conventional formulation. It can be inferred that the proposed methodology can be used to prepare a reversed PEC of chitosan and alginate with good mechanical strength, provided both the reactants are in a completely ionized form at the time of the reaction.

Ertan et al., 2007, investigated the production of α-Amylase from Aspergillus sclerotiorum under SSF conditions, and immobilized in calcium alginate beads. Effects of immobilization conditions, such as alginate concentration, $CaCl_2$ concentration, amount of loading enzyme, bead size, and amount of beads, on enzymatic activity were investigated. Optimum alginate and CaCl2 concentration were found to be 3% (w/v). Using a loading enzyme concentration of 140 U ml-1 , and bead (diameter 3 mm) amount of 0.5 g, maximum enzyme activity was observed. Beads prepared at optimum immobilization conditions were suitable for up to 7 repeated uses, losing only 35% of their initial activity. Among the various starches tested, the highest enzyme activity (96.2%) was determined in soluble potato starch hydrolysis for 120 min at 40 $^{\circ}$C.

Yagar et al., 2007, investigated some properties of immobilized a-amylase by Aspergillus sclerotiorum within calcium alginate gel beads and compared with soluble enzyme. Optimum pH and temperature were found to be 5.0 and 40.8oC, respectively, for both soluble and immobilized enzymes. The immobilized enzyme had a better Km value, but kcat/Km values were the same for both enzymes. Entrapment within calcium alginate gel beads improved, remarkably, the thermal and storage stability of α-Amylase. The half-life values of immobilized enzyme and soluble enzyme at 608oC were 164.2, and 26.2 min, respectively. The midpoint of thermal inactivation (Tm) shifted from 568oC (for soluble enzyme) to 65.48oC for immobilized enzyme. The percentages of soluble starch hydrolysis for soluble and immobilized α-Amylase were determined to be 97.5 and 92.2% for 60 mins, respectively.

Pongjanyakul and Puttipipatkhachorn, 2007, examined molecular interaction and in vitro characterization of xanthan–alginate composite gel beads. Diclofenac calcium-alginate (DCA) beads incorporated with different amounts of XG were produced. Molecular interaction between SA and XG in the composite beads and the XG–DCA beads was investigated using FTIR spectroscopy. Physical properties of the XG–DCA beads such as entrapment efficiency of diclofenac sodium (DS), thermal property, water uptake, swelling and DS release in various media were examined. XG could form intermolecular hydrogen bonding with SA in the composite beads with or without DS. Differential scanning calorimetric study indicated that XG did not affect thermal property of the DCA beads. The DS entrapment efficiency of the DCA beads increased with increasing amount of XG added. The XG–DCA beads showed higher water uptake and swelling in pH 6.8 phosphate buffer and distilled water than the DCA beads. A longer lag time and a higher DS release rate of the XG–DCA beads in pH 6.8 phosphate buffer were found.

Kumar et al., 2009, showed highly porous activated carbon (HPAC) as carrier matrix for immobilization of acid protease (AP). At pH 6.0, 250 mg acid protease g-1 HPAC was immobilized. The optimum temperature for both free and immobilized AP activities were 50º C. After incubation at 50º C, the immobilized AP maintained about 50% of its initial activity, while the free enzyme was completely inactivated. When testing the reusability of AP-HPAC combination immobilized system, a significant catalytic efficiency was maintained along more than five consecutive reaction cycles. The highly porous nature of the carbon permits significant higher loadings of enzyme, which results in a higher enzymesupport strength and increased stability. The changes in the AP, HPAC and AP-HPAC were confirmed by Fourier Transform Infrared spectroscopy (FT-IR). Furthermore, scanning electron microscopy (SEM) allowed us to observe that the morphology of the surface of HPAC and the AP-HPAC.

Namdeo and Bajpai, 2009, suggested the study of Immobilization of α-Amylase onto cellulose-coated magnetite (CCM) nanoparticles and preliminary starch degradation. The size of the CCM nanoparticles, as obtained by TEM analysis, was found to be in the range of 2.5– 22.5 nm. The

CCM nanoparticles, containing 16 and 28 wt% of cellulose, demonstrated maximum attachment of 9.1 and 16.2mg of amylase/g of CCM particles for 0.05 M concentration of periodic acid. The uptake of amylase by CCM (28) particles followed Langmuir isotherm model with maximum uptake (qmax) of 18.2 mg/g CCM (28) particles. Finally, the degradation of starch by amylase-immobilized CCM (28) nanoparticles was also investigated. The values of Michaelis constants KM and maximum degradation rate rmax were found to be 7.5×10−7 μmol ml−1 and 0.04×10−6 μmol−1 g −1 min−1 respectively. Namdeo and Bajpai, 2009, suggested the study of Immobilization of α-Amylase onto cellulose-coated magnetite (CCM) nanoparticles and preliminary starch degradation. The size of the CCM nanoparticles, as obtained by TEM analysis, was found to be in the range of 2.5– 22.5 nm. The CCM nanoparticles, containing 16 and 28 wt% of cellulose, demonstrated maximum attachment of 9.1 and 16.2mg of amylase/g of CCM particles for 0.05 M concentration of periodic acid. The uptake of amylase by CCM (28) particles followed Langmuir isotherm model with maximum uptake (qmax) of 18.2 mg/g CCM (28) particles. Finally, the degradation of starch by amylase-immobilized CCM (28) nanoparticles was also investigated. The values of Michaelis constants KM and maximum degradation rate rmax were found to be 7.5×10−7 μmol ml−1 and 0.04×10−6 μmol−1 g −1 min−1 respectively.

Mohamed et al., 2013, investigated immobilization horseradish peroxidase (HRP) on activated wool. FT-IR spectroscopy and scanning electron microscopy were used to characterize immobilized HRP. The number of ten reuses of immobilized HRP has been detected. The pH was shifted from 5.5 for soluble HRP to 5.0 for immobilized enzyme. The soluble HRP had an optimum temperature of 30ºC, which was shifted to 35º C for immobilized enzyme. The soluble HRP and immobilized HRP were thermal stable up to 35 and 45º C, respectively. The apparent kinetic constant values (Km) of soluble HRP and chitosan–HRP were 35 mM and 40 mM for guaiacol and 2.73 mM and 5.7 mM for H_2O_2, respectively. Immobilization of HRP partially protected them from metal ions compared to soluble enzyme.

Roy et al., 2013, designed novel pH-induced chitosan−gum odina complex coacervates for colon targeting. Potentiometric titration experiments established that 1:1 charge stoichiometry occurred at a Ch/GO weight ratio of 1:5. The coacervate formed at pH 4.5 displayed the highest storage modulus (G′) values. FTIR, XPS, WAXS, TGA, and DSC results suggested the strong ionic ($NH3+\cdots COO-$) bond formation between these two biopolymers. Through in vitro viability tests, the pH-induced PECs were shown to be nontoxic. In vitro biodegradation rates of their microspheres revealed insolubility in simulated gastric fluid and simulated intestinal fluid and degradation by cell associated portions of rat cecal and colonic enzymes rather than the extracellular portions. The microsphere of pH 3.0 showed the highest degradation, and LVSEM micrographs revealed notably high amount of macropores in cell-associated enzymes, in contrast to extracellular enzymes.

Guzik et al., 2014, studied immobilization of enzymes is to enhance the economics of biocatalytic processes which allows one to re-use the enzyme for an extended period of time and enables easier separation of the catalyst from the product by selecting oxidoreductases as a model enzyme.

Martínez Cuesta et al., 2015, reported that enzymes sharing a common ancestor as defined by sequence and structure similarity are grouped into families and super families. The molecular function of enzymes is defined as their ability to catalyse biochemical reactions; it is manually classified by the Enzyme Commission and robust approaches to quantitatively compare catalytic reactions are just beginning to appear.

Suzuki et al., 2016, suggested a simple method for efficient DNA ligation utilizing the heat generation of ferromagnetic particles subjected to an AC magnetic field.

