Designing a Two Component System for Enzyme Immobilization Using a Modified Chitosan Support

By

Miriam K. Mioro

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Chemistry

Program

YOUNSGTOWN STATE UNIVERSITY

August, 2020

Designing a Two Component System for Enzyme Immobilization Using a Modified Chitosan Support

Miriam K. Mioro

I hereby release this thesis to the public. I understand that this thesis will be made available from the OhioLINK ETD Centre and the Maag Library Circulation Desk for public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research.

Signature:

May 22nd, 2020

Miriam K. Mioro, Student — Date

Approvals:

May 22nd, 2020

Dr. Nina V. Stourman, Thesis Advisor — Date

May 22nd, 2020

Dr. Michael Serra, Committee Member — Date

May 22nd, 2020

Dr. Douglas Genna, Committee Member — Date

May 22nd, 2020

Dr. Salvatore A. Sanders, Dean of Graduate Studies — Date

ABSTRACT

Immobilized multienzyme systems catalyzing cascade reactions have shown to be an effective industrial strategy. These systems reduce production cost by increasing both reusability and stability of enzymes under extreme conditions. Development of a stable support for enzyme immobilization further improves the process. The objective of this study was to improve lactose hydrolysis by designing a two-component system comprised of lactase and glucose oxidase immobilized independently on a modified chitosan support. The structural stability of chitosan was enhanced by addition of fortifying agents such as activated charcoal, silica, and magnetic particles followed by crosslinking beads with glutaraldehyde. Lactase was immobilized on chitosan-magnetic beads, chitosan-charcoal beads, and chitosan-silica beads. Glucose oxidase was immobilized on chitosan-charcoal beads and chitosan-silica beads. The physical properties, immobilization efficiency, and enzymatic activity were determined for each immobilized enzyme. Both enzymes, lactase and glucose oxidase, retained their activity after immobilization. Assays performed with the combinations of the chitosan beads indicated that the immobilized enzymes are capable of catalyzing two sequential reactions - lactose hydrolysis and glucose oxidation.

ACKNOWLEDGEMENTS

I wish to express my deepest and sincere gratitude to my advisor Dr. Nina V. Stourman for giving me the opportunity to conduct research in her lab. I cannot thank her enough for the invaluable guidance she provided to me, as my professor and research advisor. Her mentorship, patience, dedication, kindness, enthusiasm and knowledge have contributed immensely to my personal and professional growth.

I would like to thank my committee members Dr. Douglas Genna and Dr. Michael Serra not only for their insightful comments and contributions to my research but also as my professors. Dr. Genna instilled in me a whole new understanding on presenting scientific research in a professional manner while Dr. Serra provided me with requisite knowledge in his Advanced Biochemistry class which I applied in my research. I am thankful to Dr. Virgil Solomon and Ray Hoff for their help with characterization studies using SEM. Thanks to Tara Pickens for laying the foundation for Enzyme Immobilization project. I appreciate the support from former and current Stourman Lab members: Taylor, Monther, Rebeca, Bailey, Dillon, Emily, Thomas, Kayla, Katelyn, Charity and Nancy. My sincere gratitude goes to YSU Department of Chemistry faculty and staff for offering me the opportunity to study, work, and making my graduate experience a memorable one.

I am extremely grateful to my family and friends for their support. Special thanks to my parents Samuel Mioro and Esther Mioro, and my sisters who have been very supportive and have encouraged me to pursue my dreams. I sincerely would not have made it this far without their love, tender care and the values they instilled in me.

Finally, I would like to thank God Almighty for the guidance, wisdom, strength, peace and good health He bestowed upon me to complete this research successfully.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abs	absorbance
BglX	β-glucosidase with unknown biological function
BSA	Bovine serum albumin
ChsnAc	Chitosan-charcoal beads
ChsnMag	Chitosan-magnetic beads
ChsnSi	Chitosan-silica beads
Chsn-CL	Crosslinked chitosan beads
Chsn-CLLN	Crosslinked chitosan beads, immobilized with lactase and treated with $NaBH_4$
Chsn-CLNL	Crosslinked chitosan beads, treated with $NaBH_4$ and immobilized with lactase
ε	Molar extinction coefficient
GA	glutaraldehyde
GO Assay	Glucose oxidase assay
GOx	glucose oxidase
IMS	Immobilized Multienzyme Systems
***o*NPGal**	ortho-nitrophenyl-β-D-galactopyranoside
***o*NP**	ortho-nitrophenol
rpm	revolutions per minute
SEM	Scanning electron microscope
TLC	Thin Layer Chromatography

CHAPTER 1. INTRODUCTION

1.1 Immobilization of Enzymes and Applications

Enzymes are essential catalysts in various biochemical processes.[1] The high substrate specificity of enzymes increases the reaction yield by reducing formation of byproducts which create unnecessary waste. In addition, enzymes are biodegradable as they are easily decomposed in the environment and this supports green chemistry. Unlike small molecule catalysts, enzymes exhibit a high level of catalytic efficiency and can operate at mild conditions of pH, temperature, and pressure.[2] For example, in the production of inverted sugar, a mixture of fructose and glucose, a biochemical process using an enzyme invertase is often preferred over a chemical process that uses an acid catalyst and requires more energy.[3]

Enzymes' properties advocate for their broad use in different industries. They have been extensively studied and utilized in scientific fields and industrial applications such as medicine for diagnostics, the food industry for hydrolysis of fats, and in pharmaceuticals for drug delivery and release, among others.[1,2] Applications of free enzymes have limitations related to their stability and reusability. Free enzymes are hard to reuse since isolating them from the reaction environment tends to be time consuming and costly. Most enzymes are unstable outside their natural environment; they are denatured and lose their catalytic activity.[2] Scientists have discovered effective ways to counteract these limitations through enzyme immobilization, a method that has been applied in industrial processes to minimize production costs. [1,2]

Immobilization is a technique where an enzyme is localized or restrained to a solid support system such as chitosan or cellulose. This provides an improved and favorable microenvironment for an enzyme resulting in the retention of its catalytic activity.[4-6] Enzyme immobilization has many advantages. It simplifies product recovery and separation since removal of the enzyme from the reaction mixture is no longer required as the enzyme is restrained in a support and does not mix with the products like the free enzyme.[3-8] Fixing an enzyme on the solid support constrains its motion and conformational changes to a three-dimensional structure, enhancing its thermal stability thereby reducing denaturation caused by temperature variations. In addition, immobilization increases the stability of an enzyme making it less sensitive to changes in pH.[1,7] Similarly, the costs incurred during an enzyme's production by microorganisms and its purification are reduced because after immobilization the enzyme can be reused multiple times. This subsequently cuts overall operation costs.[3,4]

The first immobilization incidence was immobilization of acetic acid producing bacteria on wood shavings and twigs for vinegar production.[9] In earlier reports, enzyme catalysis was carried out using single enzyme immobilization. β-fructofuranosidase, also known as invertase, was one of the first enzymes to be immobilized and used for large-scale industrial application. Invertase catalyzes sucrose hydrolysis to yield inverted sugar. The mixture is referred to as inverted sugar because its optical activity is reversed compared to the starting saccharide.[3,9] Vu et al. immobilized invertase on alginate and studied the differences between the immobilized and free enzyme.[3] They found out that after immobilization, the activity of the enzyme was retained while the thermal stability and the durability of

invertase were improved compared to the free enzyme. Hence, the immobilized invertase could be used in the production of invert syrup.[3]

1.2 Multienzyme Immobilization

Immobilized multienzyme systems (IMS) encompass several enzymes that are combined together. Usually, they are involved into specific metabolic pathways and catalyze consecutive reactions.[10,11] Multiple enzymes are preferred to single enzymes as they are able to carry out complex reactions and can be used for catalysis of cascade reactions.[10,11] Multienzyme systems ensure that once the substrate/reactant passes the initial step, the reaction goes to completion as no diffusion of the intermediates is seen outside the complex, leading to purer products and minimal contaminant formation. The fast chemical conversion rates decrease the reaction time.[10] Also, product inhibition is reduced because the products of one enzyme act as substrates for the next one and do not inhibit the preceding enzyme.[10] IMS however have some drawbacks. The process is costly and complicated.[7,8,10] Different enzymes have different optimal working conditions hence hindrance of one enzyme's activity leads to malfunctioning of the entire multienzyme system.[7,10]

There are three ways for attaining immobilized enzymatic cascades. The first is catalysis by stepwise immobilized enzymes in which different immobilized enzymes are placed in separate modules where each enzyme catalyzes one reaction. The advantage of this system is that it has flexible unit operations and it is easy to monitor and detect the stability and activity of individual enzymes. Lower catalytic efficiency and higher energy consumption are some of the limitations for this system.[4,11] The second type of cascade systems is the

mixed immobilized enzyme system where different enzymes, immobilized separately on the same or a different support are combined. The process seems complicated, but this system has some advantages, like low substrate and product inhibition. Furthermore, enzyme stability and activity detection is simple and it is easy to control proportions of each type of immobilized enzyme.[11] The third type of system, the most common type, is the co-immobilized enzyme system where several enzymes are immobilized on the same matrix. This system is known to increase reaction rates and reduce delay times, however, it is more difficult to detect enzyme stability and activity, serious substrate and product inhibition is observed, and lastly, the whole system is easily affected by the stability of individual enzymes.[4,11]

For example, Henriques[6] designed a system where β-D-galactosidase and lipase were co-immobilized on magnetic nanoparticles. Better stability was observed than in their free forms; lipase and β-galactosidase could be used over wider pH and temperature ranges after immobilization. β-Galactosidase in its free form remained active up to 30 °C, however, after immobilization the temperature range increased to 50 °C, and after treatment with glutaraldehyde, it was active up to 70 °C. [6]

Some of the factors to consider while designing an IMS are as follows: coupling pH and temperature, the properties of individual enzymes, the proportions of each enzyme and cascade bottleneck which is the rate limiting step.[11] The optimum pH of each enzyme should be considered. If enzymes have different optimum pHs, for example, one is acidic and the other is basic, then there might be a challenge in using them together within the system. Different enzymes may have different optimum temperatures and combining enzymes with different optimum temperatures affects the efficiency of the system. To

manage this issue, either single temperature control programs or variable temperatures at different time periods are employed depending on the dominant role of the immobilized enzymes.[11] The proportions of each enzyme is also very important. Different enzymes vary in their catalytic efficiencies and the quantity of each enzyme should be controlled to accommodate those variations and to ensure a smooth operation of the multienzyme system. The enzyme with the lowest catalytic efficiency forms the bottleneck and in cascade reactions, the overall rate depends on the slowest reaction. Therefore, the cascade bottleneck is a key factor in ensuring the efficiency of the system. It is important to consider the following about the rate-limiting enzyme: it should be maintained within maximum activity window and be present in appropriate proportions.[11]

Immobilized Multienzyme systems are applicable in various fields.[10-13] They are used for biofuel production, for instance, carbon dioxide conversion into methanol. They also serve in diagnosis of diseases and development of therapeutics.[10,12] For example, lysozymes and proteases have been applied in treatment of inflammation.[10] Glucose oxidase and peroxidase immobilized on SiO_2 and cross-linked with glutaraldehyde were employed to detect glucose levels in serum samples.[10] IMS are also utilized in the food industry, for instance, amylase and glycosylase have been utilized for hydrolysis of starch. In other fields, like agriculture, multienzymes systems are used for detection of pesticides and in the chemical industry - for large scale production of various compounds like syrup and amino acids.[6,10] Co-immobilized α-amylase, pectinase, and protease enzymes found their applications in waste-water pretreatment.[14]

Co-immobilized multienzyme systems can be used to design biosensors. Biosensors are analytical devices used for detection of specific chemical species by transforming a

biological signal into an electrical signal.[10,15] Lang et al.[16] designed an electrochemical sequential biosensor that was able to detect starch without measuring other chemical signals, like H_2O_2. This was done by monitoring the decrease in the amount of starch. The biosensor was designed by co-immobilizing glucoamylase that catalyzed conversion of starch to glucose, and glucose oxidase, catalyzing oxidation of glucose to gluconolactone. The enzymes were immobilized on multiwalled carbon tubes, crosslinked with glutaraldehyde, and the blocking agent bovine serum albumin was used to increase stability. [16] Heng and colleagues designed a co-immobilized system by immobilizing β-galactosidase through encapsulation within a silica matrix followed by the covalent binding of lysozyme to the silica surface using glutaraldehyde. Lysozyme was used to prevent bacterial growth during milk production.[17] It was found that co-immobilized enzymes employed to treat milk gave favorable results: high lactose hydrolysis rate and high antibacterial protection, also better operational and storage stabilities were observed with both enzymes.

1.3 Types of Support

Supports for enzyme immobilization are divided into two groups - artificial and natural polymers.[1,18] Artificial polymers are further divided into synthetic polymers and inorganic materials.[1] The synthetic polymers include polyacrylamide, polyacrylate, and polystyrene. They are insoluble and have porous surfaces where the enzyme is restrained.[1,19] An advantage of these polymers is their resistance to changes in pressure. However, they are not biocompatible with the biological molecules and can cause enzyme denaturation due to hydrophobic interactions between the artificial polymer and the protein.[19] The inorganic polymers include activated carbon, charcoal, silica, celite, ceramics, glass, and zeolites.[1]

Natural polymers include alginate, agarose, chitin, chitosan, cellulose, collagen, carrageenan, cross-linked dextran, gelatin, starch, pectin, and others.[1,19] Natural supports are usually preferred for industrial processes including drug delivery due to their non-toxicity, chemical stability, low protein denaturation, and biocompatibility. Natural supports are compatible with other biomolecules such as enzymes and are also biodegradable as they can easily decompose in the environment.[5,18] However, one drawback is that they have a low resistance to high pressure and sometimes enzyme leakage might occur due to their microporous makeup.[18]

In order to improve the performance and stability of the natural and synthetic polymer supports, modifications can be done. For instance, fortifying agents, like magnetic and non-magnetic particles including silica, charcoal, activated carbon, and crosslinking agents are used to improve the properties and stability of the supports.[10,19] The physical-chemical properties of these agents make them suitable to be used in supports. For example, the small size of silica increases the surface area to volume ratio which allows for more binding of enzymes. Magnetic particles are easy to separate from the reaction mixture.[19,20] In a recent study, Wahba[21] revealed that chitosan beads supplemented with a porogen, like sodium carbonate , increased the surface area of the beads for more enzyme loading. [21] When glutaraldehyde (GA) is used as a crosslinking agent, it enhances the stability of the support by formation of strong covalent bonds.[9,21]

Liu et al.[5] prepared crosslinked magnetic chitosan beads and immobilized glucose oxidase to aid in the oxidation of glucose and hence monitor the amounts of glucose oxidized. They found out that after immobilization, the enzyme activity was retained, the stability to changes in temperature and pH was enhanced, and the beads retained catalytic activity after

15 cycles.[5] Pospiskova et al.[20] developed simple methods for making magnetic beads. They prepared beads using chitosan and iron (II) sulfate and microwaved them to make them magnetically responsive. Glutaraldehyde, the crosslinking agent, was added to increase the beads' stability. The enzymes lactase and lipase were used as model enzymes for immobilization. They reported that both immobilized enzymes retained 100% of the initial activity after being reused eight times, no enzyme leakage was observed, and the beads were easy to isolate using a magnet.[20] Xi et al.[19] reported that immobilization of trypsin on silica coated with chitosan crosslinked with glutaraldehyde improved the properties of trypsin. The authors concluded that immobilization of the enzyme increased the enzyme's stability and resistance to changes in pH and temperature as compared to free trypsin.[19]

1.4 Methods of Immobilization

Some of the commonly known methods for enzyme immobilization include adsorption, affinity immobilization, entrapment, crosslinking, and covalent bonding.[1-5,11] Adsorption is one of the oldest, simplest, and most cost-effective immobilization techniques. It involves hydrophobic interactions, electrostatic interactions, van-der Waals forces and/or hydrogen bonding between enzymes and the support.[22] This method has limitations such as enzyme leakage, changes in enzyme conformations, and loss of its catalytic activity when subjected to harsh environments.[7]

Entrapment or encapsulation is another technique where an enzyme is localized within the membrane. The enzyme is trapped in a polymeric network but it is not directly connected to the support and this allows for transfer of only the substrates and products in and out of the membrane. Encapsulation represents an entrapment method where the enzyme is caged

(entrapped) inside of the area surrounded by a semipermeable membrane. The enzyme is restrained inside and the substrates and products diffuse in and out through the membrane.[1,7,22] The commonly known limitations of these techniques are enzyme leakage, if the pore size of the matrix is too large; and diffusion problems, if the matrix is too small for the substrate to diffuse and reach the enzyme.

Crosslinking or immobilization without supports is another method which involves the formation of intermolecular linkages between enzymes by covalent bonds. A crosslinking agent like glutaraldehyde is required as a linker between the enzymes. There are different approaches for crosslinking of enzymes. One method involves crosslinked enzymes crystals (CLECs) where glutaraldehyde is added after crystals are formed. The other method is crosslinked aggregates (CLEAs) which are an improvement of CLECs, formed by addition of salts, organic solvents, or non-ionic polymers to form enzyme aggregates capable of working in aqueous solutions.[22] Another type is crosslinked spray-dried enzymes (CSDE). Some advantages of these crosslinking methods are: they are simple to design; they minimize costs that might be incurred in using supports, and minimize enzyme leakage. Some drawbacks observed are that the system is mechanically unstable if crosslinkers are not used.[22,24] On the other hand, the use of glutaraldehyde might lead to modifications of the enzyme that affects its activity.[21-24]

Lastly, the most common and widely used method is covalent modification which involves the formation of covalent bonds between the enzyme and the support. This method is known to be very effective and prevents leakage of the enzyme. This is due to the formation of strong permanent covalent bonds that are hard to break.[1] Crosslinking agents like glutaraldehyde, dextran-aldehyde, hexamethylene-1,6-diisocyanate, and

bis(diazo)benzidine are known to increase the enzyme's stability thus maintaining the enzyme's structure and functional properties.[20,21] This method is the most studied in enzyme immobilization.[1]

1.5 Chitosan Properties

Chitosan, a waste byproduct in the shellfish industry, is commonly used as a support. It is a cationic natural polyaminosaccharide derivative of chitin.[2,25] Chitin is the second most abundant naturally occurring polysaccharide after cellulose, and it is found in the exoskeleton of marine animals like crustacean shells, shrimp, oysters, crab shells, shellfish, krill, clams, squid, and fungi among others.[1] Chitosan is a copolymer of N-acetyl-D-glucosamine and D-glucosamine. It is easily derived by N-deacetylation of chitin, a polymer of β- (1-4) linked 2 -acetamido-2-deoxy-β-D-glucose units (**Figure 1**).[2,25,26] Since chitin is not soluble in water it is deacetylated to chitosan which is soluble.[25,26]

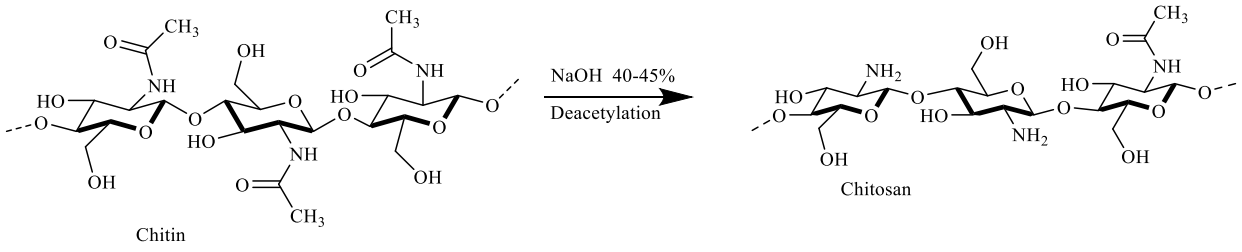

Figure 1: Chitosan synthesis from chitin.[2]

The most common industrial chitin extraction process involves three steps: deproteinization by adding alkaline solution, demineralization through treatment with an acid, and decolorization by treatment with an alkaline solution.[25,26] Deacetylation involves treatment of chitin with hydroxides at high temperatures between 120 – 150 °C which yields between 50% and 70% deacetylated chitosan. Chitosan is soluble in aqueous acidic

media when half of its amino group is deacetylated. Conversion of chitin to chitosan can also be done by enzymatic processes; however, the chemical processes are widely used because they are cost effective and, therefore, are sustainable for mass production.[25,26]

Chitosan is abundant, inexpensive, biocompatible, biodegradable, non-toxic, hydrophilic, easy to prepare, has reactive functional groups, and possesses amino and hydroxyl groups in D-glucosamine.[25,26] The functional reactive groups are important in chemical modifications and interaction with the enzyme.[2,25] Chitosan is a polyelectrolyte with a pK_a of 6.5.[2,24] In the acidic solutions of pH less than 6.5, the amino groups are protonated and they acquire a positive charge ($-NH_3^+$).[2,24] The positive charge affords antimicrobial properties through the interaction with the negative charge of the cell membrane of microorganisms.[26]

The above-mentioned characteristics make chitosan commercially viable. Li et al.[27] reported that crosslinking chitosan with glutaraldehyde exhibited antibacterial properties that inhibited the growth of *Burkholderia cepacia,* an antibiotic resistant bacterium. In addition, chitosan's solubility, ionic charge, chelating, and adsorbent properties make it applicable in various purifications. Chitosan has selective chelating properties for metals, like iron, cadmium, and magnesium. These characteristics are highly desirable in wastewater treatment.[25,26] It can be used as an adsorbent to remove textile dyes, petroleum, and, generally, in many purification processes.[2,25] Several studies demonstrated that the glutaraldehyde crosslinked magnetic chitosan nanocomposites exhibited excellent adsorption capacity for food and textile dye making them useful in the removal of the dyes from wastewater.[28,29] Moreover, chitosan has been proposed for use in designing artificial kidneys due to its permeability and high tensile strength as it does not break easily under

tension.[1] Chitosan's excellent gel forming properties are attributed to its solubility in acidic solutions and its accumulation with anions. Chitosan acidic solutions can be used to make beads or films. The beads are formed through the precipitation after chitosan is added dropwise to a basic solution.[9,21]

