Immobilization of Beta-Glycosidase BglX from *Escherichia coli* on Chitosan Gel Beads

by

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Abstract

Enzyme immobilization refers to any technique by which an enzyme is restrained or localized to a support system. This can provide retention of catalytic activity and reusability of the enzyme, both of which are important in industrial processes. Enzyme supports can be made of organic materials, typically naturally occurring polysaccharides, such as cellulose, agarose, or chitosan. The objective of this study was to immobilize beta-glycosidase BglX and its mutant E293Q on chitosan gel beads while retaining catalytic activity. The beads were fortified with activated charcoal or silica and cross-linked with glutaraldehyde to increase their mechanical stability and immobilization efficiency.

Lactase was used as a model enzyme to determine which type of chitosan gel bead was the most suitable for immobilization. The chromogenic substrate ortho-nitrophenyl-β-D-galactopyranoside (*o*NPGal) and a lactose solution were used to test the catalytic activity of immobilized lactase. Upon successfully immobilizing lactase, BglX and E293Q were tested similarly. Since BglX showed higher percent conversion of substrate, it was used in a packed bed column and the ability of the immobilized enzyme to hydrolyze the lactose present in milk whey was tested in a small-scale continuous production system.

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List of Abbreviations

Abs	absorbance
BglX	β-glucosidase with unknown biological function
BSA	Bovine serum albumin
Da	Dalton
DNA	deoxyribonucleic acid
ε	molar extinction coefficient
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
GO Assay	Glucose oxidase assay
IPTG	isopropyl-β-D-1-thiogalactopyranoside
kb	kilobase
LB	Luria-Bertani medium
MES	2-(N-morpholino)ethanesulfonic acid
OD_{600}	optical density at 600 nm
*o*NPGal	ortho-nitrophenol-β-D-galactopyranoside
pI	isoelectric point
*p*NPGlu	para-nitrophenol-β-D-glucopyranoside
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Chapter 1: Introduction

Industrial Uses of Immobilized Enzymes

Biomolecules possessing enzymatic activity are critical in sustaining life and maintaining normal, healthy function in biological systems. Enzymes are highly efficient catalysts, able to speed up the rate of a reaction by many orders of magnitude, and they exhibit a high degree of specificity, which in turn decreases by-product formation.[1] In industrial processes, the presence of by-products can necessitate a costly and time-consuming separation to purify the desired product and decreases the process's efficiency by generating unnecessary waste.[2] The development and refinement of recombinant DNA technology and protein engineering techniques in recent years have substantially increased the use of enzymes in industry.[3] Enzymes synthesized by microorganisms - such as yeast, mold, fungi, and bacteria - can be exploited for a multitude of industrial processes, including the isomerization of glucose to fructose and the production of semi-synthetic penicillins.[2]

The production of cheese relies upon either acid or, more commonly, a proteolytic enzyme, to separate the curds and whey. There is evidence of enzymes being employed for cheese production as long ago as 5500 B.C. - the tomb art of ancient Egyptians represents the milking of livestock and drying of cheese curds, and pots containing cheese have been recovered from two intact tombs.[4] Homer's *Iliad* mentions using fig juice - which contains a proteolytic enzyme, ficin, similar to rennet - to coagulate milk in the cheese making process.[4] Although people did not have a high level of understanding of enzymes, their usefulness in creating a food product had already been discovered.

Enzyme immobilization refers to any technique by which an enzyme is restrained or localized to a support system.[5] This can provide retention of catalytic activity and reusability of the enzyme, both of which are enticing for industrial processes. Immobilization often improves operational stability and reduces the cost of materials produced by biocatalysis, as the separation of product and by-product from the reaction mixture is typically unnecessary.[5]

The first known incidence of enzyme immobilization was employed for vinegar production, where the metabolism of acetic acid bacteria was utilized. This is referred to as the "quick" vinegar process and has been in use since the early 19th century. Wood shavings or twigs acted as the matrix/support upon which the acetic acid-producing bacteria are able to immobilize themselves by forming a biofilm. The alcoholic solution is trickled over the bacteria-coated matrix, and the resulting acetic acid is collected.[6]

One of the first enzymes to be immobilized for use in a large-scale industrial application was the yeast enzyme β-fructofuranosidase, which can hydrolyze β-D-fructofuranosidase-containing compounds, such as the disaccharide sucrose.[7] This enzyme is commonly referred to as invertase, and its product as inverted sugar because the mixture of products from sucrose hydrolysis have the opposite direction of optical rotation of the starting saccharide. Inverted sugar is useful to the food industry, as it tastes sweeter[8], crystallizes less easily, and retains the moisture in baked goods better than sucrose; and therefore, invertase has played an important role in the food industry, particularly when immobilized on a matrix (Figure 1-1).[9]

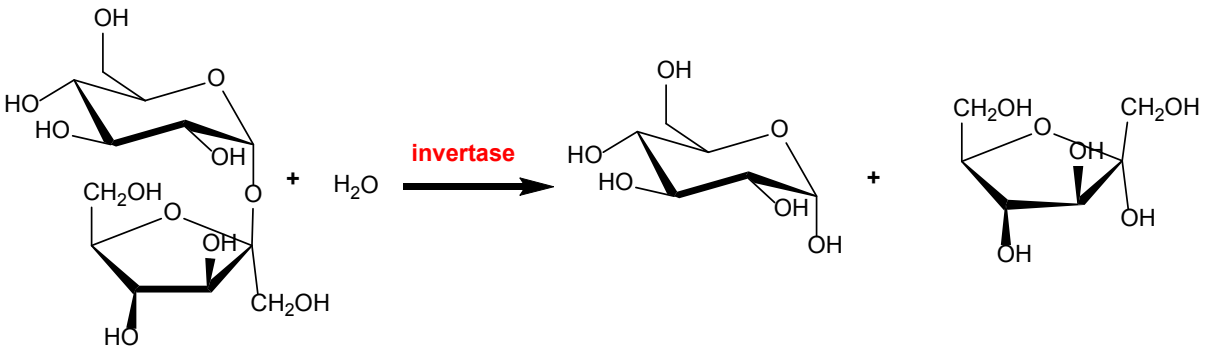


Figure 1-1: Reaction catalyzed by invertase - sucrose is hydrolyzed to glucose and fructose.

There are many advantages to using an enzyme immobilized on a matrix. Once immobilized, they are relatively easy to use. Product recovery and purification are simplified, since removal of the enzyme from the reaction mixture is no longer necessitated.[5] The reaction can take place in a wide variety of reactors, including stirred tank, fluidized, or fixed bed.[10] Often, the immobilized enzyme exhibits a wider or increased temperature and pH stability compared to that of the free enzyme.[7] Enzyme purification from microorganisms or fungi is labor intensive; however, once immobilized, an enzyme can be re-used multiple times, thus decreasing the cost associated with enzyme production and purification.[11] As with all techniques, immobilization has its disadvantages. Enzyme activity can be negatively affected by immobilization, and diffusional limitations can decrease product yield. Start-up costs are incurred when installing the necessary equipment for immobilization and trial and error is necessary to optimize the procedure.[10]

Several factors can influence the efficiency of enzymes; for example, temperature, pH, enzyme and substrate concentration, and inhibition of activity when products are accumulated.[12] Enzymes from different sources can have the same catalytic effect. To

choose the best enzyme for the application, it is necessary to consider the conditions in which it will be employed, for example, the optimum pH and temperature of the enzyme can vary depending on the source. The catalytic activity of the enzyme chosen must be retained after immobilization; therefore, it is important to choose an enzyme possessing a high catalytic activity.[7]

Choosing a support

Some properties, such as resistance to compression, hydrophilicity, microbial resistance, and cost must be considered when choosing a support.[13] Supports can be made of organic materials, typically naturally occurring polysaccharides, such as cellulose, agarose, or chitosan, or inorganic materials, like benonite or silica.[7]

The support system can either irreversibly or reversibly bind the enzyme. An example of irreversible immobilization is covalent binding, the most widely used type of immobilization, due to its high stability.[7] Entrapment and microencapsulation of the enzyme are also employed. These techniques do not actually bind the enzyme, rather it is held in place by a porous bead or fiber. Reversible immobilization is employed when it is beneficial to recover the enzyme from the reaction mixture for future use, and is not as commonly used as irreversible immobilization.[5] Simple adsorption is an easy and cheap way to reversibly immobilize an enzyme, but can prove problematic in that the enzyme is not tightly held in place and can be released into the reaction mixture. Ionic binding, affinity binding, chelation or metal binding, and disulfide bonds can all be used for reversible immobilization, but these all come with their downfalls and are not commonly

employed in industry unless all other methods of reversible and irreversible immobilization have been exhausted.[5,7]

Chitosan

Chitin is one of the most abundant naturally occurring biopolymers, making up the exoskeletons of crustaceans such as crabs and shrimp, and it is also the main component of fungal cell walls. The structure of chitin consists of repeating units of *N*-acetylglucosamine linked via a β(1→4) glycosidic bond. Upon treatment with a strong base, usually sodium hydroxide, the chitin undergoes partial deacetylation and results in chitosan, a modified structure of randomly distributed β(1→4)-linked D-glucosamine and *N*-acetyl-D-glucosamine (Figure 1-2).[14] It is well-known that the pK_a of chitosan's free amino group is between 6.0 and 7.0, depending on the degree of N-deacetylation. Chitosan's amino and hydroxyl groups greatly enhance the solubility and reactivity of the polymeric saccharide.[15]

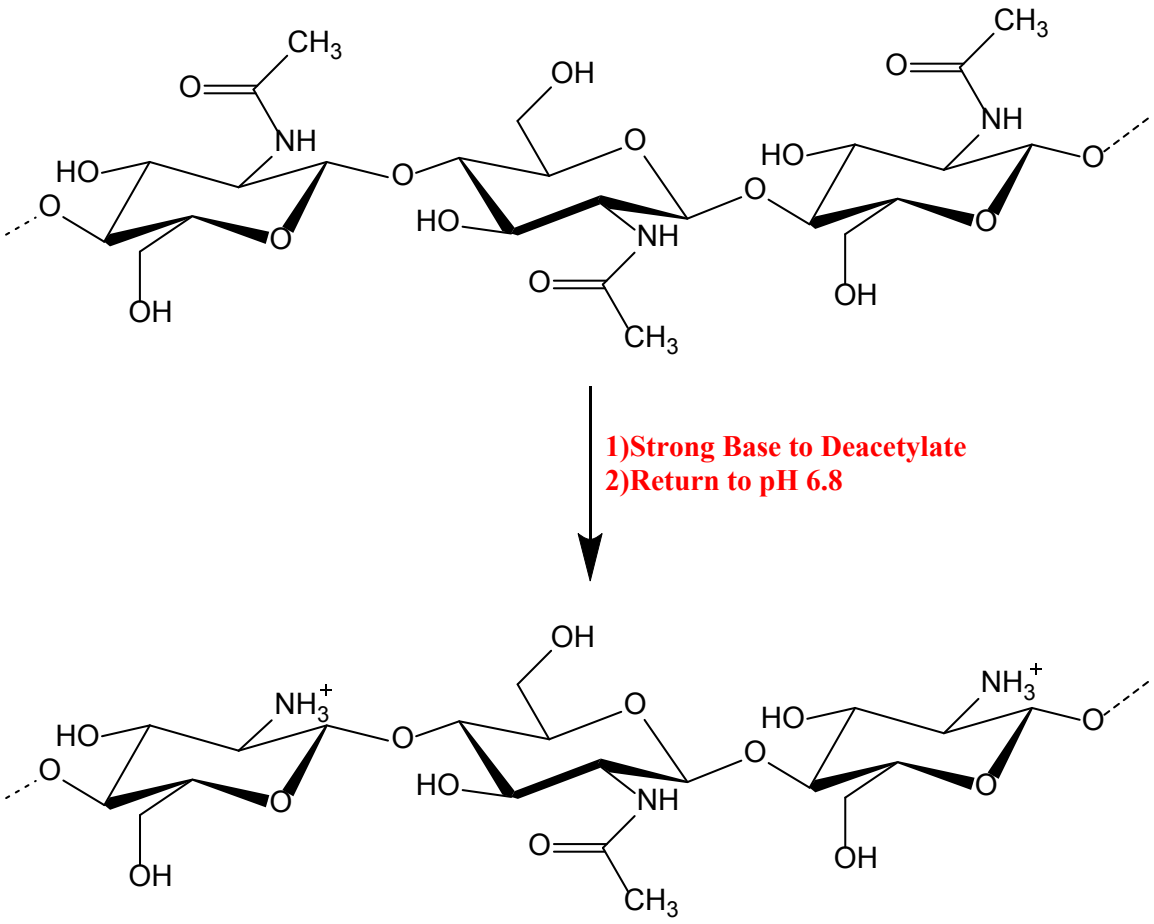


Figure 1-2: Chitin is partially (and randomly) deacetylated by treatment with a strong base, resulting in the formation of chitosan.

Chitosan has found many useful applications in biomedical, pharmaceutical, cosmetics, and food industries.[14,16] It is non-toxic to humans and animals and is unable to be digested in the gastrointestinal tract. For this reason, chitosan has been marketed as an insoluble fiber weight loss supplement; however, studies have shown there is no clinical justification for overweight people to take a chitosan supplement and the FDA issued warnings to companies advertising chitosan as a weight loss supplement.[17,18] In the biomedical industry, chitosan is used to prepare hydrogels, films, and fibers. Chitosan has

hemostatic properties and has been approved in the United States and Europe as a bandage and gauze additive.[19] Chitosan has been found useful as a support system for enzyme immobilization applications, particularly in the food industry. Its hydroxyl and amino groups make the polymer ideal for coupling to enzymes, which have been successfully immobilized on films, beads, and nanoparticles formed by using chitosan solutions.[16]

Dyes from textile industries contaminate wastewater, and many synthetic dyes are harmful to both humans and the environment. It is costly to remove dye contaminants - even at low concentrations - due to the high cost of conventionally employed adsorption techniques.[20] Chitosan is useful in the removal of both anionic and cationic dyes, due to its ability to interact via electrostatic interaction or adsorption of the dye molecules on its surface. Chitosan is an interesting option for the adsorption or chelation of toxic heavy metal ions discharged by industries that can leach into water.[21] The adsorption capacity of chitosan can be tuned by forming various chitosan composites, such as chitosan-coated cotton fibers and chitosan with magnetite.[21,22]

The surface area available for adsorption is greatly increased when the chitosan is dissolved in a dilute acid and the resulting chitosan solution is used to make beads, nanoparticles, or films.[23] Chitosan beads and microparticles are formed by precipitation or coacervation when the chitosan solution is added dropwise or blown into a basic solution. The mechanical stability of chitosan beads is improved by the addition of inert materials, like finely-ground activated charcoal or silica.[24,25] Glutaraldehyde can cross-link the chitosan beads, both improving the mechanical stability and adsorptive capacity, and

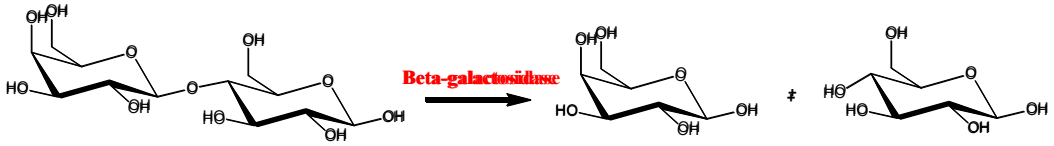
lending an antimicrobial property to the beads.[16,26,27] Chitosan's free amino groups can remain protonated up to pH 9, particularly when chemical cross-linking has occurred.[23]

Glycoside Hydrolases

The production and metabolism of carbohydrates is prevalent amongst the kingdoms; bacteria, fungi, plants and mammals all rely upon carbohydrates in some respect. The number of possible combinations of oligosaccharides that can be built from simple monomeric precursors is tremendous. There are a vast array of glycoside hydrolases exhibiting varied specificities responsible for breaking down these diverse carbohydrates.[28] The glycoside hydrolases are a widespread class of enzymes with a variety of carbohydrate-containing substrates, including cellulose, amylopectin, and lactose. These enzymes cleave the glycosidic bond linkage between two carbohydrate moieties or a carbohydrate and non-carbohydrate moiety.[29]

The enzymes exhibiting glycosidase activity are classified into families by amino acid sequence similarities. There is a direct relationship between similarities in primary structure and protein folding. The $(\alpha/\beta)_8$ barrel is the most common tertiary structural feature occurring in the glycoside hydrolase families.[30] Currently, the glycoside hydrolases (GH) are divided into 144 families.[31] Enzymes fitting into pre-existing families and some requiring the addition of a new family are constantly being discovered; the Carbohydrate-Active Enzymes Database (CAZy) has a continuously updating list of all known glycoside hydrolases. The families are sometimes grouped into clans, if there are notable sequence or folding similarities between multiple families.[32]

Glycoside hydrolases are often named using the natural substrate or the glycoside liberated by the hydrolysis.[32] For example, β-galactosidases cleave the glycosidic bond formed between galactose and another carbohydrate or organic molecule, such as the one occurring in lactose (Figure 1-3); however, this group of enzymes only exhibits activity when the glycosidic bond is in the beta configuration. In contrast, an α-glucosidase specifically hydrolyzes α-glycosidic bonds and releases a terminal glucose molecule. The configuration about the anomeric carbon is important for the enzyme's specificity; the alpha (α) designation refers to the -OH substituent being on the opposite side of the ring from the CH_2OH at the chiral center at C5' and beta (β) refers to the other anomer.[1]



Lactose **D-Galactose** **D-Glucose**

Figure 1-3: Hydrolysis of the β (1→4) glycosidic bond of lactose releases galactose and glucose. Enzymes with the ability to hydrolyze this disaccharide exhibit β-galactosidase activity.