Pandey et al., 2017, suggested application of microbial enzymes in industrial waste water treatment. Enzymes can specifically act on and remove recalcitrant pollutants by precipitation and transformation to other products and can also change the characteristics of a given waste making it more susceptible to treatment or aid in converting waste material to value added products. Immobilization increases the mechanical and thermal stability of the enzymes while decreasing the probability of enzyme leaching into solution.

Traffano-Schiffo et al., 2017, investigated stability and microstructure alginate beads containing lactase. The effect of the addition of trehalose, and arabic and guar gums and their influence on the microstructure as well as on thermal properties and molecular mobility were studied. Lactase was successfully encapsulated in alginate-Ca (II) beads, and the inclusion of trehalose was critical for activity preservation toward treatments, being improved in guar gum-containing systems. The gums increased the Tm′ values, which represents a valuable technological improvement.

Sharifi et al., 2018, studied covalent immobilization of organophosphorus hydrolase enzyme on chemically modified cellulose microfibers. The activity yield of immobilized OPH on the modified cellulose was found to be 68.32%. The kinetic parameters, Km and Vmax values, were calculated and it was determined that the catalytic efficiency of the immobilized OPH was about 4.85-fold lower than that of free enzyme. The storage, thermal, and pH stabilities of the immobilized OPH were improved compared with free counterpart. The results revealed that after incubation for 24 h at 55 °C, the soluble and immobilized OPH retained 8% and 35% of their initial activities, respectively. Furthermore, the immobilized OPH showed a 59% residual activity when used ten times repeatedly.

Chapter 3

OBJECTIVES

Objectives:

Immobilization of enzyme: Enzyme immobilization provides an excellent base for increasing availability of enzyme to the substrate with greater turnover over a considerable period of time. Different natural polymers like gum Arabica, xanthan gum, guar gum, gum odina have matrix forming and binding properties.

Stability study of the entrapped enzyme: Stability studies are performed to observe their characteristic changes after immobilization. It is very important for the compatibility study of the components used for immobilization.

The main aim of this project is to prepare entrapped α-amylase beads by using gum odina-alginate as matrix support and perform its stability studies to observe the compatibility and activity of the immobilized enzyme.

Chapter 4

MATERIALS
&
METHODS

4. Materials and Methods

4.1 Materials

4.2 Collection of gum odina

4.3 Purification of gum

4.4 Preparation of immobilized α–amylase-calcium alginate-gum odina beads

4.5 Determination of protein content

4.6 Morphological characterization

4.7 Microstructure analysis

4.7.1 SAXS

4.7.2 DSC

4.8 Stability Study:

4.8.1 Midpoint of thermal inactivation (T_m)

4.8.2 Thermal inactivation kinetics

4.8.3 Hydrolysis of starch using beads of free enzyme and gum odina-alginate beads

4. MATERIALS & METHODS:

4..1 Materials: α-Amylase, gum odina (collected natural polymer), sodium alginate, bovine serum albumin(BSA), calcium chloride, coomassie brilliant blue G, ortho-phosphoric acid, soluble starch, 3,5-dinitrosalicylic acid(DNSA), double-distilled water, sodium dihydrogen phosphate, disodium hydrogen phosphate,sodium acetate, sodium hydroxide and hydrochloric acid. All the chemicals and materials used in this experiment were of standard quality. These chemicals were purchased from Himedia, India, Merck, Germany, Loba Chemie Pvt. Ltd., Mumbai, Merck specialities Pvt. Ltd., India.

4.2 Collection of gum odina: Gum odina was obtained from the bark of the plant *Odina wodier* as clear amber like substance which was collected from tropical deciduous forests of Akra Krishnanagar, South 24 Parganas, West Bengal, India. Collected gum was dried, pulverized by a mechanical blender (Phillips, Mumbai, India) to remove extraneous materials and was kept in sterile air tight jars for further analysis.

4.3 Purification of gum: Raw gum has been purified according to the procedure suggested by Mitra et al. Crude gum (30gm) was added to Milli-Q water (150ml) and left for 24 hrs at room temperature (250°C). After overnight swelling the gum was mixed thoroughly with the help of a laboratory stirrer (REMI, RQ-121D, India) for 8 hrs. The brown viscous solution obtained was passed through muslin cloth to remove fine extraneous materials. Then acetic acid was added to the filtrate to optimize the pH to 4-5 and then ethanol (Merck, Darmstadt, Germany) was added to the mixture, which resulted in the formation of a white precipitate that was collected and titrated with absolute ethanol (Merck, Darmstadt, Germany) and dry ether (Merck, Darmstadt, Germany) in a ratio of 1 : 1. The precipitated cake obtained was filtered. This process was repeated thrice and the obtained product was dried in vacuum at room temperature, (25 °C) pulverized by a mechanical blender (Phillips, Mumbai, India) and stored in air tight jars for future use. Thus after these steps we get a polysaccharide which is free from extraneous materials. The weight of purified and crude gum was measured and taken as the numerator and denominator respectively. The percentage yield was calculated by dividing the weights and multiplying by 100.