1.6 Glycosidases

Glycosidases, also referred to as glycoside hydrolases or glycosyl hydrolases, are examples of the carbohydrate active enzymes (CAZymes) that are involved in polysaccharide degradation.[7, 30] Glycosidases are named after the type of substrate they act on, some examples of glycosidases are α-amylase, cellulases, xylanases, and β-galactosidases. Glycosidases are mainly used in the food industry and are also useful in fields like medicine and the paper industry.[7,30]

Glycosidases are primarily classified in terms of their amino acid sequences, their mode of action, and tertiary structural folds; approximately 115 families have been identified.[30] Hydrolysis of the glycosidic bond requires two amino acids present in the active site, which are usually glutamate and /or aspartate. Of the two residues one acts as a nucleophile and the other as an acid-base catalyst. Mechanistically the enzymes can be classified as either inverting or retaining glycosidases. Inverting glycosidases yield a product that has a different configuration than the substrate.[31,9] On the other hand, retaining glycosidases yield a product that has the same configuration as the substrate.[31,9]

Lactase (β-galactosidase) is a type of glycosidase that catalyzes the hydrolysis of the glyosidic bond of lactose to form glucose and galactose. Lactase is active in the pH range 2 - 7, and its optimal pH is 6, while its optimal temperature ranges from 21 to 50 °C. Lactase

is utilized in the food industry in the production of low lactose dairy products for those people who are lactose intolerant.[29] About 70% of the world adult population experiences intestinal malfunctions when they consume dairy products.[29] However, industrial applications of many glycosidases are limited by the inhibition caused by the reaction products. [29]

Bisset and Sternberg,[32] in a study of immobilization of β-glucosidase on chitosan, revealed that immobilization increased enzyme performance in catalyzing the hydrolysis of cellobiose to form two molecules of D-glucose. The stability of the immobilized enzyme outside optimal conditions was improved and it was resistant to denaturation at high temperatures above 50 ^{o}C. However, product inhibition was still a challenge encountered. The produced glucose inhibited β-glycosidase and the activity of the enzyme was reduced.[32]

Pickens[9] explored immobilization of enzymes like lactase and β-glucosidase (BglX) from *E. coli* and its mutant E293Q on chitosan solid support in the Stourman Lab. The enzymes were successfully immobilized on the chitosan support and their catalytic activity was retained. A glucose oxidase-peroxidase (GO) assay was used to determine the amount of glucose produced from the hydrolysis of lactose. Results demonstrated that BglX was inhibited by the products of lactose hydrolysis, glucose and galactose in batch production. When *ortho*-nitrophenyl -β-D-galactopyranoside was used as a substrate, high glucose concentrations reduced the activity by approximately 50%, while galactose reduced the activity by 30%. Hence it was suggested that a continuous flow system that removes product from the immobilized enzyme would increase the product yield compared to batch

production where the enzyme remains in contact with the product for the duration of the reaction.[9]

Torres and Batista-Viera[4] conducted a study on how to counteract β-galactosidase inhibition by its products through immobilization of multiple enzymes. They designed a stable system where β-galactosidase was combined with immobilized L-arabinose isomerase and D-xylose isomerase on epoxy resins Eupergit C and Eupergit C 250 L. The aim was to transform lactose into the ketohexoses D-fructose and D-tagatose. β-Galactosidase catalyzed the hydrolysis of lactose to glucose and galactose. Thereafter, the products were converted by the isomerases into ketohexoses: glucose to D-fructose catalyzed by D-xylose isomerase and galactose to D-tagatose catalyzed by L-arabinose isomerases. From the results obtained, it was concluded that co-immobilization favored higher conversions of lactose hydrolysis and reduced product inhibition.[4]

1.7 Glucose Oxidase, an Oxidoreductase

Glucose oxidase is an oxidoreductase, a class of enzymes that catalyzes the transfer of electrons from an electron donor (reductant) to an electron acceptor (oxidant). It has classification number E.C. 1.1.3.4, which means it is an oxidoreductase that uses the CH-OH as a hydrogen donor reducing oxygen to a hydrogen peroxide.[33] This enzyme is produced by some fungi species and insects. Glucose oxidase (GOx) was first discovered in *Aspergillus niger* in 1928 by Muller.[33] It is still the most commonly available source of the enzyme and the most studied. GOx is a homodimer of two identical polypeptide chains of 80 kDa that are covalently linked via disulfide bonds.[33,34] The optimum pH range of *Aspergillus niger* GOx is 4 – 7 and the optimum temperature range is 25-30 °C. The main

function of glucose oxidase is to act as an antifungal and antibacterial agent through hydrogen peroxide production during oxidation of glucose. The effectiveness has been seen mainly in microorganisms that lack catalase, an enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen.[33,34]

GOx catalyzes the oxidation of D-glucose to D-gluconic acid and hydrogen peroxide as shown in the reaction scheme below **(Figure 2).**[33,34]

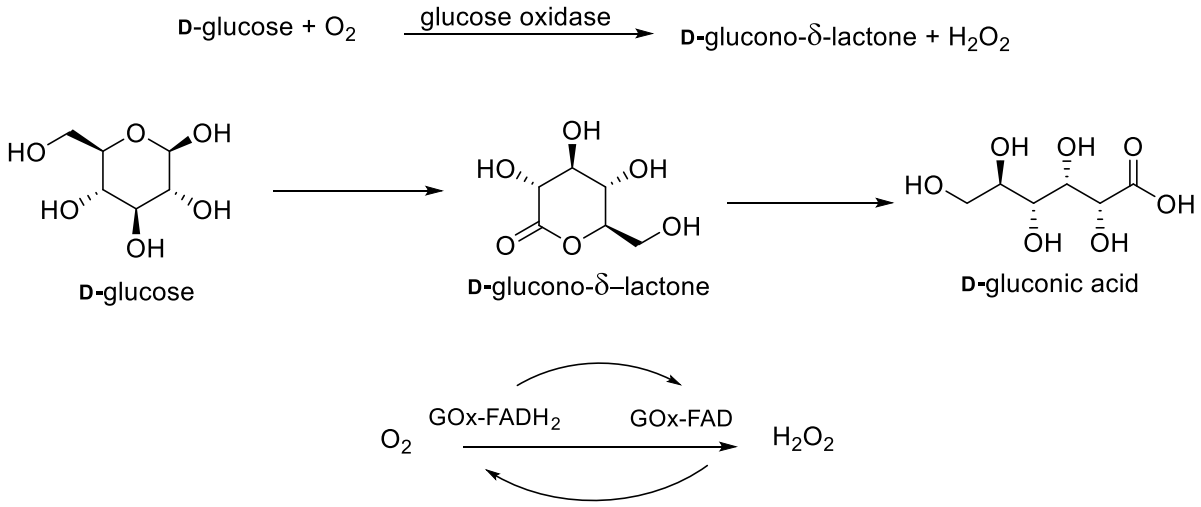

Figure 2: A representation of the glucose oxidase reaction.[33]

Glucose oxidase has been used in the food industry as a food preservation technique where the catalyzed reaction removes oxygen to generate hydrogen peroxide which is a bacteriocide.[34] It has found applications in oral care products, the hydrogen peroxide produced has a bactericidal effect on the microorganisms that might cause tooth decay. In wine production, baking, dry egg powder production GOx helps to remove oxygen before sealing bottled beverages.[33,34] Moreover, glucose oxidase is used as a glucose biosensor for patients with diabetes to monitor fluctuations in blood glucose levels. GOx is also utilized in designing biofuel cells, which convert biochemical energy into electrical energy using

biocatalysts. Additionally, glucose oxidase is applied in the production of gluconic acid, a polyhydroxycarboxylic acid. Gluconic acid is a bulk chemical in many industries such as food, beverage, and pharmaceuticals, where it is utilized as a dietary supplement. It also has found application as an industrial cleaner and as a bleaching agent in the textile industry.[33,35]

1.8 Statement of the Problem

The aim of this work is to design a two-component system comprised of lactase and glucose oxidase enzymes immobilized individually on two different modified chitosan supports. The designed system will catalyze two sequential reactions - lactose hydrolysis and glucose oxidation. This will improve lactose hydrolysis through minimization of lactase inhibition by its glucose product. Chitosan possesses most of the preferred biological and chemical characteristics for enzyme immobilization.[2,4] The chitosan gel support will be modified to improve its structural properties and stability. Activities of immobilized lactase and glucose oxidase will be measured first individually, then in combination.

1.9 Approach - General Procedure

Modification of chitosan support is achieved by addition of fortifying agents like activated charcoal, silica, and magnetic particles, followed by crosslinking of the support with glutaraldehyde. Immobilization efficiency of the enzymes lactase and glucose oxidase is determined by calculating the amount of protein bound using the Bradford assay. The activity of the immobilized enzymes is measured individually. Lactase activity is determined by measuring the amount of ortho-nitrophenolate (*o*NP) produced from the hydrolysis of a model substrate ortho-nitrophenyl-β-D-galactopyranoside (*o*NPGal).[23,36]

Glucose oxidase activity is measured using glucose coupled with a peroxidase assay using phenol/4-aminoantipyrine as the substrate.

Finally, the ability of the two immobilized enzymes, lactase and glucose oxidase, to catalyze two sequential reactions are measured using the substrate lactose. Lactose hydrolysis produces D-galactose and D-glucose which is oxidized by glucose oxidase to D-gluconic acid and hydrogen peroxide. The amount of hydrogen peroxide produced is determined by coupled assay with peroxidase using phenol/4-aminoantipyrine.[37,38] Hydrogen peroxide acts as the oxidizing agent in the oxidation of phenol/4-aminoantipyrine to *p*-quinoneimide.as shown on a proposed scheme for the two-component system below (**Figure 3**).

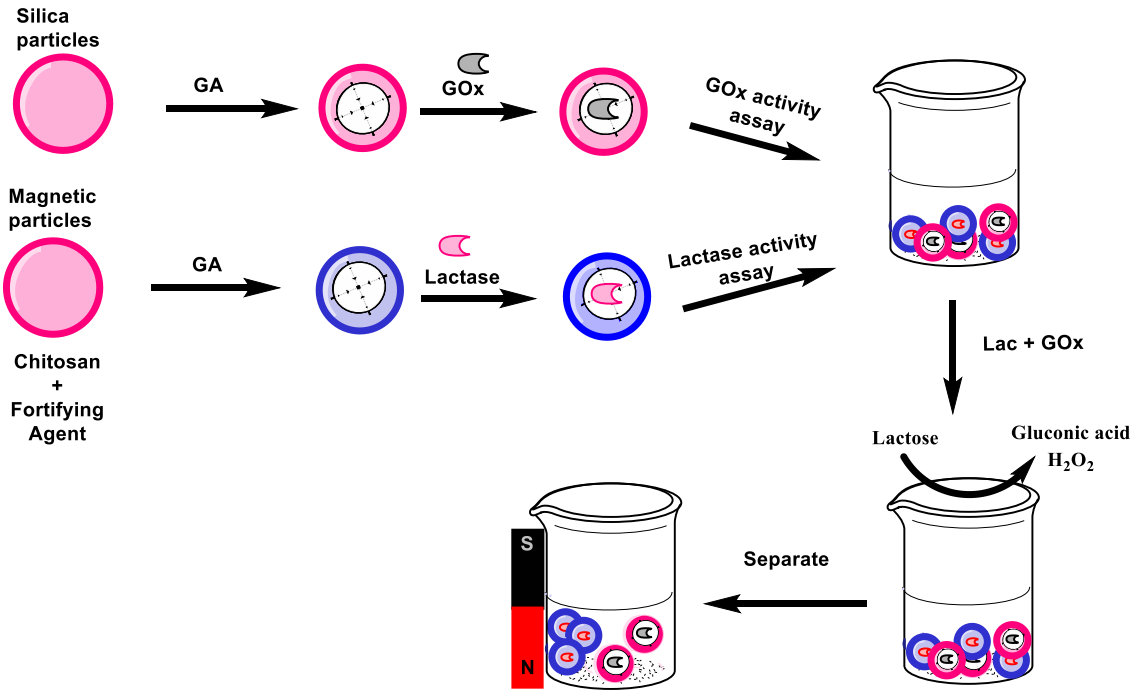

Figure 3: Creation of a proposed two-component system. Crosslinking of different chitosan beads with glutaraldehyde (GA); immobilization of enzymes lactase and glucose oxidase (GOx) on chitosan beads; individual enzymatic assays; combining the two enzymes to monitor lactose hydrolysis and glucose oxidation; separation of beads by magnet

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

Glutaraldehyde solution (Grade II, 25% in H_2O), the GO Assay kit, silica HF, activated charcoal, bovine serum albumin (BSA), glucose oxidase, D-lactose, peroxidase, phenol, 4-aminoantipyrine, and sodium borohydride were purchased from Sigma Aldrich (St. Louis, MO). Glucose was obtained from Mallinckrodt Inc. (Paris, KY). D-Galactose, *o*-nitrophenyl-β-D-galactopyranoside *(o*NPGal), acetic acid, and sodium hydroxide were purchased from Fisher Scientific (Fairlawn, NJ). Magnetic Powder was purchased from forensicsource.com/lightning powder. Lactase Dairy Relief tablets were purchased from Rite Aid (Camp Hill, PA). Chitosan powder was purchased from Bulk Supplements (Henderson, NV). Potassium phosphate monobasic anhydrous and Bradford reagent were purchased from Amresco (Solon, OH). A Hewlett Packard Agilent 8453 Photodiode Array spectrophotometer was used for all spectrophotometric analysis. MilliporeSigma™ Silica Gel 60 F254 Coated Aluminum-Backed TLC Sheets were from Fisher Scientific (Fairlawn, NJ), A JOEL JIB-4500 multi beam system SEM (scanning electron microscope) was used to examine the surface morphology of chitosan beads.

2.2 Methods

2.2.1 Preparation of Chitosan Beads

Chitosan powder (100. mg) was dissolved in 10 mL of 1.0 M acetic acid solution. It was left to stir on a magnetic stir plate for about 1 hour until all chitosan powder was dissolved and the solution had become more viscous. A NaOH/ethanol solution was prepared by

dissolving 15.0 grams of sodium hydroxide in 100 mL distilled water and then mixing with 25 mL of 95% ethanol to give a 12% (w/v)-NaOH and 20% (v/v) ethanol solution. The chitosan solution was added dropwise to the NaOH/ethanol solution. The beads were left to cure for 30-60 minutes. Distilled water was used to rinse them until a neutral pH of 7.0 was attained.

2.2.2 Preparation of Chitosan Beads Fortified with Inert Materials

A chitosan solution was prepared as previously described. Once all the chitosan powder was dissolved, 100. mg of activated charcoal was added to 10 mL of the chitosan solution to achieve a 1% (w/v) suspension and it was left to stir on a magnetic plate overnight at room temperature. The chitosan-charcoal mixture was added dropwise to the NaOH/ethanol solution. The chitosan-charcoal (ChsnAc) beads were left to cure for 30-60 minutes and later rinsed with distilled water until the pH was neutral (pH 7.0).

Chitosan-silica beads (ChsnSi) were prepared by adding 100. mg of silica HF to 10 mL of chitosan solution to make a 1% (w/v) suspension, and the beads were prepared as described above. The ChsnAc and ChsnSi beads were stored in 0.1 M potassium phosphate (KPi) buffer (pH 6.8) in the refrigerator for future use.

2.2.3 Preparation of Chitosan Beads Using Commercial Magnetic Powder

Chitosan (100. mg) was dissolved in 10 mL of 0.2 M acetic acid with continuous mixing. A magnetic stir bar was removed before 200. mg of magnetic powder was slowly added to the chitosan solution, then it was mixed by vortexing. The magnetic chitosan suspension was added dropwise to 1 M NaOH. After the chitosan-magnetic (ChsnMag) beads were formed and cured for 30-60 minutes, beads were rinsed with distilled water until a neutral

pH 7.0 was attained. The beads were then stored in 0.1 M KPi buffer (pH 6.8) in the refrigerator for future use.

2.2.4 Cross-linking Chitosan Beads with Glutaraldehyde

A 0.1% (v/v) glutaraldehyde solution (GA) in 0.1 M KPi buffer (pH 6.8) was prepared. A 50 µL scoop was used to transfer chitosan beads (ChsnAc, ChsnMag, or ChsnSi,) into individual Eppendorf tubes. The chitosan beads were incubated with glutaraldehyde solution at 4 °C with shaking: ChsnAc and ChsnSi were incubated for 90 min, ChsnMag for 120 min. Next, the glutaraldehyde solution was removed by pipetting the liquid from the Eppendorf tube that contained the beads. The beads were then rinsed with 0.1 M KPi buffer (pH 6.8) and stored in 1 mL of the same buffer in the refrigerator.

2.2.5 Treating Crosslinked Chitosan Beads with Sodium Borohydride ($NaBH_4$)

A 200 µL of aqueous sodium borohydride solution ($NaBH_4$, 1 mg/mL) was added to crosslinked chitosan beads. The mixture was gently shaken at room temperature for 40 minutes. The solution was discarded; the treated beads were rinsed with 0.1 M KPi buffer, (pH 6.8) and stored in 1 mL of the same buffer in the refrigerator.

2.2.6 Morphological Characterization of Chitosan Beads Using SEM

Morphological characterization of chitosan-silica beads was performed with the help of Dr. Solomon, Department of Mechanical and Industrial Engineering, YSU. A scanning electron microscope (SEM) was used to examine the surface morphology of non-crosslinked, non-immobilized chitosan-silica (ChsnSi) beads. The chitosan beads were air-dried on a watch glass to allow for any liquid to evaporate. Beads were fixed on a semi conductive rubber tape and then a vacuum was created to remove air so that it could not

cause any scattering of electrons, the sample was loaded on to the SEM and the morphology was examined.

2.2.7 Preparation of Lactase Solution

Lactase solution was prepared by crushing two tablets of Lactase Dairy Relief into a powder using a mortar and pestle. The powder was then dissolved in 0.1 M potassium phosphate buffer (pH 6.8). The mixture was centrifuged for 10 minutes at 6,000 x g to precipitate the insoluble particles. The pellet was discarded, and supernatant was saved as lactase solution. The lactase solution was stored in the refrigerator for future use.

2.2.8 Determination of Lactase Concentration

The concentration of lactase was determined using the Bradford assay and bovine serum albumin (BSA) was used as the standard. Four standard solutions were prepared with Bradford reagent, distilled water, and BSA (0.5 μg/μL) at varied amounts (5-35 μg). A 50 μL lactase solution was mixed with 50 μL of distilled water and 1 mL of Bradford reagent. Samples were prepared in triplicates. The samples were vortexed and incubated at room temperature for 15 minutes before taking the absorbance at 595 nm. The readings from the standard solutions were used to construct a standard curve. The line generated from these data was used to calculate the concentration of lactase.

2.2.9 General Procedure for Immobilization of Lactase on Chitosan beads

The lactase immobilization solution was prepared using 100 μL of lactase solution and 900 μL of 0.1 M KPi buffer (pH 6.8). A 50 μL scoop was used to transfer chitosan beads into individual microcentrifuge tubes. The enzyme solution was added to the chitosan beads and the tube was inverted to mix the contents. An aliquot of 100 μL of lactase solution was

removed and saved for protein analysis (before incubation). The beads were then incubated with the lactase solution for at least 16 hrs at 4 ^{o}C with shaking. After incubation, the lactase solution was removed from the beads and saved for protein analysis using the Bradford assay. The beads were stored with 1 mL of 0.1 M KPi buffer (pH 6.8) in the refrigerator. These steps were repeated for all types of chitosan beads.

2.2.10 Lactase Saturation Curve for Immobilization on Chitosan-Charcoal (ChsnAc) Beads

Chitosan-charcoal beads were prepared as described in Section 2.2.2 from 200 µL (1% w/v each) suspension. The beads were cured for 30 min in NaOH/ethanol solution and rinsed with distilled water to attain a neutral pH as previously described. Four 10-fold serial dilutions of lactase were made using 0.1 M KPi buffer (pH 6.8). ChsnAc beads were incubated with solutions prepared by mixing 100 µL aliquots of each of the lactase solution with 900 µL of 0.1 M KPi buffer (pH 6.8). The samples were prepared in duplicates and the immobilization procedure was done as previously described. Samples from before and after incubation were saved for protein analysis using the Bradford assay.

2.2.11 Lactase Activity Assay Using Substrate o-Nitrophenyl-β-D-Galactopyranoside (oNPGal)

A chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (*o*NPGal) was used to determine if lactase immobilized on chitosan beads retained activity. The activity of lactase was determined spectrophotometrically by measuring the absorbance at 405 nm of *o*-nitrophenolate which was produced during the hydrolysis of *o*NPGal (molar extinction coefficient of 3500 M^{-1} cm^{-1}). A 30 mM *o*NPGal solution was prepared in 0.1 M KPi buffer (pH 6.8). A 100 µL sample of 30 mM *o*NPGal solution was added to an Eppendorf tube with 900 µL of 0.1 M KPi buffer (pH 6.8) and chitosan beads and the timer was started

immediately. The tube was inverted gently several times and was placed horizontally on a shaker to ensure the beads were evenly exposed to solution. The spectrophotometer was set at 405 nm and blanked with 100 µL of *o*NPGal (30 mM) and 900 µL of 0.1 M KPi buffer (pH 6.8). Aliquots of 100 µL were removed from the reaction sample at intervals of 10 minutes for 30 minutes, mixed with 900 µL of 0.1 M KPi buffer (pH 6.8) and absorbances were recorded. The steps were repeated for all the samples.