There are two types of glycosidases, inverting and retaining.[33] This refers to the configuration about the anomeric carbon – inversion would lead to the product having the opposite configuration of the substrate, while retaining glycosidases would produce a molecule with the same configuration as the substrate. Both inverting and retaining glycosidases with the $(\alpha/\beta)_8$ tertiary structure have an active site at the C-terminal end of the β-barrel; however, mechanistic differences exist between inverting and retaining glycosidases (Figure 1-4).[33,34] Inverting glycosidases have an active site with two essential amino acids lying on opposite sides of the substrate-binding cleft.[29] These are amino acids with acid/base capabilities, typically glutamate or aspartate, lying approximately 9

angstroms apart.[33] One of the active site residues acts as a base and activates a water molecule by extracting a proton. The transiently formed hydroxide ion attacks at the anomeric carbon while the leaving group is stabilized by the other active site residue, which acts as an acid and donates a proton to the aglycone. Retaining glycosidases invariably have a glutamate or aspartate as the nucleophile, and most often one of these carboxylic amino acids also functions as the general acid/base.[33] The distance between these residues is significantly less than the inverting glycosidase active site, only about 5 angstroms. This is thought to be due to the formation of a covalent, glycosyl-enzyme intermediate, so it is beneficial for the active site cleft to be smaller, which positions the sugar closer to the nucleophilic residue.[29,33] The retaining mechanism proceeds via a direct nucleophilic attack at the anomeric carbon by an active site residue, and a glycosyl-enzyme intermediate is formed as the aglycone moiety is protonated by the general acid/base residue. A water molecule is activated by proton extraction by the deprotonated active site residue, and it attacks at the anomeric carbon, displacing the glycosyl-enzyme intermediate, and releasing a product that has retained the stereochemistry of the starting material.[33]

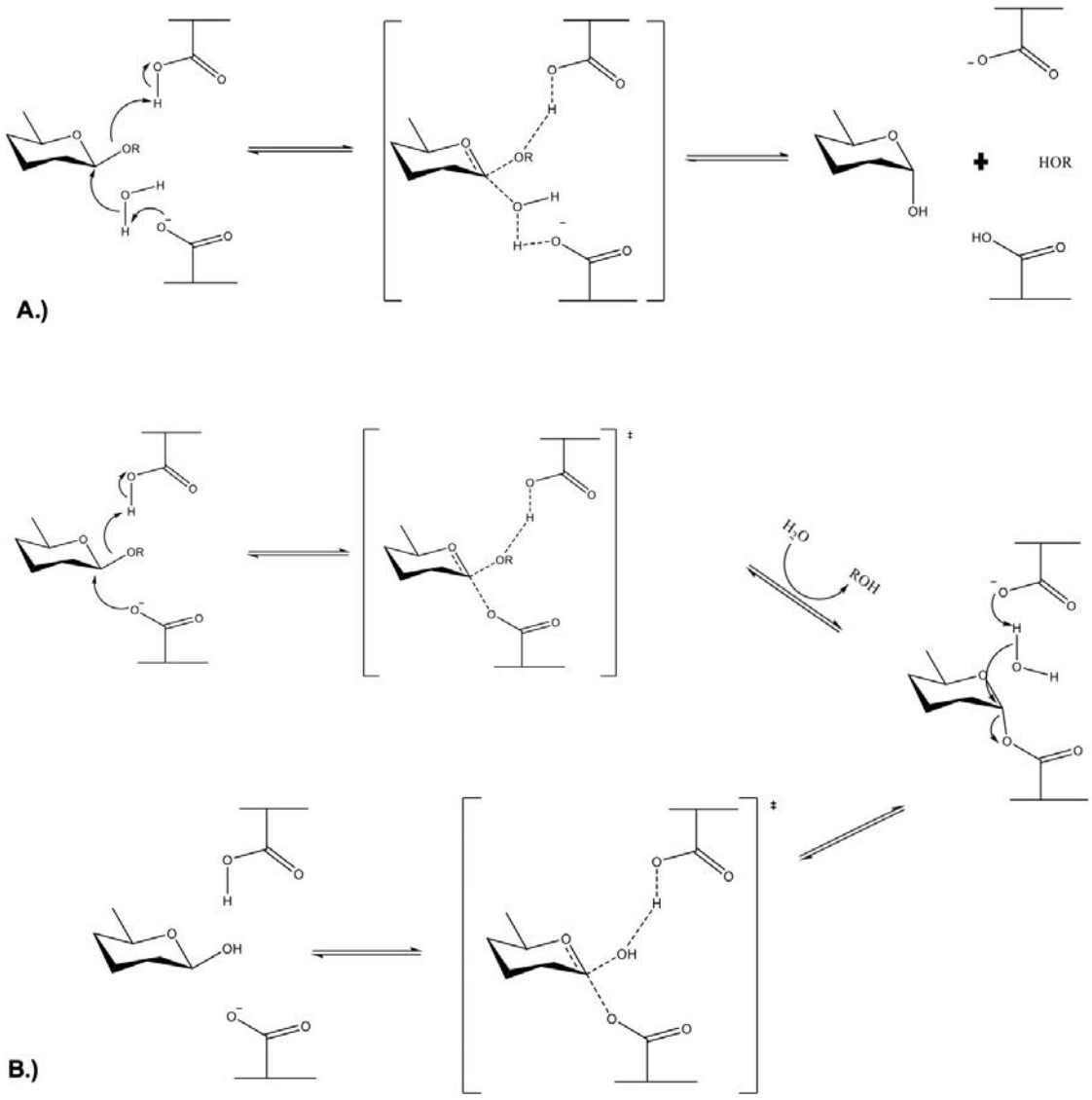


Figure 1-4: Mechanistic scheme for **a)** an inverting β-glycosidase and **b)** a retaining glycosidase. These mechanisms were first proposed by Koshland in 1953.[34]

Beta-glycosidases

β-glycosidases are a group of glycoside hydrolase enzymes that catalyze the selective cleavage of β-glycosidic bonds. These enzymes remove the non-reducing terminal glycosyl residue from oligosaccharides and other glycosides.[35] β-glycosidases occur across many living organisms including bacteria, fungi, plants, and some mammals.

The functions are varied amongst the kingdoms; for example, β-glycosidases play a role in the plant defense system. Some plants contain glycosides that, once acted upon by a β-glycosidase, release a compound such as cyanide that is toxic to other organisms. This is a clever defense mechanism employed by plants.[35] (Figure 1-5) Multiple species of legumes produce non-cyanogenic defense compounds; some examples include β-hydroxynitriles and isoflavones. β-glycosidases are proliferous in plants; a typical plant produces 40 or more isoenzymes.[35] Not all of these isoenzymes are utilized for defense; they also play a role in cell wall metabolism and the release of aromatic compounds.

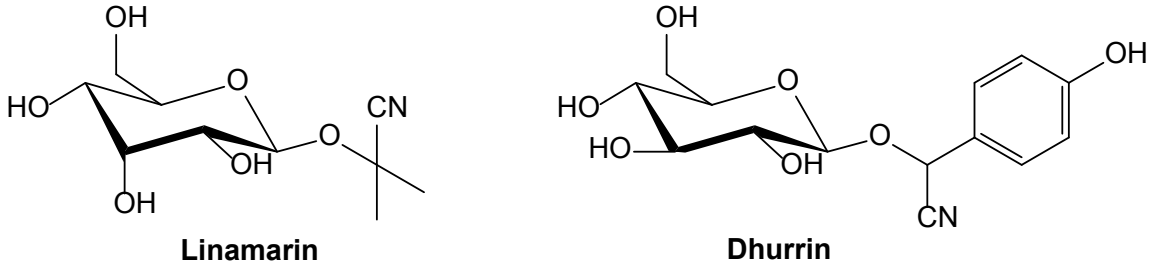


Figure 1-5: Linamarin from clover and dhurrin from sorghum are two examples of β-glycosides that, upon hydrolysis by a β-glycosidase, yield a cyanogenic compound.

Mammalian β-glycosidases are thought to play a role in the metabolism of glycolipids and glucosides. The enzyme glucosylceramidase, also referred to as β-glucosylcerebrosidase, acid-β-glucosidase, or D-glucosyl-N-acylsphingosine glycohydrolase, is well-studied.[36] It is implicated in Gaucher's disease, a lysosomal storage disease occurring when there is a deficiency in glucosylceramidase production. Gaucher's disease is a genetic disorder and the most common lysosomal storage disease; deficiency in the activity of the enzyme leads to a host of maladies, including severe neurological complications, hepatomegaly, splenomegaly, and severe pain in the joints and bones of

the hips and knees. Deficiency or the loss of activity of this enzyme leads to the abnormal accumulation of glucosylceramide in the tissues and brains of those afflicted.[36]

In bacteria, β-glycosidases participate in cellular signaling, host-pathogen interactions, and aid in the uptake of nutrients. These enzymes can hydrolyze short-chain polysaccharides and cellulose.[35] During the industrial conversion of cellulose to glucose, cellobiose disaccharide units are cleaved from the polysaccharide chain by exocellulases. Cellobiose consists of two glucose molecules linked together via a β-(1→4)-glycosidic bond. The cellobiose units are then hydrolyzed to glucose molecules using a β-glucosidase, and subsequently fermented to ethanol using yeast. Cost-efficient cellulosic ethanol production is of great interest, as it holds potential as a source of renewable energy.[37] This ability to degrade cellulose is also taken advantage of as a feed additive; "Barlican" has been used to enhance cellulose degradation *in vivo* by certain non-ruminant livestock.[38] This led to better nutrient utilization, and thus decreased the amount of feed required per animal.

The production of bacterial β-glucosidases may have potential for other applications, such as increasing the bioavailability of isoflavones found in food sources like soybeans, chickpeas, and other legumes.[35] The soybean contains more isoflavones than any other plant, in both aglycone and glycoside forms. Isoflavone aglycones have shown greater bioavailability than their glycosidic counterparts; using β-glycosidases in the enzymatic conversion of isoflavone glycosides to isoflavone aglycones could prove useful.[39] In recent years, studies have indicated that increased consumption of isoflavones could have a positive impact on human health. Isoflavones have been

investigated for their use in easing menopausal symptoms[40], treating breast cancer[41], and preventing build-up of arterial plaque[39].

Beta-Glucosidase, BglX

The enzyme BglX in *E. coli* has not been well-studied; however, based upon protein sequence alignments with 30 homologous β-glycosidases, it has been classified into the glycoside hydrolase family 3 (GH3) and its Enzyme Commission (EC) number is EC 3. 2. 1. 21.[42] This enzyme was characterized in 1996 after an open reading frame encoding for a β-glycosidase was discovered at 2225 kb on the *E. coli* chromosome.[42] The primary structure of BglX consists of 765 amino acids, 20 of which are the signal peptide.[42] Unlike homologous bacterial β-glucosidases, BglX resides in the periplasm rather than the cytoplasm. The signal peptide is cleaved once the protein reaches the periplasm and is folded into its native shape. The main function of the periplasmic enzyme and its natural substrate is unknown, but it may hydrolyze β-glucosides to release glucose for energy. It is characterized as a retaining β-glycosidase, and thus follows the Koshland double displacement mechanism shown in Figure 1-4b. Enzymes with the ability to cleave the β-galactosidic bonds are referred to as β-galactosidases.

Since the 3-dimensional structure of BglX has yet to be determined by X-ray crystallography, a sequence alignment performed using the Phyre server, compared the primary sequence of BglX to that of barley β-glycan exohydrolase isoenzyme Exol.[43] BglX showed 30% similarity to its primary structure, and so the structure of β-glycan exohydrolase isoenzyme Exol was used to predict the 3-D structure and active site of BglX. In other homologous GH3 β-glycosidases, Asp285 acts as a nucleophile in the active site

and is part of the highly-conserved Ser-Asp-Trp (SDW) motif. BglX has a partial alignment with the SDW motif, but the tryptophan is replaced by histidine.[44] Based on this information, the proposed nucleophilic active site residue for BglX is Asp287.[42] Site-directed mutagenesis was performed, and several mutants were produced, including D287N, D287G, and E293Q.[44] It is likely that D287 and D111 play a catalytic role, as the mutated enzymes showed much lower activity.[44]

A Practical Application for Immobilized β-Galactosidases

Lactose, a disaccharide, is the main carbohydrate present in dairy products and is commonly referred to as "milk sugar." In lactose, the monosaccharides galactose and glucose are bound by a β(1→4)-glycosidic bond, which can be hydrolyzed by an enzyme with β(1→4)-galactosidase activity; the mammalian form of the enzyme is lactase**.** Humans and other mammals express lactase during the gestation period and for a short time after birth. Lactase expression begins to taper off after weaning, and the down-regulation of lactase transcription is referred to as lactase non-persistence.[45] An individual lacking the ability to hydrolyze lactose is often referred to as lactose intolerant.

The most common phenotype in humans is that of lactase non-persistence. At least 70% of the world's population suffers from the inability to hydrolyze lactose; people from areas with a long history of animal husbandry and milking are more likely to be lactase persistent.[46] For example, the inhabitants of Northern European countries have been raising domesticated cattle and consuming milk and milk products for over 10,000 years, and thus are more likely to continue expressing lactase into adulthood than any other population.[45] Conversely, those of Asian descent are more likely to down-regulate

lactase production than any other population, as the milking of cattle for human consumption was not as widespread in Asian countries.[46] The inability to hydrolyze lactose was first coined "lactose intolerance" in the late nineteenth century, since the earliest research was conducted in countries with a high level of milk consumption; however, it is more correctly referred to as "lactase non-persistence," as the abnormality is the continued production of lactase into adulthood, not the lack thereof.[47]

Lactase non-persistent individuals are unable to properly digest lactose-containing dairy products. The undigested lactose travels through the small intestine undigested and into the large intestine. This causes myriad symptoms, including abdominal cramps, nausea, bloating, gas, and diarrhea. A high concentration of lactose in the large intestine causes an osmotic gradient, and water moves from the blood into the gastrointestinal tract in an effort to equilibrate the solute concentration, resulting in cramps and diarrhea. The bacteria residing in the large intestine ferment the lactose for energy and release gases as a by-product, the build-up of which causes more cramping and excessive flatulence.[48]

There is no treatment for those genetically pre-disposed to lactase non-persistence; however, solutions do exist. Dairy products treated with a β-galactosidase to hydrolyze the lactose are able to be readily consumed by those not expressing lactase. Since the 1970's, lactose-hydrolyzed milk and dairy products have been gaining in popularity. During this time, β-galactosidases of microbial origin first became available for commercial use.[49] As of 2015, the sale of lactose-free dairy products amounted to over $800 million in U.S. dollars.[50] Hydrolysis of lactose to its respective monosaccharides

results in a product sweeter in taste than that of untreated milk; to overcome this, the potassium salt of an organic acid, most commonly citrate or malate, is added to the milk after processing.[51]

Statement of Intent

The objective of this project is to immobilize BglX and its mutant E293Q on chitosan gel beads and test the activity of immobilized enzymes in hydrolysis of lactose. The enzymes will be immobilized on a matrix - chitosan beads. The chitosan will be fortified with inorganic materials, silica or activated charcoal, to increase the mechanical stability of the beads. Glutaraldehyde will be used to cross-link the beads, which has been shown to both increase mechanical stability and immobilization efficiency. First, lactase will be used as a model enzyme to test the different types of chitosan beads' ability to immobilize an enzyme. Once the lactase has been successfully immobilized, the activity of the enzyme will be tested using the model substrate ortho-nitrophenol-β-D-galactopyranoside (*o*NPGal) and lactose solution. The immobilized enzyme will also be tested for its ability to hydrolyze the lactose in milk whey.

