Characterization of β-galactosidase from *Enterobacter* sp. YSU, GalB2, and Methods to Purify and Characterize Galacto-oligosaccharides

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Abstract

Beta-galactosidases are enzymes that hydrolyze β-glycosidic bonds with galactose at the non-reducing end. These enzymes have been used in the food and pharmaceutical industries for processing waste into environmentally friendly compounds and the synthesis of prebiotics. GalB2 is a novel β-galactosidase from *Enterobacter* sp. YSU that was expressed in *Escherichia coli*. The enzyme was isolated with ammonium sulfate precipitation and purified by an anion-exchange chromatography.

The optimal pH for GalB2 was found to be 7.4. There was no loss in enzymatic activity when GalB2 was incubated at temperatures 37 ºC and 40 ºC, but activity significantly diminished after incubation at temperatures greater than 45 ºC. The window of substrates is narrow with only *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPGal) and lactose being evident. The catalytic parameters were determined for both substrates. For the substrate *o*-NPGal, the Michaelis constant, K_M was determined to be 0.18 mM and a catalytic constant, k_{cat} was 44 s^{-1}. With respect to lactose, the K_M was found to be 2.5 mM with a k_{cat} of 51 min^{-1}. Glucose inhibited the enzyme in an uncompetitive manner, but galactose demonstrated competitive inhibition of GalB2. The enzyme shows dependence from magnesium ions. Native gel electrophoresis with exposure to fluorogenic substrate 4-methylumbelliferyl-β-D-galactopyranoside indicated that enzyme is active as a dimer. No transglycosylation activity for GalB2 was observed. Transglycosylated products from immobilized commercially available lactase were confirmed using LC/ESI-MS. Attempts to isolate oligosaccharides using a silica-gel column with an acetonitrile:water eluting solvent were unsuccessful.

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List of Abbreviations

DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray-ionization
EtOH	Ethanol
Fru	Fructose
Fuc	Fucose
Gal	Galactose
GBS	Group B *Streptococcus*
GC	Gas chromatography
Glc	Glucose
GlcNAc	N-acetylglucosamine
Glu	Glutamate
GOS	Galacto-oligosaccharide
HMO	Human milk oligosaccharides
HPLC	High-performance liquid chromatography
LB	Luria-Bertani
LC	Liquid chromatography
MS	Mass spectrophotometer
Man	Mannose
MES	2-(N-morpholino)ethanesulfonic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
4-MUGal	4-methyllumbelliferyl-β-D-galactopyranoside
MWCO	Molecular weight cutoff
NMR	Nuclear magnetic resonance
o-NPGal	*o*-nitrophenol-β-D-galactopyranoside
sp.	Species
TLC	Thin layer chromatography
UV	Ultraviolet

Chapter 1: Introduction

Beta-galactosidases are enzymes that are capable of hydrolyzing β-glycosidic bonds with galactose on the non-reducing end of a sugar, some enzymes are also capable of performing transglycosylation reactions where galactose is the glycosyl donor. These enzymes exist in organisms from all kingdoms, and many have been isolated from plants, animals, and all forms of microbiota. These enzymes have found use in the food and pharmaceutical industry as they are reusable when immobilized, capable of producing prebiotic compounds, and processing waste in the dairy industry.[1-3] There is a wide range of sources of β-galactosidases with many still not characterized. The discovery of novel β-galactosidases could not only add insight into biochemical systems of the organisms that host them, but newly characterized enzymes could lead to innovations within the food and pharmaceutical industries.

1.1 Uses of β-galactosidases in industry

Beta-galactosidases are the enzymes that hydrolyze the β-glycosidic bonds of oligosaccharides, specifically those with galactose on the non-reducing end. They are found in many organisms from microbes to animals. In the food industry, microbial β-galactosidases such as ones isolated from *Kluyveromyces* and *Aspergillus* species are commonly used. These enzymes aid in the production of some desired dairy products, particularly lactose-free products. There are increasing demands for β-galactosidases that can hydrolyze lactose, whey byproduct as a means to prevent water pollution.[1] These enzymes are also relevant to human health as they possess transglycosylation activity, which can be utilized to synthesize galacto-oligosaccharides (GOS). Regarding oligosaccharides, some of their known benefits are nutrients for human gut microbiota, constipation relief, and antimicrobial and antibiofilm properties.[2-4] It has been shown that galacto-oligosaccharides are a primary component for prebiotics because of their ability to

increase the activity of bifidobacterial located in the human colon.[4] Human milk oligosaccharides (HMOs) have demonstrated potential for treating bacterial infections such as methicillin-resistant *Staphylococcus aureus* (MRSA), Group B *Streptococcus* (GBS), and *Acinetobacter baumannii*. All of these HMOs have different ways of disease intervention. HMOs have direct antimicrobial activity against *A. baumannii* but have antibiofilm activity when used against GBS or MRSA.[3]

Unfortunately, there are drawbacks in the organic synthesis of oligosaccharides due to the intricate nature of carbohydrates. Due to this, modern methods to synthesize carbohydrate-based molecules have yet to be streamlined. As of now, the synthesis of oligosaccharides is heavily reliant on protecting groups, which can be challenging for stereospecificity. Not to mention these syntheses are very involved processes with numerous steps.[5] To help in these attempts, enzymes have been sought out to be the solution as they provide regio- and stereoselectivity, reusability when immobilized, and much milder reaction conditions. Enzymes like glycoside hydrolases (GHs) can cleave glycosidic bonds. Although hydrolyzing glycosidic bonds is the main function of these enzymes, some can also perform transglycosylation reactions. The study of GHs and their transglycosylation capabilities have increased in recent years as results have shown that GHs are more advantageous in transglycosylation reactions as compared to glycosyltransferases. This is primarily due to the wide range of acceptor and donor substrates that can be utilized.[6] Glycosyltransferases are specific to the acceptor and donor pair as both bind to the enzyme that catalyzes the conjugation of the two. For glycoside hydrolases, only a single substrate is bound to the enzyme at a time. In a typical reaction for GHs, the glycosidic bond is cleaved, and the non-reducing portion of the substrate becomes bound to the enzyme as a covalent intermediate. This would then be hydrolyzed and released from the enzyme (**Figure 1**). When

transglycosylation occurs, the covalent intermediate acts as a glycosyl donor and the hydroxyls in any glycosyl acceptor act as a nucleophile and form a new glycosidic bond between the glycosyl acceptor and the covalent intermediate (**Figure 1**). A novel β-galactosidase from *Klebsiella oxytoca* ZJUH1705 demonstrated yields of galacto-oligosaccharide close to 50% when high concentrations of lactose were used.[7] Meanwhile, FpGH30 from *Fomitopsis palustris* FFPRI 0507 had a max yield of 26% of transglycosylated product when *p*-nitrophenolate-β-D-galactopyranoside was used as the substrate.[6]

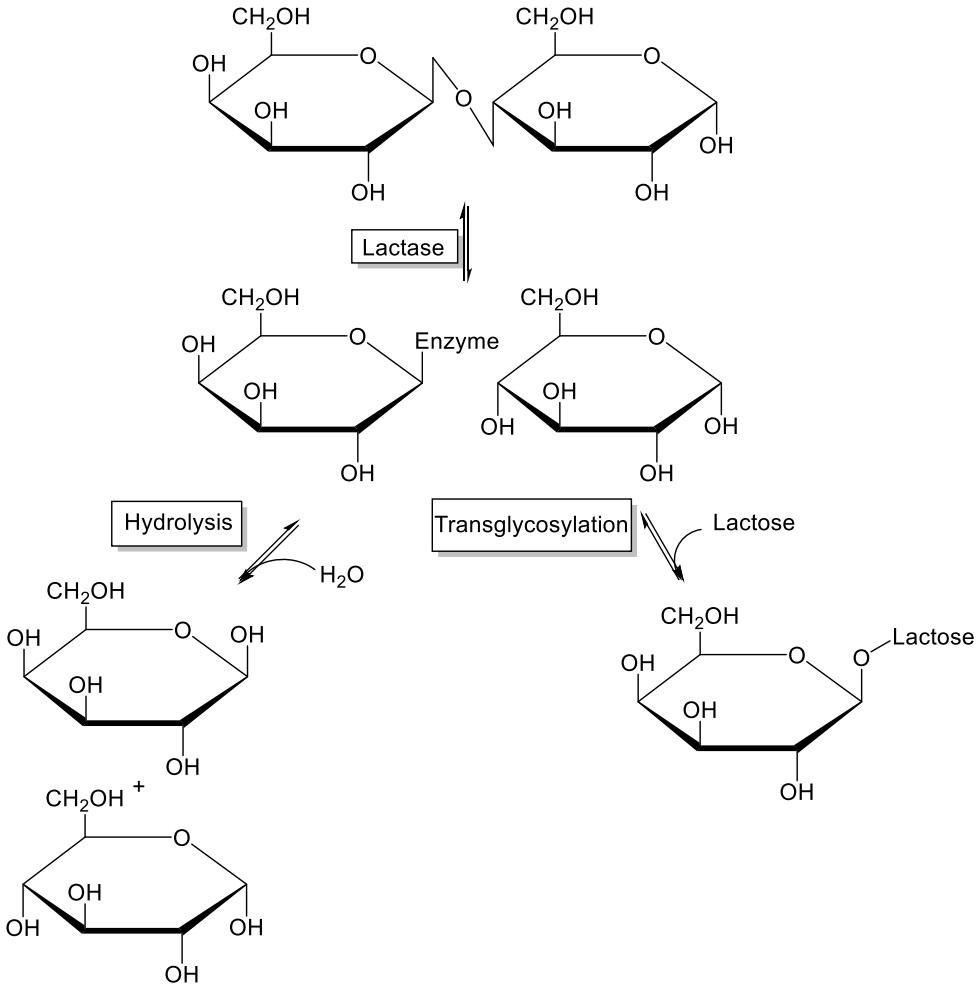


Figure 1. A comparison of the hydrolytic and transglycosylation reactions performed by the lactase enzyme.

Another benefit of using enzymes is that they can be manipulated to optimize a reaction. In cases using glycoside hydrolases, this is done to limit the secondary hydrolysis of transglycosylated products by the enzyme. For example, the Fauré group conducted a study on an endo-glycoceramidase (EGC) from the bacterium *Rhodococcus* sp. M-777 strain and performed mutagenesis to optimize the enzyme for transglycosylation.[8] They started with a point mutation, E351S, making the EGC lose hydrolytic activity and primarily catalyze transglycosylation reactions when α-fluorinated substrates were available. Following this, the EGC was subjected to random mutagenesis, and a resulting E351S-D314Y mutant synthesized complex glycosphingolipids based on β-cellobiose being the glycosyl donor with the yield up to 86%.