2.2.12 Comparing the Catalytic Activity of Free and Immobilized Lactase Enzyme

Three Eppendorf tubes containing ChsnSi beads with approximately 180 µg of immobilized lactase protein and 900 µL of 0.1 M KPi buffer (pH 6.8) were prepared. Three tubes were prepared with 100 µL of 1.80 mg/mL of free lactase and 900 µL of 0.1 M KPi buffer (pH 6.8). A 100 µL sample of *o*NPGal (30 mM) substrate was added to an Eppendorf tube with ChsnSi beads and timer was started immediately. The tube was inverted gently several times and was placed horizontally on a shaker to ensure the beads are evenly exposed to solution. The spectrophotometer was set at 405 nm and blanked with 100 µL of 30 mM *o*NPGal and 900 µL of 0.1 M KPi buffer (pH 6.8). Aliquots of 100 µL were removed from the reaction sample at intervals of 10 minutes for 60 minutes, added to a cuvette containing 900 µL 0.1 M KPi buffer (pH 6.8), mixed by inversion, and the absorbance was recorded. The procedure was repeated for all the samples.

2.2.13 Stability and Reusability of Immobilized Lactase

To assess the reusability of immobilized lactase, the lactase activity test was performed as previously described using chromogenic substrate *o*NPGal in Section 2.2.10. The same procedure was repeated consecutively nine times with the same batch of beads. Beads were

washed with potassium phosphate buffer between assays. The samples were prepared in triplicates as shown in **Figure 4** below.

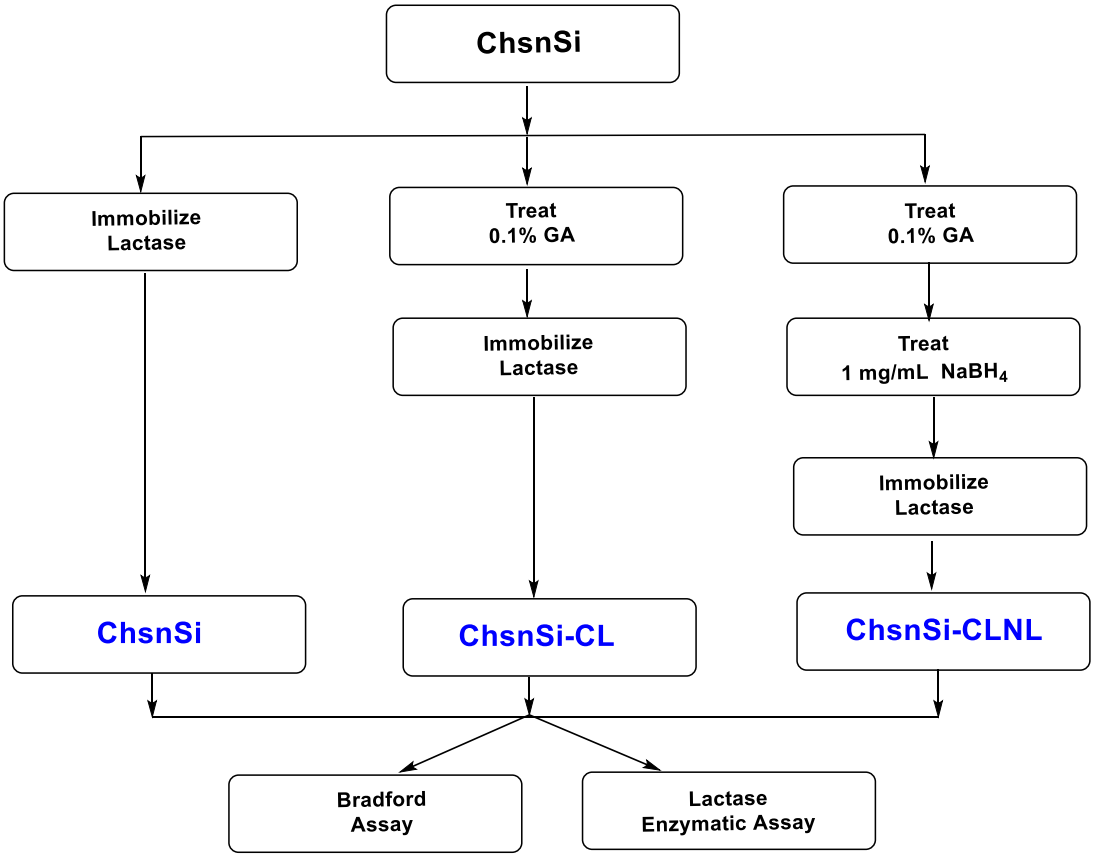

Figure 4: Procedure for preparing ChsnSi Beads with different glutaraldehyde and sodium borohydride treatments.

2.2.14 Effect of Glutaraldehyde and $NaBH_4$ on the Immobilization and Catalytic Activity of Lactase

Chitosan-charcoal (ChsnAc) beads were subjected to different treatments as outlined in the scheme below (**Figure 5**). Samples were prepared for each condition. Crosslinking, treatment with sodium borohydride, and enzyme immobilization were done as previously described.

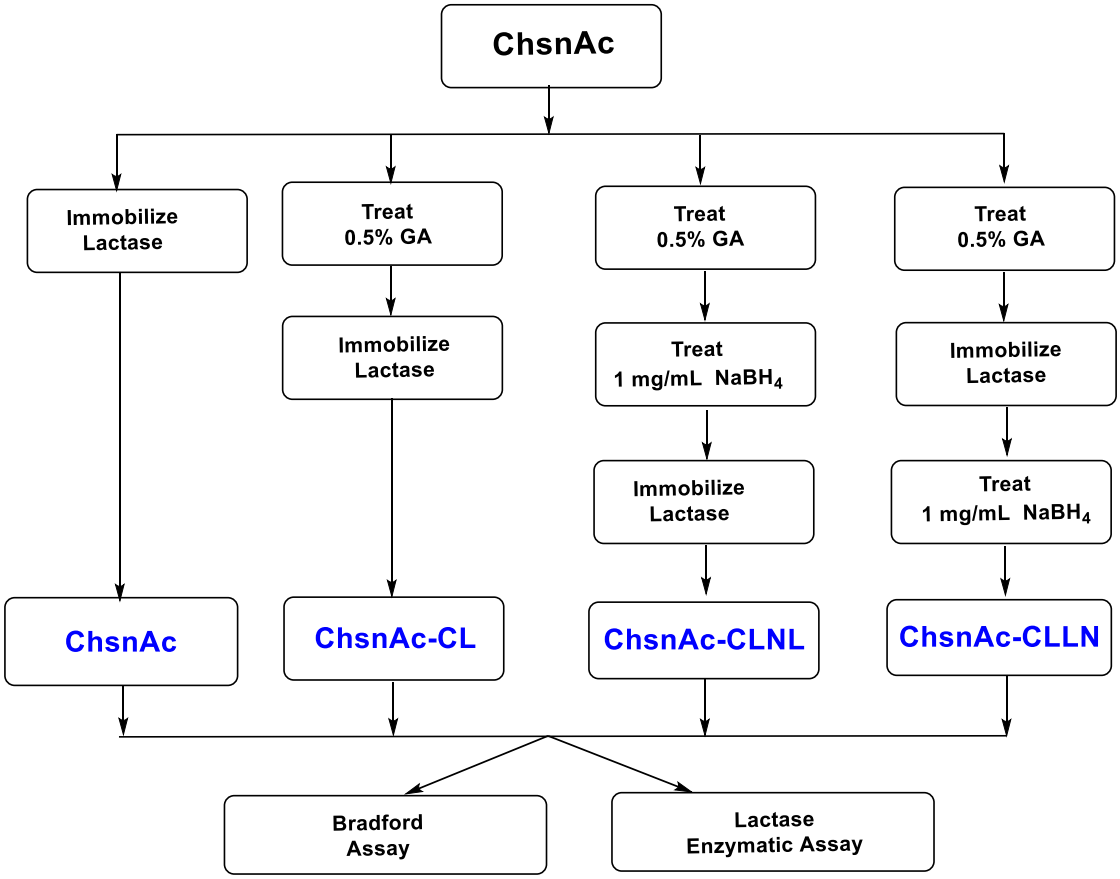

Figure 5: Procedure for preparing ChsnAc Beads with different glutaraldehyde and sodium borohydride treatments.

2.2.15 Hydrolysis of Lactose in Whey Catalyzed by Lactase

Whey was prepared by centrifugation of skim milk at 11,000 x g for 30 minutes, adjusting the pH of the supernatant to 4.6 with hydrochloric acid, and isolating the whey by centrifugation at 11,000 x g for 20 minutes. One milliliter of the whey sample was added to an Eppendorf tube that had lactase immobilized on chitosan-silica (ChsnSi) beads. The samples were incubated for 48 hours and 50 µL aliquots were collected after 10 minutes, 1, 3, 6, 24, and 48 hours. The samples were stored in clean Eppendorf tubes until analysis by thin layer chromatography (TLC).

Samples were spotted on Silica Gel 60 F254 Coated Aluminum-Backed plates and developed in a solvent system consisting of 1-butanol : acetic acid : water (2:1:1). Whey, glucose, and galactose were used as standards. To observe carbohydrates, the TLC plate was sprayed with a methanol - sulfuric acid mixture (95:5 ratio by volume), allowed to dry, then the plate was heated on hot plate for spots to develop.

2.2.16 Effect of pH on Lactose Hydrolysis Catalyzed by Immobilized and Free Lactase

The ability of free and immobilized lactase to hydrolyze lactose across a range of pH (5.0, 6.0, 6.8, and 8.0) was monitored using TLC. One milliliter of the 400 mM lactose sample was added to Eppendorf tubes that had chitosan-silica (ChsnSi) beads immobilized with lactase. The samples were incubated for 48 hours and 50 µL aliquots were collected after 10 minutes, 1, 3, 6, 24, and 48 hours, the samples were stored in clean Eppendorf tubes until analysis by TLC. TLC was developed as described previously in **Section 2.2.15.**

2.2.17 Glucose Solution Preparation

Glucose oxidase (GOx) solutions were prepared with concentrations 10, 20, 100 and 200 mg/mL in 0.1 M KPi buffer (pH 6.8).

2.2.18 General Procedure for Immobilization of Glucose Oxidase on Chitosan Beads

The glucose oxidase immobilization solution was prepared using 100 µL of 100 mg/mL GOx solution and 900 µL of 0.1 M KPi buffer (pH 6.8). A 50 µL scoop was used to transfer chitosan beads into individual microcentrifuge tubes. The enzyme solution was added to the chitosan beads and the tube was inverted to mix the contents. An aliquot of 100 µL of GOx solution was removed and saved for protein analysis (before incubation). The beads were then incubated with the GOx solution for at least 16 hrs at 4 ^{o}C with shaking. After

incubation, the GOx solution was removed from the beads and saved for protein analysis using the Bradford assay. The beads were stored with 1 mL of 0.1 M KPi buffer (pH 6.8) in the refrigerator. The steps were repeated for all types of chitosan beads.

2.2.19 Glucose Oxidase Activity Assay Using Substrate Glucose

2.2.19.1 Immobilized Glucose Oxidase with Free Peroxidase in a Separate Reaction

The activity of glucose oxidase was determined using a coupled assay with peroxidase. The absorbance of *p*-quinoneimide which was produced during the oxidation of phenol/4-aminoantipyrine by H_2O_2 in the presence of peroxidase was measured spectrophotometrically at 510 nm (molar extinction coefficient of 740 M^{-1} cm^{-1}). The phenol/4-aminoantipyrine reagent was prepared by dissolving 8.00 g of phenol in distilled water followed by addition of 0.250 g of 4-aminoantipyrine to give a phenol (170 mM)/4-aminoantipyrine (2.5mM) solution. A 1000 µL aliquot of glucose (5 or 50 mM stock concentration) was added to an Eppendorf tube with GOx immobilized chitosan beads and a timer was started immediately. The tube was inverted gently several times and was placed horizontally on a shaker to ensure that the beads were evenly exposed to solution. The spectrophotometer was set at 510 nm and blanked with 1800 µL of 0.1 M KPi buffer (pH 6.8), 1 mL phenol/4-aminoantipyrine reagent, 100 µL of peroxidase (0.1 mg/mL) and 100 µL of glucose (50 mM). Aliquots of 100 µL were removed from the reaction sample at intervals of 10 minutes for 30 minutes and added to a test tube containing 1800 µL of 0.1 M KPi buffer (pH 6.8), 1 mL phenol/4-aminoantipyrine, and 100 µL of peroxidase (0.1 mg/mL). The mixture was incubated for 20 – 30 minutes and the absorbance was recorded at 510 nm. Samples were prepared in triplicates.

2.2.19.2 Combining Immobilized Glucose Oxidase with Free Peroxidase

To monitor hydrogen peroxide reduction in real time, peroxidase and phenol/4-aminoantipyrine were added to glucose oxidase immobilized on chitosan-charcoal (ChsnAc beads). Glucose was used as a substrate. A 50 µL sample of (50 mM) substrate was added to an Eppendorf tube with chitosan beads 900 µL of 0.1 M KPi buffer (pH 6.8), 500 mL phenol/4-aminoantipyrine and 50 µL of peroxidase (0.1 mg/mL) and timer was started immediately. The tube was inverted gently several times and was placed horizontally on a shaker to ensure the even exposure of beads to solution, and. the color change was observed over time. The samples were prepared in duplicates.

2.2.20 Enzymatic Activity Assay of Lactase - Glucose Oxidase Combination

2.2.20.1 Combining Immobilized Lactase and Glucose Oxidase

Using a spatula, chitosan beads immobilized with glucose oxidase were transferred to a tube containing chitosan beads immobilized with lactase. A 1000 µL sample of 48 mM lactose was added to an Eppendorf tube with the combination of chitosan beads and a timer was started immediately. The tube was inverted gently several times and was placed horizontally on a shaker to ensure the even exposure of the beads to solution. The spectrophotometer was set at 510 nm and blanked with 1800 µL of 0.1 M KPi buffer (pH 6.8), 1 mL phenol/4-aminoantipyrine, 100 µL of peroxidase (0.1 mg/mL), and 100 µL of lactose (48 mM). Aliquots of 100 µL were removed from the reaction samples at intervals of 10 minutes for 30 minutes and added to a test tube and containing 1800 µL of 0.1 M KPi buffer (pH 6.8), 1 mL phenol/4-aminoantipyrine and 100 µL of peroxidase (0.1 mg/mL).

The mixture was incubated for 20 – 30 minutes to allow for the reaction to take place and the absorbance was recorded at 510 nm. Samples were prepared in triplicates.

2.2.20.2 Combining Immobilized Lactase and Glucose Oxidase with Free Peroxidase

Using a spatula, ChsnAc beads immobilized with glucose oxidase were transferred to a tube containing ChsnMag beads immobilized with lactase. A 50 µL sample of lactose (48 mM) substrate was added to an Eppendorf tube with chitosan beads, 900 µL of 0.1 M KPi buffer (pH 6.8), 500 µL phenol/4-aminoantipyrine, and 50 µL of peroxidase (0.1 mg/mL), timer was started immediately. The tube was inverted gently several times and was placed horizontally on a shaker to ensure the even exposure of beads to solution. A color change was observed over time. The samples were prepared in duplicates.

2.2.21 GO Assay to Determine whether Products of Glucose Oxidation Reacted with Chitosan-Charcoal Beads

Three glucose standard solutions were prepared by mixing 900 µL GO reagent, 50 µL distilled water, and 50 µL of glucose solution at different concentrations (0.5 mM, 1 mM, and 2 mM). The samples were prepared in duplicate. The samples were incubated for 30 minutes at room temperature before taking the absorbance at 540 nm. The spectrophotometer was blanked at 540 nm with 900 µL of GO reagent and 50 µL of water. The readings from the standard solutions were used to construct a standard curve. The line generated from this data was used to calculate the concentration of glucose. Then 1000 µL of 2 mM glucose solution was added to beads immobilized with glucose oxidase and the timer was started immediately. The tube was inverted gently several times and it was placed horizontally on a shaker. An aliquot of 50 µL was removed from the tube at intervals of 10

minutes for 30 minutes and added to 900 µL of GO reagent and 50 µL of water. The samples were then incubated for 30 minutes and absorbances were recorded at 540 nm.

2.2.22 Determining whether Chitosan-charcoal (ChsnAc) Beads Absorbed H_2O_2

One milliliter of hydrogen peroxide (2 mM) was added to an Eppendorf tube containing ChsnAc beads and placed horizontally on an orbital shaker. Samples of 100 µL were taken immediately after adding hydrogen peroxide and at intervals of 10 minutes for 30 minutes. The samples were added to a reaction mixture containing 1800 µL of 0.1 M KPi buffer (pH 6.8), 1000 µL phenol/4-aminoantipyrine, and 100 µL of peroxidase (0.1 mg/mL). Absorbance at 510 nm was recorded. Samples were prepared in triplicates.

2.2.23 Determining whether Chitosan-charcoal (ChsnAc) or Chitosan-silica (ChsnSi) Beads Absorbed H_2O_2

One milliliter of hydrogen peroxide (2 mM) was added to an Eppendorf tube containing ChsnAc or ChsnSi beads and placed horizontally on an orbital shaker. Samples of 100 µL were taken immediately after adding hydrogen peroxide and at intervals of 10 min for 30 min. The samples were added to a reaction mixture as previously described in **Section 2.2.22.** Absorbance at 510 nm was recorded for each sample.

2.2.24 Enzyme Activity Assay after Combining Immobilized Lactase and Glucose Oxidase on ChsnSi Beads

Activity assays after combining the two types of beads were performed as described in **Section 2.2.20.**

2.2.25 Enzyme Activity Assay after Combining Immobilized Lactase and Glucose Oxidase on ChscMag and ChsnSi Beads

Activity assays after combining the two types of beads were performed as described in **Section 2.2.20.**

CHAPTER 3. RESULTS AND DISCUSSION

The aim of this work was to improve lactose hydrolysis by designing a two-component system comprising of lactase and glucose oxidase enzymes, immobilized individually on two different modified chitosan supports. The designed system catalyzes two sequential reactions - lactose hydrolysis and glucose oxidation. This improves lactose hydrolysis through minimization of lactase inhibition by its product glucose. The ability of lactase to hydrolyze lactose and glucose oxidase to oxidize glucose was measured first individually then in combination.

The chitosan support was modified to improve its structural properties by addition of fortifying agents: activated charcoal, silica, magnetic particles, and crosslinking of the support with glutaraldehyde. Immobilization efficiency of the enzymes lactase and glucose oxidase was determined by calculating the amount of protein bound using the Bradford assay. The activity of the immobilized enzymes was first measured individually. Lactase activity was determined by measuring the amount of ortho-nitrophenolate (*o*NP) produced from the hydrolysis of a model substrate ortho-nitrophenyl-β-D-galactopyranoside (*o*NPGal).[23,36] Glucose oxidase activity was measured using glucose and was coupled with a peroxidase assay using phenol/4-aminoantipyrine as a substrate.

Lastly, the ability of the two immobilized enzymes, lactase and glucose oxidase, to catalyze sequential reactions, lactose hydrolysis and glucose oxidation, was determined. Lactose hydrolysis produces galactose and glucose which is oxidized by glucose oxidase to gluconic acid and hydrogen peroxide. The amount of hydrogen peroxide was determined using a coupled assay with peroxidase and phenol/4-aminoantipyrine.[37,38]

3.1 Formation of Chitosan Beads

Chitosan polymer is soluble in acidic solutions and the resulting gel-like solution can be used to make hydrogel beads. All chitosan beads were prepared from the chitosan solution in acetic acid. To improve chitosan beads' performance, structural make up, and stability, they were fortified with the following inorganic materials: activated charcoal, silica, and magnetic particles.[10,19] The fortifying agents' properties, like small size and porosity, render a large surface area to volume ratio to bind greater quantities of enzyme. Addition of fortifying agents increases the beads' durability allowing their use over a long period of time. Also, the ability of fortifying agents to be evenly dispersed within the matrix improves the stability of the reaction system.[16] For example, silica particles have been used because of their small size, high surface area, ordered arrangement, and high stability to mechanical and chemical forces. Magnetic particles are biocompatible and they are easy to separate.[5] After the addition of a fortifying agent, the resulting chitosan suspension was dispersed dropwise in a basic solution. Upon contact with the sodium hydroxide, the chitosan formed donut-shaped structures through the chitosan-hydroxide interaction (**Figure 6 & 7**).[21] Chitosan beads fortified with magnetic particles formed round magnetic responsive beads (**Figure 6B**).[20] Chitosan's structural stability was further improved by the crosslinking agent glutaraldehyde (GA). Crosslinking (CL) chitosan with aldehydes, like glutaraldehyde, leads to formation of strong covalent bonds and an increase in the internal surface area that improves the immobilization efficiency, the color of the chitosan-silica beads darkens after incubation with glutaraldehyde, this confirmed that cross-linking had occurred as the –C=N- imine bond acts as a chromophore. **Figure 7D** shows non-crosslinked ChsnSi and crosslinked ChsnSi-CL beads.[9,21]

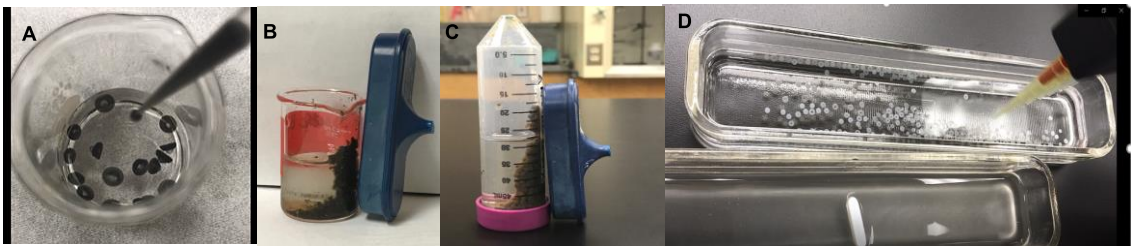

Figure 6: Preparation of chitosan beads. **A:** Chitosan-charcoal (ChsnAc) beads; **B:** Chitosan-magnetic (ChsnMag) beads; **C:** Magnetically responsive beads after 4 weeks in the refrigerator; **D:** Chitosan-silica (ChsnSi) beads.