Once the ability of the beads to both immobilize an enzyme and maintain enzymatic activity have been confirmed, BglX and its mutant E293Q will be subjected to similar tests. BglX and E293Q may be inhibited by the products of lactose hydrolysis. Inhibition studies will be performed to determine if there is a significant decrease in catalytic activity when glucose or galactose are added to the reaction mixture. If the activity of these enzymes is inhibited, it is hypothesized that a continuous production set-up would yield more product than that of batch production.

In order to compare efficiency of batch and continuous production, a small-scale continuous production system will be built using an Arduino motherboard and miniature peristaltic pump. The type of chitosan beads used in the packed bed column of the continuous production system will be determined by comparing the immobilization efficiency of the various types of beads. The percent hydrolysis of the substrates by BglX and its mutant E293Q will be compared, and the enzyme with higher percent conversion of substrate to products will be used in the continuous production of galactose and glucose from lactose.

Chapter 2: Materials and Methods

Materials

LB broth, SOC broth, agar, IPTG, Acryl/Bis^{TM} 29:1 (40% solution), streptomycin sulfate, ampicillin sodium salt, ammonium sulfate, tris hydrochloride, MES free acid monohydrate, TEMED, EZ-vision loading dye, TG-SDS 10x liquid concentrate, sodium dodecyl sulfate, and Coomassie brilliant blue G-250 were purchased from Amresco (Solon, OH). Q-Sepharose Fast Flow was purchased from GE Healthcare (Pittsburgh, PA). Glutaraldehyde solution (Grade II, 25% in H_2O), ammonium persulfate, the GO Assay kit, silica HF, activated charcoal, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). Glucose was obtained from Mallinckredt Inc. (Paris, KY). Galactose and Bradford reagent were purchased from Fisher Scientific (Fairlawn, NJ). Lactase enzyme tablets were purchased from Rite Aid. Chitosan powder was purchased from Bulk Supplements (Henderson, NV). Protein molecular weight markers and SDS-PAGE Mini Protean II apparatus were obtained from Bio-Rad (Hercules, CA). An Amicon ultra-centrifugal filters with a 30k MWCO was used for concentrating the protein. A Hewlett Packard Agilent 8453 Photodiode Array spectrophotometer was used for all spectrophotometric analysis.

Methods

Production of BglX and its Mutant E293Q

Former YSU students Devin Kelly and Carol Ann Pitcairn completed the cloning of the *bglX* gene into the *pET20b* DNA vector and its transformation into competent

BL21(DE3) *E. coli* cells. The BglX mutant E293Q was prepared by former student Lorna Ngo, who completed the mutagenesis of the *bglX* gene and the subsequent transformation of the *pET20b* vector carrying mutated gene into competent BL21(DE3) *E. coli* cells.

BglX and its mutant were expressed as follows. To 50 mL of LB media, ampicillin was added to a final concentration of 100 µg/mL and the media was inoculated with BL21(DE3) *E. coli* cells harboring either the pET20-*bglX* plasmid or the mutated pET20-*bglX* plasmid. These cultures were grown overnight in an incubator set at 37 °C, with shaking at 200 rpm. Then, 12 mL of the overnight cultures were used to inoculate each 1.2 L flask of LB media, containing 100 µg/mL ampicillin. The large cultures were grown in an incubator at 37 °C, with shaking at 200 rpm. When the OD_{600} was between 0.6 – 0.8, indicating the exponential phase of cell growth, each culture was induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 0.3 mM. The cultures were incubated overnight at 37°C, with shaking at 200 rpm. Cells were collected by centrifugation for 10 min at 6,000 x g and the resulting pellets were stored at 4 °C until further use.

Initial Protein Purification with Streptomycin Sulfate Treatment and Precipitation by Ammonium Sulfate

The pelleted cells were resuspended in 10 mM Tris, 2 mM EDTA buffer, pH 7.0 and stirred for 60 min. The cells were lysed by 15 cycles of 30 s sonication followed by stirring on ice for 60 s. The lysate was centrifuged to collect cell debris for 30 min at 11,000 x g. To the supernatant, streptomycin dissolved in 3 mL of the resuspension buffer was added

dropwise over 10 min to achieve 1% w/v, while stirring on ice. The solution was centrifuged similarly as before, and to the supernatant ammonium sulfate was added to 75% saturation over 20 min, while stirring on ice. After centrifugation the pellet was stored at 4 °C until further use.

20 μL samples were retained after each centrifugation and trace amounts of the pellets after sonication, the streptomycin treatment, and 75% ammonium sulfate precipitation were dissolved in 20 mM Tris buffer, pH 7.4. These samples were mixed with 2x SDS protein loading buffer in a 1:1 ratio. The samples were then heated at 95 °C for 5 min, cooled to room temperature, then analyzed using SDS-PAGE.

Protein Purification with Q-Sepharose FF Anion Exchange Chromatography

Q-Sepharose FF anion exchange column chromatography was used to further purify the protein. The pellet obtained from 75% ammonium sulfate precipitation was dissolved in a minimal volume of 20 mM Tris buffer, pH 7.4 and allowed to dialyze overnight at 4 °C against 2 L of the same buffer.

The dialyzed protein solution was centrifuged at 11,000 x g for 10 min to remove any remaining precipitate. The supernatant was then loaded onto a Q-Sepharose anion exchange column (3 x 9.5 cm) that had been equilibrated with 500 mL of 20 mM Tris buffer, pH 7.4. The column was washed with 500 mL of 20 mM Tris buffer, pH 7.4, buffer and the protein was eluted using a 400 mL linear salt gradient, 0 – 400 mM NaCl in the same buffer and 3.5 mL fractions were collected. The collected fractions were analyzed using UV-Vis spectrophotometry at 280 nm to determine which fractions contained a high concentration of protein. 20 μL samples of fractions with a high absorbance at 280 nm

were mixed with 2x SDS protein loading buffer in a 1:1 ratio, heated at 95 °C for 5 min, cooled to room temperature, and analyzed using SDS-PAGE. Fractions containing BglX (or BglX E293Q) were combined and concentrated using a centrifugal filtration device with MWCO of 30 kDa.

Preparation of Lactase Solution

Two Rite Aid Dairy Relief tablets containing lactase were crushed with mortar and pestle. The resulting powder was mixed with 10 mL 150 mM MES buffer, pH 6.8 and transferred to 2 mL Eppendorf tubes. The insoluble portion was separated from the solution using centrifugation at 8000 x g for 3 min. The solution was transferred into Eppendorf tubes and stored at 4°C.

BglX, E293Q, and Lactase Concentration Determinations

The concentrations of BglX was determined using the Bradford Assay with bovine serum albumin (BSA) used as the standard. Four standard solutions were prepared with Bradford reagent, distilled water, and BSA at varied amounts (10-70 μg). The concentrated BglX was diluted ten times with 20 mM Tris buffer, pH 7.4, then 10 μL of the diluted protein solution was mixed with 90 μL of distilled water and 3 mL of Bradford reagent. The samples were then 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 this data was used to calculate the concentration of BglX. The concentration of E293Q and the previously prepared lactase solution were determined similarly.

Beer's Law ($A=\varepsilon\iota C$) was used to confirm the concentration of BglX using the extinction coefficient (ε)82,740 M^{-1} cm^{-1}. The absorbance of the BglX solution at 280 nm was recorded and used to calculate the concentration of the protein. The concentration of E293Q was determined similarly. This analysis was not performed for the lactase solution, as the extinction coefficient was unknown.

Inhibition Studies

In a 1 mL quartz cuvette, the control was prepared with 850 µL of 20 mM Tris buffer, pH 7.4 and 100 µL of model substrate *o*-nitrophenyl-β-D-galactopyranoside (*o*NPGal) was used to blank the spectrophotometer at 405 nm. Next, 50 µL of 10 µM BglX was added, mixed, and the cuvette was placed in the spectrophotometer. The reaction was monitored at 405 nm for 150 s with a reading taken in 15 s intervals. The inhibition reactions were prepared by mixing 750 µL of 20 mM Tris buffer, pH 7.4 with 100 µL of 12.5 mM *o*NPGal and 100 µL of the inhibitor (1.25 M glucose or galactose) and used to blank the instrument before addition of 50 µL of 10 µM enzyme. The reaction was monitored at 405 nm for 150 s with a reading taken in 15 s intervals. This procedure was repeated using 50 mM *p*NPGlc.

Formation of Chitosan Beads

To form the chitosan beads, 1.00 g of chitosan powder was dissolved in 100 mL 1.0 M acetic acid and stirred for 3 hours until all chitosan was dissolved and the viscosity of the solution had increased. A NaOH solution was prepared by dissolving 15.0 g NaOH pellets in 100 mL of distilled water mixed with 25 mL 95% ethanol. The chitosan solution

was then added dropwise to the NaOH solution and the resulting beads (ChtR) were allowed to cure for 30 min before rinsing with distilled water until the pH was about 7.0, then 150 mM MES buffer (pH 6.8) was used for the final rinse and storage at 4°C.

Formation of Chitosan Beads Fortified with Inert Materials

The chitosan beads were fortified with either activated charcoal (ChtAC) or silica HF (ChtS). The chitosan solution was prepared as previously described. A 1% (w/v) solution was prepared by adding 100 mg of either activated charcoal or silica HF to 10 mL of the chitosan solution and allowed to stir overnight at room temperature. The solution was then pipetted into the NaOH solution similar to the preparation of the unfortified beads.

Determining the Relationship of Volume of Chitosan Gel Suspension and Weight of Chitosan Gel Beads

This was determined by adding 100 μL ChtR, ChtS, or ChtAC solution dropwise to a 2 mL Eppendorf tube containing NaOH. The beads were cured for 30 min and rinsed as previously described. After the final rinse, any remaining buffer was carefully removed from the Eppendorf tube. The weight of the beads was recorded. This procedure was repeated three times for each bead type.

Cross-linking Chitosan Beads with Glutaraldehyde

A 0.05% v/v glutaraldehyde solution in 50 mM cold sodium phosphate buffer, pH 7.0 was prepared. In a 2 mL Eppendorf tube, 50 mg of chitosan beads and 100 μL of 0.05% v/v glutaraldehyde solution were mixed gently with a pipette tip. This solution was gently

shaken at 4°C for 90 min. The cross-linked beads were rinsed and stored in 150 mM MES buffer, pH 6.8.

Adsorption of Methylene Blue Dye by ChtR and ChtR-CL Beads

A study of the beads' ability to adsorb methylene blue dye was conducted by placing 50 mg of ChtR or ChtR-CL beads in a 2 mL Eppendorf tube with 500 μL of 25 mg/L methylene blue dye. The tubes were shaken overnight at 25 °C. Methylene blue dye has a maximum absorbance at 664 nm. The absorbance at 664 nm of the methylene blue dye solution before and after incubation with the ChtR or ChtR-CL beads was recorded.

Examination of Chitosan Beads using a Light Microscope

The buffer was removed from the chitosan beads and a single bead was placed on a microscope slide and covered with a cover slip. The bead was viewed at 40x magnification and 100x magnification. The images were captured using a camera.

Immobilization of Lactase on Unlinked and Cross-Linked Chitosan Gel Beads

Lactase was immobilized on both unlinked and cross-linked chitosan beads to determine if there was a difference in their immobilization efficiency. In 2 mL Eppendorf tubes, 50 mg of ChtS or ChtS-CL beads were placed along with 100 μL of lactase solution and 900 μL 150 mM MES buffer, pH 6.8. The tubes were incubated overnight, with shaking, at 4°C. The solution was removed from the beads and the beads were rinsed two times with 1.5 mL of 150 mM MES buffer, pH 6.8. Samples of the incubation solution and the washes were saved to be analyzed via the Bradford Assay.

Batch Production of o-Nitrophenolate and Galactose by Lactase Immobilized on Chitosan Gel Beads

The chromogenic substrate *o*NPGal was used to test the lactase for its ability to hydrolyze *o*NPGal after immobilization on chitosan gel beads. In a 2 mL Eppendorf tube, 100 μL of 12.5 mM *o*NPGal was added to 50 mg of ChtS-CL-lactase beads in 900 μL of 150 mM MES buffer, pH 6.8. The solution was mixed by gently inverting the tube. A 50 μL sample was removed from the tube in 10 min intervals (10 – 60 min) and an additional sample was removed at 75 min. The spectrophotometer was blanked with 950 μL of 150 mM MES buffer, pH 6.8. The reaction sample was added to the buffer and mixed before the absorbance was recorded at 405 nm. This procedure was repeated for each sample.

Batch Production of Glucose and Galactose by Lactase Immobilized on Chitosan Gel Beads

A lactose solution was used to test the efficiency of the immobilized lactase to hydrolyze lactose to glucose and galactose. The amount of glucose in solution was quantified using the GO Assay. The assay cocktail was prepared according to the instructions provided by Sigma-Aldrich, and contained glucose oxidase, peroxidase, and *o*-dianisidine. 100 μL of 12.5 mM lactose was added to 50 mg of ChtS-CL beads with immobilized lactase in 200 μL of 150 mM MES buffer and incubated at room temperature for 60 min. Then, a 50 μL sample of the solution was removed from the reaction mixture. The sample was added to a tube containing 900 μL of GO Assay cocktail and 50 μL of de-ionized water. After 30 min of incubation, the absorbance was tested at 430 nm.

Immobilization of BglX and its mutant E293Q on Chitosan Gel Beads

BglX and E293Q were immobilized on all bead types, both unlinked and cross-linked, to determine if there was a difference in their immobilization efficiency. In 2 mL Eppendorf tubes, 50 mg of each bead type were placed along with 100 μL of BglX or E293Q and 900 μL 150 mM MES buffer, pH 6.8. The tubes were incubated overnight, with shaking, at 4°C. The solution was removed, and the beads were then rinsed two times with 1.5 mL of 150 mM MES buffer, pH 6.8. Samples of the incubation solution and the washes were saved to be analyzed via the Bradford Assay, similarly to the procedure used in the determination of the percent of lactase immobilized on chitosan gel beads.

Batch Production of o-Nitrophenolate and Galactose by BglX Immobilized on Chitosan Gel Beads

The chromogenic substrate *o*NPGal was used to test BglX for its ability to hydrolyze *o*NPGal after immobilization on chitosan gel beads. In a 2 mL Eppendorf tube, 100 μL of 12.5 mM *o*NPGal was added to 50 mg of ChtS-CL beads in 900 μL of 150 mM MES buffer, pH 6.8. The solution was mixed by gently inverting the tube several times. A 50 μL sample was removed from the tube in 10 min intervals (10 – 60 min) and an additional sample was removed at 75 min. The spectrophotometer was blanked with 950 μL of 150 mM MES buffer, pH 6.8. The reaction sample was added to the buffer and mixed before the absorbance was recorded at 405 nm. This procedure was repeated for each sample.

Batch Production of Glucose and Galactose from Lactose by BglX and E293Q Immobilized on Chitosan Gel Beads

A lactose solution was used to test the efficiency of the immobilized BglX and E293Q to hydrolyze lactose to glucose and galactose. The amount of glucose in solution was quantified using the GO Assay, and the reactions were prepared similarly to the procedure described for the batch production of glucose and galactose from lactose by immobilized lactase. A 50 µL sample of the solution was removed from the reaction mixture at 60 min. Each sample was added to a tube containing 900 µL of GO Assay cocktail and 50 µL of de-ionized water. After 30 min of incubation with the GO Assay cocktail, the absorbance of the solution was tested at 430 nm. All cross-linked bead types were tested similarly.

Preparation of Whey Solution

5 drops of glacial acetic acid were added to 5 mL of whole cow's milk. The solution was incubated at room temperature for 5 min to separate the milk into curds and whey. The curdled milk was centrifuged at 8,000 x g for 1 min. The whey supernatant was collected, and its pH was adjusted to 7 with 1.0 M NaOH for further use.