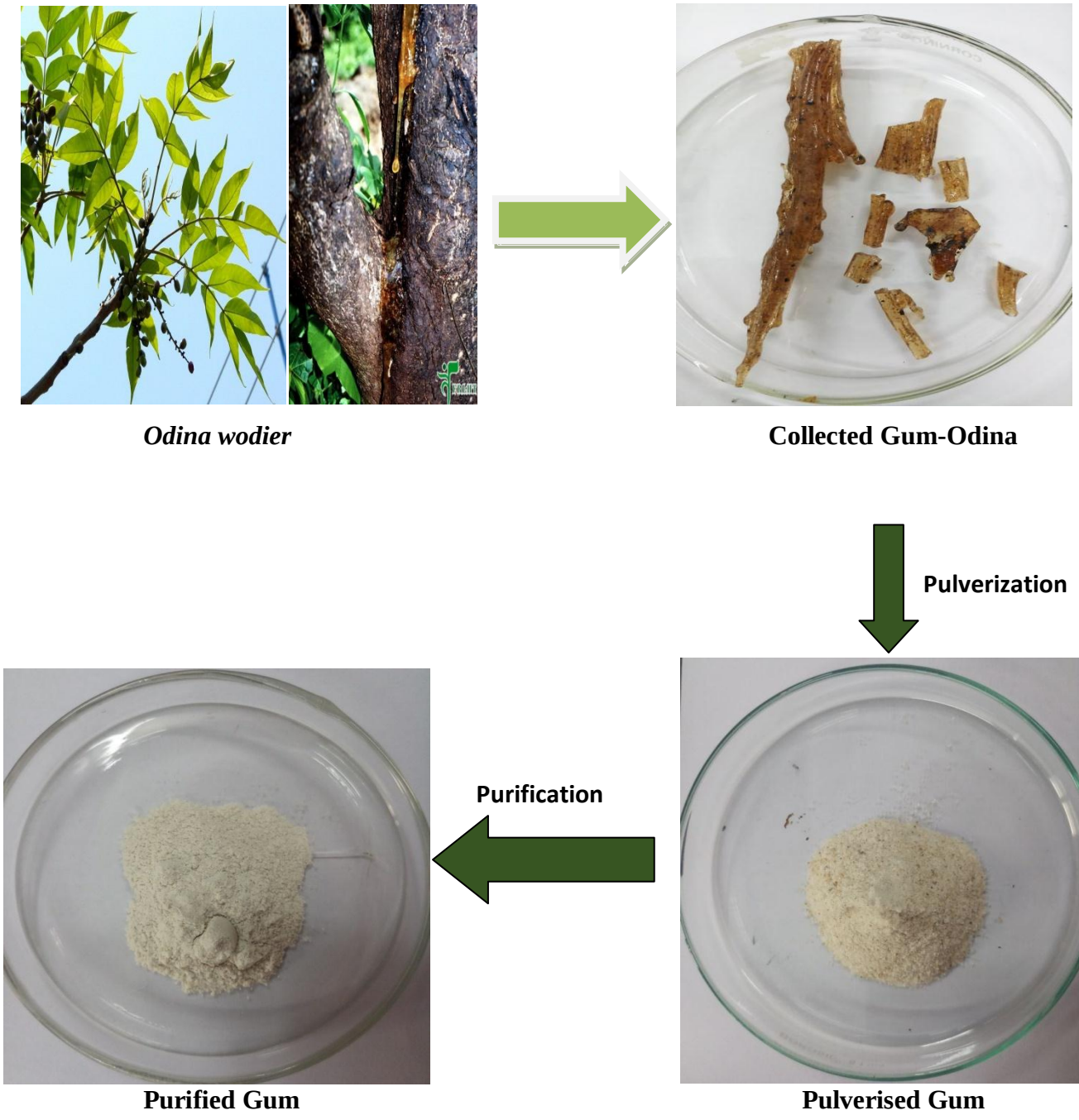

Fig: 7 Schematic representation of gum purification

4.4 Preparation of immobilized α–amylase calcium alginate-gum odina beads: The calcium alginate-gum odina beads containing α–amylase was prepared by using ionotropic gelation method. Calcium chloride ($CaCl_2$) will be used as cross-linker in ionotropic gelation. Keeping in mind that the isoelectric point of the enzyme is 5.1 (MacGREGOR, 1978) and the pKa values of alginate are 3.38 and 3.65 (Traffano-Schiffo et al., 2017), enzyme solution (2% gm/ml) was prepared by adding enzyme powder to acetate buffer of pH 4 at 25^{o}C in order to obtain an electrostatic interaction between the alginate (negatively charged) and the enzyme (positively charged). Then, Gum odina (0%-2% gm/ml) was added to enzyme solution and mixed with stirring for 10 min at 1000 rpm using a magnetic stirrer (Remi Motors, India). After that, Sodium alginate (3%-5% mg/ml) was added to the mixture and was mixed with stirring for 10 min at1000 rpm using a magnetic stirrer (Remi Motors, India). The final sodium alginate-gum odina gels containing α–amylase was ultrasonicated for 5 min for debubbling. The resulting dispersion was dropped via an 18-gauge needle drop wise into 3%-5% (w/v) $CaCl_2$ solution. Added droplets will be retained in the $CaCl_2$ solution for 30 min to complete the curing reaction and to produce rigid beads. The wet beads were collected by filtration, and washed two times with distilled water and will be dried to constant weight at 25^{o}C for 48 hrs. The calcium alginate-Gum odina beads containing α–amylase were stored in a dessicator until used. The washing solution was subjected for protein determination. Immobilization efficiency is defined as follows: Immobilization efficiency (%) = [(Theoretical enzyme loaded − Amount of enzyme leached in calcium chloride solution and washings)/Theoretical enzyme loading] × 100 (Sharma et al., 2014).

Table 1: Different concentration of gums (sodium alginate and gum odina) and different hardening times tried.

Sl No.	Sample name	Ratio of Gum odina & alginate(G:A)	Hardening Time	Conc. of $CaCl_2$ (W/V)	Amt. Of Enzyme (W/V)
1.	S_1	0:1	30 mins	3%	2%
2.	S_2	1:9	30 mins	3%	2%
3.	S_3	1:4	30 mins	3%	2%
4.	S_4	2:3	30 mins	3%	2%
5.	S_5	1:1	30 mins	3%	2%
6.	S_6	3:2	30 mins	3%	2%
7.	S_7	0:1	60 mins	5%	2%
8.	S_8	1:9	60 mins	5%	2%
9.	S_9	1:4	60 mins	5%	2%
10.	S_{10}	2:3	60 mins	5%	2%
11.	S_{11}	1:1	60 mins	5%	2%
12.	S_{12}	3:2	60 mins	5%	2%

4.5 Determination of protein content: Determination of protein contents of enzyme and washings was done by Bradford method. Approximately 1ml of diluted enzyme was added to 1ml of 0.1(M) phosphate buffer pH5.6 followed by addition of 5ml Bradfords reagent (0.1g of comassie brilliant blue G250 was dissolved in 50ml of ethanol followed by addition of 100ml 85% w/v phosphoric acid and volume will be made upto 1lt.) with uniform mixing. Then it was kept for at least 2 mins for complete colour development. Absorbance will be measured at 595 nm against blank. A standard calibration curve of absorbance versus concentration in µg/ml of protein (BSA) was plotted (Sharma et al., 2014).

4.6 Morphological characterization: Scanning electron microscopy was performed to examine the surface morphology and internal environment of the prepared microspheres. SEM images

were taken with a magnification of 50x to 400x, in a high pressure environment, using a high energy beam of electrons (1000 kV) (Zhang J et al. 2008).

4.7 Microstructure analysis: Cross-linking and gelation of alginate was widely used for immobilization of bio-entities, from macromolecules to metazoans. Beyond its high biocompatibility, by selection of the type of alginate and synthesis conditions, the pore size, degradation rate, and ultimately release kinetics can be finely controlled, allowing the design of controlled release systems. Proteins immobilized in alginate matrices are released by two mechanisms: (i) diffusion of the protein through the pores of the polymer network and (ii) degradation of the polymer network (Traffano-Schiffo et al., 2017).

4.7.1 SAXS: The microstructure characterization was performed by small-angle X-ray scattering (LNLS SAXS2 beamline, Rigaku, Japan, SmartLab 9kW, λ= 0.1488nm. the wave vector range was $0.096nm^{-1} < q < 2.856nm^{-1}$. All the beads were analysed showed isotropic scattering and will be modeled as a fractal system composed of rod like structures, although the rigorous interpretation of experimental results as indicating "fractality" requires many orders of magnitude of power-law scaling. Three parameters will be analyzed: (i) $\alpha 1$, the fractal dimension at distances higher than R, which describes the multiplicity of the junction zone, at $q < 0.28$; (ii) $\alpha 2$, the fractal dimension at distances lower than R, at $q > 0.55$, describing the degreeof compactness within the rods. Parameters $\alpha 1$ and $\alpha 2$ were evaluatedfrom the slope of the scattering intensity averaged along azimuthal angles versus the scattering vector q in the log−log scale. All measurements were made in triplicate (Traffano-Schiffo et al., 2017).

4.7.2 DSC: The endothermal melting peak were determined by differential scanning calorimetry (TA Instruments, USA; Q 10). The instrument was calibrated using standard compounds (indium and zinc) of defined melting point and heat of melting. All measurements were made in duplicate with 14−23 mg of sample mass (10 beads), using hermetically sealed aluminum pans of 40 µl inner volume (Mettler), heated from −90 to 90 °C at 10 °C/min; an empty crucible was used as a reference. Measurements were made in duplicate.

4.8 STABILITY STUDY:

4.8.1 Midpoint of thermal inactivation, (Tm): Activity loss, as a function of temperature, was performed in acetate buffer (0.1 M, pH 5.6). Both free and entrapped enzyme samples were incubated for 60 min at different temperatures in the range of 20–80^{o}C. After cooling, residual activity were measured (at 55^{o}C) by transferring an aliquot to the assay mixture. The midpoint of thermal inactivation (Tm), where the activity was diminished by 50%, will be calculated from the plot of percent residual activity versus temperature. Activity of the un-incubated enzyme was considered as 100% (Sharma et al., 2014).

4.8.2 Thermal inactivation kinetics: Kinetics of thermal inactivation of free and entrapped enzyme was studied at different temperatures in the range of 50-70°C. Enzyme samples (0.1M acetate buffer, pH 5.0) were incubated at the test temperature and aliquots were withdrawn at appropriate time intervals. Then it was cooled immediately in an ice bath and residual activity was measured. Activity of the un-incubated enzyme was considered as 100%. The inactivation rate constant, K_{in} was calculated by a semi-logarithmic plot of residual activity versus time.Then the temperature dependence of K_{in} was analysed from the Arrhenius plot to obtain the inactivation parameters (Sharma et al., 2014).

4.8.3 Hydrolysis of starch using beads of free enzymes and gum odina-alginate beads: The hydrolysis of starches from different sources (soluble potato, un-modified wheat, rice) were studied at 60°C in a batch system containing 0.1 M, pH 5.6 acetate buffer, 1% gelatinized starches each, and immobilized enzyme. After different time intervals (10, 30, 60, and 120 mins) of incubation at 60°C, samples were removed and tested (Jadhav and Singhal, 2014).