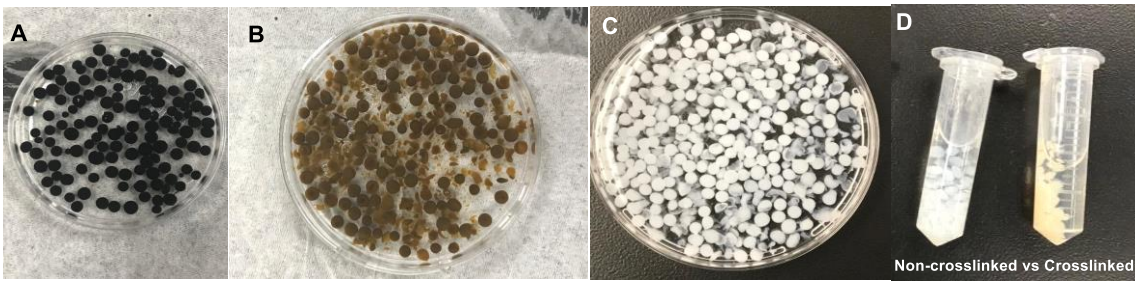

Figure 7: Fortified chitosan beads **A:** Chitosan-charcoal (ChsnAc) beads; **B:** Chitosan-magnetic (ChsnMag) beads; **C:** Chitosan-silica (ChsnSi) beads; **D:** Non-Crosslinked and Crosslinked (CL) ChsnSi beads.

3.2 Morphological Characterization of Chitosan Beads Using Scanning Electron Microscope (SEM)

The scanning electron microscope (SEM) was used to examine the surface morphology of non-crosslinked chitosan-silica (ChsnSi) beads. The chitosan beads were air-dried before examining them using SEM. **Figure 8** presents the SEM images illustrating the surface morphology of non-crosslinked ChsnSi beads at four different magnifications (30x, 90x, 550x, and 2000x). The SEM images confirmed that chitosan beads had a donut-like shape. The SEM images also demonstrated that chitosan fortified with silica forms beads with a rough morphology in which the silica particles are evenly distributed throughout the chitosan beads. These findings are consistent with the results of the previous studies by Li et al.[27]

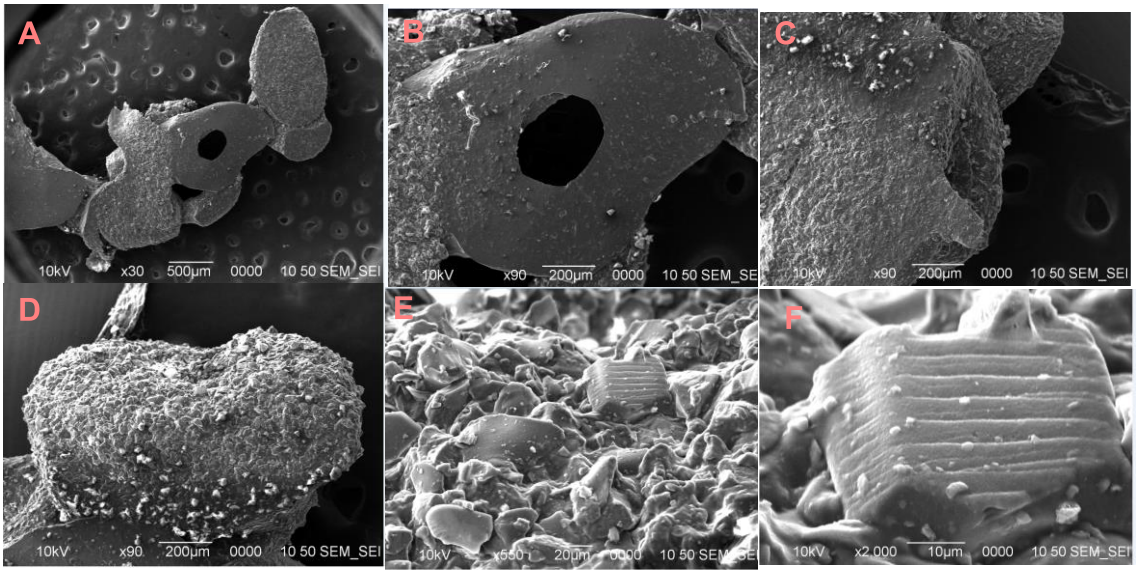

Figure 8: Images of Chitosan-silica (ChsnSi) beads examined on SEM at different resolutions (30x – 2000x). **A, B:** Chitosan bead showing a donut shape at 30x and 90x magnification; **C, D:** Chitosan bead image showing a donut shape from a side view and distribution of silica on the surface at 90x magnification; **E:** Image showing a rough surface morphology and the distribution of silica on the bead at x550x magnification **F:** Image showing silica on the bead at 2000x magnification.

3.3 Determination of Lactase Concentration

The concentration of the lactase solution prepared by dissolving the content of two Rite Aid Dairy Relief tablets in 10 mL of potassium phosphate buffer, pH 6.8 was determined using the Bradford assay. Bovine serum albumin (BSA) was used as the standard, and the absorbance of the samples with a known amount of BSA was recorded at 595 nm. A standard calibration curve was generated by plotting the absorbances against the amount of BSA (**Figure 9**). The line equation and correlation coefficient (R^2) were obtained, and the line equation was used to calculate the amount of protein in the prepared lactase solution. The concentration of the lactase was calculated by dividing the amount of protein by the volume of lactase added to the Bradford sample and multiplied by the dilution factor. The concentration of lactase in the prepared solution was determined as 1.80 mg/mL.

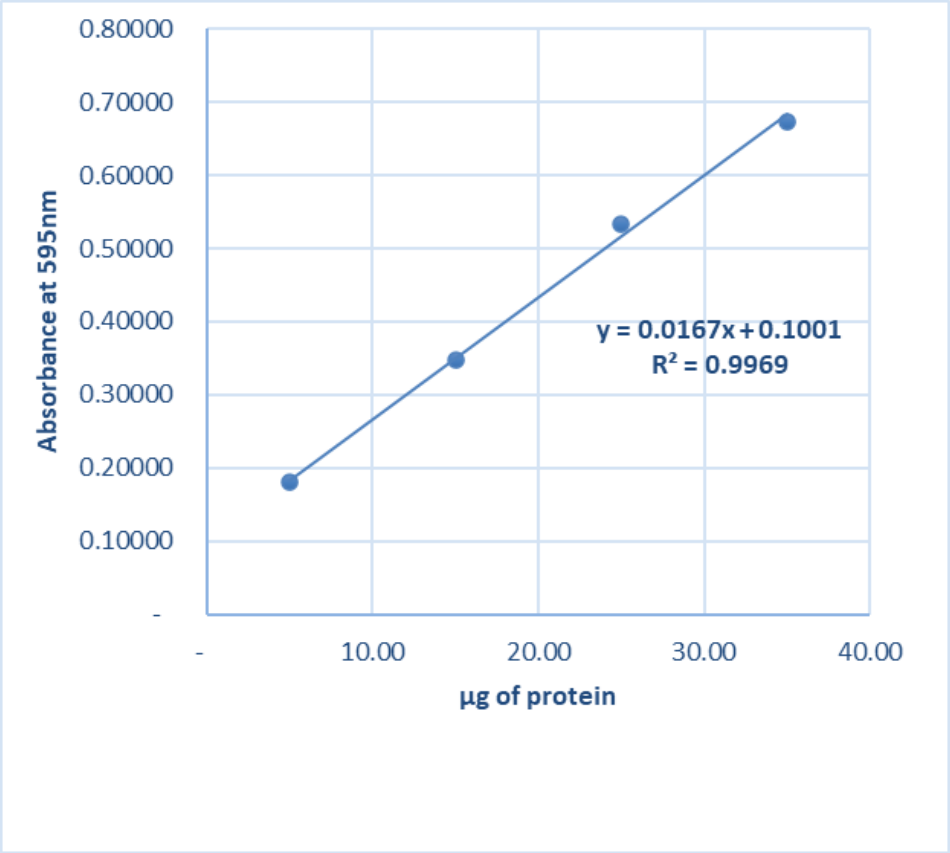

Figure 9: Bradford assay calibration curve.

3.4 Immobilization of Lactase on Chitosan Beads

Chitosan beads were incubated with 1.80 mg/mL solution of lactase. Samples of lactase collected before and after incubation were used to determine the protein content by the Bradford assay. The standard curve was prepared, and the line equation was used to calculate the amount of lactase present in the samples. The amount of lactase immobilized on the beads was calculated by subtracting the amount of lactase remaining after incubation from the total amount of protein contained in the tube before incubation. Analysis of data shown in **Table 1** indicates that lactase was immobilized successfully on non-crosslinked and crosslinked chitosan beads. The total amount of protein bound by the ChsnAc,

ChsnAc-CL, ChsnMag, ChsnMag-CL, ChsnSi, and ChsnSi-CL beads ranged between 106 and 370 µg. These results are consistent with li et al report.[9,23]

Table 1: Amount of lactase bound on chitosan beads

Chitosan beads	Initial amount of lactase in solution (µg)	Amount of lactase bound (µg)
ChsnAc	1010	209 ± 32
ChsnAc-CL	975	220 ± 35
ChsnMag	827	193 ± 34
ChsnMag-CL	787	370 ± 30
ChsnSi	518	106 ± 30
ChsnSi-CL	518	254 ± 31

3.5 Lactase Saturation Curve for Immobilization on Chitosan-Charcoal (ChsnAc) Beads

To evaluate the maximum amount of lactase that could bind to chitosan-charcoal (ChsnAc) beads, the beads were incubated with increasing amounts of lactase from 15 to 925 µg. Samples of lactase collected before and after incubation were used to analyze the protein content by the Bradford assay. The amount of lactase immobilized on the beads was calculated as previously described in **Section 3.4.** Analysis of data shown in **Table 2** indicates that lactase was immobilized successfully on ChsnAc beads. The total amount of protein bound by the beads ranged between 15 to 169 µg. Hence, the higher amount of lactase in solution led to the higher amount bound until the maximum amount of enzyme that can bind is reached. The graph in **Figure 10** shows a plateau when the beads were saturated with enzyme.

Table 2: Amount of lactase bound on chitosan-charcoal (ChsnAc) beads

Sample	Initial amount of lactase in solution (µg)	Amount of lactase bound (µg)
1	925	169 ±3
2	555	150 ± 25
3	255	116 ± 23
4	85	58 ± 4
5	15	15 ± 5

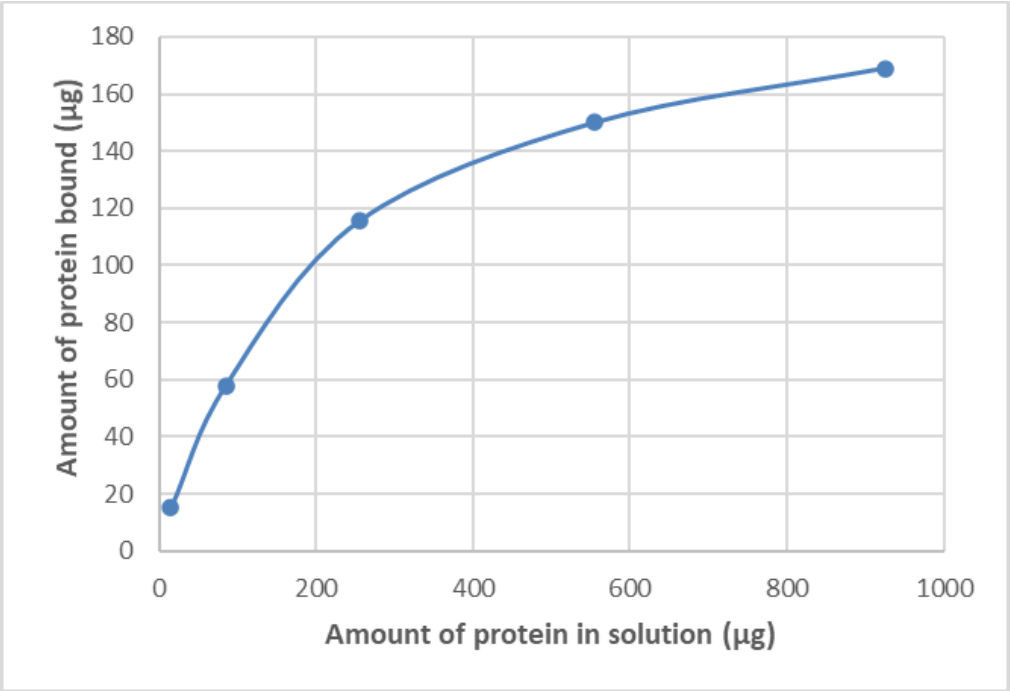

Figure 10: Relationship between the amount of protein bound to the beads and the amount of protein in solution.

3.6 Lactase Catalytic Activity Assay Using Substrate *o*NPGal

The lactase enzymatic assay with the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (*o*NPGal) was done to test if immobilized lactase was able to hydrolyze the β-glycosidic bond formed by D-galactose as shown in **Figure 11**.

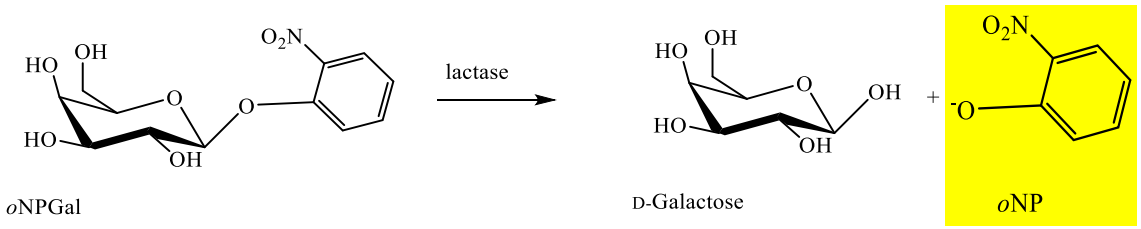

Figure 11: Conversion of *o*NPGal to *o*NP and D-galactose catalyzed by lactase.

The substrate was incubated with lactase immobilized on chitosan beads. A sample of the reaction mixture was removed every 10 minutes (10 – 30 minutes) and added to KPi buffer, pH 6.8. A color change from colorless to yellow indicated that hydrolysis had taken place and *o*NP, which is yellow in color, had been formed **(Figure 12B)**. The absorbance at 405 nm was recorded, which indicates the presence of *o*NP and the percent conversion was calculated using **Equation 1 & 2** shown below.

Equation 1: $[oNP]\ (mM) = \left(\frac{A\ 405}{E\ x\ l}\right)\ x\ 10^3\ x\ df$

Equation 2: $Percent\ conversion\ of\ oNPGal\ = \left(\frac{[oNP]}{[oNPGal]i}\right) x\ 100\%$

where [*o*NP] – concentration of *o*NP formed, A_{405} = absorbance at 405 nm, E = extinction coefficient of *o*NP, 3055 $M^{-1}cm^{-1}$, l - pathlength = 1 cm, and multiplication by 10^3 is to convert M into mM, df -dilution factor, [*o*NPGal]*i* – initial concentration of *o*NPGal added to beads.

The results indicated **(Figures 13-15)** that immobilized lactase retained activity in both non-crosslinked and crosslinked chitosan beads: ChsnAc, ChsnMag, and ChsnSi. The percent conversion of the substrate increased steadily over 30 minutes upon contact with the immobilized enzyme. After 30 minutes the percent conversion seen in ChsnAc,

ChsnAc-CL, ChsnMag, ChsnMag-CL, ChsnSi, and ChsnSi-CL beads ranged between 22 and 40% (**Table 3)**.

As indicated in **Figure 16** and **Table 4,** the amount of hydrolyzed *o*NPGal was proportional to the amount of enzyme bound to beads. The crosslinked beads were more stable and could be reused multiple times. ChsnMag had a slightly higher percent conversion compared to ChsnAc and ChsnSi. These findings are consistent with Chen et al.[23]

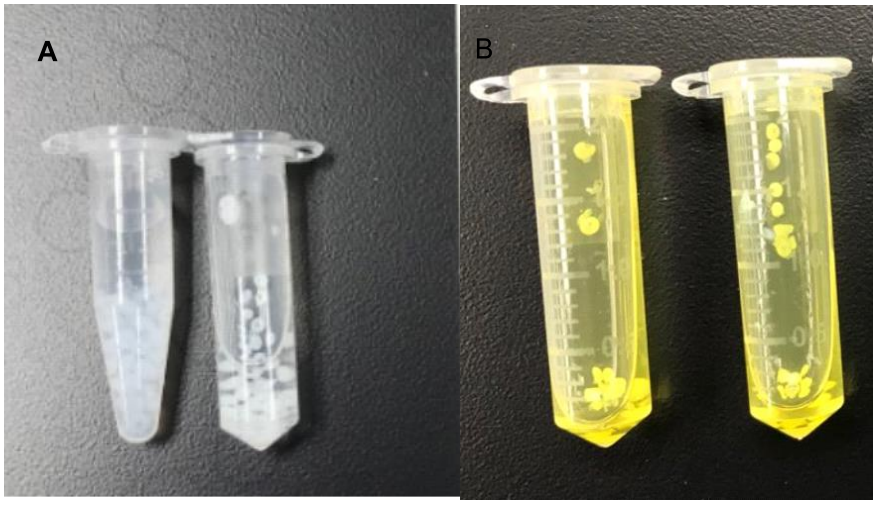

Figure 12: Immobilization and enzymatic assay of lactase. **A:** ChsnSi beads immobilized with enzyme lactase; **B:** Hydrolysis of *o*NPGal catalyzed by immobilized lactase on ChsnSi beads leads to formation of the yellow product *o*NP and D-galactose.

Table 3: Percent of *o*NPGal hydrolyzed by lactase immobilized on crosslinked and non-crosslinked chitosan beads

Chitosan Beads	**% Conversion of *o*NPGal**		
	10 min	**20 min**	**30 min**
ChsnAc	9 ± 2	18.2 ± 2	22 ± 2
ChsnAc-CL	24 ± 4	29 ± 2	29± 4
ChsnMag	25± 4	34 ± 4	38± 4
ChsnMag-CL	33± 4	39± 4	40± 4
ChsnSi	17± 8	24 ± 8	28± 8
ChsnSi-CL	21 ± 6	26 ± 4	30± 10

Table 4: Percent of *o*NPGal hydrolyzed by different amounts of lactase immobilized on non-crosslinked ChsnAc beads

Amount of lactase bound (µg)	% Conversion of *o*NPGal		
	10 min	20 min	30 min
169	13± 1	18 ± 4	20 ± 3
150	7.9 ± 1	13± 1	16± 4
116	7.8 ± 1	10.9 ± 3	12± 4
58	5 ± 2	11.2 ± 4	15± 4
15	0± 0.2	2 ± 1	6± 8

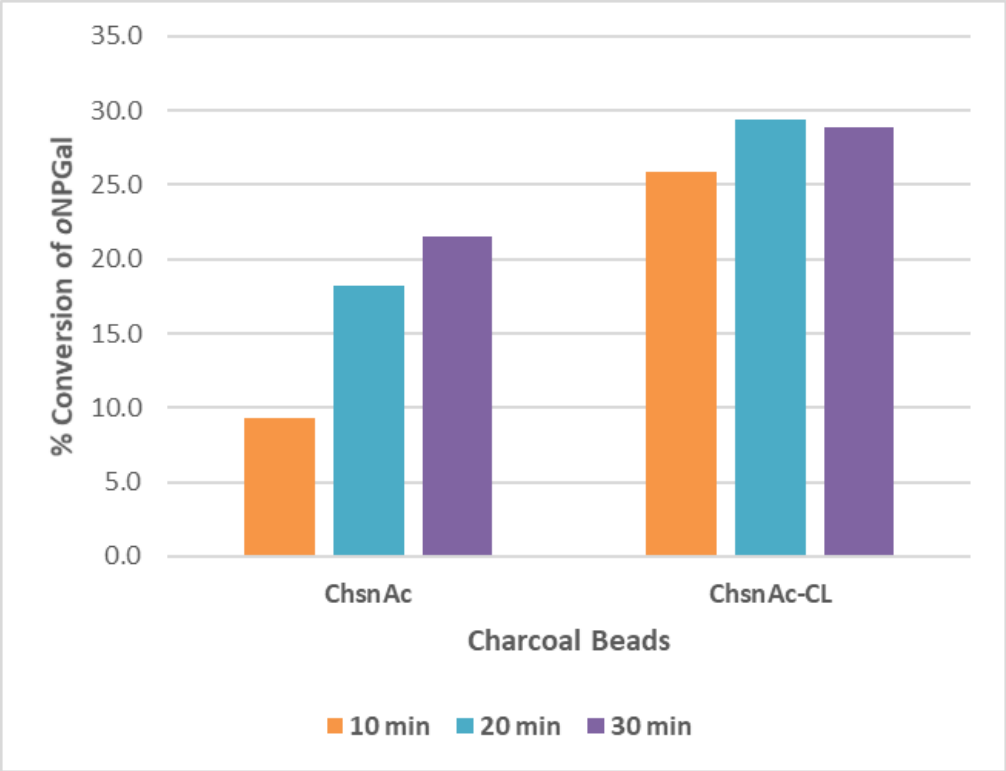

Figure 13: Average percent conversion of *o*NPGal catalyzed by lactase immobilized on non-crosslinked and crosslinked ChsnAc beads.