Determining the Concentration of Lactose in Whey

A 250 µL sample of the whey solution was incubated with 25 µL of a ten times dilution of the lactase solution. 50 µL samples were removed after 30 min, 60 min and 48 hours and added to a tube containing 900 µL of GO Assay cocktail and 50 µL of de-ionized water. These solutions were incubated 30 min and then the absorbance at 430

nm was recorded. The same procedure was done for a lactose solution of known concentration.

Assembly of Continuous Production System

The TA7291P was placed on the breadboard, then the following were connected: an Arduino GND to pin 1 on the TA7291P and to the 12V GND, the Arduino (+)5V to the TA7291P pin 7 (Vcc), the Arduino D5 to the TA7291P pin 5 (IN1), the Arduino D6 to the TA7291P pin 6 (IN2), the Arduino D13 to R1 (for the external LED), R1 to (+) leg of the LED, the (-) leg of the LED to GND, the TA7291P pin 2 (OUT1) to the (+) terminal of the pump, the TA7291P pin 4 (Vref) and pin 8 (Vs) to (+)12V, the TA7291P pin 10 (OUT2) to the (-) terminal of the pump. The TA7291P pin 3 and pin 9 were not used (Figure 2-1).

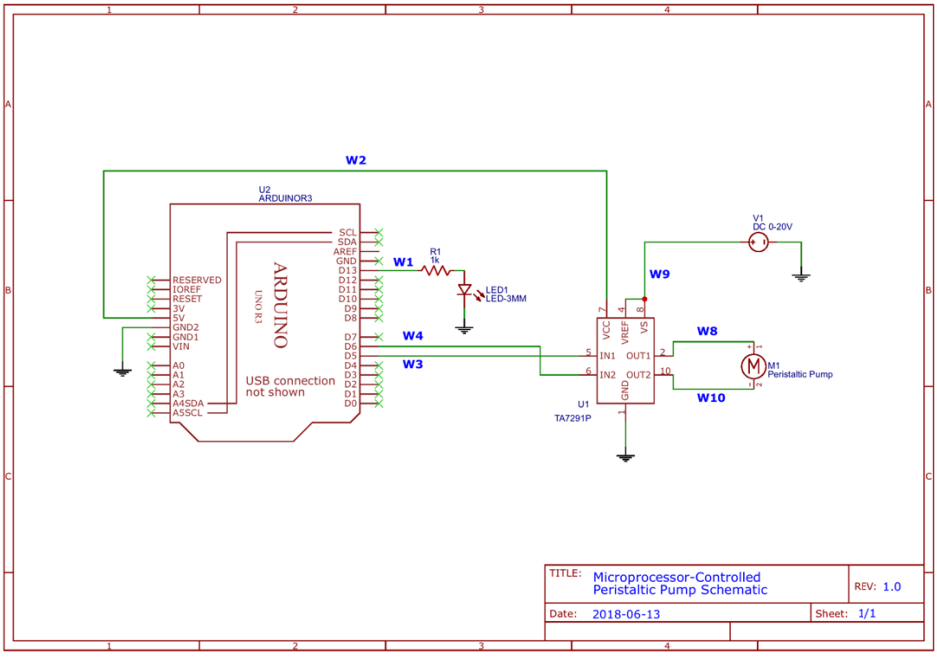


Figure 2-1: Circuit diagram for connecting the Arduino motherboard and the peristaltic pump. The USB connection (not shown) was used to power the Arduino motherboard and run the software.

Calibration of the Continuous Production System

The minimum flow rate (mL/min) for Pumps 1 and 2 was determined by recording the volume of 150 mM MES buffer pumped through each pump during a 1 min interval. This was repeated five times for each pump and the minimum flow rate (mL/min) was calculated as the average of those trials.

Initial Test of Immobilized BglX in Continuous Hydrolysis of oNPGal

The substrate, 12.5 mM *o*NPGal, was pumped through Pump 1 into the column (1.5 x 5.0 cm) packed with BglX immobilized on ChtS-CL beads for 10 s and left in contact with the immobilized enzyme for 10 min before being pumped out of the column into a collection vessel. A 100 µL sample was mixed with 900 µL of 150 mM MES buffer, pH 6.8, and the absorbance at 405 nm was recorded to determine if the immobilized BglX hydrolyzed the substrate.

Determination of Volume of Buffer Necessary to Remove Remaining Substrate from the Column and Pump Lines

After performing the initial tests of the continuous hydrolysis of *o*NPGal, any residual substrate and/or products were removed from the packed bed of chitosan beads by pumping 150 MES buffer, pH 6.8 through the column. 10 mL of the buffer was pumped by Pump 1 into the column and out of the column by Pump 2 into a collection vessel. A sample was taken for analysis at 405 nm after every 10 mL of buffer had been collected off the column. The absorbance at 405 nm approached zero when the residual products had been removed from the column.

Testing Immobilized BglX ChtS-CL Beads in Column for the Ability to be Used Repeatedly

To determine if BglX immobilized on ChtS-CL beads could be used repeatedly, 2.10 mL of 12.5 mM *o*NPGal was pumped into the column and held for 10 min before being pumped off into a collection vessel. The column was washed with 130 mL of 150 mM MES buffer, pH 6.8 to remove any residual products or substrate. This procedure was repeated three times.

Continuous Hydrolysis of oNPGal by BglX Immobilized on ChtS-CL beads

To test if BglX would hydrolyze model substrate *o*NPGal when immobilized on ChtS-CL beads in a continuous production system, 10 mL of 12.5 mM *o*NPGal were pumped through the column and into a collection vessel. A 100 μL sample was removed. In a quartz cuvette, 50 μL of the retained sample and 950 μL of 150 mM MES buffer, pH 6.8 were mixed and the absorbance at 405 nm was recorded. This procedure was repeated ten times.

Continuous Hydrolysis of Lactose by BglX Immobilized on ChtS-CL Beads

To test if BglX is capable of hydrolysis of lactose when immobilized on ChtS-CL beads in a continuous production system, 10 mL of a 12.5 mM lactose solution or a 9 mM lactose in whey solution was pumped through the column and into a collection vessel. A 100 μL sample was removed and saved for future analysis. The GO Assay was performed to determine the amount of glucose liberated by the immobilized enzyme; 50 μL of the sample was added to 900 μL of GO reagent and 50 μL of distilled water. After 30 min of incubation, the absorbance at 430 nm was recorded. This procedure was repeated ten

times for each substrate. The column was washed with 130 mL of 150 mM MES buffer before the other substrate was used.

Chapter 3: Results

Initial Protein Purification with Streptomycin Sulfate Treatment and Precipitation by Ammonium Sulfate

The native BglX and its mutant E293Q were expressed similarly. Samples were taken at each step of the purification and were analyzed using SDS-PAGE. The BL21(DE3) cells that expressed BglX or BglX E293Q were harvested by centrifugation and resuspended in 10 mM Tris buffer, 2 mM EDTA, pH 7.0, which disrupted the outer membrane structure. The resuspended cells were sonicated to further disrupt the cell membrane and centrifuged. The supernatant after sonication (Figure 3-1, Lane 1) was treated with 1% streptomycin sulfate to precipitate all nucleic acids and centrifuged to remove the nucleic acids from the solution (Lane 3). The supernatant from the streptomycin was treated with 75% ammonium sulfate saturation to precipitate proteins, including BglX, from the solution. Lane 4 shows that most proteins were precipitated at a 75% ammonium sulfate saturation and remained in the pellet, which was used for further purification.

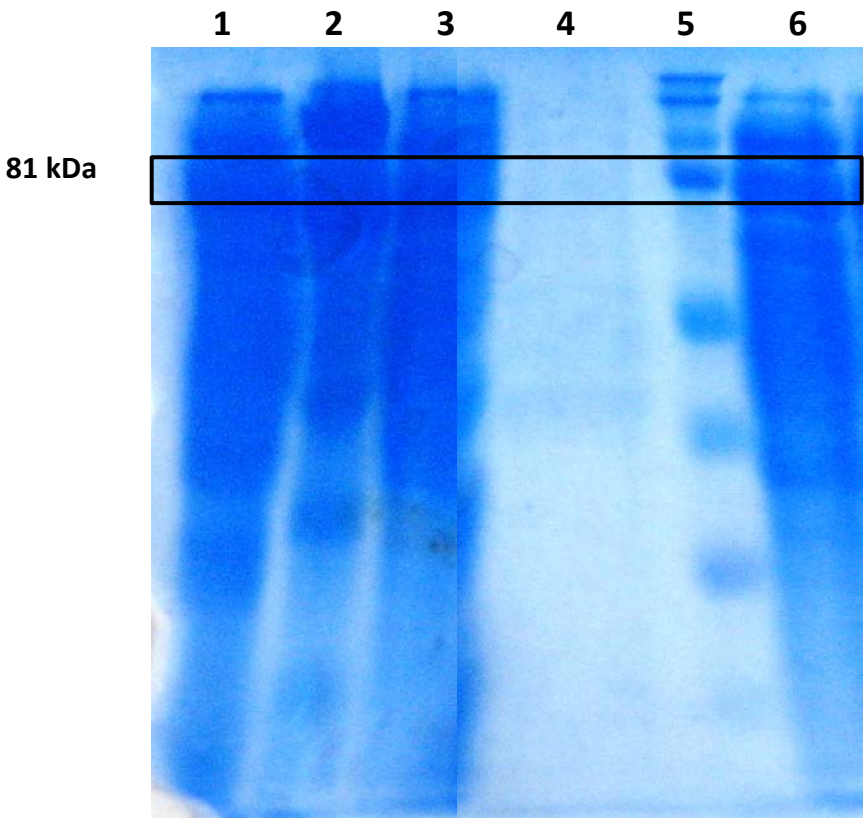


Figure 3-1: 10% SDS-PAGE gel from the initial steps undertaken to purify BglX using streptomycin sulfate treatment and precipitation by ammonium sulfate.
Lane 1: supernatant after sonication. Lane 2: pellet after sonication. Lane 3: supernatant from streptomycin. Lane 4: pellet from streptomycin. Lane 5: Molecular weight marker. Lane 6: Pellet from 75% ammonium sulfate.

Protein Purification with Q-Sepharose FF Anion Exchange Column Chromatography

The pellets obtained at 75% ammonium sulfate saturation were resuspended in 20 mM Tris buffer, pH 7.4 and dialyzed against the same buffer overnight at 4 °C. After dialysis, the sample was loaded onto a Q-Sepharose FF anion exchange column. The proteins were eluted using an increasing (0 – 400 mM) gradient of NaCl in 20 mM Tris buffer, pH 7.4. The absorbance of collected fractions at 280 nm was measured (Figure 3-2) and SDS-PAGE analysis was performed for fractions 44 – 66 (Figure 3-3). Fractions

44 – 64 showed a high absorbance at 280 nm and a band of 81 kDa on SDS-PAGE. Those fractions were pooled and concentrated using a centrifugal filter with MWCO of 30 kDa.

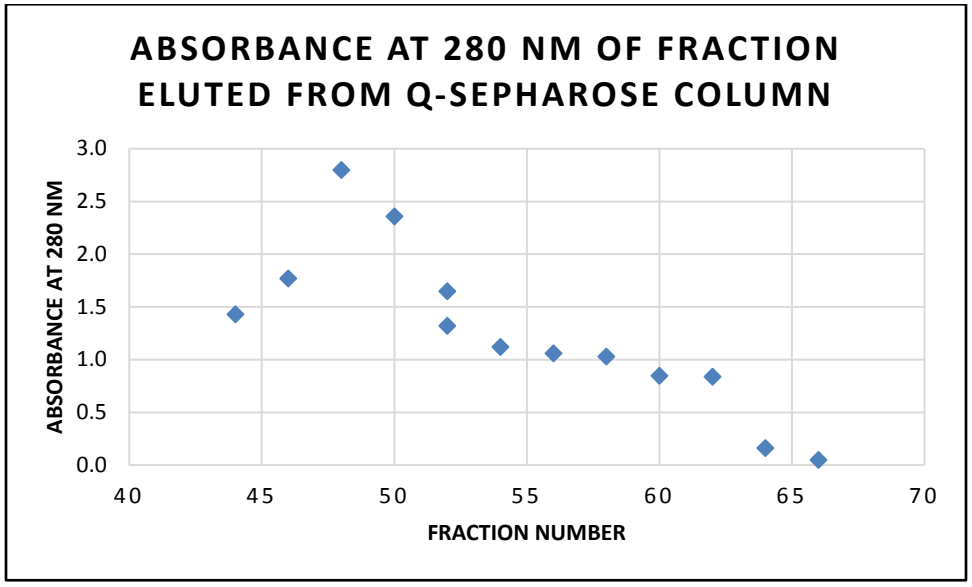


Figure 3-2: Graph of absorbance at 280 nm of fractions eluted from Q-Sepharose FF anion exchange column chromatography during BglX purification.

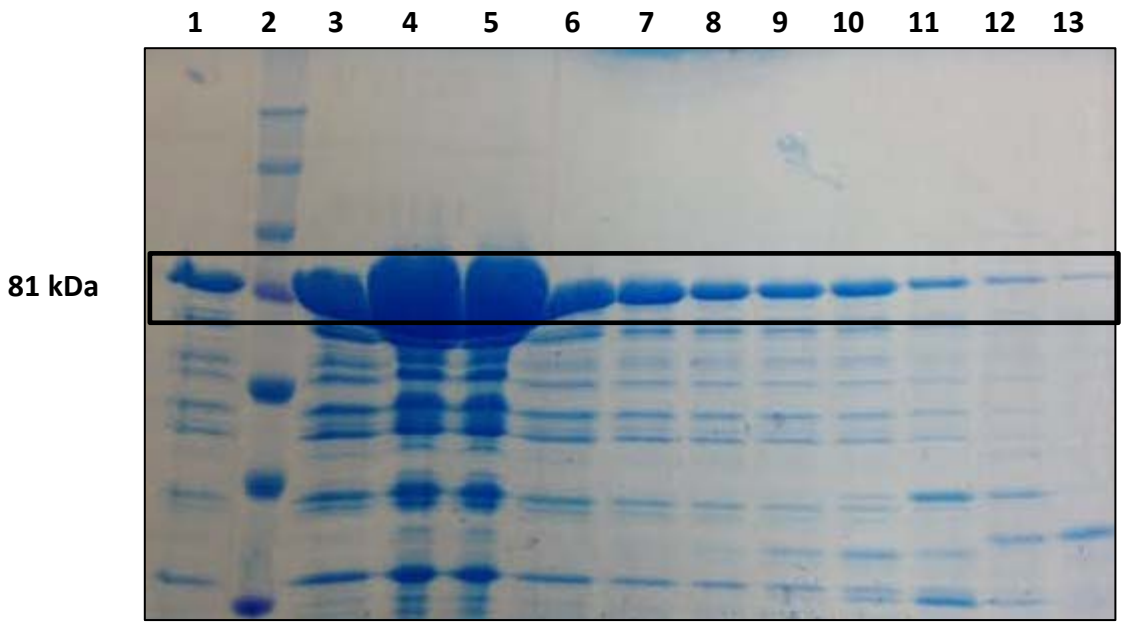


Figure 3-3: 10 % SDS-PAGE gel of fractions collected during Q-Sepharose FF anion exchange chromatography for BglX. Lane 1: fraction 44. Lane 2: molecular weight marker. Lane 3: fraction 46. Lane 4: fraction 48. Lane 5: fraction 50. Lane 6: fraction 52. Lane 7: fraction 54. Lane 8: fraction 56. Lane 9: fraction 58. Lane 10: fraction 60. Lane 11: fraction 62. Lane 12: fraction 64. Lane 13: fraction 66.

BglX, E293Q, and Lactase Concentration Determinations

The concentrations of the lactase solution prepared from the Rite Aid Dairy Relief tablets, the purified BglX, and its mutant E293Q were determined using the Bradford Assay. BSA was used as the standard, and the absorbance of the samples with known amount of BSA was measured at 595 nm. This was plotted against the amount of BSA added (Figure 3-4) and a calibration curve was generated. The line equation and correlation coefficient (R^2) were obtained, and the line equation was solved for the amount of protein (μg) in the prepared lactase solution, BglX, E293Q (Table 3-1). The concentrations of the three proteins were calculated similarly, by dividing the amount of protein by the volume added to the sample. The concentration of the prepared lactase

solution was 51 mg/mL. The concentration of BglX and E293Q were 30 and 34 mg/mL, respectively.