Chapter 5

RESULTS

&

DISSCUSSION

5. Results & Discussion

5.1 Enzyme encapsulation efficiency (%)

5.2 Surface morpholology analysis

5.3 Microstructure analysis

5.3.1 Small angle x-ray scattering (SAXS)

5.3.2 DSC

5.4 Stability study:

5.4.1 Midpoint of thermal inactivation (T_m)

5.4.2 Thermal inactivation kinetics

5.4.3 Hydrolysis of starch using beads of free enzyme and α-amylase gum odina-alginate beads

5.1 Enzyme encapsulation efficiency (%): Entrapment of enzyme within calcium alginate beads and gum odina beads was within range of 52.15±3.16 to 83.4±3.75. This result suggested that enzyme encapsulation efficiency was increased with the increase of both the amount of sodium alginate and gum odina due to high viscosity of polymer solution. High viscosity solution prevents leaching of enzyme during beads preparation thus resulting in enhanced encapsulation efficiency (Sharma et al., 2014).

5.2 Surface morpholology analysis: The surface morphology of formulated beads was analyzed by using scanning electron microscopy (SEM). The SEM images showed that beads possessed a homogeneous and compact structure with good spherical shape. Fig 8.b showed that various tiny pores are present on the surface of beads which allowed water uptake by capillary action and swelling (Traffano-schiffo et al.). This analysis was performed by using Quanta 250 FEG.

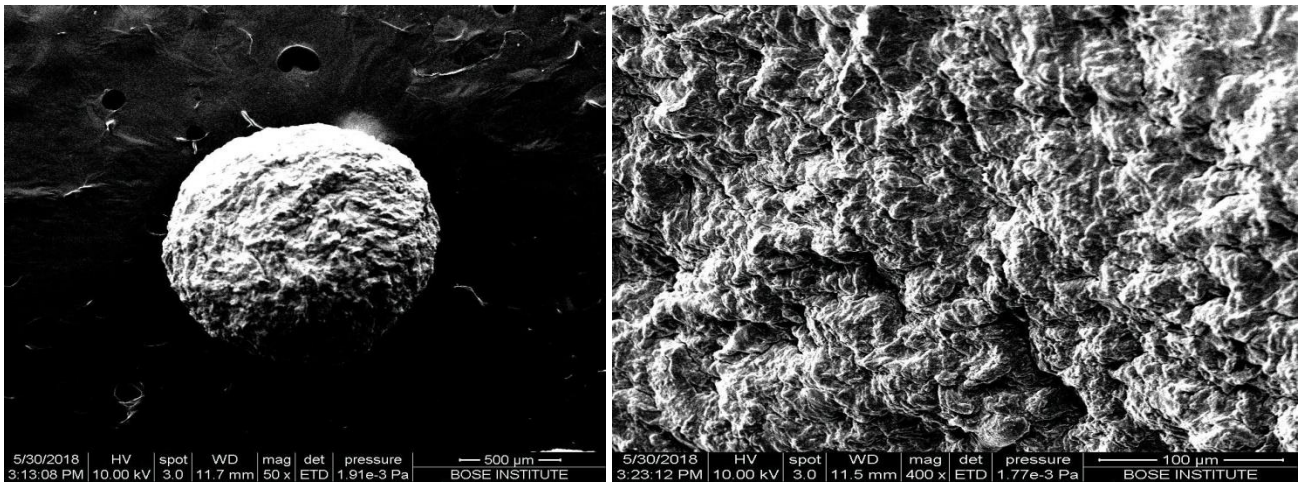

Fig 8. SEM images of (a) microsphere and b) presence of pores on surface of microsphere.

5.3 Microstructure Analysis:

5.3.1. Small angle x-ray scattering (SAXS):

Fig 9. below shows the graph between Scattering Intensity [I(q)] as a function of the scattering wave vector (q). The microstructure of beads can be found out by dividing the scattering curves into three regions of low, intermediate and high q values. At low q values, the parameter α_1, signifies the interconnectivity within the molecules. At intermediate q values, α_2, characterizes the compactness within the molecules and at high q values, the parameter α_3, is indicative of the connectivity between polymer chains, forming dimers. This analysis was performed by using Rigaku, Japan, SmartLab 9kW, λ= 0.1488nm. the wave vector range was $0.096nm^{-1} < q < 2.856nm^{-1}$.

Table 2: Values of parameter α_1, α_2 & α_3 for sample 1, sample 2 & sample 3

sample	α_1	α_2	α_3
Sample 1 (enzyme)	0.284	0.318	2.836
Sample 2 (enzyme + alginate)	1.566	0.962	1.27
Sample 3 (enzyme + alginate + gum odina)	1.87	2.36	0.37

From the above data, it was observed that α_3 value for free enzyme (sample 1) was highest indicating the formation of dimer by the enzyme. The gradually increasing values of α_2 from 0.318 to 2.36 indicates that the presence of gum (gum-odina) increases the compactness within the molecules, i.e., the molecules are more densely arranged within the microsphere. Low α_1 value (close to 1) as depicted in sample 1 is indicative of randomly oriented molecules. α_1 values close to 2 signifies interconnections between molecules as evident in sample 3. Also, the higher the value of α_1 the more the interconnections (Traffano-Schiffo et al., 2017).