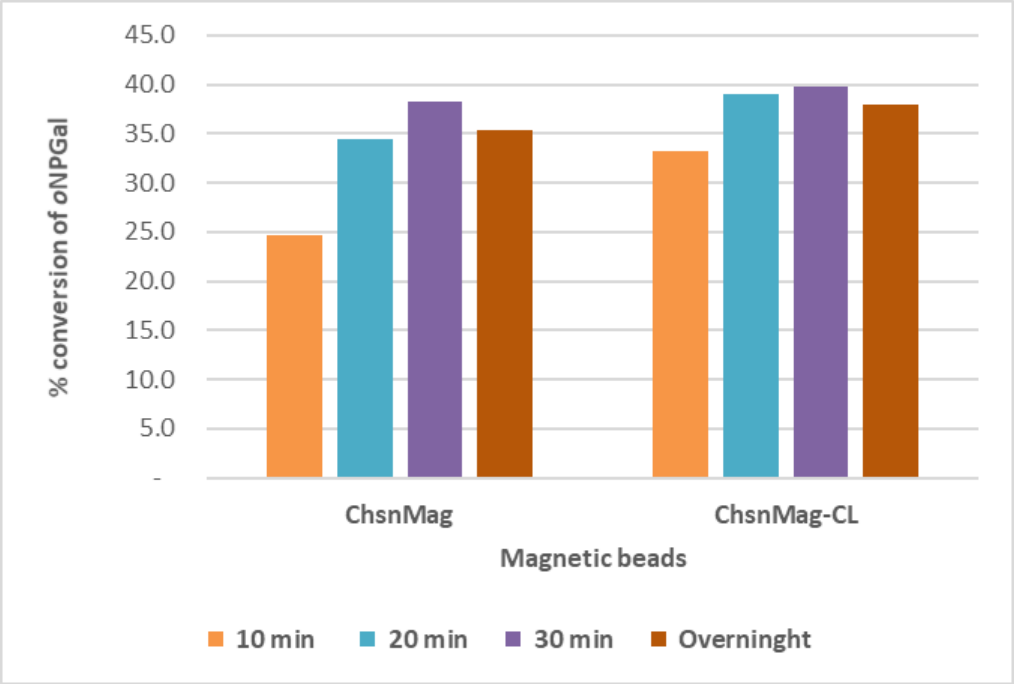

Figure 14: Average percent conversion of *o*NPGal catalyzed by lactase immobilized on non-crosslinked and crosslinked ChsnMag beads.

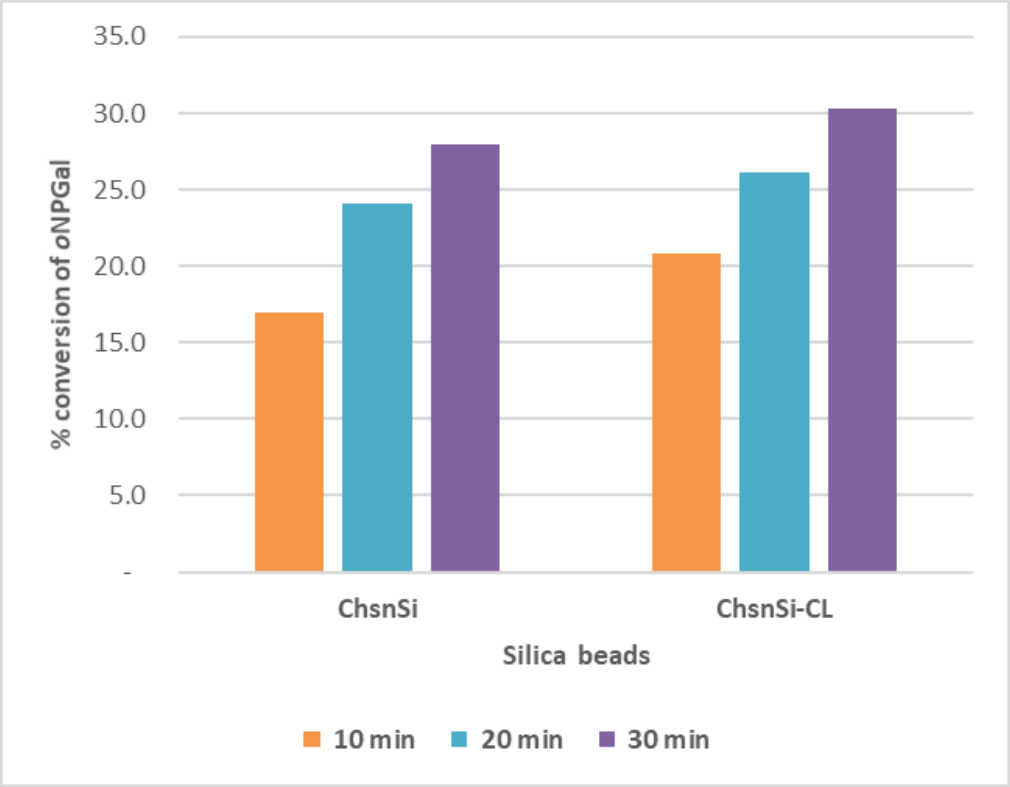

Figure 15: Average percent conversion of *o*NPGal catalyzed by lactase immobilized on non-crosslinked and crosslinked ChsnSi beads.

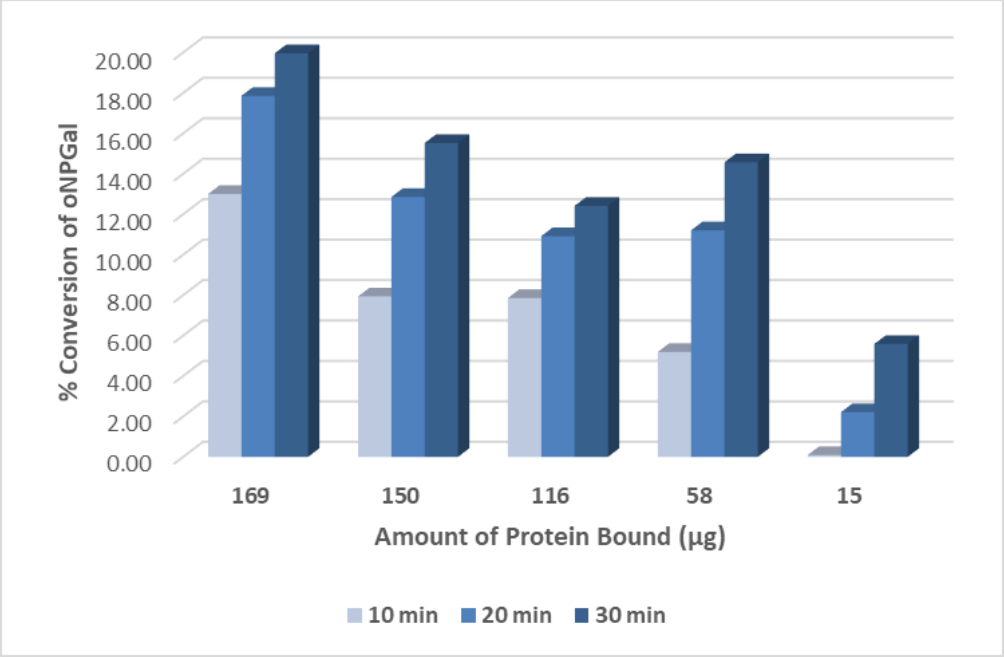

Figure 16: Average percent conversion of *o*NPGal catalyzed by different amounts of lactase immobilized on non-crosslinked ChsnAc beads.

3.7 Comparison of the Catalytic Activity of Free Enzyme and Immobilized Lactase Enzyme

The lactase was successfully immobilized on chitosan-silica beads as previously described in **Section 3.4** and retained catalytic activity. The chromogenic substrate *o*NPGal was used to compare the enzyme activity of free and immobilized lactase. *o*NPGal was incubated for 60 min with a solution of lactase or lactase immobilized on ChsnSi beads. Aliquots from the reactions were removed every 10 min. The absorbance at 405 nm was recorded and the extinction coefficient (3055 M^{-1} cm^{-1}) was used to calculate the concentration of *o*NP as described in **Section 3.6**. For free enzyme, the percent conversion increased rapidly during the first 10 minutes to about 40% and at 40-60 minutes the curve plateaued after converting about 45% of the substrate, see **Figure 17** below. This might be a result of product inhibition of the lactase enzyme that affected its activity.[4] On the other hand, with the

immobilized enzyme the percent conversion of the substrate increased steadily from 10 – 60 minutes. It takes a longer time for products to be formed, after 10 minutes only about 5% of the available substrate had been hydrolyzed to products. After 60 minutes about 38% of substrate had been hydrolyzed and it also started to plateau close to 40%. The slow increase might be due to the hindrance of the support, making it harder for the substrate to diffuse to the enzyme's active site.[10] With the free enzyme, the substrate can easily access the active site and can be hydrolyzed quickly. The advantage of using the immobilized enzyme is that it can be reused multiple times as compared to free enzyme and can be separated, hence, it is economical.[5]

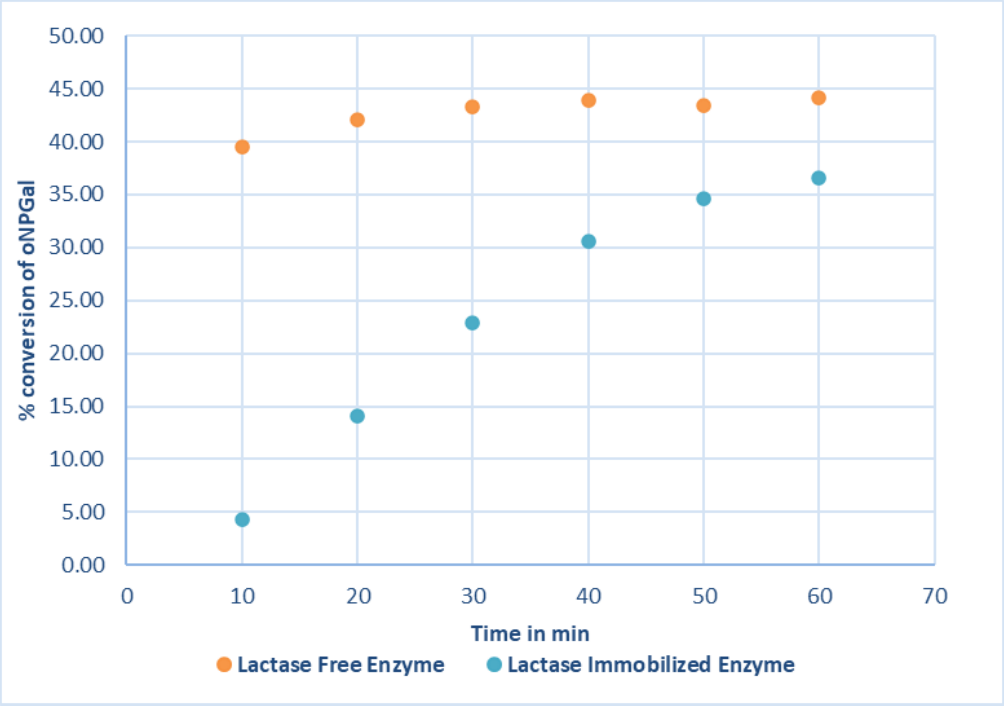

Figure 17: Percent conversion of *o*NPGal catalyzed by free and immobilized lactase.

3.8 Determining the Stability and Reusability of Lactase Immobilized on Chitosan-Silica Beads

The experiment was done to determine the stability and the reusability of beads. Different sets of ChsnSi beads were prepared in different conditions. ChsnSi beads were immobilized

with lactase, ChsnSi-CL beads were crosslinked with glutaraldehyde (GA) and then immobilized with lactase and, lastly, ChsnSi-CLNL beads were crosslinked with GA, treated with $NaBH_4$, and then immobilized with lactase. An enzyme activity assay was performed as previously described using chromogenic substrate *o*NPGal. The same procedure was repeated nine times. Absorbances at 405 nm were recorded from which the percent conversion was calculated as previously described in **Section 3.6.**

As exhibited in **Figure 18,** the immobilized lactase retained activity and ChsnSi, ChsnSi-CL, ChsnSi-CLNL hydrolyzed about 7%, 20% and 25% of the substrate, respectively, after eight cycles. The control beads that were not crosslinked demonstrated poor mechanical stability. Some of the beads were fragmented during the enzymatic reactions that took place with shaking, and some were lost during washing that was performed between the enzymatic cycles. This resulted in a decrease in the enzymatic activity, and the percent conversion of substrate to products reduced from 23% during the first cycle to lower than 10% in the fourth cycle. These findings are consistent with Wahba's report.[21] In contrast, the ChsnSi-CL and ChsnSi-CLNL beads were stable, did not fragment, and were able to hydrolyze more than 18% of a substrate within 30 minutes of the reaction during the ninth cycle. The higher stability could be ascribed to the covalent bonds formed after crosslinking the beads with glutaraldehyde. For the ChsnSi-CLNL beads, the higher conversion rate could be attributed to treatment with $NaBH_4$ that reduces any free reactive aldehyde groups that might hinder the catalytic activity.[39] Studies have shown that immobilized beads can be reused multiple times and crosslinking by covalent linkages increases the stability of the beads and the enzyme.[5,21,23]

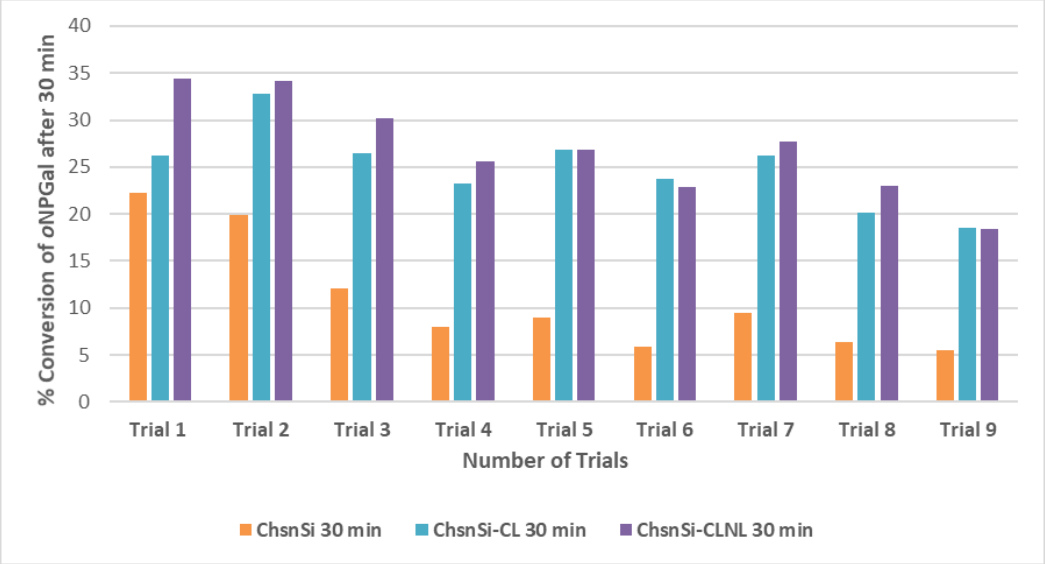

Figure 18: Percent conversion of *o*NPGal catalyzed by lactase during nine trials

3.9 Effects of Glutaraldehyde and $NaBH_4$ on the Immobilization of Lactase

The experiment was done to determine if crosslinking chitosan (ChsnAc and ChsnSi) beads with glutaraldehyde and treatment with $NaBH_4$ had any effect on the immobilization efficiency. Different sets of ChsnAc beads were prepared in different conditions. ChsnAc beads were immobilized with lactase. ChsnAc-CL beads were crosslinked with glutaraldehyde (GA), and then immobilized with lactase. ChsnAc-CLLN beads were crosslinked with GA, immobilized with lactase, then treated with $NaBH_4$. Lastly, ChsnAc-CLNL beads were crosslinked with GA, treated with $NaBH_4$, and then immobilized with lactase. Several types of the ChsnSi beads were prepared in the same way.

According to **Tables 5 and 6,** crosslinked chitosan beads bound more enzyme compared to the non-crosslinked beads. The amount of protein bound on different ChsnAc and ChsnSi beads ranged between 209 and 530 µg and from 103 to 162 µg, respectively. Treatment with sodium borohydride had no effect on the amount of protein immobilized.

Table 5: Average amount of lactase bound on chitosan-charcoal (0.5% GA) beads

ChsnAc beads	Initial amount of lactase in solution (µg)	Amount of lactase bound (µg)
ChsnAc	1010	209 ± 7
ChsnAc-CL	975	530 ±90
ChsnAc-CLLN	970	516 ±59
ChsnAc-CLNL	970	475 ±9

Table 6: Average amount of lactase bound on chitosan-silica beads crosslinked with 0.1% GA.

Chitosan beads	Initial amount of lactase in solution (µg)	Amount of lactase bound (µg)
ChsnSi	746	103± 17
ChsnSi-CL	683	162±57
ChsnSi-CLNL	619	124±59

3.10 Effect of Glutaraldehyde and $NaBH_4$ on the Catalytic Activity of Lactase

The experiment was done to determine if crosslinking chitosan (ChsnAc and ChsnSi) beads with glutaraldehyde and treatment with $NaBH_4$ had any effect on the catalytic activity of lactase. An enzyme activity assay was performed as previously described using chromogenic substrate *o*NPGal. Absorbances at 405 nm were recorded from which the percent conversion was calculated.

As exhibited in **Tables 7 and 8** and **Figures 19 and 20** the immobilized lactase retained activity, and after 30 minutes the percentage of *o*NPGal hydrolyzed on chitosan-charcoal beads and chitosan-silica beads ranged between 22% and 34% for all bead types. The non-crosslinked beads ChsnAc and ChsnSi demonstrated a lower conversion rate compared to their respective crosslinked beads. The higher catalytic activity could be ascribed to the larger amount of protein bound to the crosslinked beads, hence more enzyme is available to hydrolyze *o*NPGal.[5,20,21] For the ChsnAc-CLLN, ChsnAc-CLNL, and ChsnSi-CLNL

bead types, more protein being bound and treatment with $NaBH_4$ that reduces any free reactive aldehyde groups resulted in a slightly higher catalytic activity.[39] Studies have shown that treatment of crosslinked beads with $NaBH_4$ increases the catalytic efficiency slightly.[5,21] Also, crosslinking and treatment with $NaBH_4$ before or after immobilization of lactase (ChsnAc-CLLN, ChsnAc-CLNL) had no effect on the catalytic activity of lactase immobilized on different ChsnAc beads.

Table 7: Percent of *o*NPGal hydrolyzed by immobilized lactase on chitosan-charcoal (0.5% GA) beads

Chitosan Beads	% Conversion of oNPGal		
	10 min	20 min	30 min
ChsnAc	9± 2	18± 1	22± 2
ChsnAc-CL	26± 2	29± 2	29± 3
ChsnAc-CLLN	27± 2	33± 1	33± 2
ChsnAc-CLNL	31± 1	34± 3	34± 3

Table 8: Percent of *o*NPGal hydrolyzed by immobilized lactase on chitosan-silica (0.1% GA) beads

Chitosan Beads	% Conversion of oNPGal		
	10 min	20 min	30 min
ChsnSi	8± 2	17± 1	22±2
ChsnSi-CL	14± 1	23± 2	26±1
ChsnSi-CLNL	25± 2	32± 1	34±2

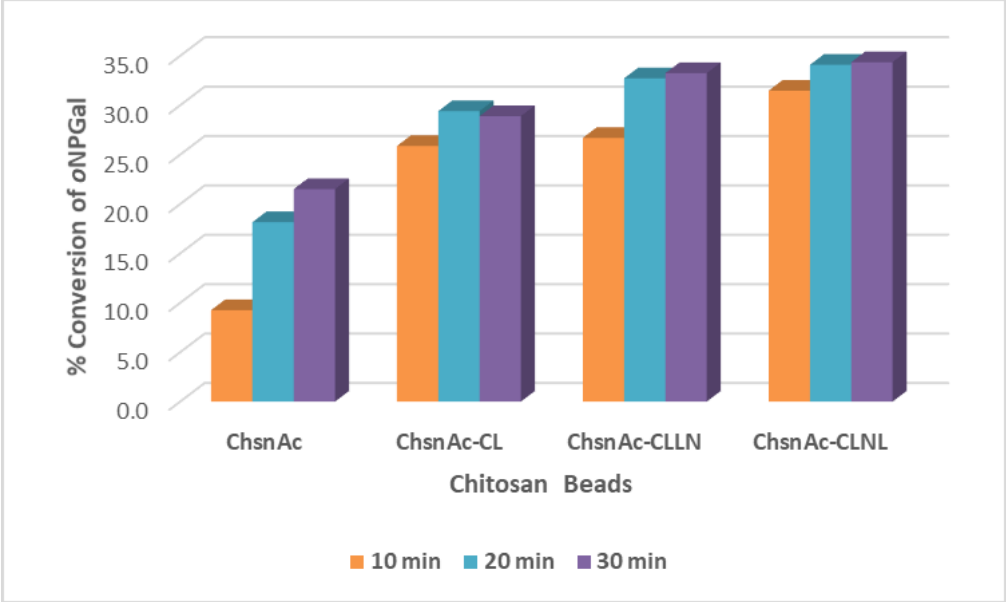

Figure 19: Average percent conversion of *o*NPGal catalyzed by lactase immobilized on non-crosslinked and crosslinked (0.5% GA) ChsnAc beads.

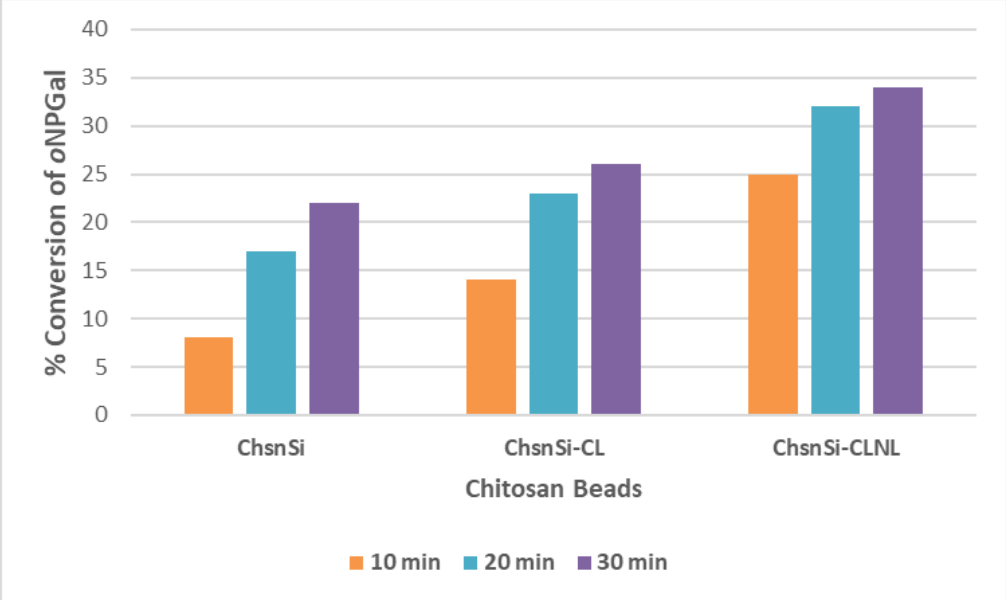

Figure 20: Average percent conversion of *o*NPGal catalyzed by lactase immobilized on non-crosslinked and crosslinked (0.1% GA) ChsnSi beads.