The concentrations of BglX and E293Q were also calculated using Beer's Law; the absorbance at 280 nm, the extinction coefficient (82,740 M^{-1} cm^{-1}), and the pathlength (1 cm) were used to solve for molarity. The molarity of BglX and E293Q solutions were 383 and 432 μM. Using the molar mass of the protein (81,000 g mol^{-1}), the concentration was converted to mg/mL. The concentration of BglX and E293Q calculated by this method were 33 and 36 mg/mL, which was in strong agreement with the results from the Bradford Assay.

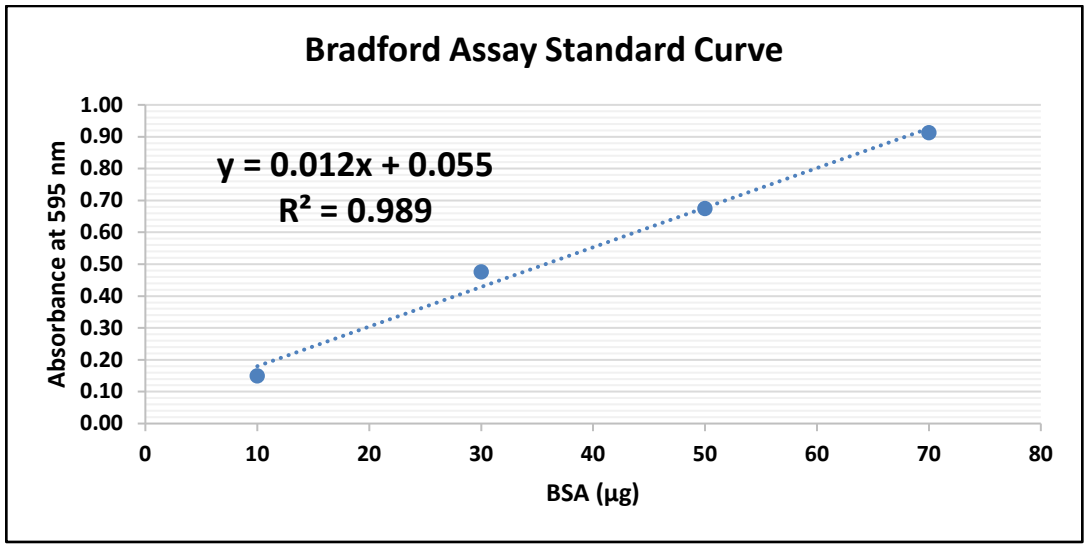


Figure 3-4: Bradford Assay standard curve**.** Standard curve for determining micrograms of protein in solution using the Bradford Assay.

Table 3-1: Protein concentration determined from the Bradford Assay and Beer's Law

Protein	Concentration (mg/mL) Bradford Assay	Concentration (mg/mL) Beer's Law	Concentration (µM)
Lactase	51	N/A	N/A
BglX	30	33	383
E293Q	34	36	432

Inhibition of BglX

During batch production of glucose and galactose from lactose, the products of hydrolysis build-up in the reaction vessel. This can decrease the activity of some enzymes. To test whether the activity of BglX is affected by the products of lactose hydrolysis inhibition studies were conducted.

The chromogenic substrates *o*NPGal and *p*NPGlc were used, which, after hydrolysis, forms galactose and the *o*-nitrophenolate ion or glucose and the *p*-nitrophenolate ion. Both the *o*-nitrophenolate (ε 3055 M^{-1} cm^{-1}) and the *p*-nitrophenolate (ε 10440 M^{-1} cm^{-1}) ions absorb at 405 nm (Figure 3-5). The activity of BglX can be analyzed by monitoring the increase in the absorbance at that wavelength. A control reaction was performed without the addition of any inhibitor for comparison to reactions in the presence of an inhibitor.

The percent of activity retained was calculated by comparing the rate of the reactions containing inhibitor to that of the control. When using *o*NPGal as the substrate, there was a significant decrease in activity upon addition of glucose or galactose – a 49% and 52% decrease in activity, respectively (Figure 3-6). When *p*NPGlc was used as the substrate, there was a 75% decrease in activity when glucose was added to the reaction

mixture, while upon the addition of galactose, only a 19% decrease in activity was observed (Figure 3-7).

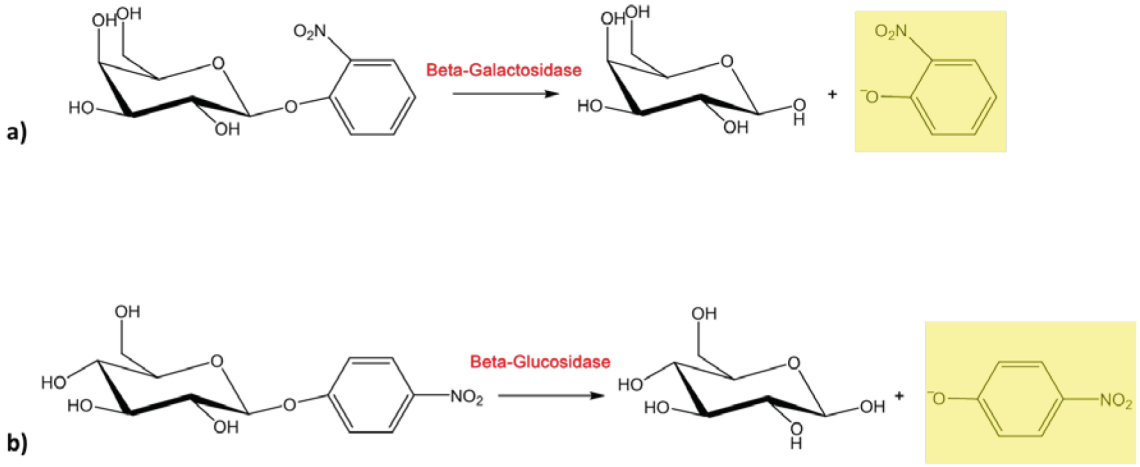


Figure 3-5: Hydrolysis of the chromogenic substrates **a)** *o*NPGal and **b)** *p*NPGlc produces the *o*-nitrophenolate or *p*-nitrophenolate species, which absorb at 405 nm**.**

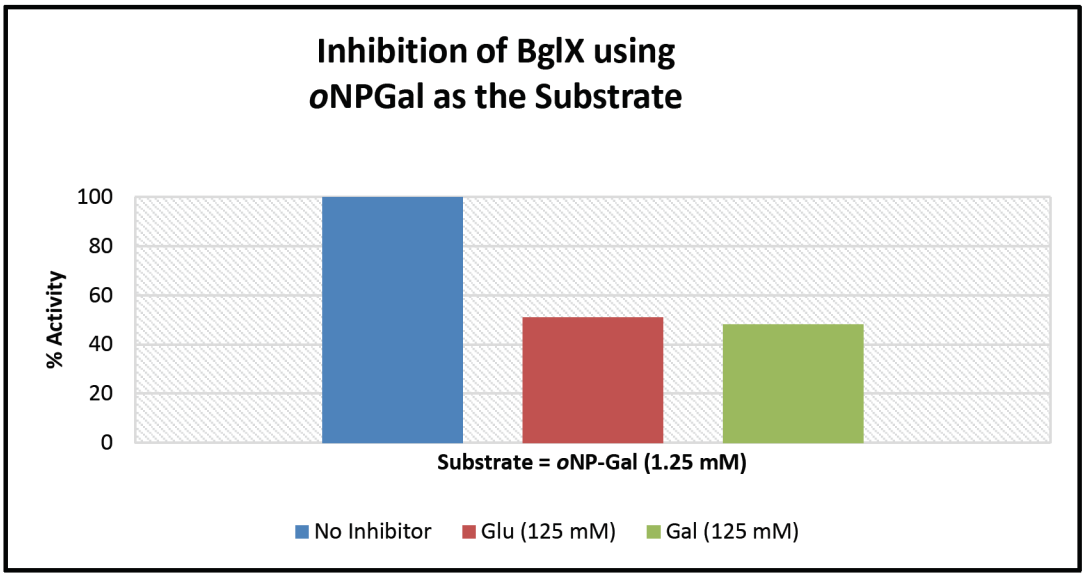


Figure 3-6: Effect of glucose and galactose on activity of BglX with *o*NPGal.
The activity of BglX decreased when *o*NPGal was used as the substrate and either 125 mM glucose or 125 mM galactose was added to the reaction mixture.

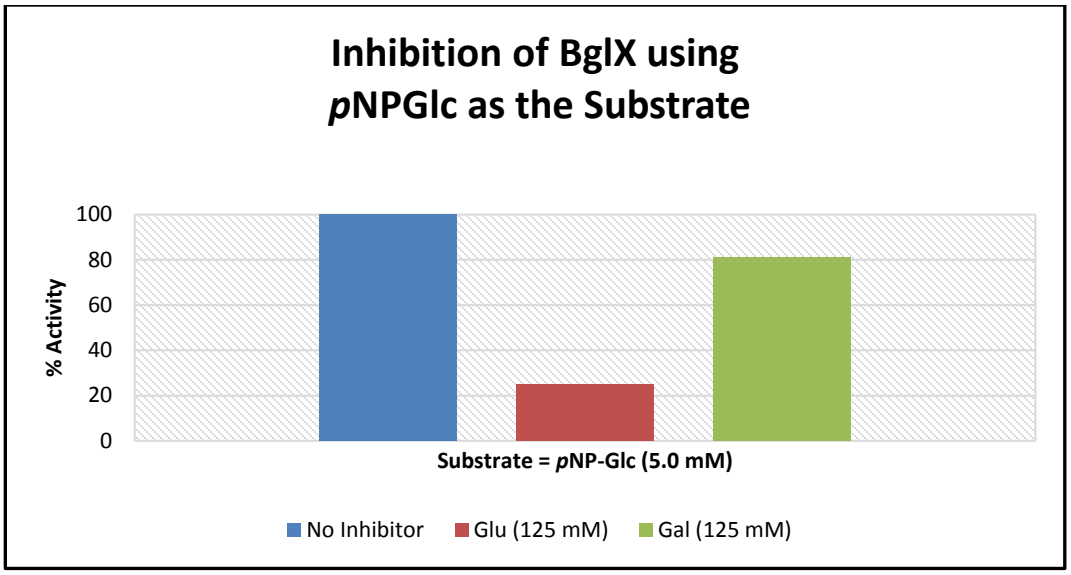


Figure 3-7: Effect of glucose and galactose on activity of BglX with *p*NPGlc. The activity of BglX decreased when *p*NPGlc was used as the substrate and either glucose or galactose was added to the reaction mixture.

Formation of Chitosan Beads, Fortification with Inert Materials, Determination of the Volume of Chitosan Gel Suspension: Weight of Beads Formed Relationship, and Cross-linking with Glutaraldehyde

The chitosan beads (ChtR) were formed by first dissolving powdered chitosan in a dilute acetic acid solution. The solubilized chitosan was then added dropwise to a NaOH solution, and the chitosan beads were formed. After curing the beads in the basic solution for 30 min, the beads were rinsed with distilled water until neutral pH was reached and stored in a buffered solution, pH 6.8 at 4 °C for further use. The chitosan beads fortified with inert materials were formed similarly, except for the addition of either activated charcoal (ChtAC) or silica HF (ChtS) into the chitosan solution before bead formation (Figure 3-8 a, c, d). The relationship between the volume of chitosan gel suspension (ChtR, ChtS, and ChtAC) and the weight of beads formed was determined to standardize the weight of beads in the reaction mixtures. Each bead type was formed from the same

volume of solution, the buffer was removed, and the weight was recorded. This was done three times for each type of bead and the average value was taken (Table 3-2). A portion of chitosan beads of each type was cross-linked by incubation in a dilute glutaraldehyde solution. This resulted in formation of ChtR-CL, ChtAC-CL, and ChtS-CL beads (Figure 3-8 b).

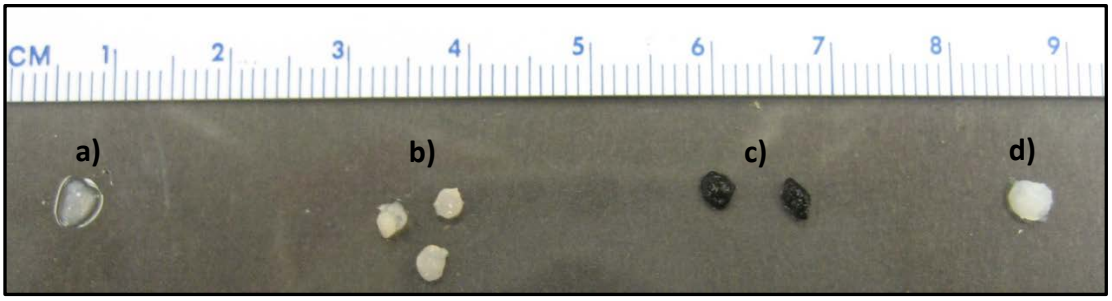


Figure 3-8: Chitosan gel beads. Chitosan gel beads are formed by dissolving chitosan powder in acetic acid, then adding dropwise to concentrated NaOH solution. From left to right: **a)** ChtR, **b)** ChtS-CL, **c)** ChtAC, and **d)** ChtS beads.

Table 3-2: Relationship of the Volume of Chitosan Gel Suspension and the Weight of Beads Formed

Bead Type	Volume of Chitosan Gel Suspension (µL)	Weight of Beads Formed (mg)
ChtR	100	35
ChtS	100	37
ChtAC	100	59

Adsorption of Methylene Blue Dye by ChtR and ChtR-CL Beads

Cross-linking of chitosan can affect the porosity of the beads and, as a result, change their ability to adsorb other molecules. Chitosan's free amino groups interact with glutaraldehyde, forming a Schiff base. To determine if the chitosan gel beads were cross-linked by treatment with the glutaraldehyde solution, an adsorption study using

methylene blue dye was conducted. The ChtR and ChtR-CL beads were incubated at 25 °C with methylene blue dye overnight with shaking. Absorbance of the dye solution was analyzed at 664 nm before and after overnight incubation with beads. The ChtR beads adsorbed 91% of the dye in solution, while the ChtR-CL beads adsorbed 85% of the dye.

Examination of Chitosan Beads using a Light Microscope

For each type of chitosan gel bead, a single chitosan bead was examined under the light microscope. The ChtR and ChtS beads did not provide enough contrast to view using the light microscope. The ChtAC bead was viewed at both 40x and 100x magnification (Figure 3-9).

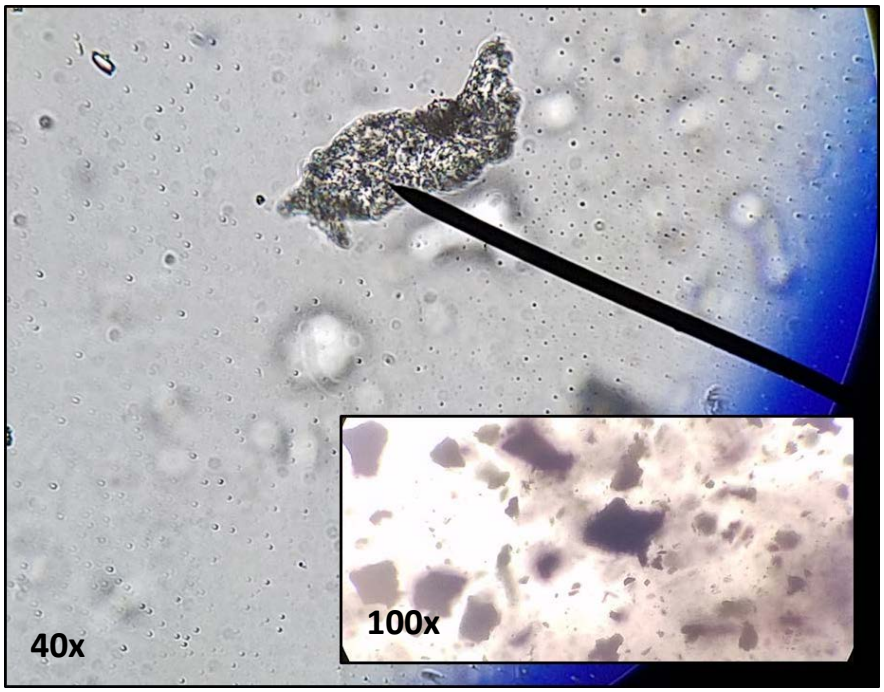


Figure 3-9: Photograph of a ChtAC bead viewed with a light microscope. The ChtAC bead, viewed at both 40x and 100x magnification (inset). These pictures were taken using a light microscope and camera.