1.2 β-galactosidase structure

The structure determination of β-galactosidases historically started with *lacZ* β-galactosidase from *Escherichia coli*. It was discovered to have a quaternary structure consisting of four identical polypeptide chains with 1023 amino acids in each.[9] Although it is homotetrameric, the enzyme will remain active in a dimeric state. As long as metal ions such as sodium and magnesium, and thiol reagents like 2-mercaptoethanol and dithiothreitol, were present in ample amounts in solution, the dimer remained stable and did not dissociate into monomers. Sodium ions are necessary for enzymatic activity along with two magnesium ions per subunit that are bound in the active site, while other magnesium ions are believed to have structural relevance. The thiols help prevent the formation of disulfide bridges that would cause strain and deformation of the overall structure.[10] Some hypotheses propose that β-galactosidase evolved from a triose phosphate isomerase (TIM) as the central domain of the β-galactosidase houses the same barrel structure.[9]

1.3 Metal ion cofactors and their effects on β-galactosidases

Many β-galactosidases are metalloenzymes and require metal ions for activity and structural stability, but metal ions vary among the kinds of enzymes. As previously mentioned, sodium and magnesium ions were associated with the *lacZ* β-galactosidase from *E. coli*. It has also been reported that *lacZ* β-galactosidase from *E. coli* requires Mg^{2+} or Mn^{2+} for full catalysis and the active sites require Na^{+} and K^{+} as it ligates the O-6 hydroxyl of galactose.[11] In a kinetic study to assess the impact of divalent metal ions on the enzyme from *Anthrobacter oxydans* SB , the addition of Mn^{2+} resulted in the highest V_{max} for the enzyme (55.98 μM of *o*-nitrophenol-β-D-galactopyranoside hydrolyzed mg^{-1} protein min^{-1}) as compared to Fe^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, and Cu^{2+}.[12] In a similar study done with β-galactosidase from *Rhizomucor* sp, Co^{2+} was observed to increase the activity of the enzyme by 33% relative to the control, while Hg^{2+} completely inhibited the enzyme. Other divalent metal ions that cause significant inhibition were Mn^{2+}, Fe^{2+}, and Zn^{2+}.[13]

In metal ion assays, a chelating agent, ethylenediaminetetraacetic acid (EDTA), is commonly included. When EDTA traps divalent metal ions, it usually decreases the activity of a β-galactosidase, but the amplitude of impact varies. For example, the catalytic activity of β-galactosidase from *Bacillus* sp MTCC 3088 was not affected when treated with EDTA, demonstrating that the enzyme is not a metalloenzyme, but when the β-galactosidase from strawberries was treated with EDTA, the enzyme became inactive.[13,14]

1.4 Substrates of β-galactosidases

Lactose has been characterized as the natural substrate of many β-galactosidases, giving the common name of the enzyme, lactase. It has been noted that the enzyme has great specificity for

D-galactose at the non-reducing end of the glycosidic bond being cleaved due to the stereochemistry of the hydroxyls found at positions 2, 3, and 4, but minimal specificity for the reducing end.[9] With this, various chromogenic substrates were developed such as X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPGal), and *p*-nitrophenyl-β-D-galactopyranoside (*p*-NPGal) (**Figure 2**). These disaccharide-like derivates are colorless, but when hydrolyzed, the portion of the molecule on the "reducing-end" gives off a color. In the case of X-gal hydrolysis, two equivalents of the indole react forming 5,5'-dibromo-4,4'-dichloro-indigo resulting in the development of a blue color. Both *o*-NPGal and *p*-NPGal are regularly used in assays with β-galactosidases as the use of these nitrophenol-containing compounds is simple. The hydrolysis of either *o*-NPGal or *p*-NPGal results in the release of the nitrophenolate product which absorbs light around 420 nm.[9,15,16]

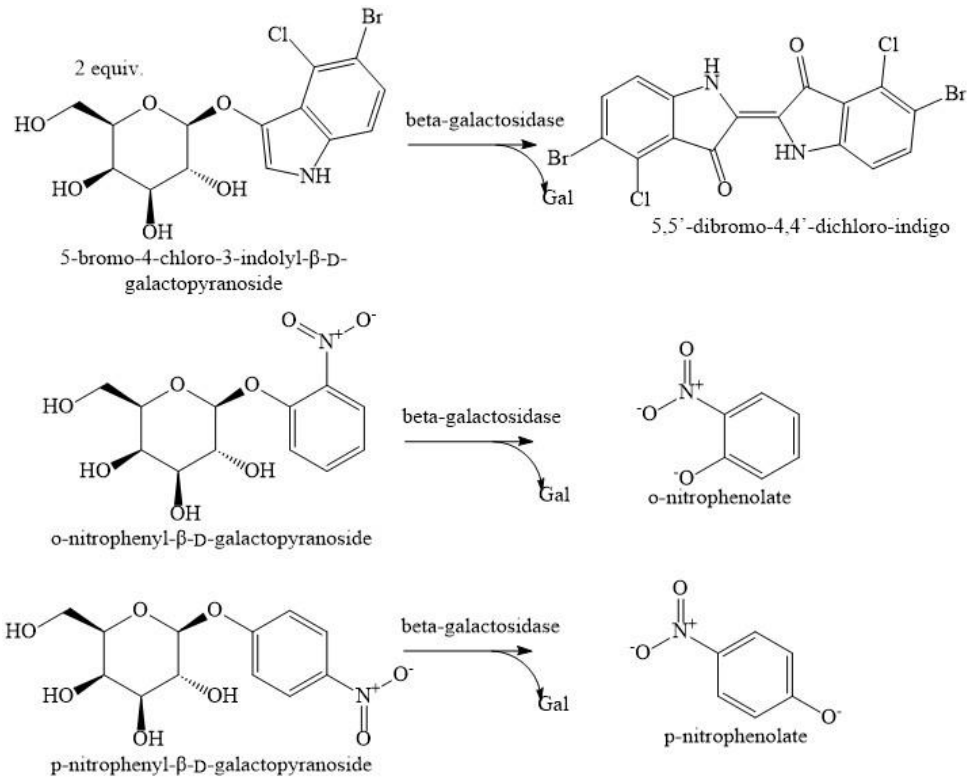


Figure 2. Hydrolysis of chromogenic substrates with β-galactosidase (Gal = D-galactose).

1.5 Effect of pH and temperature on β-galactosidases' activity

Temperature and pH are important parameters as enzymes are sensitive to these, but the optimal temperature and pH for a given enzyme is dependent on the source. Increasing the enzyme's temperature can ultimately reach a point at which the hydrogen bonding within a protein is broken due to the intense vibration of the bonds.[18] In one study it was found that incubation of β-galactosidase from *B. longum* RD47 for one hour at the temperatures between between 35 ºC and 37 ºC did not affect its activity while decreased activity was observed when the enzyme was exposed to the temperatures outside of this range for an hour.[18] It was observed that a major loss of activity occurred after 45 ºC. Asraf et al. referenced several bacteria that showcased the wide variety of thermal stability of β-galactosidases.[19] For example, after a 9-hour incubation the β-galactosidase from *Arthrobacter* sp. 32c was reported to have a thermostability range of 0-60 ºC, while the β-galactosidase from *A. acidocaldarius* has a thermostability range of 40-90 ºC.

The pH of the environment can greatly affect the activity of an enzyme. Enzymes are dependent on certain ionization states of their active site residues and changes to the ionization states can decrease enzymatic activity, even to the point of inactivation. For example, β-galactosidase from *E. coli* has two glutamate residues, Glu461 and Glu537, that act as acid-base and nucleophilic catalysts, respectively. The binding of galactose in the active site requires Glu537 to remain deprotonated to act as a nucleophile to form a covalent intermediate (**Figure 3**). If the enzyme is in a more acidic environment, Glu537 is more likely to become protonated, thus, preventing the stabilization of galactosyl oxonium species.[9] Roberts et al. found that lactase from the small intestine of horses had an optimal pH of 6.0 when lactose was the substrate but its activity significantly decreased below a pH of 5.0.[20] A β-galactosidase from *Bacillus* sp. BPTK4 showed

maximal activity with *o*-NPGal at a pH of 7.0, and low activity was found at both pH 5.0 and 9.0.[21]

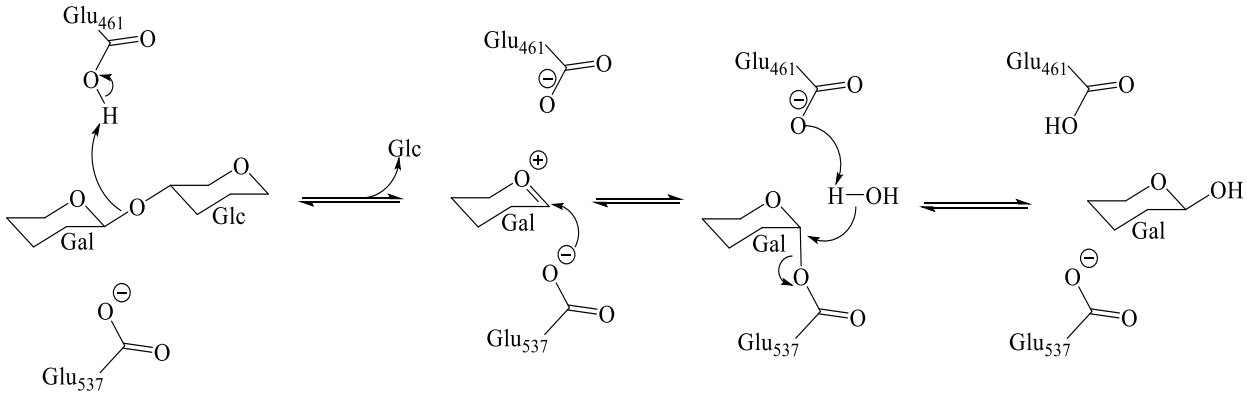


Figure 3. The mechanism of hydrolysis of lactose by β-galactosidase from *E. coli* as adapted from Ref. 9.

1.6 Immobilization of β-galactosidases

In industrial chemical reactions, the destabilization of enzymes is a fundamental problem. As previously mentioned, enzymes are sensitive to pH and temperature, in addition, within industrial settings factors such as salts, surfactants, and organic solvents can disrupt the efficiency and stability of enzymes. Therefore, efforts to create carrier materials for enzymes have been made to aid in the stability and reusability of enzymes.[19,22] For β-galactosidases, many techniques of immobilization have been explored, this includes adsorption, covalent binding, entrapment, and aggregation by cross-linking. Two methods have shown promise for galacto-oligosaccharide synthesis: entrapment and cross-linking. Both chitosan and alginate beads have been utilized to entrap β-galactosidases. Combining this method with cross-linking by glutaraldehyde has been shown to aid in galacto-oligosaccharide synthesis using β-galactosidase from *Lactococcus lactis*.[22] Klein et al. reported that glutaraldehyde-stabilized chitosan in a

packed-bed reactor increased the operational pH range, operational temperature, and thermal stability of β-galactosidase from *Kluyveromyces lactis* compared to the free enzyme.[23] Enzyme immobilization resulted in an operational pH range from 6.5 to 8.0 with 70% remaining activity, about 55.8% of remaining activity after an 8-hour incubation at 55 ºC, and higher activity across the temperature range of 10-70 ºC compared to that of the free enzyme.

1.7 Characterization of oligosaccharides

A big challenge when it comes to oligosaccharides is still rooted in the characterization of transglycosylated products synthesized by enzymes. The longer the carbohydrate chain, the more complex its analysis and structural determination. This requires methods and instrumentation that can purify such intricate compounds and analyze them with high sensitivity and great accuracy. The two most common approaches for the characterization of oligosaccharides are nuclear magnetic resonance (NMR) and mass spectrometry (MS), where mass spectrometry is usually coupled with gas chromatography (GC) or high-performance liquid chromatography (HPLC).[24-28] GC-MS is not ideal for oligosaccharide analysis due to the sensitivity issues of the method and the necessity of having volatile oligosaccharide samples.[3] Methods using LC/MS are more favorable as the sensitivity of ESI-MS is high with reports of femtomolar concentrations and parts per million with the option of derivatized and underivatized methods.[2,25,26] *N*-glycans like Gal(β1→4)GlcNAc(β1→2)Man(α1→6)[Gal(β1→4)GlcNAc(β1→2)-Man(α1→3)]Man(β1→4)GlcNAc(β1→4)GlcNAc, and Gal(β1→6)Man(α1→6)[Man(α1→3)]Man(β1→4)GlcNAc(β1→4)[Fuc(α1→6)]GlcNAc have been characterized using nano-LC-ESI-MS in femtomole quantities.[25] Advancements in NMR-based methods have been tremendous, moving from standard NMR to modern approaches that use correlated spectra to enhance the sensitivity and accuracy of structural analysis.[2,24-28] For

example, Abe et al. characterized 11 trisaccharides from sugar beet molasses via NMR techniques.[27] Some of the trisaccharides characterized were Gal(α1→6)Fru(2→1)Glc, Man(β1→3)Glc(α1→2)Fru, and Gal(α1→2)Glu(α1→2)Fru, to name a few.

1.8 Research problem

Organic synthesis of oligosaccharides is costly and inefficient. The use of β-galactosidases is often relied on as an alternative route. This method has many advantages, but enzymes are also sensitive to their environment, such as temperature and pH. The novel β-galactosidase, GalB2, from *Enterobacter* sp. YSU could be an enzyme capable of producing galacto-oligosaccharides through transglycosylation. Ultimately, the biochemical characterization of the enzyme will add insight to the ever-growing research on β-galactosidases. Structural analysis of oligosaccharides is challenging as potential isomers are numerous and increase as the length of the sugar polymers increases. Although enzymes aid in the selectivity of a product, it is often not specific to one isomer. Efforts in column chromatography and LC/ESI-MS methods will benefit overall methodologies used in oligosaccharide characterization.