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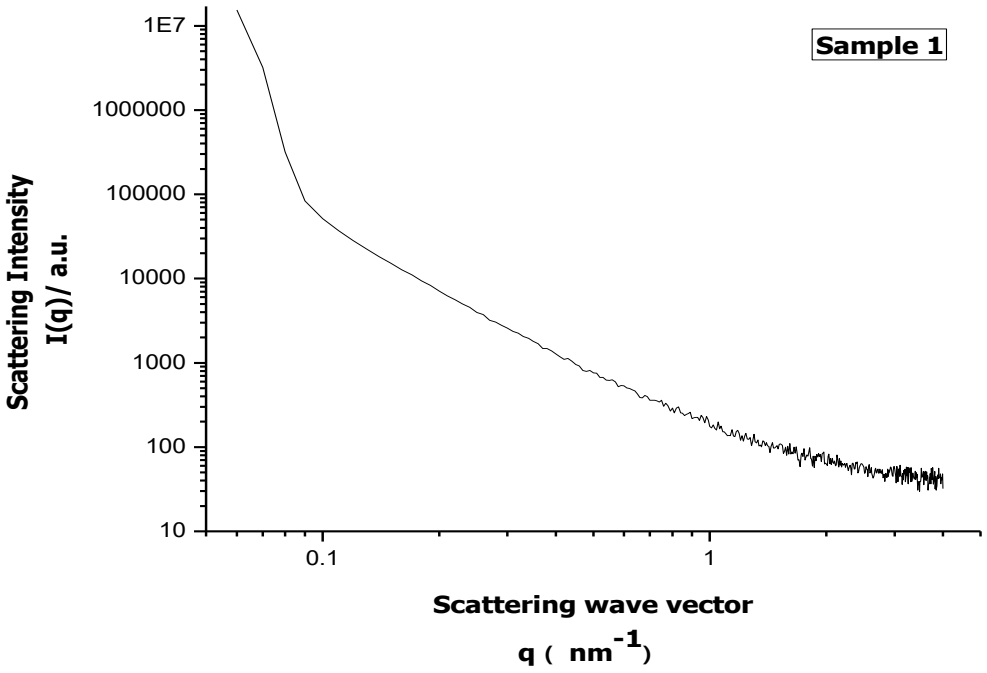
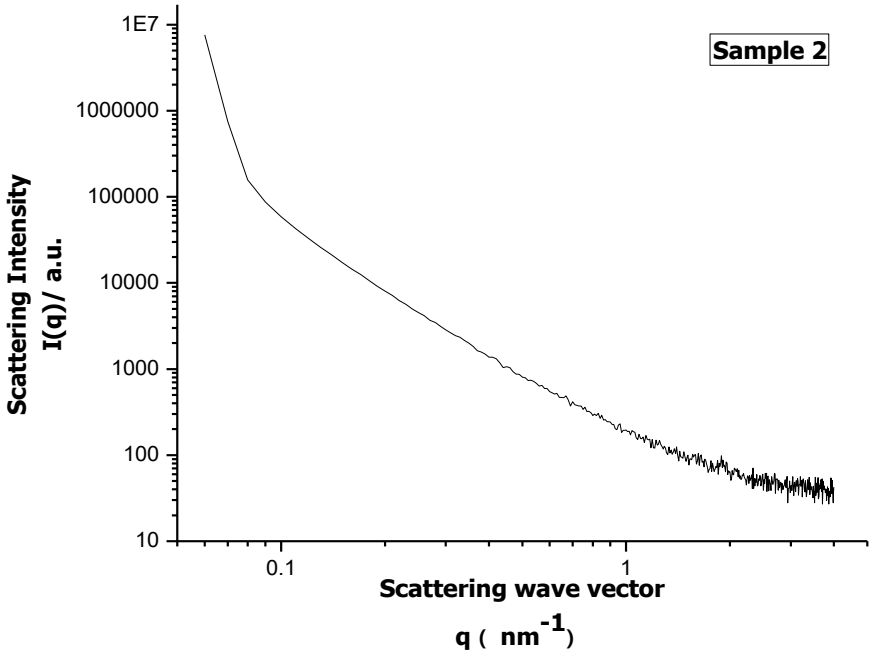
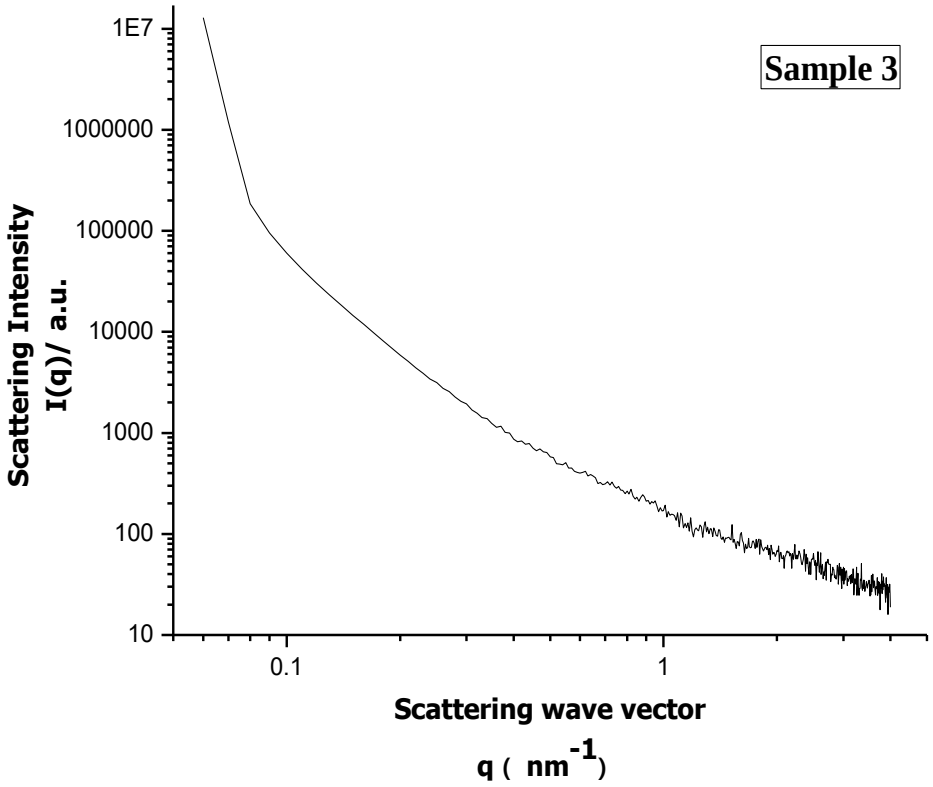


(a) sample 1



(a) Sample 2

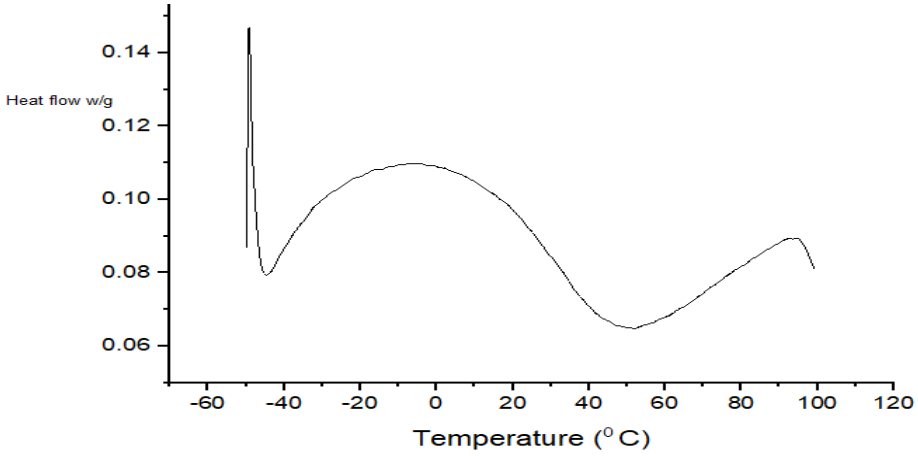


(b) Sample 3

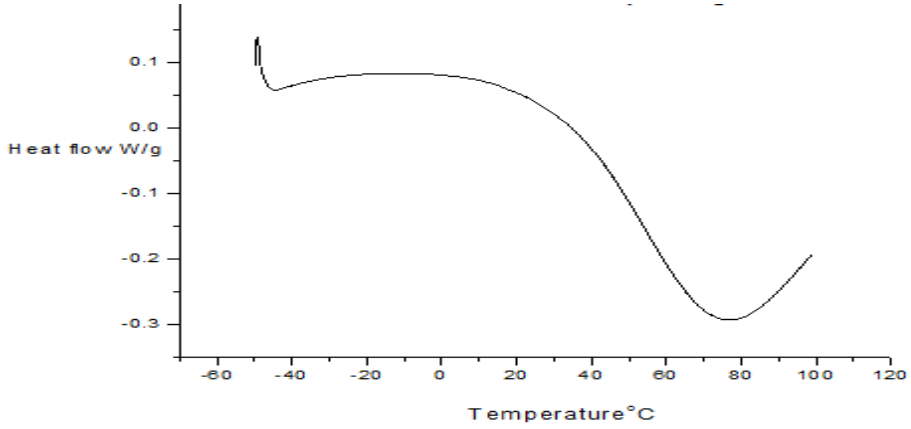
Fig. 9. SAXS graphs of (a) free enzyme, (b)alginate-enzyme bead, (c) alginate enzyme gum-odina bead

5.3.2 DSC

Fig 10. represented the DSC thermogram of alpha amylase, α- amylase gum-odina alginate beads. In case of α-amylase sharp endothermic peak was observed at 47.19˚C which was due to melting transition.The final formulation showed a broad peak at 74.98˚C. The absence of a enzyme peak indicates that enzyme was amorphous or was present as a solid solution inside the polymeric matrix (Traffano-Schiffo et al., 2017) .this analysis was performed by using TA Instruments, USA, Q 10. Samples were heated from -90º C to 90° C. measusements were made with 15-25 mg samples.



(a)



(b)

Fig 10. (a) DSC graph of α amylase (b) DSC graph of formulated α amylase gum odina alginate beads.

5.4 stability study:

5.4.1. Midpoint of thermal inactivation(T_m): Midpoint of thermal inactivation shifted from 63.4°C (free enzyme) to 76.8 for the entrapped amylase within beads. Fig. 11 exhibited that amylase loaded beads showed highest temperature stability against free enzyme(Sharma et al., 2014). .