Immobilization of Lactase on Cross-linked and Unlinked Chitosan Gel Beads

The previously prepared lactase solution was incubated overnight at 4 °C with ChtS or ChtS-CL beads so the immobilization efficiency of unlinked and cross-linked beads could be compared. After the overnight incubation period of the protein with the beads, the incubation solution was removed, and the beads were washed twice with buffer to take out any protein that had not been immobilized and a sample was retained from each wash. The samples were then analyzed for protein content using the Bradford Assay. The standard curve was prepared as previously described (Figure 3-4). The line equation was solved for the amount of lactase present in the prepared lactase solution, remaining in the solution after incubation, and lost during the two washes.

The amount of lactase immobilized on the beads was calculated by subtracting the amount of lactase remaining in the incubation and wash mixtures from the total amount of protein contained in the Eppendorf tube. The percent immobilization of lactase on the ChtS or ChtS-CL beads was calculated as 99.1% for ChtS beads and 99.5% for ChtS-CL beads

Batch Production of o-Nitrophenolate and Galactose by Lactase Immobilized on Chitosan Gel Beads

To determine if the lactase immobilized on chitosan gel beads retained its catalytic activity, the chromogenic substrate *o*NPGal was used in micro-scale batch production. The substrate was incubated with lactase immobilized on ChtS-CL beads. A sample of the reaction mixture was removed every 10 min (10 – 60 min) and one additional sample at 75 min. The absorbance at 405 nm was recorded, as this is the absorbance shift which

indicates hydrolysis of the substrate had occurred by an increase of *o*NP in the solution. Using the extinction coefficient (3055 M^{-1} cm^{-1}) for *o*NP, the concentration of *o*NP, and thus *o*NPGal hydrolyzed was calculated (Figure 3-10). The percent conversion of the substrate increased steadily from 10 – 50 min of contact with the immobilized enzyme. After 75 minutes of contact with the beads, 27% of the available substrate had been converted into products.

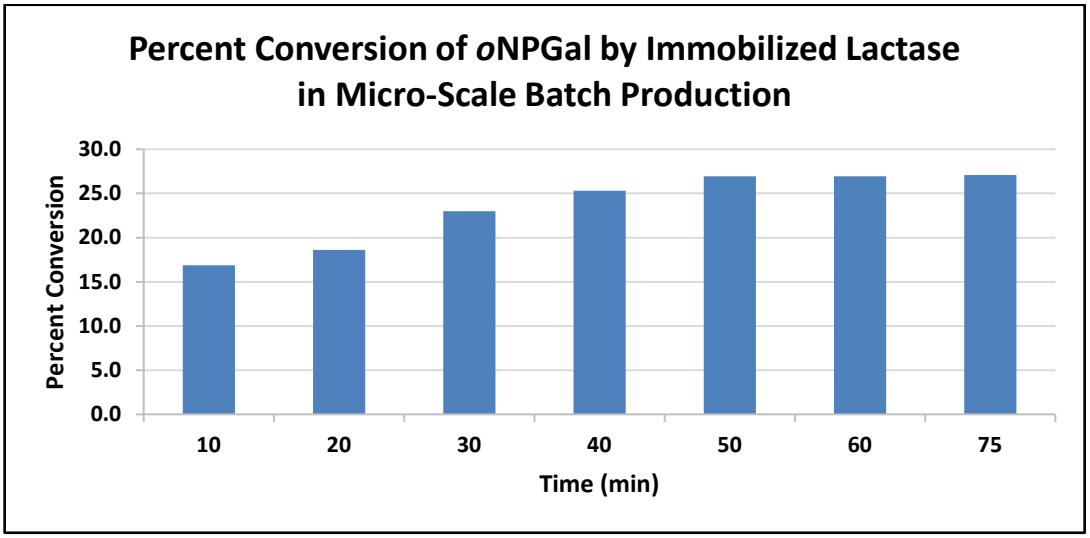


Figure 3-10: Percent conversion of *o*NPGal by immobilized lactase in micro-scale batch production. *o*NPGal is converted to *o*NP and Gal by lactase immobilized on ChtS-CL beads in micro-scale batch production

Batch Production of Glucose and Galactose by Lactase Immobilized on Chitosan Gel Beads

A prepared lactose solution was used as the substrate for batch production of glucose and galactose by lactase immobilized on unlinked and cross-linked chitosan beads. After incubation for 60 min with the immobilized lactase, the concentration of glucose in the solution was determined with the GO Assay cocktail containing glucose oxidase, peroxidase, and the reduced form of *o*-dianisidine. When *o*-dianisidine undergoes its first oxidation, it has a maximum absorbance at 430 nm. The amount of

oxidized *o*-dianisidine in the solution is directly proportional to the amount of free glucose (Figure 3-11).

A glucose standard curve was constructed (Figure 3–12) using the absorbance at 430 nm and the known concentration of glucose in the standards (0.015 – 0.150 mM). The line equation generated from these data points was used to calculate the percent of lactose that was converted to glucose and galactose after 60 min of contact with the immobilized lactase (Figure 3-13). The enzyme immobilized on the ChtS-CL beads exhibited the highest percent conversion of lactose, 29%.

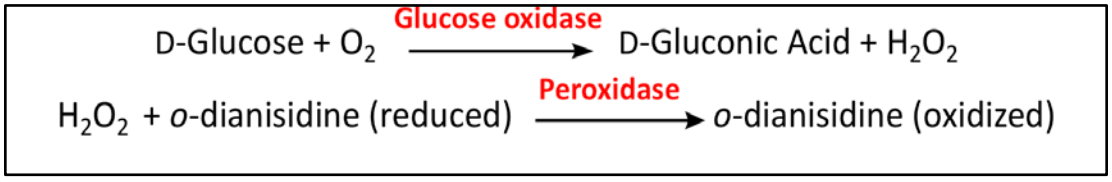


Figure 3-11: The reaction scheme for the GO Assay; the oxidized *o*-dianisidine absorbs at 430 nm.

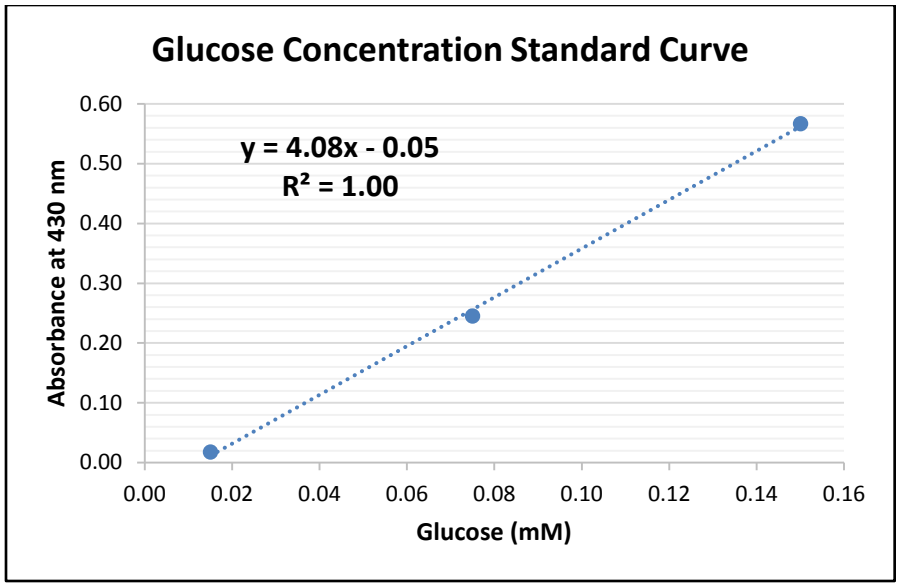


Figure 3-12: Glucose concentration standard curve. Standard curve generated by recording the absorbance at 430 nm of samples with a known concentration of glucose.

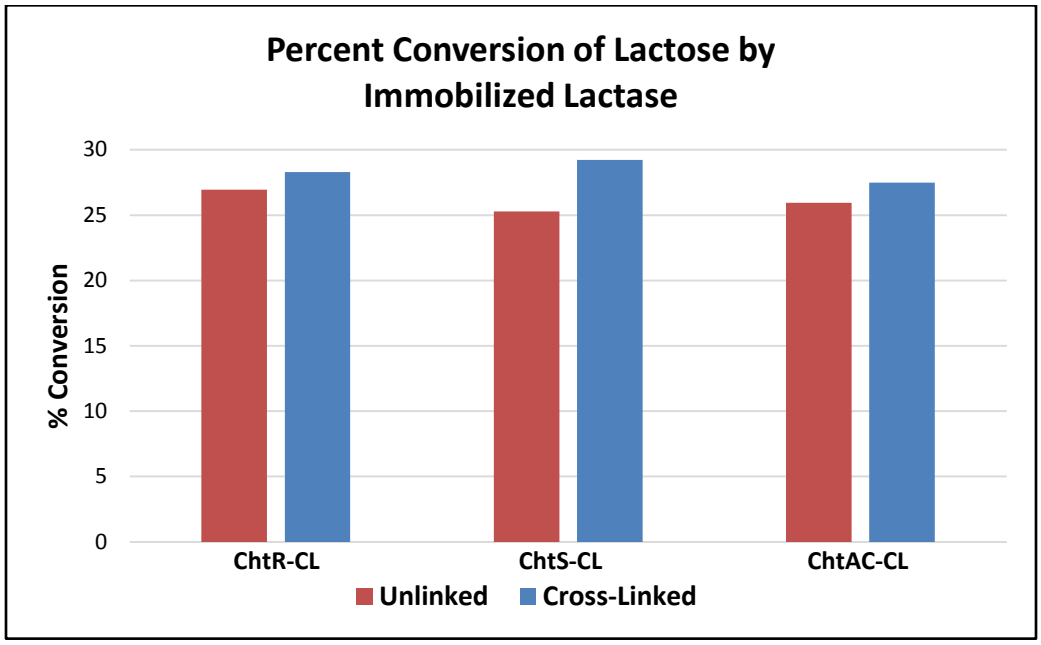


Figure 3-13: Percent conversion of lactose to Glc and Gal by lactase immobilized on unlinked and cross-linked chitosan beads.

Immobilization of BglX and its mutant E293Q on Cross-linked Chitosan Gel Beads

The previously prepared BglX or E293Q solutions were incubated overnight at 4 °C with ChtR-CL, ChtS-CL, or ChtAC-CL beads so the immobilization efficiency of the cross-linked beads and the different enzymes could be compared. Samples from the overnight incubation mixture and each of two washes with buffer were retained and analyzed for protein content using the Bradford Assay, similarly to when the percent of immobilization of lactase was described. The standard curve was prepared as previously described (Figure 3-4). The line equation was solved for the amount of BglX or E293Q remaining in the solution after incubation and lost during the two washes. The percent immobilization of BglX or E293Q on the various cross-linked beads was calculated by dividing the micrograms of protein immobilized by the total amount of protein in the incubation solution (Table 3-3).

Table 3-3: Percent Immobilization of BglX and E293Q mutant on chitosan beads

Enzyme	ChtR-CL	ChtS-CL	ChtAC-CL
BglX	97.6	98.4	91.2
E293Q	97.1	97.9	89.6

Batch Production of o-Nitrophenolate and Galactose by BglX Immobilized on Chitosan Gel Beads

To test if BglX retained catalytic activity after immobilization on chitosan gel beads, the chromogenic substrate *o*NPGal was used. The substrate was incubated with BglX immobilized on ChtS-CL beads. A sample of the reaction mixture was removed every 10 min (10 – 60 min) and one additional sample at 75 min, and the absorbance at 405 nm

was recorded for each sample. The percent conversion of the substrate was calculated similarly to the batch production of *o*-nitrophenolate and galactose by lactase immobilized on chitosan beads (Figure 3-14). After 75 minutes of contact with the beads, 5% of the available substrate had been hydrolyzed.

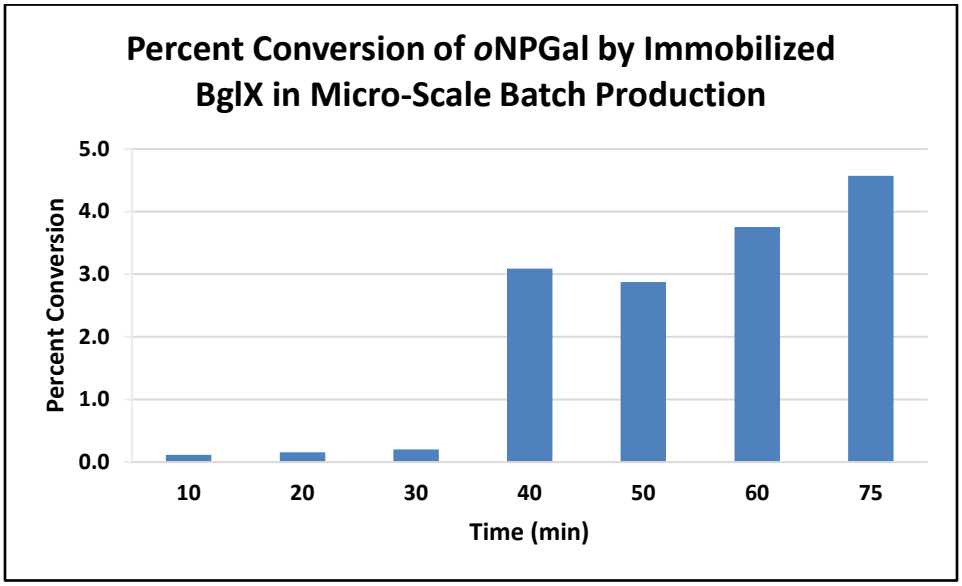


Figure 3-14: Percent conversion of *o*NPGal in micro-scale batch production. *o*NPGal is converted to *o*NP and Gal by BglX immobilized on ChtS-CL beads.

Batch Production of Glucose and Galactose from Lactose by Immobilized BglX and E293Q

The enzymes BglX and E293Q were immobilized on cross-linked ChtR, ChtS, and ChtAC chitosan gel beads. The samples were treated similarly to the batch production of glucose and galactose by immobilized lactase at 60 min. The GO Assay was performed and the absorbance at 430 nm was recorded for all of the samples.

A glucose standard curve was constructed (Figure 3–12.) The line equation generated from these data points was used to calculate the percent of the lactose that

was converted to glucose and galactose by the immobilized enzyme (Figure 3-15). The BglX immobilized on the ChtS-CL beads exhibited the highest percent conversion, 15%.

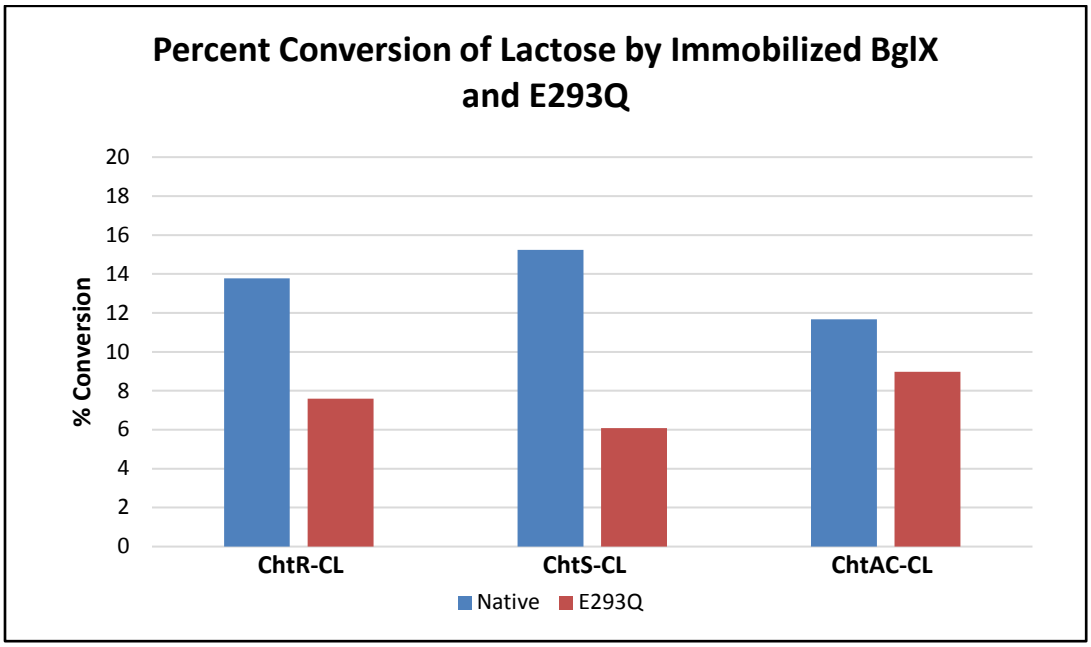


Figure 3-15: Percent conversion of lactose by immobilized BglX and E293Q. Small-scale batch production of glucose and galactose from lactose catalyzed by immobilized BglX and E293Q on the various types of cross-linked chitosan gel beads.