1.9 Objectives of the research

The aim of this research was to gain insight into a novel β-galactosidase, GalB2 from *Enterobacter* sp. YSU, and to develop a method to characterize oligosaccharides obtained via enzymatic transglycosylation. A biochemical characterization of GalB2 was done to assess various properties of the enzyme such as thermal stability, optimal pH, substrate specificity, transglycosylation activity, effects of metal ions, potential inhibitors, and quaternary structure. Transglycosylation was also evaluated using commercially available lactase. This was used as a

model system for transglycosylation for developing a method to characterize synthesized oligosaccharides.

Chapter 2: Materials and Methods

2.1 Materials

Dr. Caguiat (Youngstown State University) generously gifted the DNA plasmid *pET20b-lacZ2*.The boric acid was acquired from Matheson Coleman and Bell (Norwood, OH). D-glucose was purchased from Mallinckrodt Inc. (Paris, KY). The 200-500 μm, 60 Å porosity silica gel was obtained from Sorbtech (Sorbent Technologies, Norcross, GA). D-galactose, sulfuric acid, ethanol, 1-butanol, acetic acid, orcinol, and magnesium chloride were obtained from Fisher Scientific Company (Fair Lawn, NJ). Raffinose pentahydrate, *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPGal), D-lactose, glutaraldehyde (25% in H_2O), silica HF powder, HPLC grade water, HPLC grade methanol, peroxidase, bovine serum albumin, and 4-methylumbelliferyl-β-D-galactopyranoside (4-MUGal) were purchased from Sigma-Aldrich (St. Louis, MO). Laminarin was acquired from Spectrum Chemical Manufacturing Company (New Brunswick, NJ). Lactase supplement was purchased from Rite Aid (Camp Hill, PA). Chitosan powder was purchased from Bulk Supplements (Henderson, NV). Q-Sepharose was obtained from Amersham Biosciences (Piscataway, NJ). Acetonitrile was obtained from Acros Organics (Geel, Belgium). Potassium phosphate monobasic, sodium phosphate monobasic, Luria-Bertani (LB) medium, ammonium sulfate, streptomycin sulfate, sodium chloride, ampilcillin sodium salt, Tris-HCl, MES, sodium acetate, glucose oxidase, and coomassie brilliant blue G-250 were purchased from Amresco (Solon, OH). The TLC plates were purchased from Merck KGaA (Darmstadt, Germany). The UV light transilluminator, gel electrophoresis equipment, and thermal cycler were from Bio-Rad (Hercules, California). Mass spectrometry was carried out on

a Bruker Esquire-LC ion trap (Billerica, MA), which was connected to a Hewlett Packard series 1100 LC (Agilent Technologies, Santa Clara, CA). The Rezex RPM-Monosaccharide, 00H-0135-K0, 300 x 7.80 mm column was purchased from Phenomenex (Torrance, CA). An Avanti J-25 I centrifuge by Beckman Coulter (Brea, CA) and Sorvall RT6000B Refrigerated centrifuge by Du Pont (Wilmington, DE) were used in enzyme purification. All spectrophotometric measurements were conducted on a Hewlett Packard 8453 photodiode array spectrophotometer (Agilent Technologies, Santa Clara, CA).

2.2 Methods

2.2.1 GalB2 expression and purification

Escherichia coli BL21(DE3) cells containing *pET20b-lacZ2* plasmid were cultured in 50 mL of Luria-Bertani (LB) broth containing 100 µg/mL ampicillin. The culture was placed in a rotary shaker at 200 rpm at 37 ºC overnight. From the starter culture, 100-fold dilutions were made by adding 12 mL of starter culture into each of three flasks with LB/ampicillin media, reaching a final volume of 1.2 L. These were then placed in the rotary shaker at 200 rpm at 37 ºC and left overnight for protein expression. The cells were harvested by centrifugation at 6,000 x g for 10 minutes. After centrifugation, the supernatant was removed, and the pellets were stored at -25 ºC. To free cellular contents the pellets were transferred to a beaker and suspended in 60 mL of 50 mM sodium phosphate buffer, pH 7.4. The beaker was then placed in an ice-water bath for sonication. The sonication was done in cycles of 30 seconds followed by 60 seconds of stirring for a total of 15 cycles. Once the cycles were completed, the contents of the beaker were centrifuged at 11,000 x g for 20 minutes at 4 ºC. Nucleic acids were precipitated using streptomycin sulfate, which was added to the supernatant to form a 1% (m/v) solution and then centrifuged for 20 minutes at 11,000 x g at 4 ºC. Protein was precipitated from the supernatant by

adding ammonium sulfate to reach 45% saturation. The mixture was centrifuged at 11,000 x g for 20 minutes at 4 ºC. The pellet was removed, resuspended in 50 mM sodium phosphate buffer, pH 7.4, and was dialyzed against 2 L of 50 mM sodium phosphate buffer, pH 7.4 in 12,000-14,000 MWCO tubing for 39 hours at 4 ºC.

2.2.2 Ion-exchange Chromatography

A column (15 x 2.5 cm) filled with Q Sepharose fast flow was equilibrated with 50 mM sodium phosphate buffer, pH 7.4. The dialyzed protein solution was loaded onto the column, the column was washed with 200 mL of 50 mM sodium phosphate buffer, pH 7.4, and a sodium chloride gradient ranging from 0-500 mM with a total volume of 200 mL was applied. Fractions were collected in 7 mL portions. After the salt gradient, a 1 M sodium chloride in 50 mM sodium phosphate buffer, pH 7.4 wash was done, followed by a 1.5 M sodium chloride in 50 mM sodium phosphate buffer, pH 7.4 wash. Of the fractions collected, the odd-numbered fractions were analyzed for absorbance at 280 nm. The fractions with the greatest absorbance at 280 nm were then tested on a spot plate with the model substrate *o*-NPGal for β-galactosidase activity. The fractions with β-galactosidase activity were combined.

2.2.3 Determination of protein concentration

The combined fractions were dialyzed against 50 mM sodium phosphate buffer, pH 7.4, then the dialyzed solution was transferred to a 30,000 MWCO Millipore centrifugal unit to concentrate the protein solution. Once this was complete, 10 µL of a concentrated protein solution was added to 990 µL of 50 mM sodium phosphate buffer, pH 7.4, and the absorbance was evaluated at 280 nm. Using the extinction coefficient for GalB2, 268,710 $M^{-1}cm^{-1}$, and the Beer-Lambert equation, the protein concentration was calculated.

2.2.4 Evaluation of optimal pH for GalB2

Buffer solutions were prepared in 50 mM concentrations with a pH ranging from 5.0 to 9.0. The following buffers were used: sodium acetate (pH 5), MES (pH 6), sodium phosphate (pH 7), Tris (pH 8), and borate (pH 9). A 2.6 μM GalB2 solution and a 30 mM *o*-NPGal solution were prepared in each of the buffers.

To assess the impact of pH, 890 μL of the 50 mM buffer, 10 μL of a 2.64 μM GalB2 solution at a given pH, and 100 μL of 30 mM *o*-NPGal in the same respective buffer were mixed in a 3-mL cuvette and incubated for 90 seconds. Then, 2 mL of 0.5 M sodium carbonate was added to stop hydrolysis and the absorbance at 405 nm was measured. The spectrophotometer was blanked for every pH using a 3-mL cuvette containing 2 mL of 0.5 M sodium carbonate, 900 μL of buffer, and 100 μL of 30 mM *o*-NPGal in the respective buffer. All experiments were done in triplicate and the absorbances at 405 nm were averaged.

2.2.5 Determination of enzymatic activity of GalB2 using model substrate *o*-NPGal

The model substrate *o*-NPGal was used to evaluate the hydrolytic activity of GalB2. In a 1-mL cuvette, 890 μL of 50 mM sodium phosphate buffer, pH 7.4 was mixed with 100 μL of *o*-NPGal and used to blank the spectrophotometer at 405 nm, The reaction was initiated by the addition of 10 μL of a 2.6 μM enzyme solution, reaching an enzyme concentration of 26 nM in the reaction mixture. The reaction was monitored for the change in absorbance at 405 nm for 90 seconds every 10 seconds. The reactions were run in triplicate with substrate concentration in the reaction mixture ranging from 0.02 mM to 3.0 mM. The average change in absorbance at each substrate concentration was then used to calculate the initial velocity using the extinction coefficient of *o*-

nitrophenolate, 3055 $M^{-1}cm^{-1}$, as this is the chromophore produced. A Michaelis-Menten plot was made from the data collected and K_M, V_{max}, and k_{cat} were determined.

2.2.6 Inhibitory effect of galactose

To test the effect of galactose on GalB2 activity, a 1 M galactose solution in 50 mM sodium phosphate buffer, pH 7.4 was made. The reaction mixture consisted of 800 µL of 50 mM sodium phosphate buffer, pH 7.4, 90 µL of 1 M galactose solution, and 10 µL of a 2.6 µM enzyme solution. The assay mixture was incubated for one minute at room temperature then 100 µL of 30 mM *o*-NPGal was added to initiate the reaction. The reaction was monitored for the change in absorbance at 405 nm over 90 seconds. The change in absorbance was used to calculate the rate of reaction and then compared to that of the control, with no added galactose. Upon confirmation of inhibition, the concentration of galactose in the reaction mixture was varied to find the concentration that resulted in the loss of 25% and 50% of the rate of reaction given by the control. Meanwhile, the concentration of *o*-NPGal remained constant. It was found that 150 mM was the concentration of galactose that decreased the rate of reaction by 25% and 330 mM decreased the rate by 50%.

The activity of the enzyme in the presence of 150 mM and 330 mM galactose was evaluated while changing the *o*-NPGal concentration. In the case of 150 mM galactose, the reaction mixture consisted of 740 µL of sodium phosphate buffer, pH 7.4, 150 µL of 1 M galactose in sodium phosphate buffer, pH 7.4, 10 µL of a 2.6 µM enzyme solution, and 100 µL of *o*-NPGal ranging from 0.13 to 3.0 mM. The order of addition follows the same procedure described previously, allowing galactose to bind to the enzyme for one minute before initiating the reaction with *o*-NPGal. The reaction was monitored at 405 nm for 90 seconds. The data collected was then used to generate a Lineweaver-Burk plot and compared to the uninhibited enzyme.

2.2.7 Inhibitory effect of glucose

To test the effect of glucose on GalB2, a 1 M glucose solution in 50 mM sodium phosphate buffer, pH 7.4 was used. The reaction mixture consisted of 800 µL of 50 mM in sodium phosphate buffer, pH 7.4, 90 µL of 1 M glucose solution, and 10 µL of a 2.6 µM enzyme solution. This mixture was incubated at room temperature for 1 minute. After 1 minute, 100 µL of 30 mM *o*-NPGal was added to initiate the reaction. The change in absorbance at 405 nm was monitored over 90 seconds. The rate of reaction was calculated from the data collected and was compared to the uninhibited enzyme. After confirmation of inhibition, the concentration of glucose in the reaction mixture was varied to find the concentration that decreased the activity by 25% with respect to the uninhibited enzyme. It was found that a glucose concentration of 90 mM decreased the activity of GalB2 by 25%.

The activity of the enzyme using 90 mM glucose was evaluated while maintaining the concentration of glucose and varying the concentration of *o*-NPGal in the reaction mixture from 0.08 to 3.0 mM. The reaction mixture consisted of 800 µL of 50 mM in sodium phosphate buffer, pH 7.4, 90 µL of 1 M glucose solution in 50 mM sodium phosphate, pH 7.4, and 10 µL of a 2.6 µM enzyme solution. This mixture was incubated at room temperature for one minute. After one minute, *o*-NPGal was added to begin the reaction. The reaction was monitored over 90 seconds for the change in absorbance at 405 nm. The rate of reaction was calculated, and the data was used to form a Lineweaver-Burk plot to compare the results to the uninhibited enzyme.

2.2.8 Dependency from metal ion cofactors

To test dependency from magnesium ions, first, a control was established using 890 µL of 50 mM sodium phosphate buffer, pH 7.4, 100 µL of 30 mM *o*-NPGal in 50 mM sodium phosphate buffer, pH 7.4, and 10 µL of 2.6 µM GalB2. The reaction was monitored via the change in absorbance at 405 nm for 90 seconds, which was done in triplicate and the results were averaged.