3.11 Hydrolysis of Lactose in Whey (pH 4.6) Using Immobilized and Free Lactase

The ability of lactase to hydrolyze lactose in whey to form glucose and galactose was monitored using thin layer chromatography (TLC). Whey was prepared by centrifugation of skim milk, adjusting the pH of the supernatant to 4.6, and isolating whey by centrifugation. Whey was incubated at room temperature with ChsnSi beads immobilized with lactase; aliquots were collected at different time points between 10 minutes and 48 hrs and saved for analysis . Whey was also incubated with free lactase and aliquots collected at different times within 48 hours were immediately spotted on the TLC plate. The results demonstrated (**Figure 21A**), that after 10 minutes of incubation light spots that corresponds the positions of standards for D-glucose and D-galactose begin to appear showing the formation of products. The lactose spot at the beginning is very dark but becomes lighter with time as the D-glucose and D-galactose spots become darker and at 48 hours almost all lactose was hydrolyzed. In conclusion, the results show that lactose in whey was hydrolyzed by the immobilized lactase. Also, the TLC method can be used to monitor the hydrolysis of lactose. It is interesting to note, that the TLC results also suggest the possibility of using the immobilized lactase in the synthesis of oligosaccharides. Similar observations were made when an experiment was done to compare the lactose hydrolysis using free and immobilized lactase with monitoring by TLC (**Figure 21B**). The only difference observed is that free lactase hydrolyzed lactose after 24 hours while immobilized enzyme required 48 hours to complete the reaction.

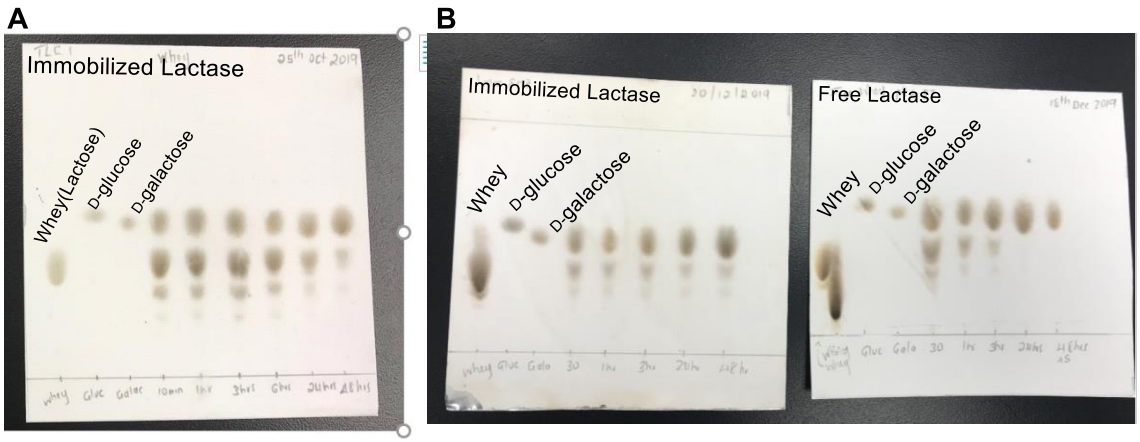

Figure 21: Hydrolysis of lactose in whey by immobilized and free lactase **A:** TLC plate showing hydrolysis of lactose in whey by lactase immobilized on ChsnSi beads at 30 min, 1, 3, 24, and 48 hrs.; **B:** TLC plate showing hydrolysis of lactose in whey by free and immobilized lactase on ChsnSi beads at 30 min, 1, 3, 24, and 48 hrs.

3.12 Hydrolysis of Lactose at Different pH Using Immobilized and Free Lactase

The ability of lactase to hydrolyze lactose was tested at different pH values - 5.0, 6.0, 6.8, and 8.0, and was monitored using TLC. The experiment was done as previously described in lactose hydrolysis in whey (**Section 3.11**). The results observed for free and immobilized lactase (**Figure 22)** demonstrated that at pH 6.0, 6.8 and 8.0 after 30 minutes of incubation light spots began to appear that corresponded to the positions of standards for glucose and galactose showing the formation of products. At 48 hours almost all lactose was hydrolyzed. At pH 5.0 all lactose was hydrolyzed as early as within the first 30 minutes. At pH 8.0 in addition to the lactose hydrolysis, transglycosylation products were observed as spots below lactose. Several spots observed with the immobilized lactase at pH 8 after 3 hours and pH 5.0 after 1 hour and 3 hours are most likely due to some contamination of the samples. Additional experiments should be done to get cleaner results. In conclusion, the results show that lactose was hydrolyzed by both free and immobilized lactase. At 48

hours the reaction was completed at all pH values tested. Also, the synthesis of oligosaccharides is seen to be favored more at pH 8.0.

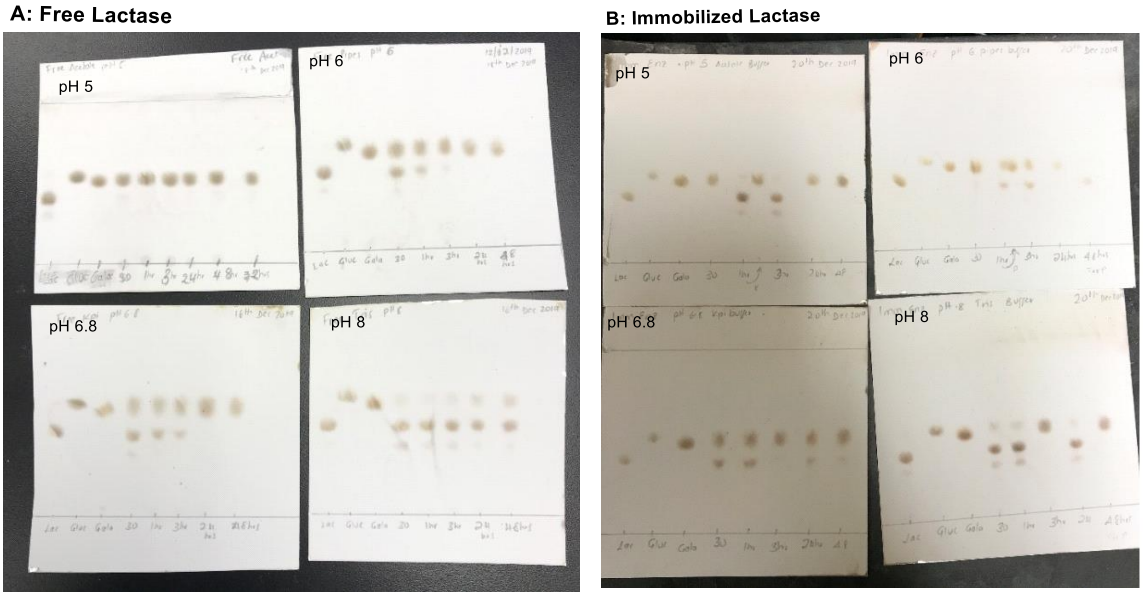

Figure 22: Hydrolysis of lactose in whey by lactase at pH 5.0, 6.0, 6.8 and 8.0. **A:** TLC plate showing hydrolysis of lactose by free lactase at 30 min, 1, 3, 24, and 48 hrs. **B:** TLC plate showing hydrolysis of lactose by lactase immobilized on ChsnSi beads at 30 min, 1, 3, 24, and 48 hrs.

3.13 Immobilization of Glucose Oxidase on Chitosan Beads

Glucose oxidase immobilization was done as previously described for lactase immobilization (Section 3.4). Analysis of data shown in **Table 9** indicates that glucose oxidase was immobilized successfully on chitosan beads, and the amount immobilized ranged between 126 and 280 µg.

Table 9: Amount of glucose oxidase bound on chitosan (ChsnAc) beads

Chitosan beads	Initial amount of glucose oxidase in solution (µg)	Amount of glucose oxidase bound (µg)
ChsnAc	738	126 ± 20
ChsnAc-CL	772	176 ± 30
ChsnSi	478	128 ± 34
ChsnSi-CL	434	280 ± 30

Chitosan-charcoal beads were prepared and incubated with solutions of different concentration of glucose oxidase ranging from 10 – 200 mg/mL. The amount of protein bound was calculated as previously described. According to **Table 10**, glucose oxidase was successfully bound on the chitosan support. The total amount of protein bound was initially proportional to the protein concentration in solution and then plateaued at concentrations above 100 mg/mL, the amount of protein bound ranged between 57 and 138 µg.

Table 10: Amount of glucose oxidase bound on chitosan-charcoal beads

Sample	Initial amount of glucose oxidase in solution (µg)	Amount of glucose oxidase bound (µg)
1	64 ± 1	57 ± 4
2	168 ± 7	82 ± 6
3	717 ±13	176 ± 13
4	947 ± 7	138 ± 20

3.14 Evaluation of Catalytic Activity of Immobilized Glucose Oxidase Using Substrate Glucose

3.14.1 Immobilized Glucose Oxidase with Free Peroxidase in a Separate Reaction

The glucose oxidase enzymatic assay was conducted using D-glucose substrate to test the ability of immobilized enzyme to oxidize D-glucose to D-gluconic acid and H_2O_2, as shown in the reaction scheme below (**Figure 23)**.

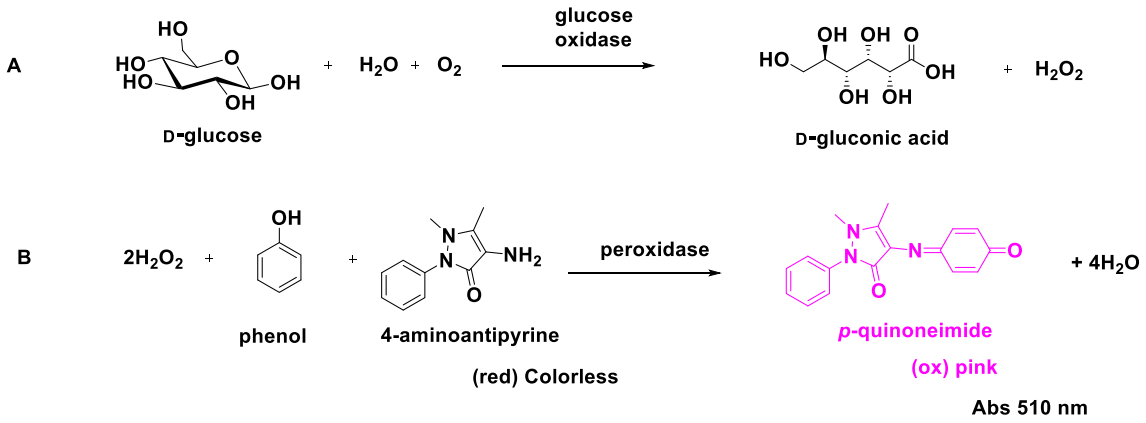

Figure 23: Reaction summary of glucose oxidation. **A**: D-glucose oxidation to D-gluconic acid and H_2O_2 catalyzed by glucose oxidase; **B:** Oxidation of phenol/4-aminoantipyrine by hydrogen peroxide to form *p*-quinoneimide catalyzed by peroxidase.[37]

D-Glucose was incubated with immobilized enzyme and aliquots were taken after 30 minutes. The samples were added to a reaction mixture containing phenol/4-aminoantipyrine and peroxidase. The H_2O_2 oxidizes phenol/4-aminoantipyrine in the presence of peroxidase to form a pink product *p*-quinoneimide (**Figure 24**). The absorbance at 510 nm was recorded, which is the absorbance of *p*-quinoneimide. The extinction coefficient (740 M^{-1} cm^{-1}) was used to calculate the present conversion as shown in **Equation 3 & 4** below.

Equation 3: $$[p-quinoneimide]\ (mM) = \left(\frac{\text{A } 510}{E\ x\ l}\right)\ x\ 10^3\ x\ df$$

Equation 4: $$Percent\ oxidation\ of\ Glucose = \left(\frac{[p-quinoneimide]}{[Glucose]\ i}\ x\ 2\right)\ x\ 100\%$$

where [*p*-quinoneimide] – concentration of *p*-quinoneimide, A_{510} - absorbance at 510 nm, E - extinction coefficient of *p*-quinoneimide, 740 $M^{-1}cm^{-1}$, *l* - pathlength = 1 cm, and multiplication by 10^3 is to convert M into mM, df - dilution factor, [Glucose]*i* – initial concentration of glucose added to reaction mixture.

From the results shown **(Figures 25 - 27**) and (**Tables 11 and 12)**, immobilized glucose oxidase retained activity in both non-crosslinked and crosslinked chitosan beads, ChsnAc and ChsnSi. The results also indicate a slightly lower conversion rate observed with the crosslinked beads compared to the non-crosslinked beads. These findings are consistent with the findings of Liu et al.[5]As shown in **Figure 24B,** the color intensity formed in the presence of non-crosslinked beads is slightly higher compared to crosslinked beads. The percent conversions after 30 minutes ranged between 3 and 90% between different types of beads, with ChsnAc showing the anomalously low amount of hydrogen peroxide. Most likely, activated charcoal affects hydrogen peroxide. This makes it more difficult to follow the reaction using the coupled assay with peroxidase; therefore, additional experiments were done to test whether charcoal containing beads affects hydrogen peroxide concentration.

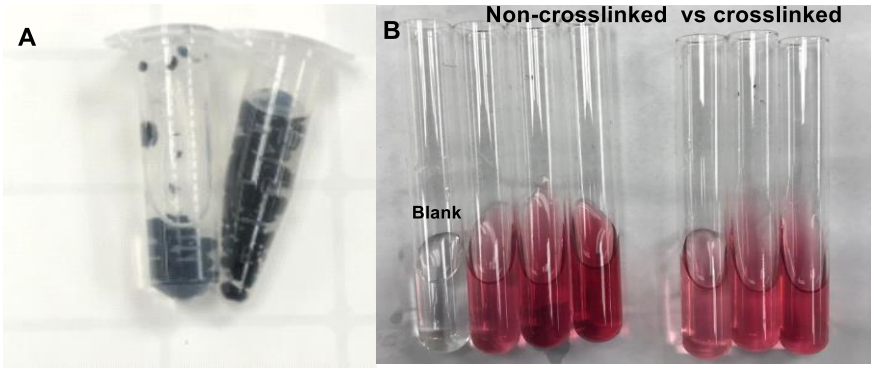

Figure 24: Immobilization and enzymatic assay of glucose oxidase **A:** ChsnAc beads immobilized with glucose oxidase enzyme; **B:** Oxidation of D-glucose to D-gluconic acid and H_2O_2 coupled with oxidation of phenol/4-aminoantipyrine by H_2O_2 catalyzed by peroxidase to form pink *p*-quinoneimide after 10, 20, and 30 minutes of non-crosslinked and crosslinked beads.

Table 11: Percent oxidation of D-glucose catalyzed by glucose oxidase immobilized on chitosan beads

Chitosan Beads	% Oxidation of D-glucose		
	10 min	**20 min**	**30 min**
ChsnAc	11 ± 5	8 ± 4	5 ± 5*
ChsnAc-CL	9 ± 6	6 ± 5	3 ± 5*
ChsnSi	61 ± 8	81 ± 8	90 ± 8
ChsnSi-CL	46 ± 6	30 ± 4	29 ± 10

* Some components of the reaction mixture might be adsorbed by the charcoal in the beads over time, this results in a decrease of absorbance at 510 nm, and, consequently, leads to the negative % conversion values.

Table 12: Percent oxidation of D-glucose catalyzed by glucose oxidase immobilized on non-crosslinked ChsnAc beads

Amount of glucose oxidase bound (µg)	% Oxidation of D-glucose		
	10 min	**20 min**	**30 min**
46	5.0 ± 6	16.2 ± 4	16.2 ± 1
82	5.8 ± 4	15.3 ± 5	15.3± 1
176	6.9 ± 4	7.6 ± 3	7.6± 2
138	6.2 ± 4	7.4 ± 3	7.4 ± 2

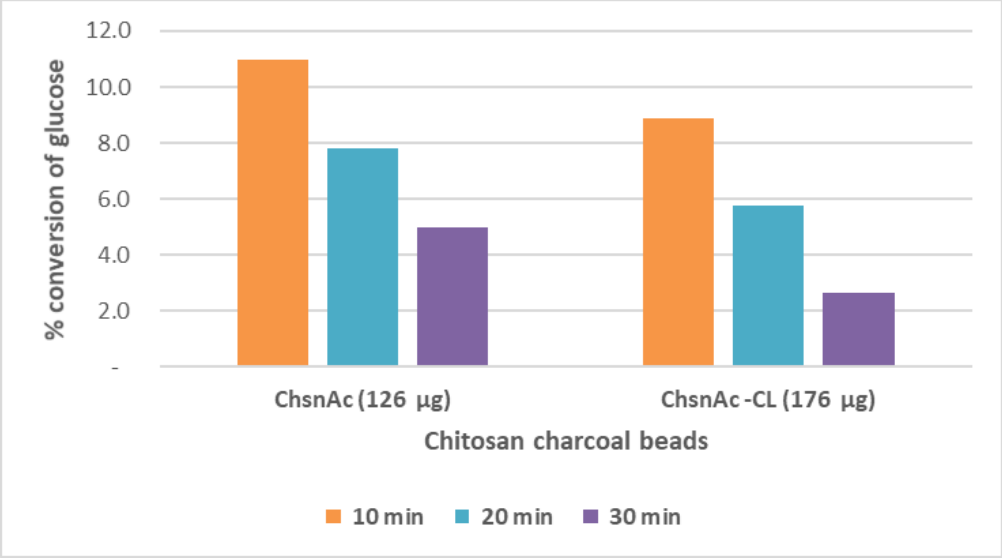

Figure 25: Average percent oxidation of D-glucose catalyzed by glucose oxidase immobilized on non-crosslinked and crosslinked ChsnAc beads.

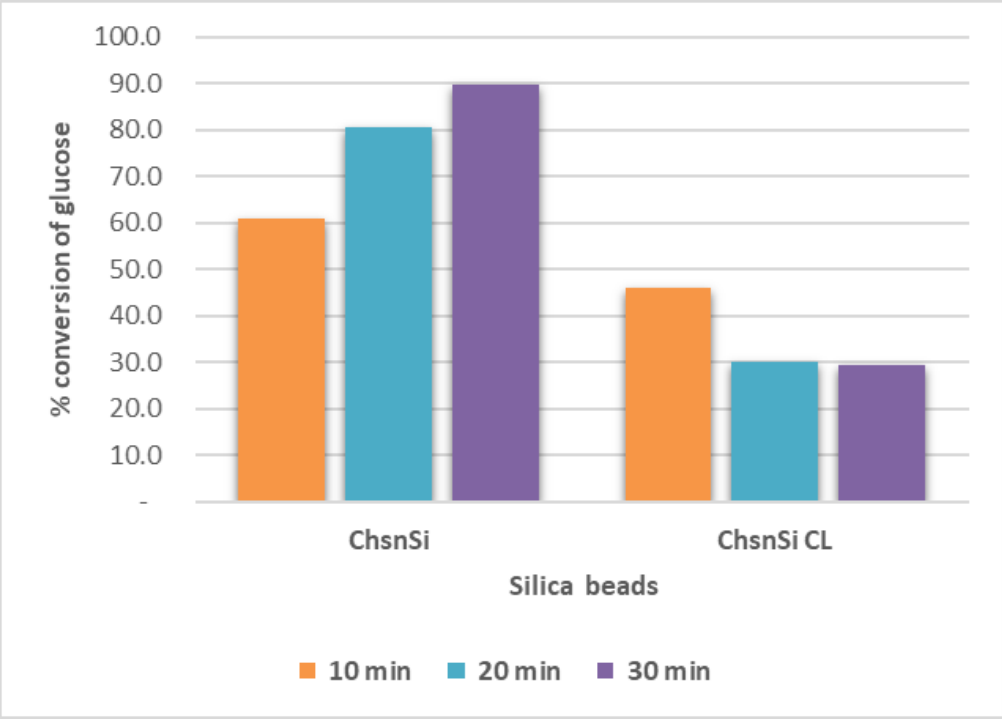

Figure 26: Average percent oxidation of D-glucose catalyzed by glucose oxidase immobilized on non-crosslinked and crosslinked ChsnSi beads.

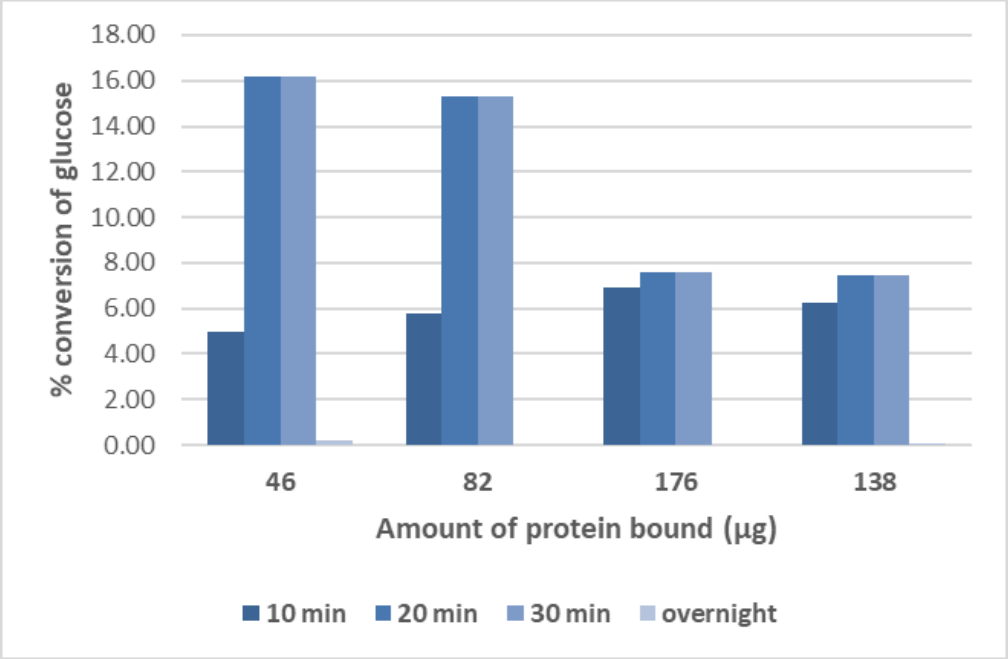

Figure 27: Average percent oxidation of D-glucose catalyzed by different amounts of glucose oxidase immobilized on non-crosslinked ChsnAc beads.