Determining the Concentration of Lactose in Whey

The whey solution was incubated with lactase, so the concentration of lactose present could be determined. After 30 min, 60 min, and 48 hours of incubation with the enzyme, a sample was removed and promptly added to the GO Assay reagent. The absorbance at 430 nm was recorded for each sample and the line equation from the calibration curve (Figure 3-12) was used to determine the concentration of glucose in the reaction mixture. This procedure was performed similarly with the lactose of known concentration (12.5 mM) to elucidate whether complete hydrolysis of the substrate had occurred. After 48 hours of incubation with the lactase, the percent hydrolysis of the

lactose solution was 98% (Figure 3-16). The concentration of the glucose in the reaction mixture was 12.3 mM. The sample removed from the reaction using milk whey as the substrate had a concentration of 9.0 mM after the 48-hour incubation period. This was used as the concentration of lactose in the whey solution.

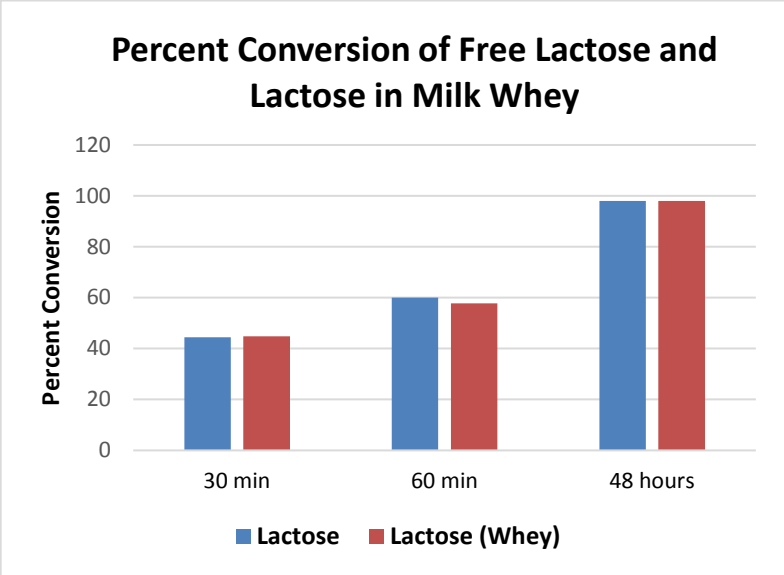


Figure 3-16: Percent conversion of free lactose and lactose in milk whey. The concentration of lactose in the whey solution was determined by comparing the percent hydrolysis of lactose to the hydrolysis of lactose in a solution with known concentration.

Assembly of Continuous Production System

The continuous production system was assembled with two pumps; Pump 1, which pumped the substrate into the column packed with immobilized BglX enzyme on ChtS-CL beads and Pump 2, which pumped the products from the column into a collection vessel (Figure 3-17). The Arduino motherboard was powered by the laptop via a USB power source and Pump 1 was powered using a variable-voltage (2 – 20 V) power source. Pump 2 was powered using a standard wall AC power outlet.

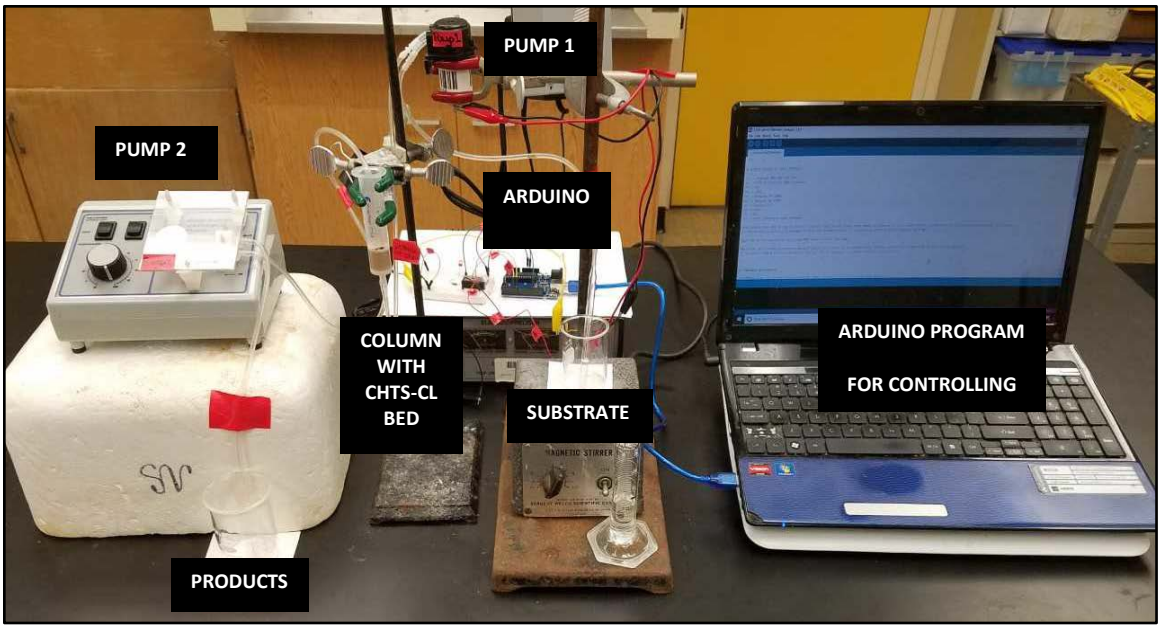


Figure 3-16: Continuous production system set-up. The peristaltic pump system was used to pump substrate into the column and then remove the products to a collection vessel.

Calibration of the Continuous Production System

The minimum flow rate (mL/min) for each pump was calculated as the average of 5 trials (Table 3-4). The minimum flow rate for Pump 1 was 12.5 ± 0.2 mL/min and for Pump 2 was 10.5 ± 0.1 mL/min.

Table 3-4: Flow Rate (mL/min) for Pump 1 and Pump 2

Pump 1		**Pump 2**	
Trial #	**mL/min**	**Trial #**	**mL/min**
1	12.8	1	10.3
2	12.1	2	10.2
3	12.6	3	10.4
4	12.4	4	10.5
5	12.4	5	10.4
Ave (mL/min)		**Ave (mL/min)**	
12.5 ± 0.2		10.5 ± 0.1	

Initial Tests of Immobilized BglX in Continuous Production of oNP

A test was performed to test the efficiency of the immobilized BglX in the packed bed column to hydrolyze the substrate *o*NPGal. The percentage of substrate hydrolyzed after 10 minutes of contact time was 27%.

Determine Volume of Buffer Necessary to Remove Remaining Substrate from the Column and Pump Lines

After performing hydrolysis of the chromogenic substrate *o*NPGal, the column packed with immobilized BglX on ChtS-CL beads was washed with buffer to remove any remaining product. The absorbance at 405 nm, the extinction coefficient (3055 M^{-1} cm^{-1}) and the volume analyzed (50 µL) were used to calculate the concentration of *o*NP

remaining in the wash solutions. The concentration of residual *o*NP approached zero after 130 mL of buffer were run through the column (Figure 3-18).

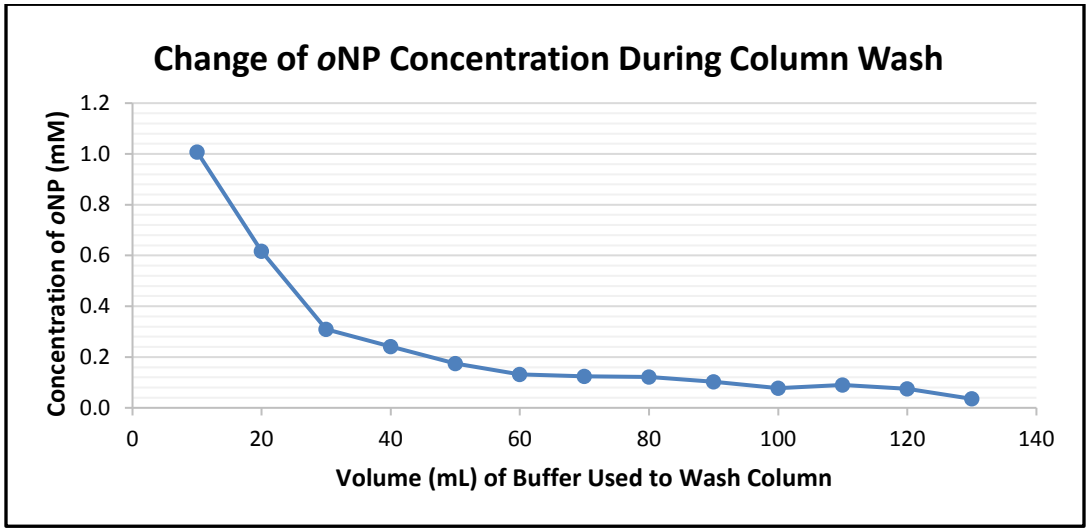


Figure 3-17: Change of *o*NP concentration during column wash.
The total volume of buffer needed to wash residual oNP from the column was 130 mL.

Testing Immobilized BglX ChtS-CL Beads in Column for the Ability to be Used Repeatedly

The immobilized BglX was tested for reusability by pumping the substrate *o*NPGal into the column, collecting the products for analysis, and washing the column with buffer to remove any residual products. The concentration of *o*NP in the solution collected from the pump was calculated by using the absorbance at 405 nm and the extinction coefficient (3055 M^{-1} cm^{-1}). The percent conversion of substrate for Trials 1 – 3 were 36%, 35%, and 36%, respectively (Figure 3-19).

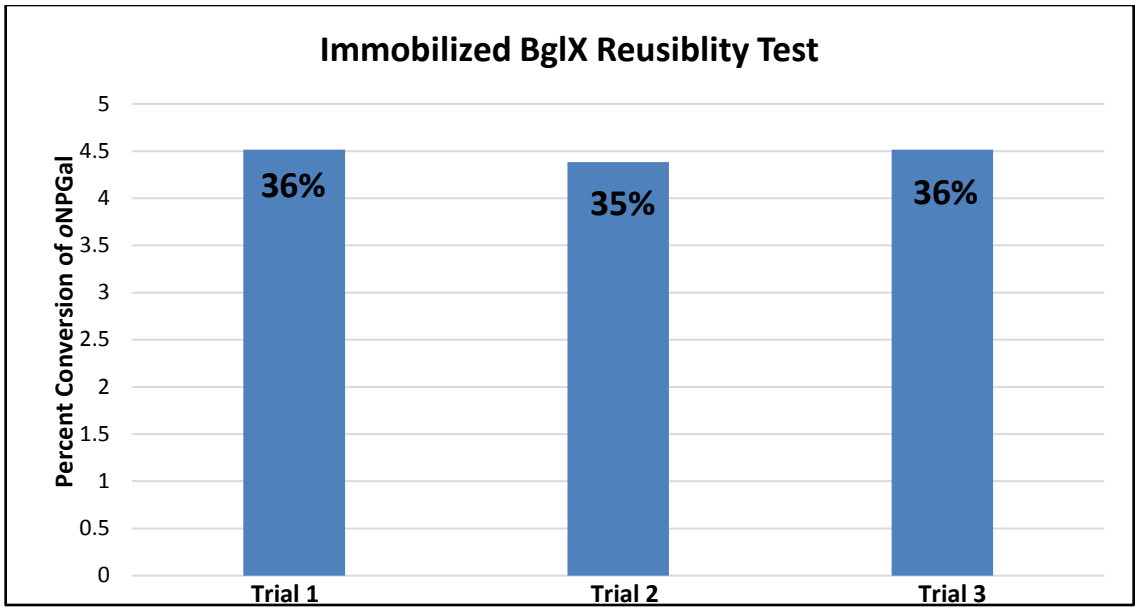


Figure 3-18: Immobilized BglX reusability trials. The percent hydrolyzed *o*NPGal catalyzed by BglX immobilized on ChtS-CL beads, the column was washed with 130 mL of buffer between trials.

Continuous Hydrolysis of oNPGal by BglX Immobilized on ChtS-CL beads

The substrate *o*NPGal was pumped through the bed of immobilized BglX on ChtS-CL beads and a sample was removed for analysis. This was repeated for the same substrate ten times (Figure 3-20). The samples were analyzed at 405 nm, the characteristic absorbance of the product, *o*NP. The concentration of *o*NP in the samples was calculated using the absorbance at 405 nm and the extinction coefficient (3055 M^{-1} cm^{-1}) (Figure 3 - 26). After ten passes through the column containing the immobilized BglX, the percent conversion of *o*NPGal to *o*NP and Gal was 30%.

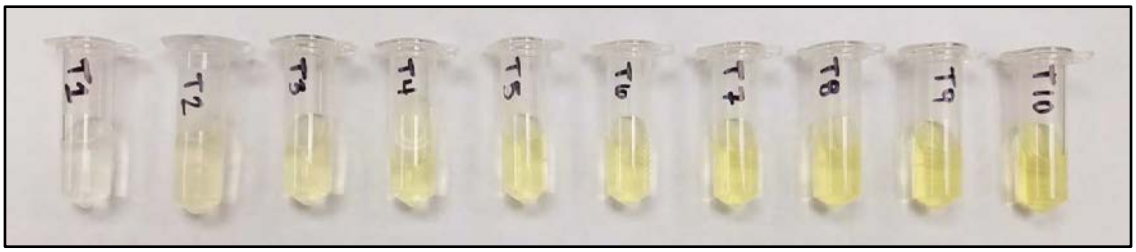


Figure 3-19: Samples collected from continuous production of *o*NP by immobilized BglX. The samples were retained for analysis at 405 nm.

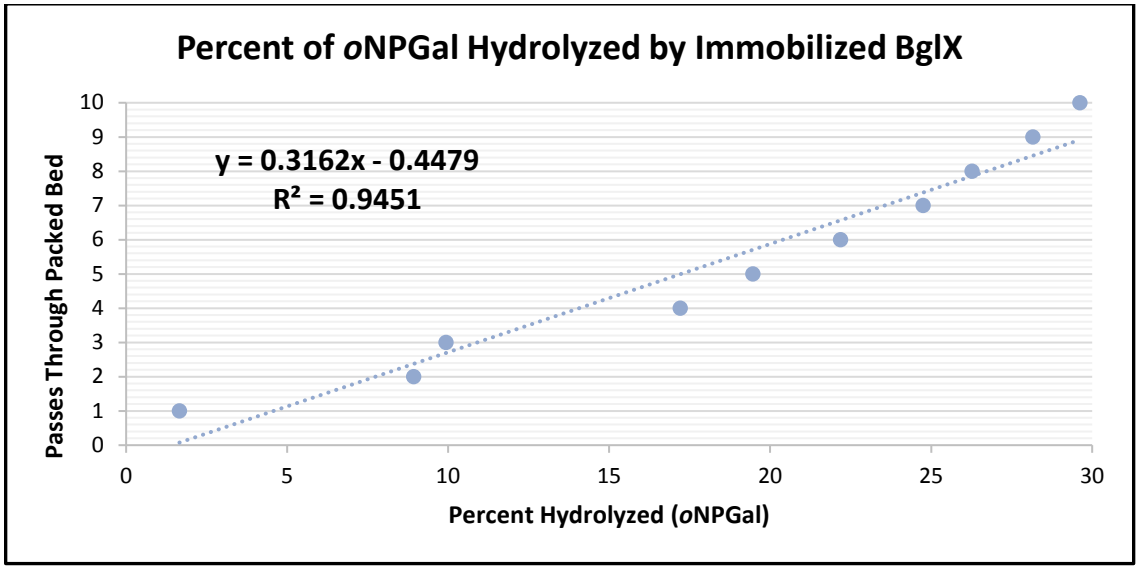


Figure 3-20: Percent of *o*NPGal hydrolyzed by BglX immobilized on ChtS-CL beads in a continuous production system.