Ethylenediaminetetraacetic acid (EDTA) was used as a chelating agent to strip divalent metal cofactors from GalB2. To do so, 50 µL of a 50 µM GalB2 solution was added to 700 µL of a 50 mM sodium phosphate buffer, pH 7.4, and 200 µL of 0.25 M EDTA in 50 mM sodium phosphate buffer, pH 7.4. The mixture was left to incubate for 35 minutes at room temperature. After incubation, the activity was tested by mixing 890 µL 50 mM sodium phosphate buffer, pH 7.4, 100 µL of 30 mM *o*-NPGal in 50 mM sodium phosphate buffer, pH 7.4, and 10 µL of the EDTA-treated GalB2 solution in a 1-mL cuvette. The change in absorbance at 405 nm was monitored for 90 seconds.

Once evidence showed that incubation with EDTA decreased the activity of the enzyme, the dependency from magnesium was assessed by reintroducing it to GalB2. This was achieved by mixing 690 µL of 50 mM sodium phosphate buffer, pH 7.4, 10 µL of the EDTA-treated GalB2 solution, and 200 µL of 1 M magnesium chloride in 50 mM sodium phosphate buffer, pH 7.4, in an Eppendorf tube and incubating the solution for 35 minutes at room temperature. After incubation, the mixture was transferred to a 1-mL cuvette where 100 µL of 30 mM *o*-NPGal in 50 mM sodium phosphate buffer, pH 7.4, was added to begin the reaction. The reaction was done in triplicate and monitored for the change in absorbance at 405 nm for 90 seconds.

2.2.9 Thermal stability of GalB2

Into three 0.2 mL PCR tubes, 100 µL of 2.6 mM GalB2 solution was added, respectively. The tubes were then placed in a Bio-Rad thermal cycler and incubated at 37 ºC for 20 minutes. After incubation, the tubes were allowed to cool to room temperature. The activity was assessed by *o*-NPGal hydrolysis by GalB2. Into a 1-mL cuvette, 890 µL of 50 mM sodium phosphate buffer, pH 7.4, was mixed with 100 µL of 30 mM *o*-NPGal in 50 mM sodium phosphate buffer, pH 7.4, and used to blank the spectrophotometer. The pre-incubated solution of GalB2 (10 µL) was added last to initiate the reaction. The reaction was monitored for the change in absorbance at 405 nm for 90 seconds. The assay was performed in triplicate for GalB2 incubated at 40 ºC, 45 ºC, 50 ºC, and 55 ºC.

2.2.10 Substrate specificity of GalB2

To test the substrate specificity of GalB2, 30 mM solutions were prepared in 50 mM sodium phosphate buffer, pH 7.4. The saccharides tested were lactose, raffinose, and laminarin. The general reaction mixture was prepared in an Eppendorf tube and consisted of 890 µL of 50 mM sodium phosphate buffer, pH 7.4, 100 µL of 30 mM substrate, and 10 µL of a 2.6 µM GalB2 solution. The reaction mixture was analyzed by TLC after 30 minutes and overnight. The reaction mixture was spotted on the TLC plate alongside standards such as glucose, lactose, raffinose, and laminarin to assess the occurrence of hydrolysis. The plate was developed in a 2:1:1 (v/v/v) 1-butanol:acetic acid:water solution, dried in the air, sprayed with 5% sulfuric acid in ethanol containing a small quantity of orcinol, and placed on a hot plate until spots appeared.

2.2.11 Lactose hydrolysis by GalB2

A glucose standard curve was constructed as follows. A stock solution of 10 mM glucose in 50 mM sodium phosphate buffer, pH 7.4, was prepared and was used to make 5, 2.5, and 1 mM glucose solutions in 50 mM sodium phosphate buffer, pH 7.4. For each glucose concentration, 50 µL of solution was added to a reaction cocktail, which contained 1.75 mL of 50 mM sodium phosphate buffer, pH 7.4, 1 mL of phenol/4-aminoantipyrine (170 mM/2.5 mM in distilled water), 100 µL of glucose oxidase (1 mg/mL in 50 mM sodium phosphate buffer, pH 7.4), and 100 µL peroxidase (1 mg/mL in 50 mM sodium phosphate buffer, pH 7.4). The mixture was incubated for 40 minutes in a 3-mL cuvette, and then the absorbance at 510 nm was measured using a spectrophotometer, which was blanked with the reaction cocktail. This was done in triplicate and averaged. The averaged data was then used to plot a standard curve.

To assess lactose hydrolysis by GalB2, a reaction mixture consisting of 775 µL of 50 mM sodium phosphate buffer, pH 7.4, 200 µL of varying lactose concentrations in 50 mM sodium phosphate buffer, pH 7.4, and 25 µL of 2.6 µM GalB2 in 50 mM sodium phosphate buffer, pH 7.4, was placed in an Eppendorf tube. The mixture was incubated for 20 minutes at room temperature and then heated at 95° C for 3 minutes. Next, 50 µL of the heated reaction mixture was added to the cocktail reagent described previously. After 40 minutes, the mixture was analyzed for absorbance at 510 nm using a spectrophotometer. This was done in triplicate and averaged. The rates of the reaction were calculated using the glucose standard curve and used to construct a Michaelis-Menten and a Lineweaver-Burk plot.

2.2.12 Assessment of quaternary structure of GalB2

Four lanes of a native polyacrylamide gel with 4.5% stacking gel and 6% resolving gel, with at least one lane separation, were loaded with 15 µL of a GalB2/native loading dye solution (30 µL of 10 µM GalB2 and 30 µL of native loading dye) each. The gel was then run for about 50 minutes at 200 V in the native gel running buffer. Upon completion of electrophoresis, the gel was divided in half. One half was developed with Coomassie brilliant blue stain for protein detection, and the other half was assessed for enzymatic activity using 4-MUGal. For enzymatic assessment, the gel was incubated in 50 mM sodium phosphate buffer, pH 7.4, for 10 minutes at room temperature. Following this, the buffer was removed, and the gel was incubated in a 5 mM 4-MUGal solution in 50 mM sodium phosphate buffer, pH 7.4, for 20 minutes at room temperature. Finally, this gel portion was assessed under UV light.

2.2.13 Formation of chitosan-silica beads for protein immobilization

In a 50 mL beaker, 100 mg of chitosan powder was mixed with 10 mL of 1 M acetic acid until the powder was dissolved. Then, 100 mg of silica HF gel was added and mixed well. To form the beads, the solution was carefully added dropwise using a micropipette to a petri dish containing a solution of 12% (m/v) NaOH in 20% (v/v) EtOH. Once all the chitosan-silica mixture was used, the beads were transferred to a beaker. The basic solution was carefully removed, and the beads were washed with deionized water until the solution had a pH of ~7.

The enforcement of the chitosan-silica beads was done using glutaraldehyde to crosslink chitosan. To do so, a 0.1% aqueous glutaraldehyde solution (25 mL) was made from a stock solution of 25% glutaraldehyde in water. The deionized water was removed from the beaker containing the chitosan-silica beads and replaced with the 0.1% aqueous glutaraldehyde solution

(25 mL). The mixture was then shaken at 100 rpm overnight at 4 ºC. After incubation, the glutaraldehyde solution was removed, and the beads were washed with deionized water until the solution containing the beads reached a pH ~7.

2.2.14 Preparation and immobilization of lactase and GalB2 on chitosan-silica beads

Using a mortar and pestle, 2 tablets from the commercially available Fast Acting Dairy Relief were ground into a powder and mixed with 10 mL of 100 mM potassium phosphate buffer, pH 6.8. The solution was centrifuged at 4,000 rpm for 10 minutes. The supernatant was saved and stored at 4 ºC, while the pellet was discarded.

To immobilize lactase, the water was removed from the beaker containing the glutaraldehyde cross-linked chitosan-silica beads and replaced with 10 mL of lactase solution and 25 mL of 100 mM potassium phosphate buffer, pH 6.8. This solution was shaken at 100 rpm and allowed to incubate at 4 ºC overnight. After incubation, the solution was removed and saved, and the beads were rinsed with deionized water. Finally, the immobilized lactase was stored at 4 ºC.

To immobilize GalB2, 30 chitosan-silica beads cross-linked with glutaraldehyde were placed into an Eppendorf tube. Then, 30 µL of the concentrated GalB2 solution were added and allowed to incubate overnight at 4 °C while being shaken at 100 rpm. After incubation, the remaining enzyme solution was removed, transferred to a separate Eppendorf tube, and stored at 4 °C. Finally, 1 mL of 50 mM sodium phosphate buffer, pH 7.4 was added to the Eppendorf containing the immobilized GalB2 and the tube was stored at 4 °C.

2.2.15 Transglycosylation capability of lactase from lactose intolerance supplement

Six chitosan-silica beads containing immobilized lactase were placed in an Eppendorf tube and 1 mL of a 40% (v/v) lactose solution in 50 mM sodium phosphate buffer, pH 7.4, was added. The

Eppendorf tube was inverted once to mix. The tube was placed in a 40 ºC water bath for 3 hours. Aliquots of 100 µL were taken every 20 minutes and immediately placed in the refrigerator at 4º C until further testing. For analysis, the aliquots along with standard solutions of lactose, glucose, and galactose were spotted on a TLC plate and developed using a 2:1:1 (v/v/v) 1-butanol:acetic acid:water solution. The plate was allowed to dry, then sprayed with 5% sulfuric acid in ethanol solution with orcinol and placed on a hot plate. The sugars appeared on the plate as black and/or purple spots and were compared to the standards.

2.2.16 Thermal stability of immobilized GalB2

Six chitosan-silica beads containing immobilized GalB2 were placed into an Eppendorf tube containing 900 µL of 50 mM sodium phosphate buffer, pH 7.4, and equilibrated for 5 minutes. The tube was then placed in a 45 ºC water bath and incubated for 10 minutes. After incubation, the Eppendorf tube was removed from the water bath and allowed to cool in a room-temperature water bath for 25 minutes. Finally, the Eppendorf tube was removed from the water bath, and 100 µL of 30 mM *o*-NPGal in 50 mM sodium phosphate buffer, pH 7.4, was added. The reaction was carried out for 10 minutes, then the solution was transferred to a 1-mL cuvette and the absorbance was measured at 405 nm. The spectrophotometer was blanked with 890 µL of 50 mM sodium phosphate buffer, pH 7.4, mixed with 100 µL of 30 mM *o*-NPGal in 50 mM sodium phosphate buffer, pH 7.4. This was done for temperatures of 50 ºC, 55 ºC, and 60 ºC. Each temperature was tested in triplicate and the absorbances at 405 nm were averaged.

2.2.17 Transglycosylation potential of immobilized GalB2

Twelve chitosan-silica beads containing immobilized GalB2 were placed in an Eppendorf tube and 1 mL of 350 mM lactose solution in 50 mM sodium phosphate buffer, pH 7.4, was added.

The Eppendorf tube was inverted once to mix. The tube was placed in a 40 ºC water bath for 2 hours. Aliquots of 100 µL were taken every 20 minutes and immediately placed in the refrigerator at 4 ºC until further testing. For analysis, the aliquots were diluted 10-fold with 50 mM sodium phosphate buffer, pH 7.4, and were spotted on a TLC plate, along with standard solutions of lactose and raffinose, and the TLC plate was developed using a 2:1:1 (v/v/v) 1-butanol:acetic acid:water solution. The plate was allowed to dry, then sprayed with 5% sulfuric acid in ethanol solution containing orcinol and placed on a hot plate. The sugars appeared on the plate as black and/or purple spots.