3.15 Stability and Reusability of Crosslinked vs Non-crosslinked Beads with Immobilized Glucose Oxidase

The purpose of the experiment was to compare the stability of crosslinked and non-crosslinked beads. Three individual activity tests were done on ChsnAc beads and ChsnSi beads immobilized with glucose oxidase beads. The non-crosslinked beads disintegrated and dissolved during the enzymatic reactions that involved shaking. As shown in **Figure 29**, the ChsnAc were seen to dissolve and some of the ChsnAc and ChsnSi beads were lost during the washing done between the enzymatic cycles. On the other hand, the crosslinked beads were very stable and did not dissolve. The stability of the crosslinked beads was attributed to the covalent bonds formed during crosslinking with glutaraldehyde.[23]

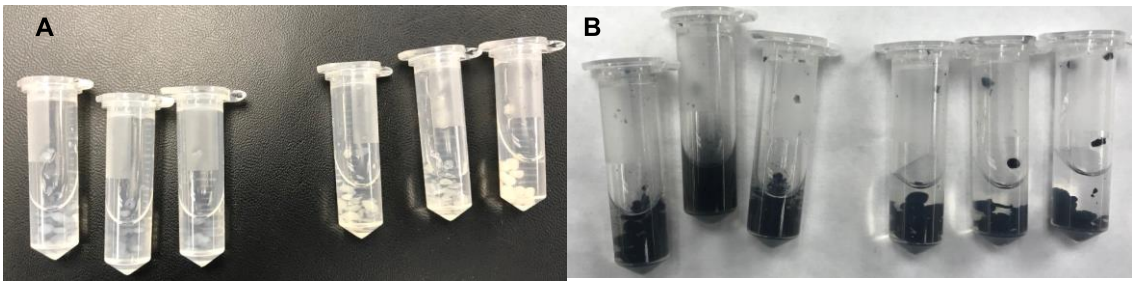

Figure 28: Stability of chitosan beads immobilized with glucose oxidase. **A:** Non-crosslinked and crosslinked (CL) ChsnSi beads; **B:** Non-crosslinked and crosslinked (CL) ChsnAc beads.

3.16 GO Assay to Confirm that the Products of Glucose Oxidation React with Chitosan-Charcoal (ChsnAc) Beads

Since the overnight incubation of D-glucose with ChsnAc beads immobilized with glucose oxidase demonstrated very low concentrations of hydrogen peroxide, the experiment was conducted as an independent way to evaluate the amount of D-glucose oxidized by the immobilized enzyme. Glucose does not have a characteristic absorbance; therefore, a discontinuous coupled enzyme assay was conducted using a Glucose Oxidase (GO) assay. The GO assay is a quantitative enzymatic assay used for the determination of glucose concentrations. It is composed of glucose oxidase, horseradish peroxidase, and *o*-dianisidine. Glucose oxidase enzyme oxidizes glucose to gluconic acid and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide reacts with *o*-dianisidine to produce a brownish colored product. The oxidized form of *o*-dianisidine has a characteristic absorbance at 540 nm, the absorbance intensity correlates with the amount of glucose present. Below is a representation of the reaction scheme (**Figure 30)**.

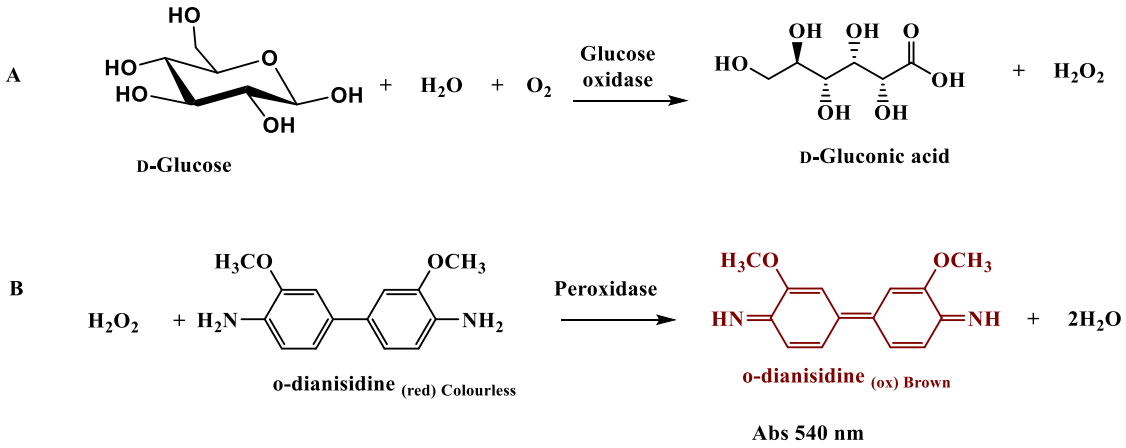

Figure 29: Reaction summary of glucose oxidation in GO assay. **A:** Glucose oxidation to D-gluconic acid and H_2O_2 catalyzed by glucose oxidase; **B:** Oxidation of o-dianisidine by H_2O_2 catalyzed by peroxidase.

A GO assay was used to quantify the amount of glucose left in the reaction mixture after incubation with GOx immobilized on chitosan-charcoal beads. A glucose standard curve was generated **Figure 31**. D-Glucose was incubated with glucose oxidase immobilized on ChsnAc beads. Aliquots were removed after 10 minutes and 30 minutes and added to a sample with GO reagent and water, and then incubated for 30 minutes. Thereafter, the absorbance at 540 nm was recorded and used to calculate the amount of D-glucose remaining in the solution. The data shown in **Table 13** and **Figure 32** indicate that the D-glucose concentration in the reaction mixture decreased over time, as it was being oxidized to D-gluconic acid and H_2O_2. After 10 minutes the D-glucose concentration in samples 1 and 2 was 1.23 and 1.25 µg/µL and after 30 minutes it decreased to 0.97 and 0.92 µg/µL, respectively. The decrease in D-glucose concentration confirms its oxidation by immobilized enzyme; however, there was no correlation with the amount of H_2O_2 detected. One of the explanations for this discrepancy was the possibility of H_2O_2 decomposition or adsorption by the chitosan-charcoal. Further experiments were performed to confirm this hypothesis.

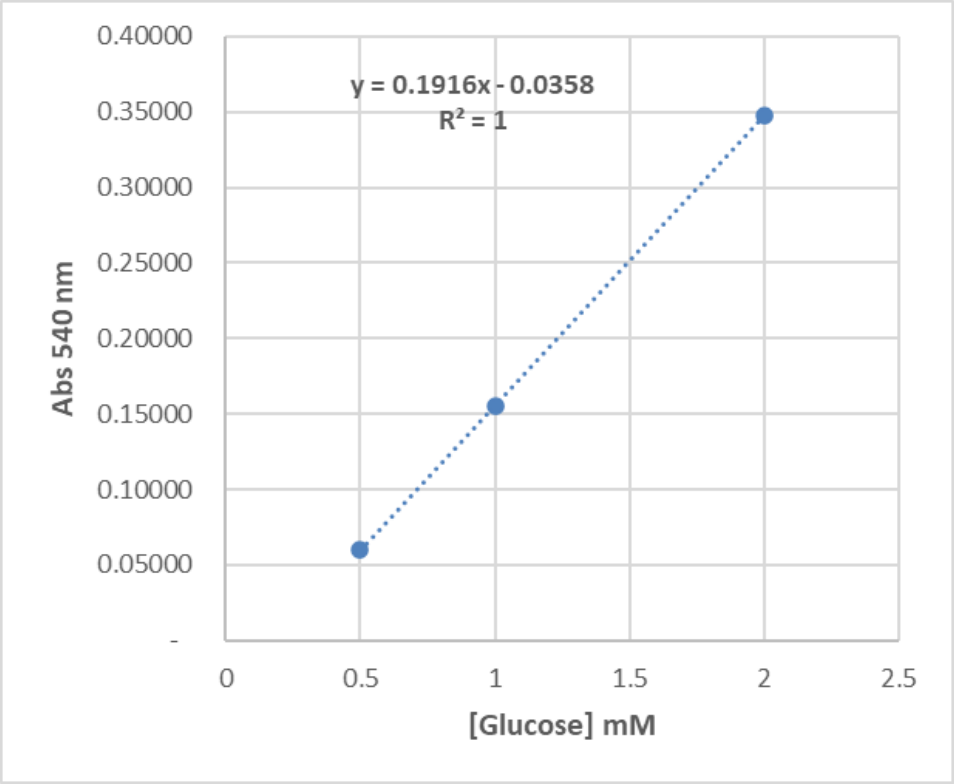

Figure 30: D-Glucose standard curve generated to calculate amount of glucose.

Table 13: Summary of the results from the GO assay experiment

ChsnAc beads	Amount of glucose remaining (µg)	
	10 min	**30 min**
Sample 1 -(136 µg)	61.71	48.52
Sample 2 -(127 µg)	62.29	46.05

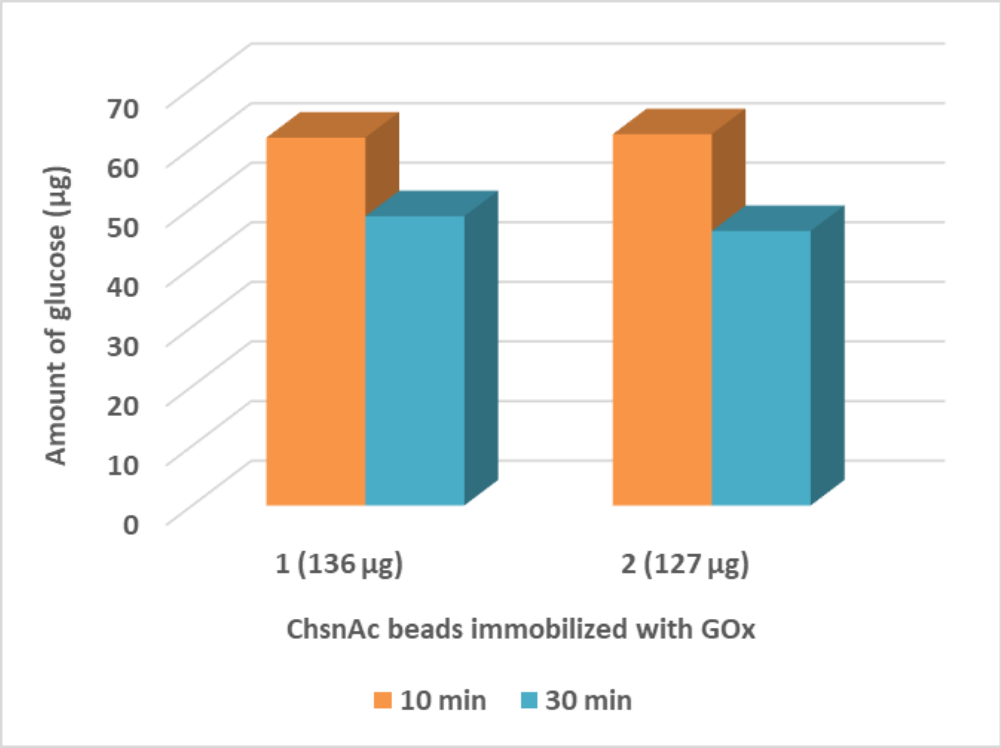

Figure 31: Summary of the results from the GO assay experiment.

3.17 Determining whether H_2O_2 Was Affected by Chitosan-charcoal Beads

The purpose of this experiment was to determine the possibility of H_2O_2 adsorption by the chitosan-charcoal. Hydrogen peroxide was incubated with ChsnAc beads that were not immobilized with any enzyme. Aliquots from the reaction mixture were removed every 10 minutes and added to a reaction mixture containing phenol, 4-aminoantipyrine, and peroxidase to quantify the amount of hydrogen peroxide in the solution. The absorbance at 510 nm was recorded, which is the absorbance of *p*-quinoneimide. The extinction coefficient (740 M^{-1} cm^{-1}) was used to calculate the concentration of H_2O_2 as described previously in **Equation 3, Section 3.14**.The results show **(Figure 33)** that the H_2O_2 concentration dropped progressively over 30 minutes upon contact with the ChsnAc beads, and it was concluded, that hydrogen peroxide was affected by the ChsnAc beads. To confirm the effect of chitosan-charcoal beads on H_2O_2 further experiments were performed.

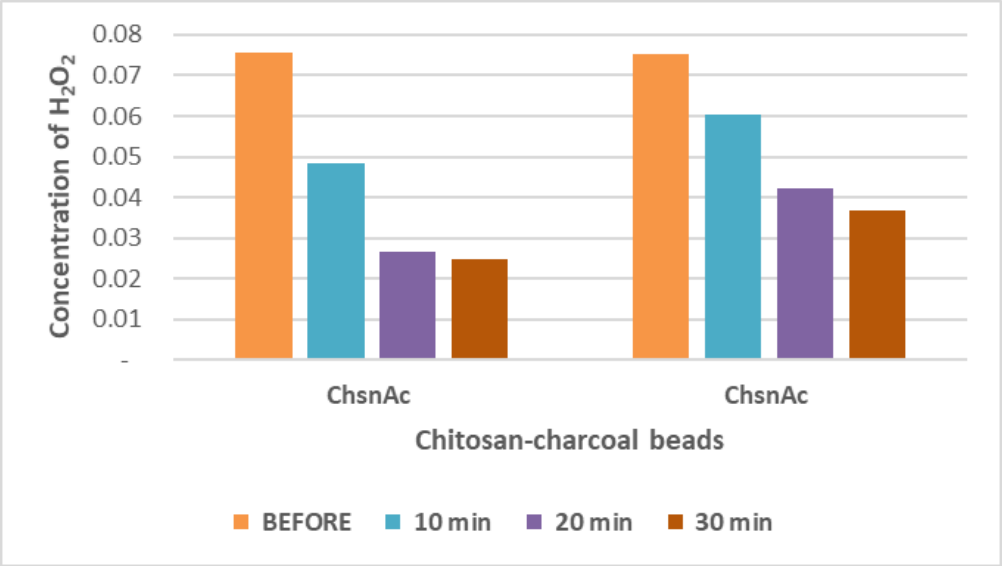

Figure 32: Concentration of H_2O_2 left after incubation with ChsnAc beads.

3.18 Determining whether H_2O_2 Was Affected by Chitosan-charcoal Beads or Chitosan- Silica Beads

To test whether H_2O_2 is affected by ChsnSi beads, H_2O_2 was incubated separately with chitosan-charcoal and chitosan-silica beads that were not immobilized with any enzyme. The procedure was done as previously described (**Section 3.17**). According to **Figure 34**, in the presence of ChsnAc beads the H_2O_2 concentration dropped progressively, while it was not affected during incubation with the ChsnSi beads. From the results obtained with the GO assay (Sections 3.16) and this experiment, it was concluded that the activated charcoal in the chitosan beads affects H_2O_2. Studies show that activated charcoal is used to adsorb H_2O_2 as a toxic component in the environment.[40] Due to these complications with quantifying the amount of hydrolyzed lactose through H_2O_2, a glucose oxidation byproduct; we decided to use ChsnSi beads for immobilization of glucose oxidase instead of ChsnAc.

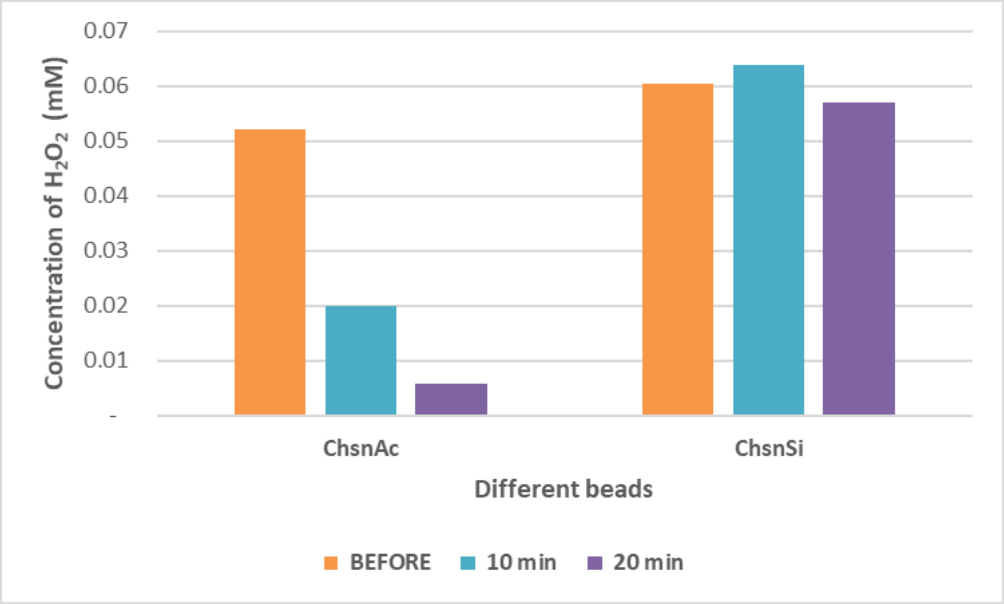

Figure 33: Concentration of H_2O_2 left after incubation with ChsnAc and ChsnSi beads.

3.19 Combining Immobilized Glucose Oxidase with Free Peroxidase

To monitor hydrogen peroxide reduction in real time, glucose substrate was added to an Eppendorf tube with glucose oxidase immobilized on ChsnAc beads, peroxidase and phenol/4-aminoantipyrine. After about 10 minutes a color change was observed. The contents in the tube changed from colorless to pink and after 30 minutes a deep pink color developed **(Figure 28**). The formation of the pink color indicated that the reaction had occurred and glucose oxidase was able to oxidize glucose to D-gluconic acid and H_2O_2 which oxidized phenol/4-aminoantipyrine to form pink *p*-quinoneimide in the presence of peroxidase.

Figure 34: Hydrogen peroxide reduction in real time through: oxidation of D-glucose to D-gluconic acid and H_2O_2 coupled with oxidation of phenol/4-aminoantipyrine by H_2O_2 catalyzed by peroxidase to form pink *p*-quinoneimide.

3.20 Testing the Two-Component (Immobilized Lactase and Glucose Oxidase) System for Hydrolysis of Lactose

3.20.1 Combining Immobilized Lactase and Glucose Oxidase

The experiment was performed to determine if the combination of immobilized lactase and glucose oxidase catalyzes sequential reactions: lactose hydrolysis and glucose oxidation. A summary of the reaction sequences is given in **Figure 35**. Lactose substrate was incubated with two enzyme systems: **I)** lactase and glucose oxidase immobilized separately on crosslinked and non-crosslinked ChsnSi beads; and **II)** lactase, immobilized on non-crosslinked ChsnMag beads and glucose oxidase immobilized on non-crosslinked ChsnSi beads. Aliquots from the reaction mixtures were removed every 10 minutes and added to a reaction mixture containing phenol, 4-aminoantipyrine, and peroxidase. The absorbance at 510 nm was recorded, which is the absorbance of *p*-quinoneimide. The extinction

coefficient (740 M^{-1} cm^{-1}) was used to calculate the conversion rate as described previously using **Equations 3 and 4** replacing $[glucose]_i$ with $[lactose]_i$ in this case.

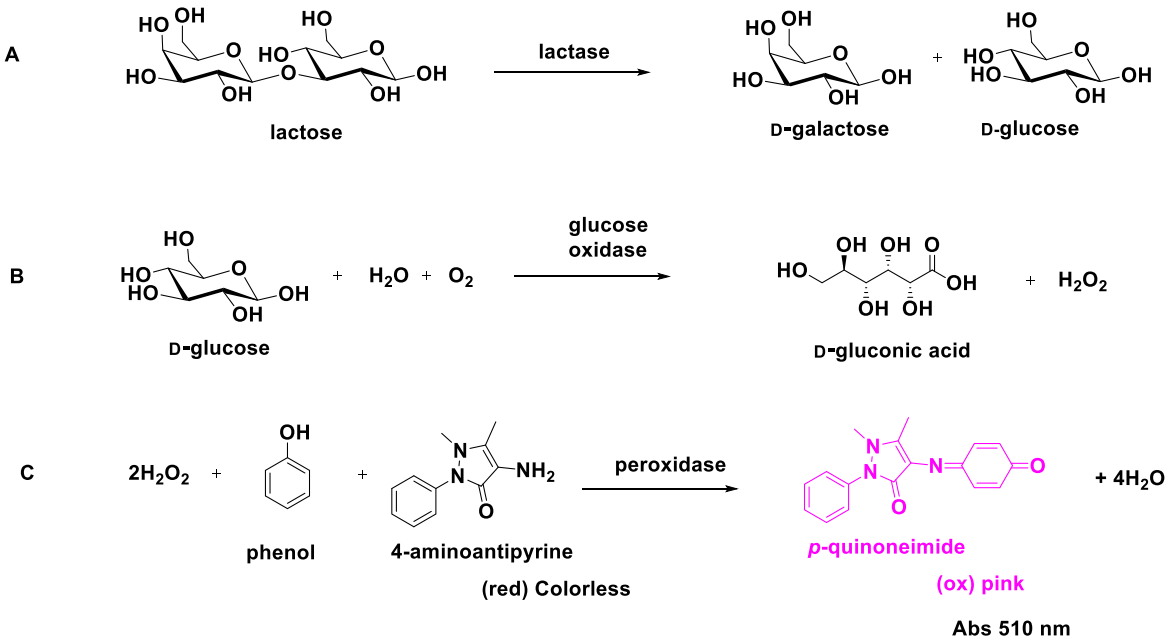

Figure 35: Coupled assay reaction summary. **A:** Lactose hydrolysis to D-glucose and D-galactose catalyzed by lactase. **B:** Oxidation of D-glucose to D-gluconic acid and hydrogen peroxide catalyzed by glucose oxidase. **C:** Oxidation of phenol/4-aminoantipyrine by hydrogen peroxide catalyzed by peroxidase.[37,38]

The results indicate (**Figures 36 - 38**) that the percent of lactose hydrolyzed increased steadily over 30 minutes. Our data confirmed that the designed two-component system with immobilized glucose oxidase and lactase on ChsnSi and ChsnMag beads retained activity and catalyzed the sequential reactions –-lactose hydrolysis and glucose oxidation. The combination of ChsnMag and ChsnSi showed a higher conversion rate compared to ChsnSi beads. The amount of substrate consumed by enzymes immobilized on the crosslinked beads was lower compared to the non-crosslinked beads (**Figure 37**). Since the beads also had a different amount of immobilized enzymes, these observations require

additional tests to determine whether the crosslinking of chitosan-silica beads affects the enzyme activity in the two-component system or some other factors may play a role.