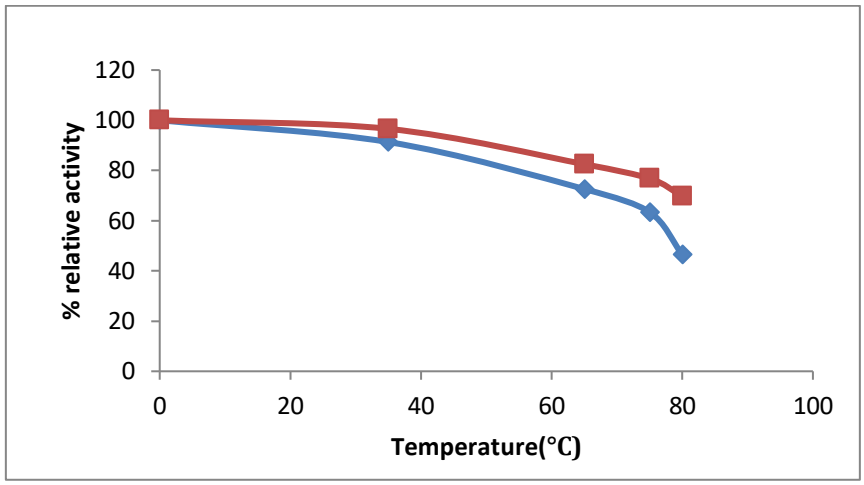
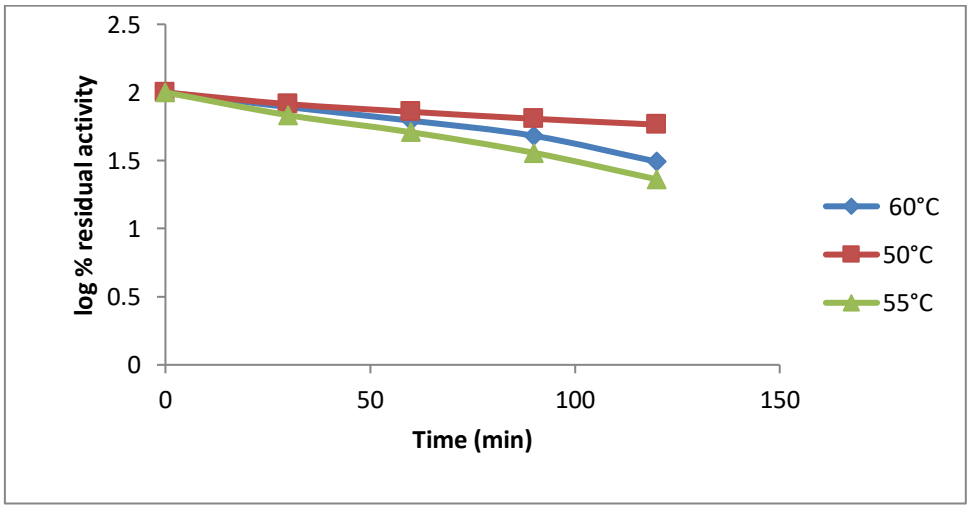
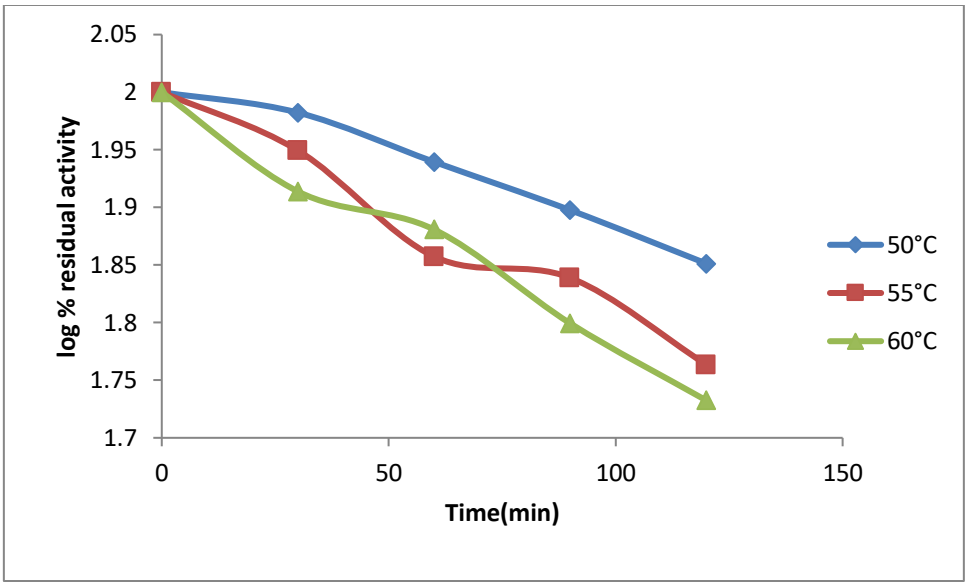


Fig 11: Mipoint of thermal inactivation for free and entrapped α-amylase in gum odina alginate beads.

5.4.2 Thermal inactivation kinetics:



(a) Free enzyme



(b) Entrapped Enzyme

Fig. 12 Thermal inactivation kinetic graph for a) free enzyme, b) enzyme entrapped in gum-odina alginate beads

Table 3: Thermodynamic parameters of free enzyme and formulation

Temperature (Kelvin)	$K_m \times 10^{-4}$ (S^{-1}) Free enzyme	Half Life (min) Free enzyme	$K_m \times 10^{-4}$ (S^{-1}) Entrapped enzyme	Half – Life (min) Entrapped enzyme
323	2.16	53.47	1.09	105.96
328	3.13	36.90	2.18	52.98
333	8.95	12.90	5.34	20.47

Thermal inactivation parameters for free and entrapped enzyme within polymer were determined by incubation at different temperature range from 50-60ºC. Residual activity was measured against function of time. Enzyme inactivation followed first order kinetics. Half life of entrapped enzyme was higher than the value of free enzyme. Half life of the enzyme increased from 20.47 to 105.96 min after entrapment within gum odina alginate beads indicating stabilization of enzyme(Sharma et al., 2014).

5.4.3. Hydrolysis of starch using beads of free enzyme and gum odina-alginate beads: The hydrolysis of starches from different sources like soluble potato, rice, maize, arrowroot was investigated both in presence and absence of polymer. The best hydrolysis activity was observed form soluble potatoes, arrowroot, maize, rice (80.3,72, 67.6, 59.26) in absent of polymer gum-odina at the end of 120 minutes. In the same time interval lower hydrolysis rate was observed for potatoes, arrowroot, maize, rice (45.3, 39, 42.73, 33.9) in present of gum odina which retard rapid hydrolysis of potatoes. From these result it had been concluded that hydrolysis pattern of starches totally depend on the type of polymer and along with properties of enzyme (Jadhav and Singhal, 2014).