2.2.18 Column chromatography of saccharides

A 5-mL glass pipette, 0.3 cm in diameter, was filled with silica gel (200-500 µm, 60 Å porosity) to a height of 12.6 cm. The column was equilibrated with a 4:1 (v/v) acetonitrile:water solution. Standard sugar solutions for glucose, lactose, and raffinose were made at 50 mM concentrations in 4:1 (v/v) acetonitrile:water and were added to the column as 100 µL aliquots. Fractions of 0.5 mL were collected and spotted on a TLC plate. The plate was then sprayed with 5% sulfuric acid in ethanol containing orcinol and placed on a hot plate. If carbohydrates were present, they showed on the TLC plate as black spots for higher concentrations and purple for lower concentrations. The fractions showing carbohydrates were then spotted on a TLC plate, alongside carbohydrate standards and developed in a 2:1:1 (v/v/v) 1-butanol:acetic acid:water solvent system. A TLC plate was then sprayed with 5% sulfuric acid in ethanol containing orcinol and heated on a hot plate.

2.2.19 LC/ESI-MS analysis of saccharides

A Bruker Esquire-LC with solvents fed via an HP Agilent 1100 HPLC was used for mass spectral analysis. In this case, the flow of solvents bypassed the column RPM-Monosaccharide column and aided the delivery of analyte to the ion trap. To find the optimal tuning for each saccharide, 50 mM solutions of glucose, galactose, lactose, and raffinose were prepared in deionized water. A volume of 250 µL of each sample was analyzed via direct injection in positive ion mode. The mobile phase of the LC was a 1:1 HPLC grade water:HPLC grade methanol solution. The flow rate was 0.1 mL/min.

Once the tunings were obtained, 50 µL aliquots of the transglycosylation reaction were analyzed in positive ion mode by direct injection under the optimum tuning found for disaccharides: a flow rate of 0.1 mL/min, nebulizer set to 30.0 psi, a dry gas flow of 8.00 L/min, a dry temperature of 325º C, skimmer 1 set to 41.5 V, the octupole set to 2.97 V, and the drive trap set to 47.3. Since ESI-MS commonly forms sodium adducts, $[M+Na]^+$, peaks of interest were at a *m/z* = 365, 527, and 689 for di-, tri-, and tetrasaccharides, respectively.

Chapter 3: Results and Discussion

This study on β-galactosidase GalB2, a protein from *Enterobacter* sp. YSU was done in an effort to understand the biochemical capabilities of the enzyme along with its basic characterization. The β-galactosidase was expressed in *E. coli* cells, isolated, and purified into a concentrated solution which was used to perform all the tests described above. General characteristics of the enzyme such as optimal pH, thermal stability, and quaternary structure were investigated. The kinetic parameters were evaluated using the model substrate *o*-NPGal and lactose. Galactose and glucose were examined for their inhibitory effects on the enzyme's activity. GalB2 was

investigated for its potential to perform transglycosylation reactions using lactose as a substrate. Attempts to isolate and characterize synthesized oligosaccharides were done based on previous efforts in the Stourman lab using techniques such as column chromatography and ESI-MS.

3.1 Expression and purification of GalB2

The *E. coli* BL21(DE3) cells containing the *pET20b-lacZ2* plasmid were used to express the β-galactosidase, GalB2. The cells were grown at 37 ºC in an LB-broth containing ampicillin and harvested by centrifugation. To isolate GalB2, the pellet was sonicated and then centrifuged to remove cell debris from the supernatant. The supernatant was treated with streptomycin sulfate to precipitate DNA. The precipitate was removed by centrifugation, and then ammonium sulfate was added to the supernatant until 45% saturation to precipitate proteins. The ammonium sulfate was dialyzed out and the remaining content was loaded onto a Q-Sepharose, a strong anion exchanger, column, equilibrated with 50 mM sodium phosphate buffer, pH 7.4. The pI for GalB2 was calculated to be 5.53 using the ProtParam tool, therefore, the enzyme would carry a negative charge and bind to the column at a pH of 7.4. The proteins were eluted with a sodium chloride gradient. The absorbance of fractions collected from the Q-Sepharose column was measured at 280 nm. The fractions with the highest absorbances were tested for galactosidase activity by placing a drop on a spot plate and adding *o*-NPGal. Any spot that turned yellow indicated the presence of GalB2 because the hydrolysis of *o*-NPGal releases *o*-nitrophenolate (**Figure 4**). These fractions were then combined and concentrated. About 1-mL of the concentrated solution was obtained. The protein concentration of 66 μM was calculated based on the absorbance at 280 nm and the extinction coefficient for GalB2, 268,710 $M^{-1}cm^{-1}$, which was calculated from the protein sequence using the ProtParam tool.[29]

Figure 4. Spot plates were used for a visual test of β-galactosidase activity. Small amounts of solution from the Q-Sepharose fractions that showed high absorbance at 280 nm were mixed with a small amount of *o*-NPGal. The fraction number corresponds to the spot on the plate.

3.2 Evaluation of GalB2 optimal pH

To assess the optimal pH for GalB2, the enzymatic activity was tested using buffers with a pH ranging from 5.0 to 9.0. GalB2 solutions were made with a concentration of 2.6 μM for each pH, along with solutions of the substrate, *o*-NPGal, which had a concentration of 30 mM. The substrate was mixed with enzyme and buffer and incubated for 90 seconds. After this, 0.5 M sodium carbonate was added to stop the reaction. The solution was then transferred to a 3-mL cuvette and the absorbance at 405 nm was measured. The activity tests for each pH were conducted in triplicate with the average A_{405} being plotted against the pH (**Figure 5**). The absorbance at 405 nm is proportional to the concentration of *o*-nitrophenolate, however, the extent of *o*-nitrophenolate deprotonation depends on the pH. To eliminate the phenol ionization differences between various pH, sodium carbonate was added at the end of the reaction to raise the pH and ensure the complete deprotonation of *o*-nitrophenol. From the data collected, GalB2 demonstrated an optimum pH of 7.4. Within ±2 pH units of 7.4, the activity of the enzyme was

lower, and acidic conditions had a greater negative effect on the hydrolytic activity than basic conditions.

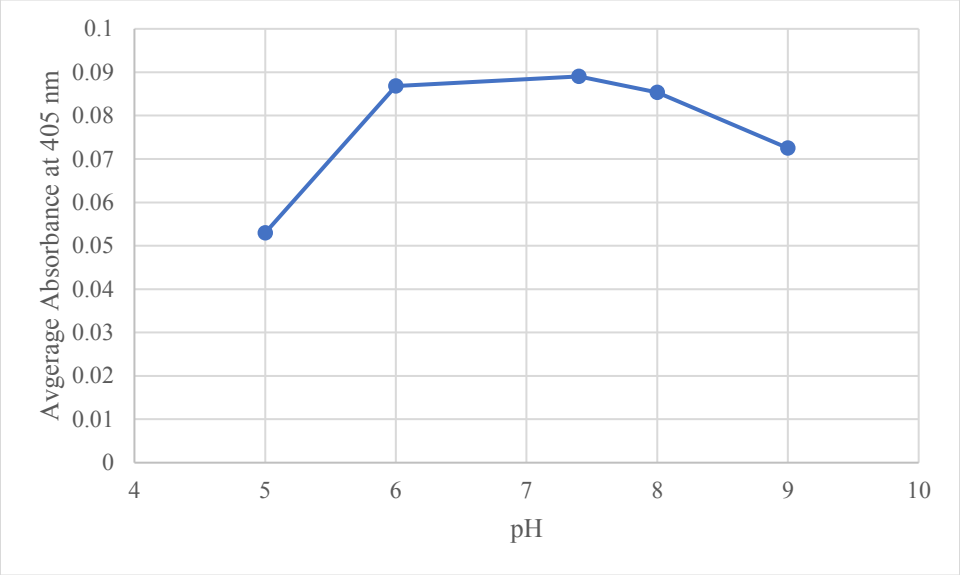


Figure 5. A plot showing the impact of pH on the hydrolytic activity of GalB2 with *o*-NPGal as a substrate.

3.3 Enzymatic activity test with *o*-NPGal as a substrate

The hydrolytic activity for GalB2 was tested using the model substrate, *o*-NPGal. Not only does this give visual evidence of enzyme activity but the change in absorbance at 405 nm can be measured and can be used to calculate the reaction rate to construct a Michaelis-Menten plot and determine the enzyme's kinetic parameters. Therefore, the reaction was monitored using a UV-Vis spectrophotometer for the change in absorbance at 405 nm over 90 seconds. This data was then used to calculate the initial reaction rate at a given substrate concentration using the extinction coefficient of *o*-nitrophenolate, 3055 $M^{-1}cm^{-1}$.

Based on the data collected, the Michaelis-Menten plot (**Figure 6**) revealed that GalB2 had a maximal velocity, V_{max} of 1.17 µM/s. With a ½ V_{max} of 0.585 µM/s, the Michaelis constant, K_M,

the concentration of substrate to reach ½ $V_{max,}$ for *o*-NPGal was determined to be 0.18 mM. To find a catalytic constant, k_{cat}, the V_{max}, 1.17 µM/s, was divided by the enzyme concentration of 0.026 µM, resulting in a k_{cat} of 44 s^{-1}.

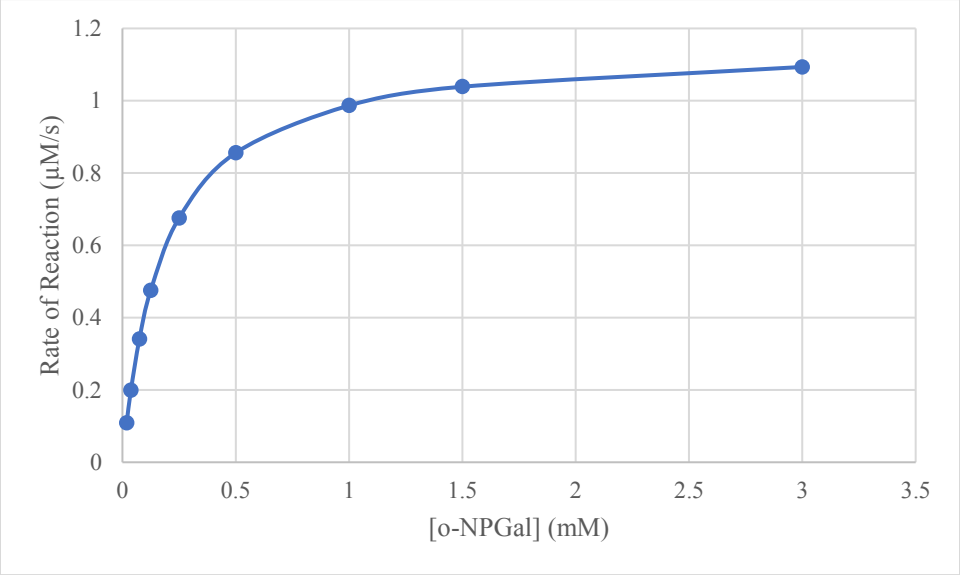


Figure 6. A Michaelis-Menten plot for the GalB2 catalyzed hydrolysis of *o*-NPGal.

3.4 Inhibitory effect of galactose and glucose

The inhibitory effect of galactose and glucose on the GalB2-catalyzed hydrolysis of *o*-NPGal was tested. The enzyme was subjected to varying concentrations of inhibitor while the enzyme concentration remained at 0.026 µM and the *o*-NPGal concentration was maintained at 3 mM. First, the concentrations of galactose that reduced the rate of reaction by 25% and 50% were determined as 150 mM and 330 mM, respectively. The enzyme activity was then tested for each concentration of galactose with concentrations of *o*-NPGal within the reaction mixture ranging from 0.13-3.0 mM. The change in absorbance at 405 nm was used to calculate the rate of reaction and to build Lineweaver-Burk plots for the inhibited enzyme and the control (uninhibited enzyme).

For glucose, the same approach was taken except the concentration that decreased activity by 50% was not used as it was too high (1 M glucose). Therefore, only the concentration of glucose in the reaction mixture that resulted in a 25 % decrease of the reaction rate was used and found to be 90 mM. As with the prior experiment with galactose, the reaction rates were determined with a constant concentration of glucose at 90 mM and an enzyme concentration of 0.026 μM. The same range (0.13-3.0 mM) of *o*-NPGal substrate concentrations was tested, and the change in absorbance at 405 nm was monitored. The rate of reaction was calculated based on this data and the extinction coefficient of 3055 $M^{-1}cm^{-1}$ for *o*-nitrophenolate. A Lineweaver-Burk plot was constructed and the uninhibited enzyme and glucose-inhibited enzyme were compared (**Figure 7**).