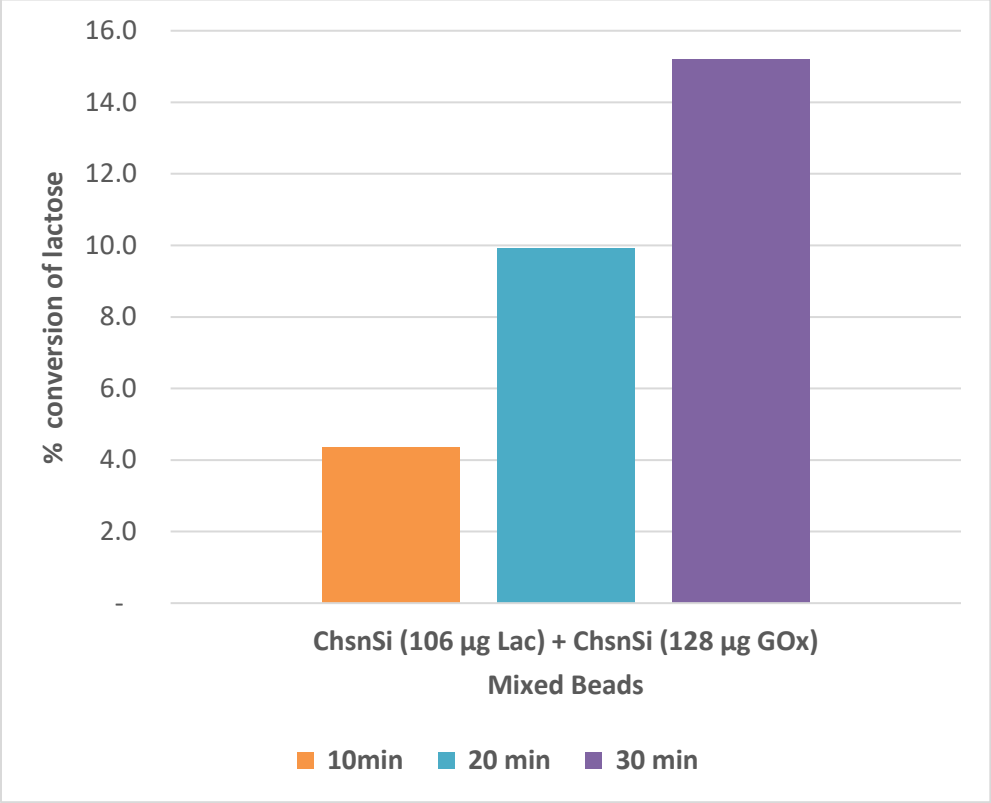

Figure 36: Lactose hydrolysis and D-glucose oxidation catalyzed by lactase and glucose oxidase immobilized separately on chitosan-silica beads.

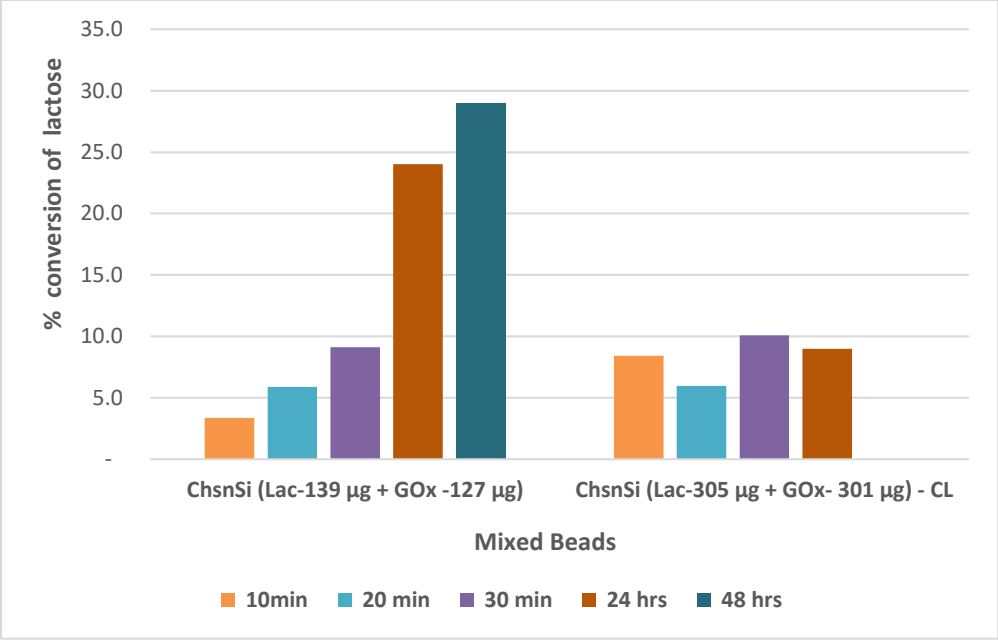

Figure 37: Lactose hydrolysis and D-glucose oxidation catalyzed by lactase and glucose oxidase immobilized separately on crosslinked and non-crosslinked chitosan-silica beads.

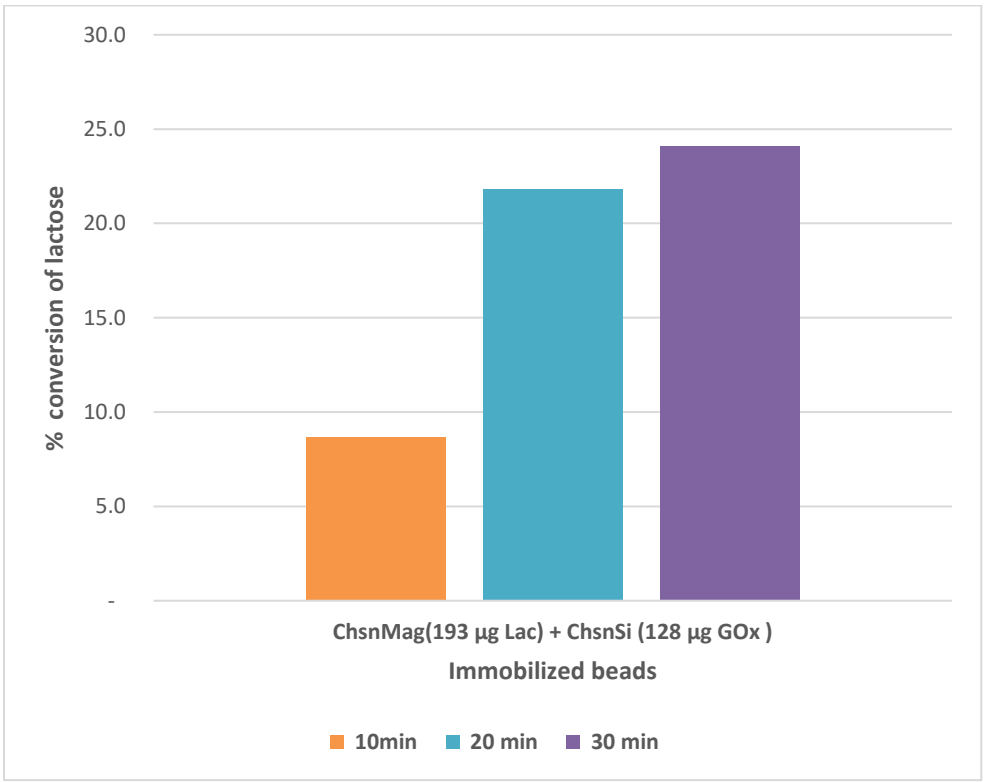

Figure 38: Lactose hydrolysis and D-glucose oxidation catalyzed by lactase immobilized on magnetic chitosan beads and glucose oxidase immobilized on chitosan - silica beads.

3.20.2 Combining Immobilized Lactase and Glucose Oxidase with Free Peroxidase

This coupled enzyme assay was done to confirm the activity of the combination of immobilized lactase – GOx. ChsnAc beads immobilized with glucose oxidase were mixed with ChsnMag beads immobilized with lactase. Lactose substrate was added to an Eppendorf tube with chitosan beads, KPi buffer, phenol, 4-aminoantipyrine, and peroxidase. The color change from colorless to pink indicated the formation of pink *p*-quinoneimide **(Figure 39)**. These results confirmed that the designed two-component system with immobilized glucose oxidase and lactase on ChsnAc and ChsnMag beads retained activity and catalyzed the sequential reactions –-lactose hydrolysis and glucose oxidation.

Figure 39: Lactose hydrolysis and D-glucose oxidation catalyzed by lactase immobilized on magnetic chitosan beads and glucose oxidase immobilized on chitosan – charcoal beads and oxidation of phenol/4-aminoantipyrine catalyzed by free peroxidase to form pink *p*-quinoneimide

CHAPTER 4. CONCLUSION

This research attempted to improve lactose hydrolysis using an immobilized multienzyme system. The chitosan gel bead formation involved dissolving the chitosan powder in an acidic solution and adding it dropwise to a basic solution. The chitosan structural stability was improved by addition of fortifying agents such as activated charcoal, silica, and magnetic particles followed by crosslinking beads with glutaraldehyde.

Lactase was immobilized successfully on six different types of chitosan beads with retention of its catalytic activity. Crosslinked beads were able to bind more enzyme and showed a slightly higher percent conversion of *o*NPGal compared to the non-crosslinked beads. Furthermore, treatment with $NaBH_4$ had no effect on the immobilization efficiency but the catalytic activity observed in these types of beads was slightly higher. Immobilized lactase could be reused up to nine times in contrast to the free enzyme that is not reusable. Lactase was also able to hydrolyze lactose in whey and TLC was used to monitor the hydrolysis reaction.

Glucose oxidase was immobilized on four types of chitosan beads successfully and the catalytic activity was retained. Similar to lactase immobilization, crosslinked beads were able to bind more enzyme compared to the non-crosslinked beads. However, a slightly lower percent oxidation of glucose was observed in crosslinked beads compared to the non-crosslinked beads. ChsnAc showed an unusual low percent conversion, most likely hydrogen peroxide formed during oxidation of glucose in the presence of GOx was adsorbed by activated charcoal in the chitosan beads. As a result, the reaction became more challenging to follow, therefore, ChsnAc beads were replaced with ChsnSi beads for immobilization of glucose oxidase.

Assays performed with the combination of the two types of chitosan beads indicated that systems consisting of immobilized lactase and glucose oxidase catalyzed two sequential reactions:- lactose hydrolysis and glucose oxidation. The Combination of ChsnMag and ChsnSi showed a higher conversion rate compared to ChsnSi beads. The designed two-component system can help improve lactose hydrolysis for industrial production of lactose-free products. Further investigation will be required to understand and compare the efficiency of lactose conversion in a column versus batch production.

The characterization of all bead types using SEM could provide a better understanding of the morphology of the chitosan support. This will help in designing a more efficient enzyme immobilization system with improved retention of catalytic activity. Also, any possibility of cross-contamination after combining immobilized lactase and glucose oxidase to different supports requires further investigation. Additionally, a continuous stepwise multienzyme system consisting of immobilized lactase and glucose oxidase should be tested for lactose hydrolysis. Ability of modified chitosan beads to work in multiple cycles is important in this process. A stepwise multienzyme system might have some advantages compared to batch, if one enzyme is losing its catalytic activity, it could be detected and the enzyme could be replaced.

CHAPTER 5. REFERENCES

1. Datta, S.; Christena R.; Rajaram Y. R. S. Enzyme Immobilization: an Overview on Techniques and Support Materials. *Biotech*. **2013,** 3, 1-9

2. Krajewska, B. Application of Chitin and Chitosan Based Materials for Enzyme Immobilization. *Enzyme Microb. Technol*. **2004**, 35, 126-139

3. Vu, T. K. H.; Le, V. V. M. Biochemical Studies on the Immobilization of the Enzyme Invertase (EC.3.2.1.26) in Alginate Gel and Its Kinetics. *ASEAN Food Journal*. **2008,** 15 (1), 73-78

4. Torres, P.; Batista-Viera, F. Immobilized Trienzymatic System with Enhanced Stabilization for the Biotransformation of Lactose. *Molecules* **2017**, 22, 284,1-15

5. Liu, Z.; Wang, H.; Li, C.; Jiang, Y., Yu, G.; Mu, X.; Biocompatible Magnetic Cellulose-Chitosan Hybrid Gel Microspheres Reconstituted from Ionic Liquids for Enzyme Immobilization. *J. Mater. Chem.* **2012**, 22, 15085-15091

6. Henriques, O, R; Bork, A. J; Fernandez-Lorente, G; Guisan, M. J; Furigo, A; Oliviera, D; Pessela,C. B. Co-Immobilization of Lipases and β-D-galactosidase onto Magnetic Nanoparticles Supports: Biochemical Characterization. *Molecular Catalysis.* **2018,** 453,12-21

7. Souza, C. J. F.; Garcia-Rojas E.E.; Favaro-Trindade C.S. Lactase (Beta-Galactosidase) immobilization by complex formation: Impact of Biopolymers on Enzyme Activity. *Food Hydrocolloids* **2018,** 83, 88-96

8. Brena, B.; Gonzalez -Pombo, P.; Batista-Viera, F. Immobilization of Enzymes and Cells; Methods in Biotechnology; Humana Press: New York City, NY, **2006,** 15-50.

9. Pickens, T. Immobilization of Beta-Glycosidase BglX from *Escherichia coli* on Chitosan Gel Beads. Master of Science Thesis, Youngstown State University, **2018.**

10. Ren, S; Li, C; Jiao, X; Jia S; Jiang, Y; Bilal, M; Cui, J. Recent progress in Multi Enzyme Co-Immobilization. *Chem. Eng. J.* **2019,** 373*,* 1254-1278.

11. Ji, Q; Wang, B; Tan, J; Zhu, L; Li, L. Immobilized Multi enzymatic Systems for Catalysis of Cascade Reactions. *Process Biochem.* **2016,** 51,1193-1203

12. Onal, S; Zihniglu, F; Encapsulation of Insulin in Chitosan-Coated Alginate Beads: Oral Therapeutic Peptide Delivery. *Artif Cell, Blood Sub.* **2009**, 30, 229-237

13. Jia F; Narasimhan, B; Mallapragada, S. Materials- Based Strategies for Multiple Enzyme -Immobilization and Co-immobilization. A review. *Biotech. and Bioeng.* **2014,** 111 (2), 209-222.

14. Gur, D. S.; Idil, N.; Aksoz, N. Optimization of Enzyme Co-Immobilization with Sodium Alginate and Glutaraldehyde-Activated Chitosan Beads. *Appl Biochem Biotechnol*. **2018**, 184, 538-552

15. Memon H. A; Ding, R; Yuan, Q; Liang H; Wei, Y. Coordination of GMP ligand with Cu to enhance the multi enzymes stability and Substrate Specificity by Co-immobilization process. *Biochem. Engin. J.* **2018,** 136, 102-108.

16. Lang, Q; Yin, L; Shi, J; Li, L; Xia, L; Liu, A. Co-Immobilization of Glucoamylase and Glucose Oxidase for Electrochemical Sequential Enzyme Electrode for Starch Biosensor and Biofuel Cell. *Biosens. Bioelectron.* **2014**, 51 , 158 -163.

17. Li, H; Li, S; Tian, P; Wu, Z; Li, Z; Prevention of Bacterial Contamination of a Silica Matrix Containing Entrapped β-Galactosidase through the Action of Covalently Bound Lysozymes. *Molecules* **2017**, 22, 377, 1-13

18. Avinash A. K.; Lee S. D.; Glutaraldehyde crosslinked Magnetic Chitosan Nanocomposites: Reduction Precipitation synthesis, Characterization, and Application for Removal of Hazardous Textile Dyes. *Bioresour. Technol.* **2015**, 193,563-567

19. Xi, F.; Wu, J.; Jia, Z.; Lin, X. Preparation and characterization of Trypsin Immobilized on Silica Gel Supported Macroporous Chitosan Bead. *Process Biochem.* **2005**, 40, 2833-2840

20. Pospiskova K., Safarik.I. Low-cost, easy-to-prepare magnetic chitosan microparticles for enzyme immobilization. *Carbohydr Polym.* **2013,** 96,545-548

21. Wahba I. M.; Porous Chitosan Beads of Superior Mechanical Properties for the Covalent Immobilization of Enzymes. *Int. J. Biol. Macromol*. **2017**, 105, 894-904

22. Nguyen, H,H; Kim, M. An Overview of Techniques in Enzyme Immobilization. *Appl. Sci. Converg. Technol.* **2017,** 26 (6): 157 -163

23. Chen, H; Zhang, Q; Dang, Y; Shu, G. The Effect of Glutaraldehyde crosslinking on the enzyme Activity of Immobilized β-galactosidase on Chitosan Bead. *Adv. J. of Food Sci. and Technol*. **2013,** 5 (7): 932-935

24. Panwar, D; Kaira, S, G; Kapoor, M; Crosslinked Enzyme Aggregates (CLEAs) and Magnetic Nanocomposite Grafted CLEAs of GH26 endo-B-1,4-mannase: Improved Activity, Stability and Reusability. *Int. J. Biol. Macromol*. **2017,**1-11

25. Dutta, P. K.; Dutta, J.; Tripathi, V.S. Chitin and Chitosan: Chemistry, Properties and Applications. *J. Sci. Ind. Res.* **2004**, 63, 20-31

26. Muxika, A.; Etxabide, J.; Uranga, P.; Guerrero, K. Chitosan as a Bioactive Polymer: Processing, Properties and Applications. *Int. J. Biol. Macromol*. **2017**, 105, 1358-1368.

27. Li, B; Lin, C S; Zhou, Q; Fang, Y; Wang, Y; Xu, F; Han Li; Ibrahim, M; Guo, L; Xie, G; Sun, G. Synthesis, Characterization, and Antibacterial Activity of Cross-Linked Chitosan-Glutaraldehyde. *Mar. Drugs.* **2013**, 11, 1534-1552

28. Kadam A, A; Lee, D, S. Glutaraldehyde cross-linked magnetic chitosan nanocomposites: Reduction precipitation synthesis, characterization, and

application for removal of hazardous textile dyes. *Bioresour. Technol.* **2015,** 193,563-567

29. Zhoua, Z; Linb,S; Yuea,T; Leeb,T. Adsorption of food dyes from aqueous solution by glutaraldehyde cross-linked magnetic chitosan nanoparticles *J. Food. Eng*. **2014**, 126, 133–141

30. Sathya, T. A.; Khan, M. Diversity of Glycosyl Hydrolase Enzymes from Metagenome and their Application in Food Industry. *J. Food Sci.* **2014,** 79, 2149-2156

31. Chandrasekar, B.; Thomas Colby, T.; Asif Emran Khan Emon, A.E.K.; Jiang, J.; Hong, N. T.; Villamor, G. J.; Harzen, A.; Herman S. Overkleeft, S. H.; A. L. Van der Hoor, A. L. R. Broad-range Glycosidase Activity Profiling. *Mol Cell Proteomics*, **2014,** 2787 - 2800

32. Bisset, F.; Sternberg, D.; Immobilization of Aspergillus Beta-Glucosidase on Chitosan. *Appl. Environ. Microbiol.* **1978**, 35, (4) 750-755

33. Bankar, S.B; Bule,V, M; Singhal S,R; Ananthanarayan, L. Glucose oxidase an overview. *Biotechnol Adv*. **2009**, 489-501

34. Wong, C.M; Wong, H, K; Chen, D, X. Glucose oxidase: natural occurrence, function, properties and industrial applications. *Appl microbiol biotechnol*. **2008,** 78 (6) :927 -38

35. Singh, V, M; Kumar, R. Biotechnology Production of Gluconic acid: Future Implications. *Appl Microbiol Biotechnol*. **2007,** 75:713–722

36. Leksmono, C, S; Manzoni, C; Tomkins, J,E; Lucchesi, W; Cottrell, G; Lewis, P,A. Measuring Lactase Enzymatic Activity in the Teaching Lab. *J. Vis. Exp*. **2018**, 138 1-5

37. Varadaraju, C; Tamilselvan, G; Enoch, I; Selvakumar, M.P. Phenol Sensing Studies by 4-Aminoantipyrine Method - A review. *Org. and Med. Chem.* **2018,** 5(2) 1-7

38. Jones, P, F; Johnson K,E. Estimation of Phenols by the 4-Aminoantipyrine Method. II. Products from para-Substituted Alkylphenols. *Can. J. Chem*. **1973,** 51, 3733 -3737

39. Walt, R,D; Agayn, I,V. The Chemistry of Enzyme and Protein Immobilization with Glutaraldehyde.*Trends Analyt Chem*. **1994**, 13 (10): 425-430

40. Hoffman, S, P; Pine, L; Bell, S; Production of Superoxide and Hydrogen Peroxide in Medium Used to Culture Legionella pneumophilia: Catalytic Decomposition by Charcoal. *Applied. Environ. Microbiol.* **1983**, 45 (3) 784-791