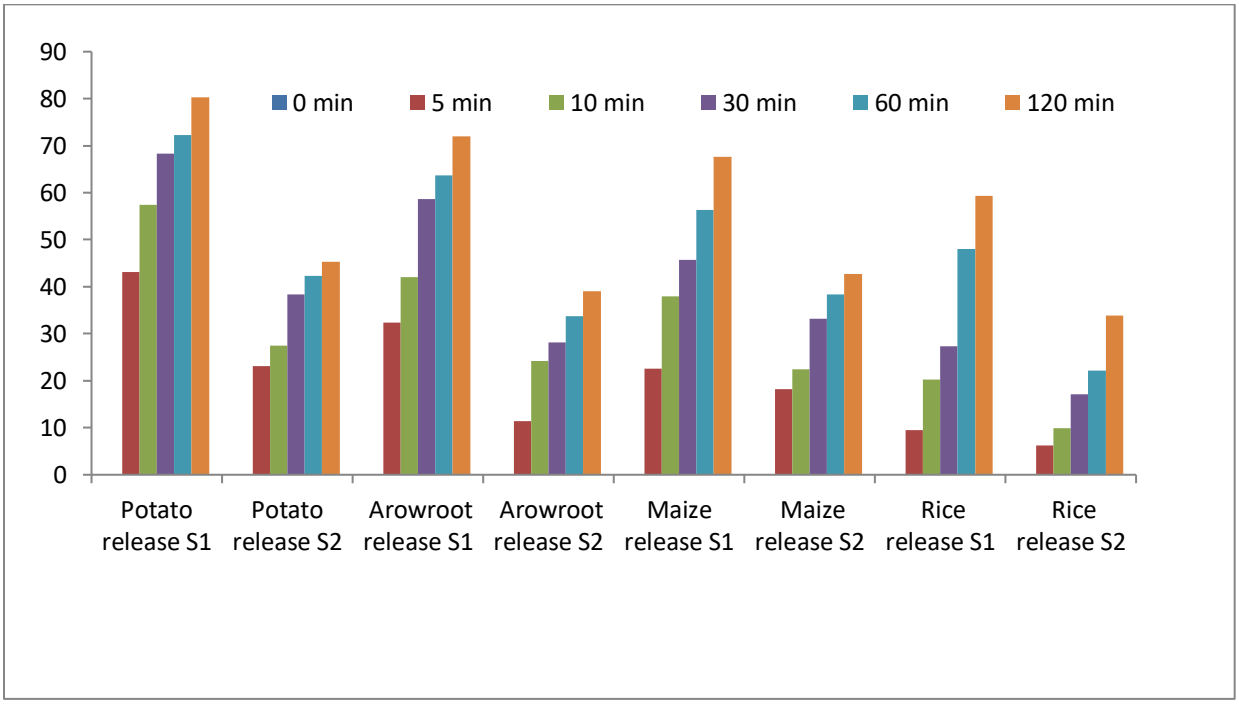


Fig 13. Hydrolysis pattern of various starches (a) potato starch in absence of polymer S1, potato starch in presence of polymer S2 (b) arrowroot in absence of polymer (S1), arrowroot in presence of polymer (S2) (c) maize in absence of polymer (S1), maize in presence of polymer(S2) (d) rice in absence of polymer(S1), rice in presence of polymer(S2)

Chapter 6

CONCLUSION

5. Conclusion

On the basis of the results found it can be concluded that the enzyme was successfully entrapped in the Gum-odina alginate beads so the immobilization was successful. The analytical parameters showed that the components of this formulation is compatible with each other without interfering the characteristics of each component. Gum-odina is a safe, nontoxic and biocompatible natural polymer used in this formulation. Incorporation of Gum-odina increased the stability of the immobilized enzyme beads. Immobilization, using natural polymer shows higher efficacy, relative activity, reusability, stability than the free enzyme and immobilized beads with-out polymer. Immobilization could be a great approach for targeted, sustained release enzyme drug delivery system in biotechnological and biomedical field. Future investigations should endeavor at adopting logistic and sensible entrapment techniques along with innovatively modified supports to improve the state of enzyme immobilization and provide new perspectives to the industrial sector as well as in pharmaceutical field.

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Chapter 7

REFFERENCES

8. References

Amid, M., Manap, Y., Zohdi, N., 2014. Microencapsulation of Purified Amylase Enzyme from Pitaya (Hylocereus polyrhizus) Peel in Arabic Gum-Chitosan using Freeze Drying. Molecules 19, 3731–3743. https://doi.org/10.3390/molecules19033731.

Amiel, D., Woo, S.L.-Y., Harwood, F.L., Akeson, W.H., 1982. The Effect of Immobilization on Collagen Turnover in Connective Tissue: A Biochemical-Biomechanical Correlation. Acta Orthopaedica Scandinavica 53, 325–332. https://doi.org/10.3109/17453678208992224.

An, J.D., Patterson, D.A., McNeil, S., Hossain, M., n.d. IMMOBILIZATION OF LIPASE ON WOOLLEN FABRICS: ENHANCED EFFECTIVENESS IN STAIN REMOVAL 33.

Betigeri, S.S., Neau, S.H., 2002. Immobilization of lipase using hydrophilic polymers in the form of hydrogel beads. Biomaterials 23, 3627–3636. https://doi.org/10.1016/S0142-9612(02)00095-9.

Das, B., De, A., Das, M., Das, S., Samanta, A., 2017. A new exploration of Dregea volubilis flowers: Focusing on antioxidant and antidiabetic properties. South African Journal of Botany 109, 16–24. https://doi.org/10.1016/j.sajb.2016.12.003.

de Lathouder, K.M., Lozano-Castelló, D., Linares-Solano, A., Wallin, S.A., Kapteijn, F., Moulijn, J.A., 2007. Carbon–ceramic composites for enzyme immobilization. Microporous and Mesoporous Materials 99, 216–223. https://doi.org/10.1016/j.micromeso.2006.08.039.

Elmarzugi, N.A., Enshasy, H.A.E., AbdulHamid, M., n.d. A AMYLASE ECONOMIC AND APPLICATION VALUE. World Journal of Pharmaceutical Research 3, 17.

Ertan, F., Yagar, H., Balkan, B., 2007. Optimization of α- Amylase Immobilization in Calcium Alginate Beads. Preparative Biochemistry and Biotechnology 37, 195–204. https://doi.org/10.1080/10826060701386679.

Evans, C, Egwim., O, Oloyede., 2011. Assessment of gum Arabica and agar gum as binders for the immobilization of α-Amylase. J Biochem Tech 3(1), 222-224.

Ganesh Kumar, A., Swarnalatha, S., Kamatchi, P., Kirubagaran, R., Perinmbam, K., Sekaran, G., 2009. Immobilization of proteolytic enzyme on highly porous activated carbon derived from rice bran. Journal of Porous Materials 16, 439–445. https://doi.org/10.1007/s10934-008-9216-9.

Ge, Y., Zhou, H., Kong, W., Tong, Y., Wang, S., Li, W., 1997. Immobilization of Glucose Isomerase and Its Application in Continuous Production of Hogh Fruvtose Syrup 98, 0273-2289.

Gombotz, W.R., Wee, S.F., 2012. Protein release from alginate matrices. Advanced Drug Delivery Reviews 64, 194–205. https://doi.org/10.1016/j.addr.2012.09.007.

Gómez, J.L., Bódalo, A., Gómez, E., Bastida, J., Hidalgo, A.M., Gómez, M., 2006. Immobilization of peroxidases on glass beads: An improved alternative for phenol removal. Enzyme and Microbial Technology 39, 1016–1022. https://doi.org/10.1016/j.enzmictec.2006.02.008.

Guzik, U., Hupert-Kocurek, K., Wojcieszyńska, D., 2014. Immobilization as a Strategy for Improving Enzyme Properties-Application to Oxidoreductases. Molecules 19, 8995– 9018. https://doi.org/10.3390/molecules19078995.

He, T., Tian, Y.-L., Qi, L., Zhang, J., Zhang, Z.-Q., 2014. Improved performance of α-amylase immobilized on poly(glycidyl methacrylate-co-ethylenedimethacrylate) beads. International Journal of Biological Macromolecules 65, 492–499. https://doi.org/10.1016/j.ijbiomac.2014.01.066.

Homaei, A.A., Sariri, R., Vianello, F., Stevanato, R., 2013. Enzyme immobilization: an update. Journal of Chemical Biology 6, 185–205. https://doi.org/10.1007/s12154-013- 0102-9.

Jadhav, S.B., Singhal, R.S., 2014. Pullulan-complexed α-amylase and glucosidase in alginate beads: Enhanced entrapment and stability. Carbohydrate Polymers 105, 49–56. https://doi.org/10.1016/j.carbpol.2014.01.066.

Jana, S., Das, A., Nayak, A.K., Sen, K.K., Basu, S.K., 2013. Aceclofenac-loaded unsaturated esterified alginate/gellan gum microspheres: In vitro and in vivo assessment. International Journal of Biological Macromolecules 57, 129–137. https://doi.org/10.1016/j.ijbiomac.2013.03.015.

Jawaheer, S., White, S.F., Rughooputh, S.D.D.V., Cullen, D.C., 2002. ENZYME STABILIZATION USING PECTIN AS A NOVEL ENTRAPMENT MATRIX IN BIOSENSORS. Analytical Letters 35, 2077–2091. https://doi.org/10.1081/AL-120014997.