The results from testing the inhibitory effect of galactose were indicative of competitive inhibition. As seen in **Figure 7**, the y-intercept, that represents $1/V_{max}$, of each line is relatively close to each other showing that V_{max} is not greatly affected by the presence of galactose. The x-intercepts, $1/-K_M$, continue to decrease as the concentration of galactose increases. This indicates the increase in K_M, representative of competitive inhibition in which higher concentrations of inhibitor requires more substrate to reach ½ V_{max}. Using the equations for the best-fit lines for 150 mM and 330 mM galactose, the V_{max} was determined to be 0.595 μM/s for 150 mM galactose and 0.549 μM/s for 330 mM galactose. Regarding K_M, 150 mM galactose was found to increase the K_M to 0.842 mM while 330 mM galactose increased it to 1.37 mM.

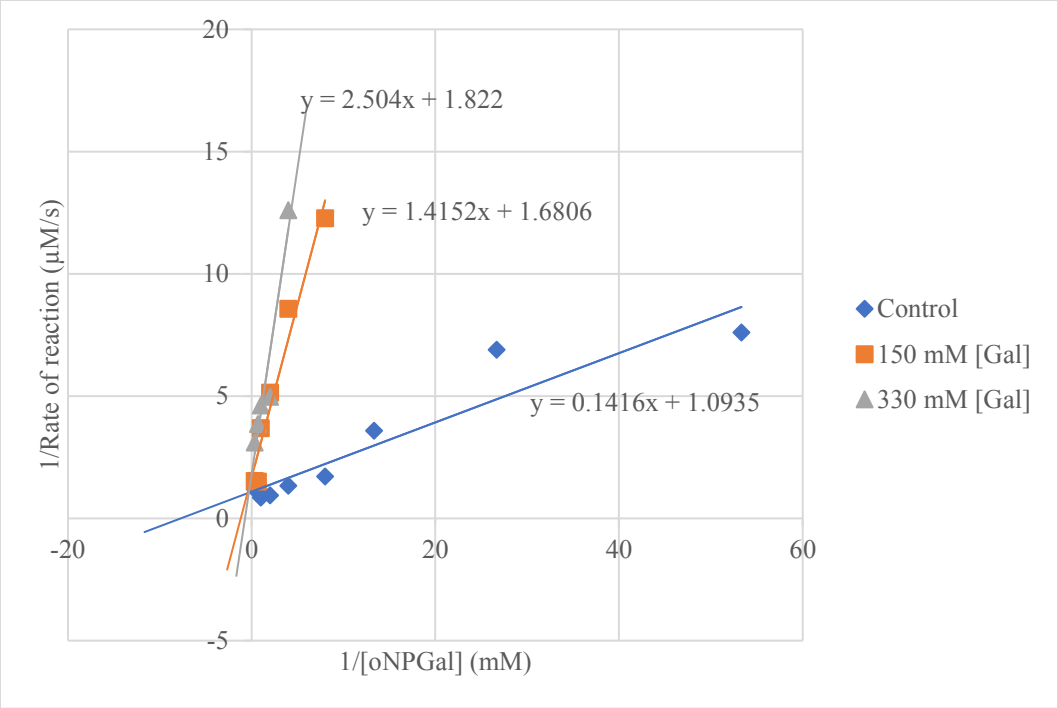


Figure 7. A Lineweaver-Burk plot of the competitive inhibition effect of galactose (Gal) on the hydrolysis of *o*-NPGal by GalB2.

Based on the results found for glucose inhibition of GalB2 (**Figure 8**), the Lineweaver-Burk plot matches the characteristics of an uncompetitive inhibitor. There $1/V_{max}$ and $1/-K_M$ were altered in a relatively proportional manner by glucose. This is evident by the parallel nature of the best-fit lines of uninhibited GalB2 and glucose-inhibited GalB2 (**Figure 8**). In the presence of 90 mM glucose, the V_{max} of the uninhibited enzyme decreased from 0.143 µM/s to 0.11 µM/s, while the K_M shifted from 0.67 mM to 0.097 mM. Both parameters decreased approximately by an 8-fold in the presence of inhibitor.

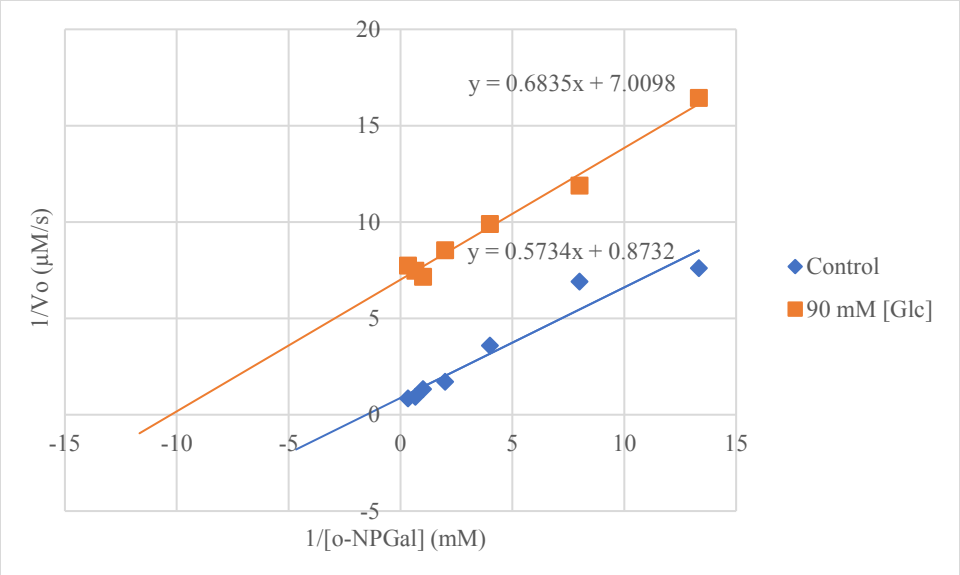


Figure 8. A Lineweaver-Burk plot of the uncompetitive inhibition effect of glucose (Glc) on the hydrolysis of *o*-NPGal by GalB2.

3.5 Effect of magnesium(II) ions on GalB2

The UniProt data for β-galactosidase from *Enterobacter cloacae* which is homologous to GalB2 indicated that sodium and magnesium ions may be cofactors for GalB2. To test dependency from the magnesium ions, GalB2 was treated with EDTA to chelate the ions, and then the hydrolytic activity of the enzyme was tested with *o*-NPGal and compared to GalB2 without EDTA. Then, magnesium chloride was added to the GalB2 sample treated with EDTA to reintroduce magnesium ions, and the activity was tested again. Sodium was not evaluated because the means to test its impact was beyond the lab's available resources. As **Figure 9** shows, when GalB2 was treated with EDTA, there was an 89% decrease in activity. With the reintroduction of magnesium ions, the activity was recovered but only 63% of activity was achieved relative to native GalB2. This means magnesium plays a critical role in the activity of GalB2, but other cofactors may also be necessary to restore the enzyme's activity to its native state.

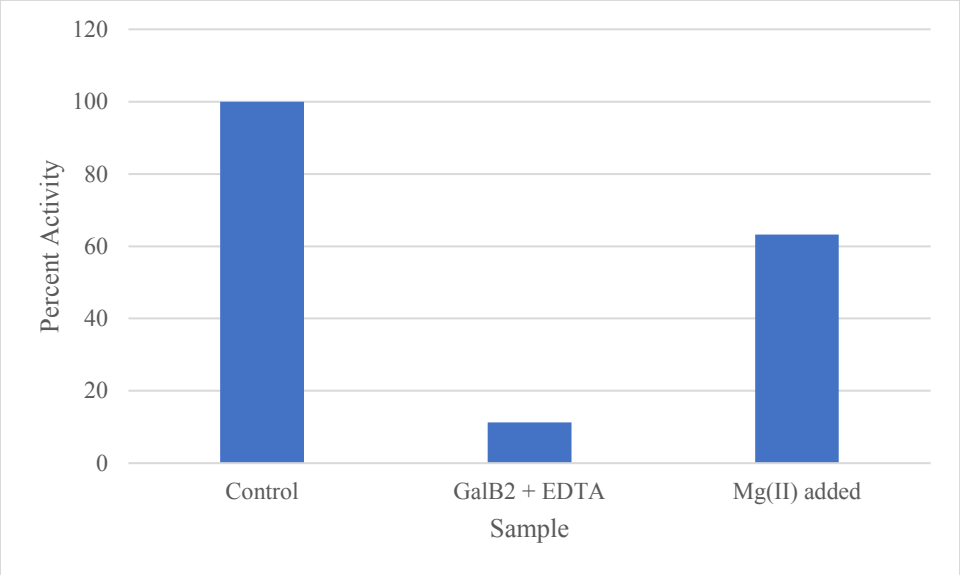


Figure 9. A bar graph depicting the Mg^{2+} ion dependency of GalB2.

3.6 Thermal stability of GalB2

The thermal stability of enzymes is an important property, especially with respect to transglycosylation as these reactions are generally done at 40 ºC or higher. Therefore, GalB2 was incubated at temperatures ranging from 37 ºC to 55 ºC independently, and the impact of temperature on the enzyme activity was investigated using *o*-NPGal as a substrate. As seen in **Figure 10**, the highest activity was demonstrated after incubation of GalB2 at 37 ºC but as the incubation temperature increased, the rate of reaction decreased. For the incubation temperature of 37 ºC to 45 ºC, there was a minimal loss in the reaction rate, 7% decrease compared to 37 °C incubation. However, for temperatures higher than 45 ºC, there was a greater loss in the reaction rate – a 99% decrease in the rate of reaction. Incubation at 55 °C led to a complete loss of enzymatic activity.

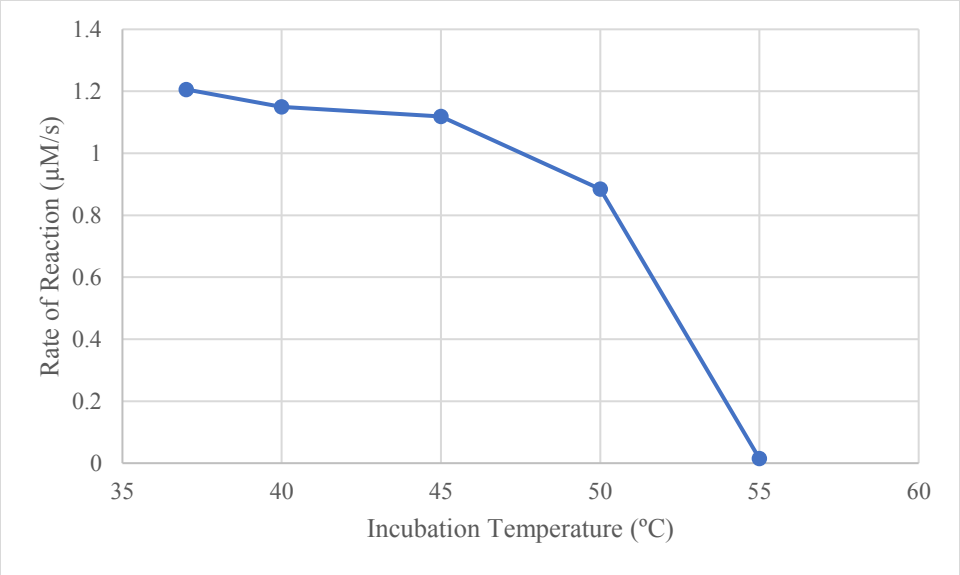


Figure 10. A plot showing the thermal stability of GalB2.

3.7 Substrate Specificity of GalB2

A substrate specificity assay was conducted to investigate what kind of glycosidic bonds could be hydrolyzed by GalB2. The substrates selected were lactose (Gal(β1→4)Glc), raffinose (Gal(α1→6)Glc(α,β1→2)Fru), and laminarin (Glc(β1→3)Glc(β1→3)Glc). Based on the rate of the hydrolysis of *o*-NPGal by GalB2, the hydrolysis of the substrates was assessed at random intervals past the initial 30 minutes of the incubation. **Figure 11a** shows that lactose hydrolysis was not detected after 30 minutes but as seen in **Figure 11d**, all the lactose had been hydrolyzed after 24 hours. Evidence of the hydrolysis of raffinose was never observed. Laminarin initially showed a small reverse comet tail (**Figure 11d**), therefore the hydrolysis of laminarin was re-evaluated after one week. The reverse comet tail appears to have moved higher than the laminarin standard after 1-week incubation, including a faint spot above lactose, as seen in **Figure 11c**. This indicates that monosaccharides were present due to hydrolysis by GalB2. Therefore, GalB2 can hydrolyze β1→4 bonds and very slowly β1→3 glycosidic bonds.