Continuous Hydrolysis of Lactose by BglX Immobilized on ChtS-CL Beads

In independent trials, the substrate lactose or whey was pumped through the bed of immobilized BglX on ChtS-CL beads and into a collection vessel. A sample was removed for analysis. This was repeated for the same substrate ten times. A glucose standard curve was constructed (Figure 3–12) using the absorbance at 430 nm. The GO Assay was used to determine the concentration of glucose in the collected samples. The line equation was solved for the concentration of glucose in the samples after each pass

through the column of immobilized BglX. The percentage of lactose hydrolyzed was 10% for the lactose solution and 7.8% for the whey solution (Figure 3-22).

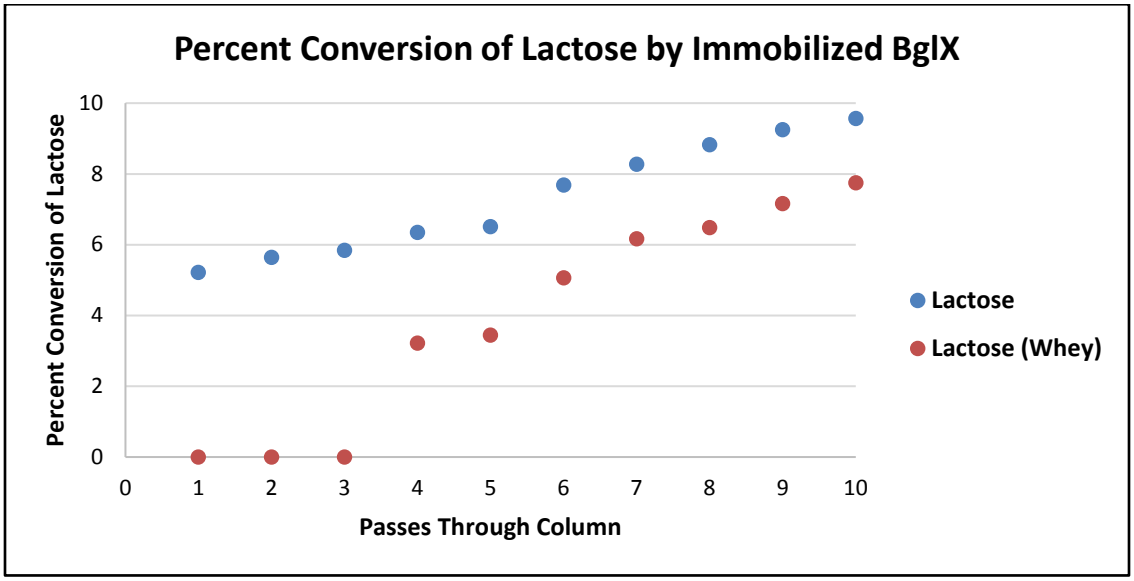


Figure 3-21: Percent conversion of lactose by immobilized BglX. The percent conversion of the lactose present in a lactose or lactose in milk whey solution. The substrates were pumped through a bed of ChtS-CL beads with immobilized BglX.

Chapter 4: Discussion

Enzyme immobilization is a useful technique for industrial applications because the enzyme retains its catalytic activity when attached to the support system (matrix), and provides a convenient way to separate the product from the immobilized enzyme, typically by filtration.[9] The immobilized enzyme can be washed free of residual substrate or products and reused. Enzyme supports are made of organic, inorganic, or organic-inorganic hybrid materials.[52] The organic material chitosan is a good option as the support system for enzymes because it is inexpensive, plentiful, has shown good immobilization efficiency, lends an antimicrobial property to the matrix, and it can be doped with inorganic materials to change its structural properties and immobilization efficiency.[14] Chitosan is soluble in dilute acidic solutions, and the resulting solution can be used to make hydrogel beads.[16] Cross-linking the polymeric strands of chitosan with aldehydes like vanillin or glutaraldehyde has been shown to increase the adsorptive capacity by increasing the internal surface area of the hydrogel beads.[53] Both batch and continuous production methods benefit from the use of immobilized enzymes; however, batch production proves less useful when the enzyme is inhibited by its products. Inhibition studies should be conducted to determine if a batch or continuous production method would prove most effective.[54]

In this study, chitosan gel beads were formed and used for enzyme immobilization. Chitosan powder was dissolved in a dilute solution of acetic acid and the beads were formed by counter-ion precipitation when the acidic chitosan solution was

added dropwise to a basic solution. A portion of the chitosan beads was fortified with either activated charcoal or silica by addition of those materials to a solution prior to bead formation (Figure 3-8).

After washing the beads with buffer until the pH decreased to 6.8, the diameter of the chitosan beads was measured as 2.8 ± 0.2 mm for ChtR beads, 2.5 ± 0.3 mm for ChtAC beads, and 3.1 ± 0.2 mm for the ChtS beads. The beads were examined using a light microscope to observe the internal characteristics by slicing them in half using a micro spatula. The nearly translucent ChtR and ChtS beads did not provide enough contrast for viewing. A ChtAC bead at 40x and 100x magnification showed that the activated charcoal was distributed throughout the bead, rather than localized to the surface (Figure 3-9).

Chemical cross-linking of chitosan was performed using glutaraldehyde, which has been proposed to occur in three ways: a) one Schiff base is formed with one of the aldehyde groups, while the pendant aldehyde group remains free to interact with other molecules[55]; b) a Schiff base is formed with two of chitosan's amino groups, which may or may not belong to the same polymeric chain[56]; and c) polymerization of the glutaraldehyde molecules occurs and a longer cross-linking chain is formed.[56] The diameter of all types of the chitosan gel beads decreased by an average of 0.3 mm after treatment with a dilute glutaraldehyde solution (Figure 3-8b,d). Cross-linking results in a reduction of the chitosan beads' diameter due to a change in the charge surface chemistry, which reduces repulsion by the weakly acidic $-NH_2$ groups, as the five-carbon alkyl glutaraldehyde molecules hold the polysaccharide chains more closely together.[57]

The color of the chitosan beads darkened after incubation in the glutaraldehyde solution, further confirming that cross-linking had occurred. The –C=N- ethylenic double bond formed acts as a chromophore, and thus the cross-linked beads are visibly darker.[58] Cross-linking was further confirmed by an adsorption study using a methylene blue dye solution. Adsorption of a cationic dye by chitosan beads is thought to occur mostly through hydrogen bonding with the free –OH and $–NH_2$ groups. The amount of dye adsorbed by the unlinked and cross-linked unfortified beads was compared. The adsorption capacity of the unlinked beads was 0.228 mg dye/g of ChtR and for the cross-linked beads 0.213 mg of dye/g of ChtR-CL. This seems to refute that cross-linking occurred, as cross-linking increases the adsorptive capacity of chitosan beads; however, since a portion of the $–NH_2$ groups are bound with glutaraldehyde, they cannot interact via hydrogen-bonding with the dye molecules, and in addition, the tertiary amines present on methylene blue dye do not react with aldehydes. Thus the decreased adsorption capacity of methylene blue dye by cross-linked chitosan beads corroborates that the chitosan beads were successfully cross-linked.

Lactase was used as a model enzyme to test the ability of the beads to both immobilize the enzyme and retain its catalytic activity. The ChtS and ChtS-CL beads were incubated overnight in a lactase solution and rinsed twice to remove any protein that was not immobilized. The amount of protein adsorbed by the beads was similar; the ChtS beads immobilized 39 μg of protein per mg of beads, while the ChtS-CL beads immobilized 40 μg of protein per mg of beads. The enzyme immobilized on ChtS-CL beads was tested for its ability to hydrolyze model substrate ortho-nitrophenyl-β-D-galactopyranoside in

micro-scale batch production, and 27% of the available substrate was hydrolyzed in 75 min of contact time. Another substrate, lactose, was tested similarly, and yielded similar results, resulting in 29% of the available substrate undergoing hydrolysis in 75 min. This indicates the enzyme did retain catalytic activity after being immobilization on the chitosan beads.

BglX and its catalytically-active mutant E293Q were produced by *E. coli* and purified using anion exchange chromatography. The purified BglX was used to determine if the enzyme was inhibited by the products of lactose hydrolysis. Chromogenic substrates *o*NPGal and *p*NPGlc were used to test the effects of glucose and galactose on the hydrolytic activity of the enzyme. The formation of *o*NP or *p*NP by the cleavage of the β(1→4) glycosidic bond of the substrates can be monitored at 405 nm. When *o*NPGal was used as the substrate, the addition of galactose or glucose to the reaction mixture resulted in a 52% and 49% reduction in activity, respectively. Activity was reduced by 19% or 75% upon the addition of galactose or glucose, respectively, to the reaction mixture when *p*NPGlc was used as the substrate. This indicates that BglX is inhibited by galactose and glucose, and therefore, a continuous production system may provide a higher yield than batch production. BglX and E293Q were immobilized on the ChtR-CL, ChtS-CL, and ChtAC-CL beads, and there was little difference in the percent of available protein adsorbed. The unfortified beads adsorbed 39 µg BglX per mg of beads or 38 µg E293Q per mg of beads. The results for the enzymes immobilized on ChtS-CL beads were the same, 39 µg protein/mg beads, while the ChtAC-CL beads adsorbed the least amount of protein, at 36 µg protein/mg beads for both BglX and E293Q. The adsorption capacity of

activated charcoal is determined by both its pore size and the electrical charge of the surface groups.[59] If the pore size is too small, it will not trap larger molecules.[59] The size of BglX and E293Q is 81 kDA, which may prove too large to fit in the pores of the activated charcoal used in this experiment. The proteins' net charge should have remained negative throughout the course of this experiment, since the isoelectric point is approximately 5.8 and the pH was maintained at 6.8. Depending on the surface charge of the activated charcoal, this could either attract or repel the protein. Analysis of the activated charcoal should be performed to determine the reason for the ChtAC-CL beads' inability to immobilize as much protein as the other beads. Pore size could be determined using SEM or BET surface analysis and elucidation of the functional groups present on the surface of activated charcoal could be determined using FT-IR.[60] The charge of the functional groups would depend on the pH of the solution.[61]

BglX was tested using the substrate *o*NPGal to determine if the immobilized enzyme still possessed catalytic abilities. After 30 min, only 0.2% of the available substrate had been hydrolyzed and by 75 min of contact time with the immobilized enzyme, only 5% of the substrate was converted into products. The mass transfer of reactants first occurs from the bulk fluid containing the substrate to the external surface of the beads. The substrate can then diffuse into the pores, where the majority of the catalytic surface lies.[62] If the majority of the enzyme does not make contact with the substrate, catalysis will not occur as readily. Since it was shown that BglX is inhibited by galactose, a build-up of the product inside the beads would have a negative effect on catalytic activity. It would be beneficial to determine the K_M of the immobilized enzyme

toward *o*NPGal, as this would provide more information about the difference in product conversion by the immobilized lactase and BglX.

Both BglX and E293Q immobilized on the cross-linked beads were tested for their ability to hydrolyze lactose. The highest percent conversion was exhibited by BglX immobilized on ChtS-CL beads (15%) followed by ChtR-CL beads (14%), which makes sense considering these beads had immobilized more enzyme than the ChtAC-CL beads. The mutant E293Q hydrolyzed about 50% less substrate than BglX, regardless of bead type. It is probable that E293Q does not have the same catalytic efficiency as the native protein, as enzyme type was the only variable in these experiments.

It was shown that BglX was successfully immobilized on chitosan gel beads and catalytic activity was maintained. The protein immobilized on the ChtS-CL beads exhibited slightly better immobilization efficiency and percent conversion of substrate than its mutant E293Q. In order to determine if continuous production would increase the product yield over that obtained by batch production, a miniature column packed with immobilized BglX on ChtS-CL beads was used. Two peristaltic pumps were employed, one to pump the substrate upwards into the packed bed and another to remove products into a collection vessel. The fluid passes through three microvalves which are sequentially opened and closed, - this process is referred to as "peristalsis."[63] The first pump was an off-the-shelf miniature integrated pump and controller manufactured by VWR ; the second pump was a Gifkunun 12 V DC dosing pump with an Arduino connector, which required the construction of a power supply circuit. To power the pump, a microprocessor-controlled power supply circuit was constructed based on a

Toshiba 7921P bridge driver integrated circuit (IC)[64] and an Arduino Uno R3 single-board microcontroller based on instructions published under a Creative Commons license.[65] The output pins on the Arduino board are limited to relatively low voltage and current draw and are only intended for use in delivering the signal from the software rather than driving a high-power device like a peristaltic pump. To address this problem, a motor driver IC was used to power the peristaltic pump under the control of the Arduino. The TA7921P can power a motor that requires a supply voltage (Vs) between 0-20V DC and draws 1.0A of current, making it appropriate for use in this application. The minimum flow rate (mL/min) of the peristaltic pumps was determined as the average of five trials. Pump 1 had a minimum flow rate of 12.5 ± 0.2 mL/min and 10.5 ± 0.1 mL/min for Pump 2. The small standard deviation associated with the flow rate of each pump indicates high dosing accuracy and reproducibility. This enabled delivery of a pre-specified volume of substrate by controlling the duration of time Pump 1 was drawing current.

The continuous production system was tested using *o*NPGal, which was pumped into the bed of the column (1.5 x 5.0 cm) and kept in contact with the immobilized enzyme for 10 min before being pumped off. The percent conversion of substrate was 27%, substantially higher than when batch production was employed. This could be due to the pressure of forcing the substrate through the column and into the pores of the beads alleviating some of the diffusional limitations seen with batch production. The beads were washed with buffer and after 130 mL were flushed through the column, the concentration of products remaining in the column approached zero.

The immobilized enzyme was then tested for its ability to be used repeatedly, while maintaining catalytic ability. Instead of holding the substrate in the packed bed, it was pumped through the column multiple times. This procedure was done three times and the beads were washed between each trial. The results were a net 36%, 35%, and 36% conversion of *o*NPGal to *o*NP and Gal, which indicates the beads can be used repeatedly, washing does not result in loss of protein, and the catalytic ability was maintained. Taken in respect to the results from the initial test of the continuous production system, these results indicate that the force exerted on the beads as the substrate is pumped through the column could decrease the effects of internal diffusion.

Lactose and a prepared whey solution were used as substrates in the continuous production system and the procedure was performed similarly to when *o*NPGal was used. This resulted in a 10% conversion of lactose and an 8% conversion of the lactose in whey. In contrast to the pure lactose solution, acid separated whey contains 0.55 – 0.75 % (w/v) protein, 0.8% (w/v) ash, and 0.04% (w/v) of lipids.[66] These molecules could interfere with the ability of the enzyme to hydrolyze the lactose, as they could adsorb to the chitosan and impart some diffusional issues. Further study is necessary to determine if immobilized BglX is a good candidate for the hydrolysis of lactose from whey.

Chapter 5: Conclusion

The chitosan gel beads were formed by dissolving powdered chitosan in a dilute acidic solution and then adding dropwise to a basic solution. Some of the beads were fortified with silica or activated charcoal, which was added to the chitosan solution before the beads were formed. Chemical cross-linking was performed by incubating the beads with glutaraldehyde. All types of the chitosan gel beads were able to successfully immobilize lactase, BglX, and E293Q while catalytic activity towards was maintained. There was no difference in the immobilization efficiency of unlinked and cross-linked beads. The chitosan beads fortified with activated charcoal immobilized the least amount of the enzymes, while the beads fortified with silica immobilized slightly more enzyme than the unfortified beads.

The immobilized enzymes were tested in micro-scale batch production of galactose and glucose from lactose. A coupled assay confirmed that immobilized BglX and E293Q are able to hydrolyze lactose; however, immobilized BglX converted more substrate to product than E293Q. The immobilized BglX retained about 14% the activity towards lactose as the free enzyme and so inhibition studies were conducting to test whether BglX is inhibited by the products of lactose hydrolysis.

Once confirming that BglX was inhibited by the presence of an abundance of galactose or glucose, the enzyme was immobilized to ChtS-CL beads and used in a continuous hydrolysis experiment. When the model substrate *o*NPGal was used, the immobilized BglX on ChtS-CL beads yielded a higher percent conversion than batch production and

achieved a substrate conversion rivaling that of the free enzyme. The continuous hydrolysis of free lactose and the lactose from whey need optimization to increase the product yield. Since the residence time in the column was low, it would be beneficial to use a pump with a significantly lower flow rate and see if this increases the percent conversion of those substrates.

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