Kolodziej, C.M., Chang, C.-W., Maynard, H.D., 2011. GlutathioneS-transferase as a general and reversible tag for surface immobilization of proteins. J. Mater. Chem. 21, 1457– 1461. https://doi.org/10.1039/C0JM02370A.

Kumar, R.S.S., Vishwanath, K.S., Singh, S.A., Rao, A.G.A., 2006. Entrapment of α-amylase in alginate beads: Single step protocol for purification and thermal stabilization. Process Biochemistry 41, 2282–2288. https://doi.org/10.1016/j.procbio.2006.05.028.

MacGREGOR, A., W., 1978. α-Amylase-I from malted barley- Physical properties and action pattern on amylose. Cereal Chem 55(5). 754-765.

Martínez Cuesta, S., Rahman, S.A., Furnham, N., Thornton, J.M., 2015. The Classification and Evolution of Enzyme Function. Biophysical Journal 109, 1082–1086. https://doi.org/10.1016/j.bpj.2015.04.020.

Mitra, D., Jena, A.K., De, A., Das, M., Das, B., Samanta, A., 2016. Prebiotic potential of gum odina and its impact on gut ecology: in vitro and in vivo assessments. Food & Function 7, 3064–3072. https://doi.org/10.1039/C5FO01581B.

Mohamed, S.A., Al-Malki, A.L., Kumosani, T.A., El-Shishtawy, R.M., 2013. Horseradish peroxidase and chitosan: Activation, immobilization and comparative results. International Journal of Biological Macromolecules 60, 295–300. https://doi.org/10.1016/j.ijbiomac.2013.06.003.

Mong Thu, T.T., Krasaekoopt, W., 2016. Encapsulation of protease from Aspergillus oryzae and lipase from Thermomyces lanuginoseus using alginate and different copolymer types. Agriculture and Natural Resources 50, 155–161. https://doi.org/10.1016/j.anres.2016.06.002.

Mulhbacher, J., McGeeney, K., Ispas-Szabo, P., Lenaerts, V., and Mateescu, M., 2002. Modified high amylose starch for im mobilization of uricase for therapeutic application. Biotechnol. Appl. Biochen 36, 163-170.

Namdeo, M., Bajpai, S.K., 2009. Immobilization of α-amylase onto cellulose-coated magnetite (CCM) nanoparticles and preliminary starch degradation study. Journal of Molecular Catalysis B: Enzymatic 59, 134–139. https://doi.org/10.1016/j.molcatb.2009.02.005.

Nayak, A.K., Das, B., Maji, R., 2012. Calcium alginate/gum Arabic beads containing glibenclamide: Development and in vitro characterization. International Journal of Biological Macromolecules 51, 1070–1078. https://doi.org/10.1016/j.ijbiomac.2012.08.021.

Pandey, K., Singh, B., Pandey, A.K., Badruddin, I.J., Pandey, S., Mishra, V.K., Jain, P.A., 2017. Application of Microbial Enzymes in Industrial Waste Water Treatment. International Journal of Current Microbiology and Applied Sciences 6, 1243–1254. https://doi.org/10.20546/ijcmas.2017.608.151.

Patel, N., Lalwani, D., Gollmer, S., Injeti, E., Sari, Y., Nesamony, J., 2016. Development and evaluation of a calcium alginate based oral ceftriaxone sodium formulation. Progress in Biomaterials 5, 117–133. https://doi.org/10.1007/s40204-016-0051-9.

Pongjanyakul, T., Puttipipatkhachorn, S., 2007. Xanthan–alginate composite gel beads: Molecular interaction and in vitro characterization. International Journal of Pharmaceutics 331, 61–71. https://doi.org/10.1016/j.ijpharm.2006.09.011.

Rahim, S.N.A., Sulaiman, A., Hamzah, F., Hamid, K.H.K., Rodhi, M.N.M., Musa, M., Edama, N.A., 2013. Enzymes Encapsulation within Calcium Alginate-clay Beads: Characterization and Application for Cassava Slurry Saccharification. Procedia Engineering 68, 411–417. https://doi.org/10.1016/j.proeng.2013.12.200.

Roy, P.S., Samanta, A., Mukherjee, M., Roy, B., Mukherjee, A., 2013. Designing Novel pH-Induced Chitosan–Gum Odina Complex Coacervates for Colon Targeting. Industrial & Engineering Chemistry Research 52, 15728–15745. https://doi.org/10.1021/ie401681t.

Sankalia, M.G., Mashru, R.C., Sankalia, J.M., Sutariya, V.B., 2007. Reversed chitosan–alginate polyelectrolyte complex for stability improvement of alpha-amylase: Optimization and physicochemical characterization. European Journal of Pharmaceutics and Biopharmaceutics 65, 215–232. https://doi.org/10.1016/j.ejpb.2006.07.014.

Sankaran, R., Show, P.L., Chang, J.-S., 2016. Biodiesel production using immobilized lipase: feasibility and challenges: Producing biodiesel from biomass oil via immobilized- lipase catalyzed alcoholysis. Biofuels, Bioproducts and Biorefining 10, 896–916. https://doi.org/10.1002/bbb.1719.

Sharifi, M., Robatjazi, S.-M., Sadri, M., Mosaabadi, J.M., 2018. Covalent immobilization of organophosphorus hydrolase enzyme on chemically modified cellulose microfibers: Statistical optimization and characterization. Reactive and Functional Polymers 124, 162–170. https://doi.org/10.1016/j.reactfunctpolym.2018.01.019.

Sharma, M., Sharma, V., Majumdar, D.K., 2014. Entrapment of α -Amylase in Agar Beads for Biocatalysis of Macromolecular Substrate. International Scholarly Research Notices 2014, 1–8. https://doi.org/10.1155/2014/936129.

Singh, V., Kumar, P., 2011. Carboxymethyl tamarind gum–silica nanohybrids for effective immobilization of amylase. Journal of Molecular Catalysis B: Enzymatic 70, 67–73. https://doi.org/10.1016/j.molcatb.2011.02.006.

Spagna, G., Pifferi, P.G., Gilioli, E., 1995. Immobilization of a pectinlyase from Aspergillus niger for application in food technology. Enzyme and Microbial Technology 17, 729– 738. https://doi.org/10.1016/0141-0229(94)01134-I.

Suzuki, M., Hayashi, H., Mizuki, T., Maekawa, T., Morimoto, H., 2016. Efficient DNA ligation by selective heating of DNA ligase with a radio frequency alternating magnetic field. Biochemistry and Biophysics Reports 8, 360–364. https://doi.org/10.1016/j.bbrep.2016.10.006.

Traffano-Schiffo, M.V., Aguirre Calvo, T.R., Castro-Giraldez, M., Fito, P.J., Santagapita, P.R., 2017. Alginate Beads Containing Lactase: Stability and Microstructure. Biomacromolecules 18, 1785–1792. https://doi.org/10.1021/acs.biomac.7b00202.

Tsen, J.-H., Lin, Y.-P., An-Erl King, V., 2004. Fermentation of banana media by using κ-carrageenan immobilized Lactobacillus acidophilus. International Journal of Food Microbiology 91, 215–220. https://doi.org/10.1016/S0168-1605(03)00376-3.

Xing, G.-W., Li, X.-W., Tian, G.-L., Ye, Y.-H., 2000. Enzymatic Peptide Synthesis in Organic Solvent with Different Zeolites as Immobilization Matrixes. Tetrahedron 56, 3517–3522. https://doi.org/10.1016/S0040-4020(00)00261-1.

Yagar, H., Ertan, F., Balkan, B., 2007. Comparison of Some Properties of Free and Immobilized α-Amylase by *Aspergillus sclerotiorum* in Calcium Alginate Gel Beads. Preparative Biochemistry and Biotechnology 38, 13–23. https://doi.org/10.1080/10826060701774304.

Zhu, Y., Gao, C., Shen, J., 2002. Surface modification of polycaprolactone with poly(methacrylic acid) and gelatin covalent immobilization for promoting its cytocompatibility. Biomaterials 23, 4889–4895. https://doi.org/10.1016/S0142-9612(02)00247-8.