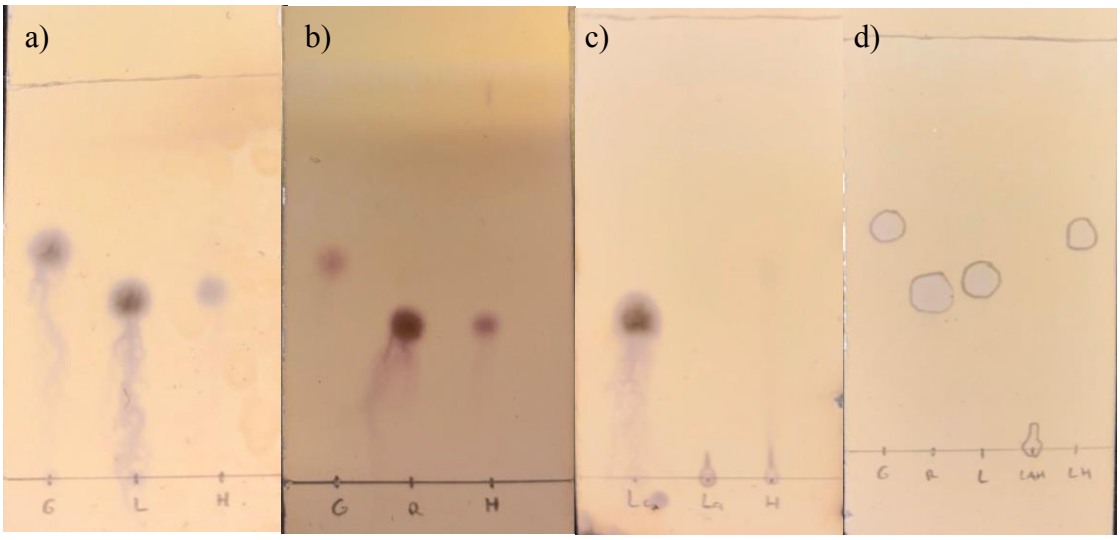
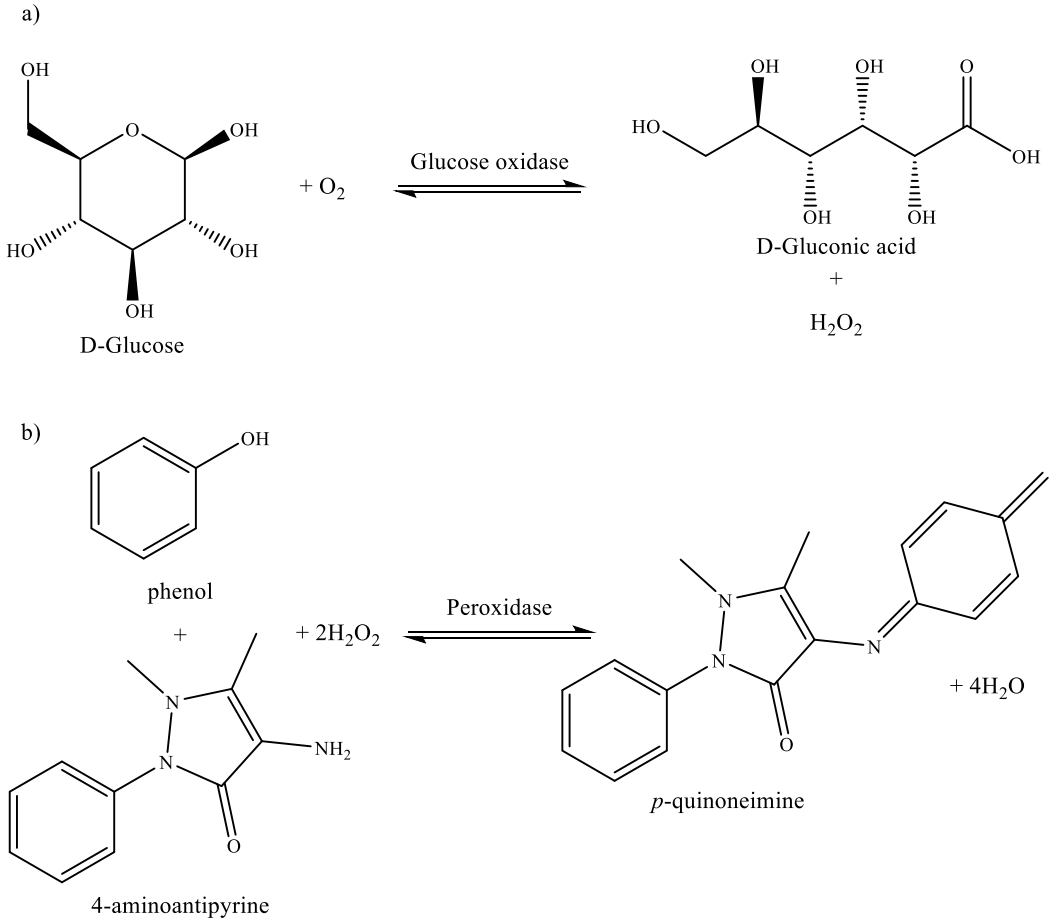


Figure 11. TLC plates of substrates lactose (11a & 11d), raffinose (11b), and laminarin (11c & 11d) when added to a GalB2 solution to see what glycosidic bonds could be hydrolyzed by the enzyme. G = glucose, L = lactose, H (plate a & b) = post-30-minute incubation with GalB2, H (plate c) = post-1-week incubation laminarin hydrolysis, R = raffinose, Lc = lactose, La = laminarin, LH = post-24-hour incubation lactose hydrolysis, LAH = post-24-hour incubation laminarin hydrolysis.

3.8 Activity assay using lactose as a substrate

Since lactose was the only oligosaccharide hydrolyzed by GalB2, the kinetic parameters for this substrate were further assessed. To evaluate the hydrolysis of lactose, a coupled assay was applied using two enzymes - glucose oxidase and peroxidase. When glucose is released from lactose by GalB2, it will be oxidized to D-gluconic acid and hydrogen peroxide will be released by glucose oxidase. The peroxidase, in the presence of hydrogen peroxide, phenol, and 4-aminoantipyrine, will form *p*-quinoneimine (**Scheme 1**), which is pink in solution and absorbs light at 510 nm. Standard solutions of glucose (1.0-5.0 mM) were used to build the calibration curve for quantification of glucose released from lactose. Each solution was mixed with the

reaction cocktail containing two enzymes and phenol/4-aminoantipyrinne. After the incubation, the absorbance of each solution was measured at 510 nm to develop a calibration curve for the lactose hydrolysis study (**Figure 12**).



Scheme 1. The coupled assay using glucose oxidase and peroxidase: a) glucose oxidase converts glucose to gluconic acid and hydrogen peroxide, and b) hydrogen peroxide being used to form *p*-quinonimine from phenol and 4-aminoantipyrine.

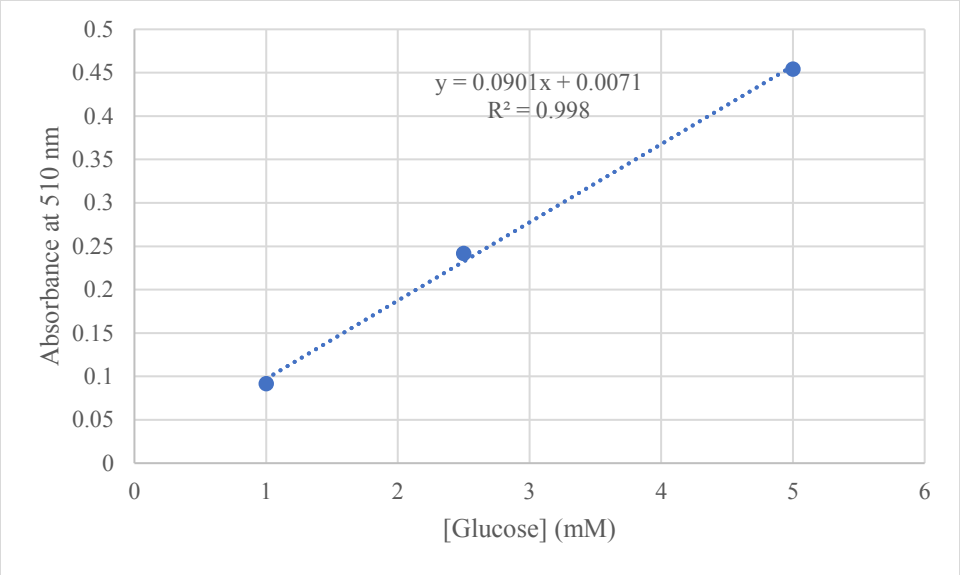


Figure 12. The calibration curve for glucose concentration.

After incubation of GalB2 with lactose, the reaction was stopped by heating the reaction mixture to denature the enzyme, then an aliquot of the reaction was mixed with the same reaction cocktail that was used for glucose quantification. The reaction rate was calculated from the amount of glucose formed over 20 minutes. To form the Michaelis-Menten and Lineweaver-Burk plots, a lactose concentration within the reaction mixture was varied from 0.5 mM to 70 mM. Using the Michaelis-Menten plot, the V_{max} was determined to be 0.055 mM/min with a K_M of 2.5 mM lactose. The k_{cat} was calculated to be 51 min^{-1}, based on the values of maximal velocity and a GalB2 concentration of 0.065 µM. Similar results were obtained from the Lineweaver-Burk plot, the V_{max} was determined to be 0.059 mM/min with a K_M of 2.9 mM lactose. The k_{cat} was calculated to be 55 min^{-1}.

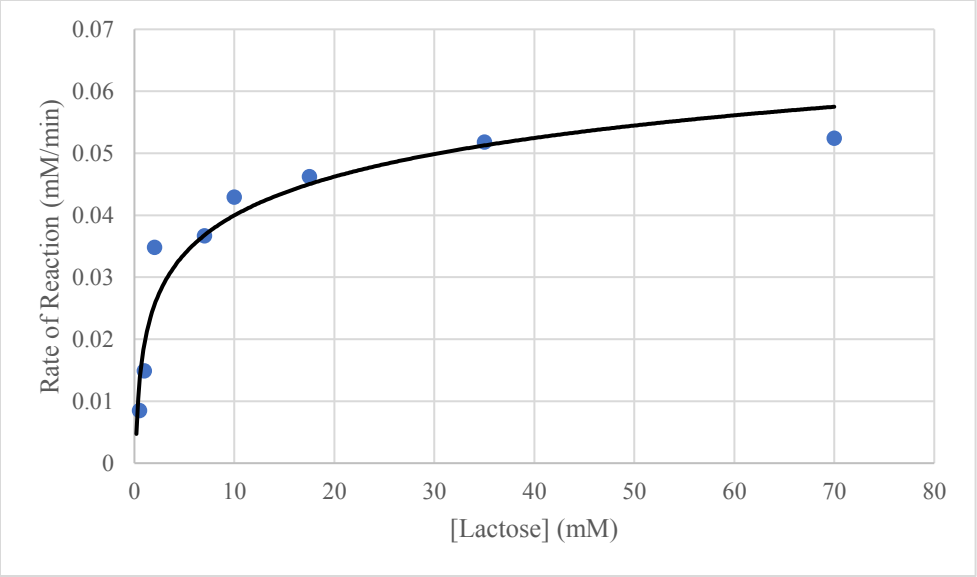


Figure 13. Michaelis-Menten plot for the hydrolysis of lactose by GalB2.

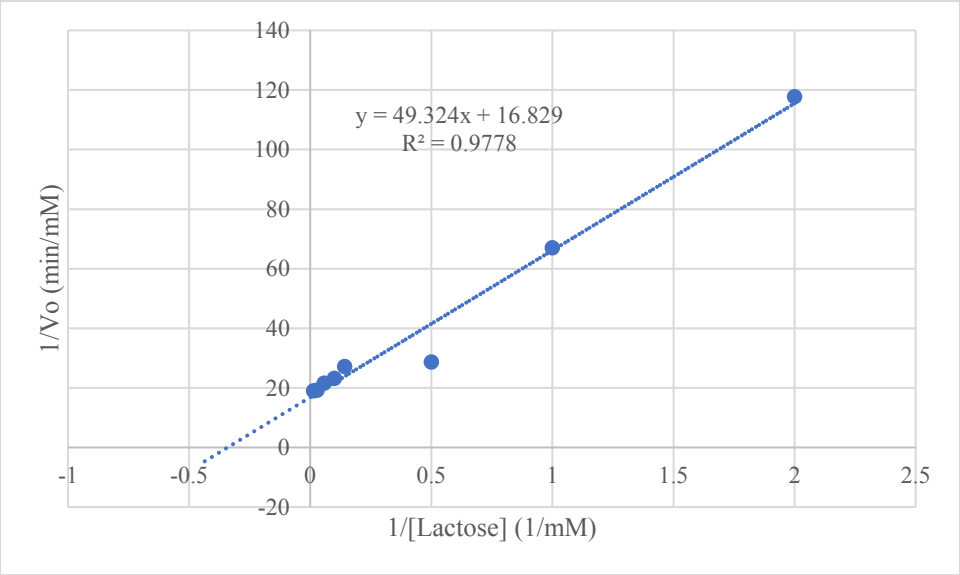


Figure 14. Lineweaver-Burk plot for the hydrolysis of lactose by GalB2.

3.9 Assessment of the quaternary structure of GalB2

Beta-galactosidase from *Enterobacter cloacae* which is homologous to GalB2 exists as a homodimer. The β-galactosidase, GalB2, could also potentially form a tetramer like that of *E.*

coli. GalB2 has a similar number of amino acid residues per monomer, 1038 residues versus 1023 amino acid residues per monomer for the *E. coli* protein.[9] To assess the oligomeric structure of GalB2, a native gel electrophoresis was used. GalB2 mixed with native loading dye was added to each of the four lanes and the gel was electrophoresed for about one hour at 200 V. After electrophoresis was complete, the gel was divided into two portions. One portion was stained with Coomassie brilliant blue to develop protein bands and the other was incubated with a 4-MUGal solution for 20 minutes and examined under UV light to assess galactosidase activity. The glowing band in **Figure 15a** is indicative of GalB2 which demonstrates galactosidase activity. Therefore, when comparing the position of the bands on the Coomassie brilliant blue stained gel (**Figure 15b**) and the position of the fluorescent band from the 4-MUGal treated gel (**Figure 15a**), the fluorescent band progresses to a point that lines up with that of the dimeric form of GalB2, meaning that only the dimer is necessary to maintain hydrolytic activity.

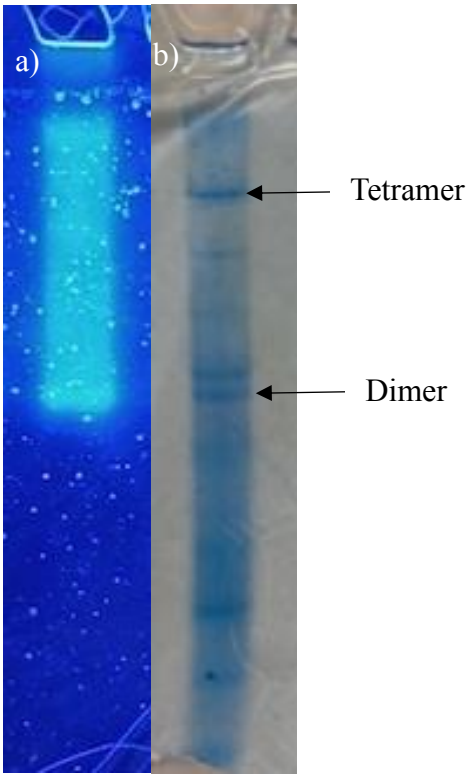


Figure 15. Assessment of the quaternary structure of GalB2 using native gel electrophoresis: a) portion of the gel treated with 4-MUGal to assess activity visualized under UV light and b) a portion of the gel stained with Coomassie brilliant blue.

3.10 Immobilization of lactase and GalB2 with glutaraldehyde cross-linked chitosan-silica beads

The chitosan-silica beads were made by releasing chitosan-silica mix into a basic alcohol solution maintaining similar bead size. The formed beads have a gel-like texture, even when cross-linked with glutaraldehyde. It was noted that there is a subtle color change of the beads when treated with glutaraldehyde, showing a yellowish tint (**Figure 16b**) compared to the initial white (**Figure 16a**). When lactase was immobilized, the beads became darker in color (**Figure 16c**), but this was not observed when GalB2 was immobilized. To test the GalB2 activity after immobilization, an observational study was done with *o*-NPGal. If GalB2 was leeching from the

beads, *o*-nitrophenolate would be expected to give off a yellow color throughout the solution, but if immobilization was successful, then *o*-nitrophenolate would be localized to the bead before it diffuses into the rest of the solution. A single bead with immobilized GalB2 was used for the test and after the introduction of *o*-NPGal, pictures were taken over the course of 5 minutes to note the progression of hydrolysis. As can be seen from left to right in **Figure 17**, the yellow color from *o*-nitrophenolate is initially localized to the bead, and over time it diffuses throughout the solution, which confirms the success of the immobilization of GalB2.

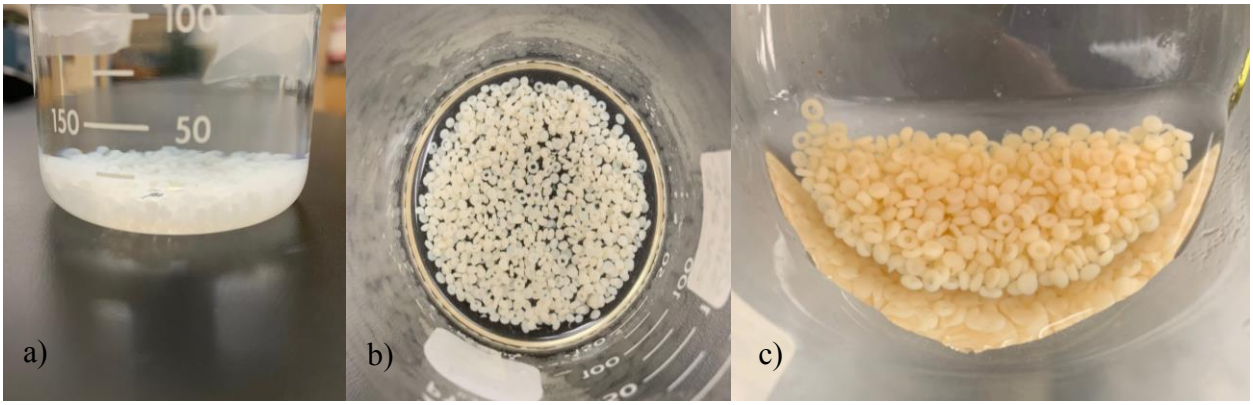


Figure 16. a) Formation of chitosan-silica beads, b) beads cross-linked with glutaraldehyde, and c) beads with immobilized lactase.

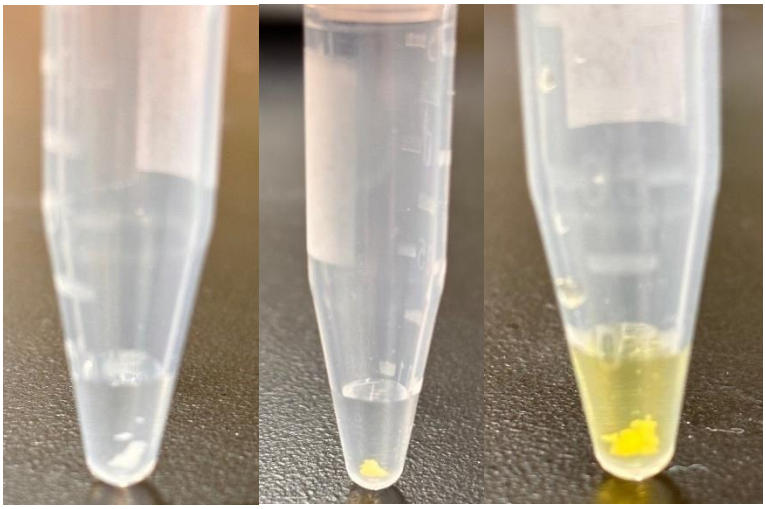


Figure 17. Confirmation of GalB2 activity after immobilization using *o*-NPGal.

3.11 Thermal stability of immobilized GalB2

As previously mentioned, the stability of the enzyme to endure temperatures above physiological temperatures is essential as this plays a major role in driving the kinetics of transglycosylation over the hydrolysis reactions catalyzed by galactosidases. Immobilized GalB2 was tested to confirm that the glutaraldehyde cross-linked chitosan-silica beads can withstand the high temperatures and potentially improve the thermal stability of GalB2. In anticipation of increasing thermal stability, the beads with immobilized enzyme were incubated at temperatures ranging from 45 ºC to 60 ºC. After incubation of the beads at a certain temperature for 10 minutes, the activity of the immobilized GalB2 was tested using o-NPGal. The beads enhanced the stability of GalB2 as it showed hydrolytic activity with the substrate *o*-NPGal when the beads were incubated at 60 ºC, when the free enzyme had essentially no activity at 55 ºC. The overall thermostability trend for immobilized GalB2 still mirrors the trend of free enzyme for temperatures greater than 45 ºC where there is a significant loss in activity.

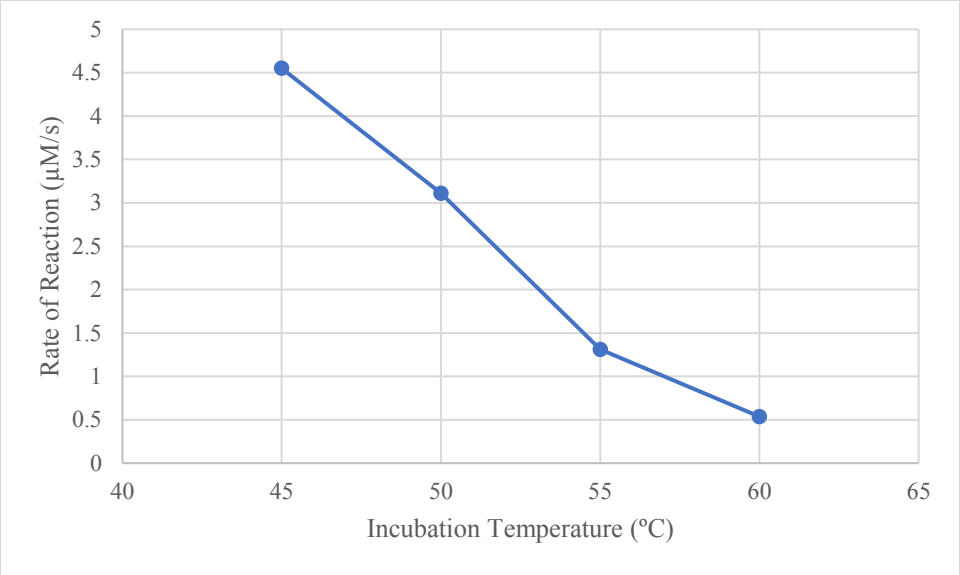


Figure 18. A plot of thermal stability of immobilized GalB2 on chitosan-silica beads cross-linked with glutaraldehyde.

3.12 Transglycosylation reactions with GalB2

In order to assess the potential for transglycosylation, immobilized GalB2 was exposed to 350 mM lactase in sodium phosphate buffer, pH 7.4, for 2 hours at 40 °C and 100 µL aliquots were taken at 20-minute intervals. The aliquots were diluted 10-fold and analyzed by TLC. Lactose and raffinose were used as standards for comparison as lactose is the starting material and raffinose is a trisaccharide. For the aliquots analyzed, all spots matched lactose and no spots were in line with raffinose. This indicates that either transglycosylation is not occurring or that the amount of oligosaccharides potentially synthesized is too small to be detected via TLC.

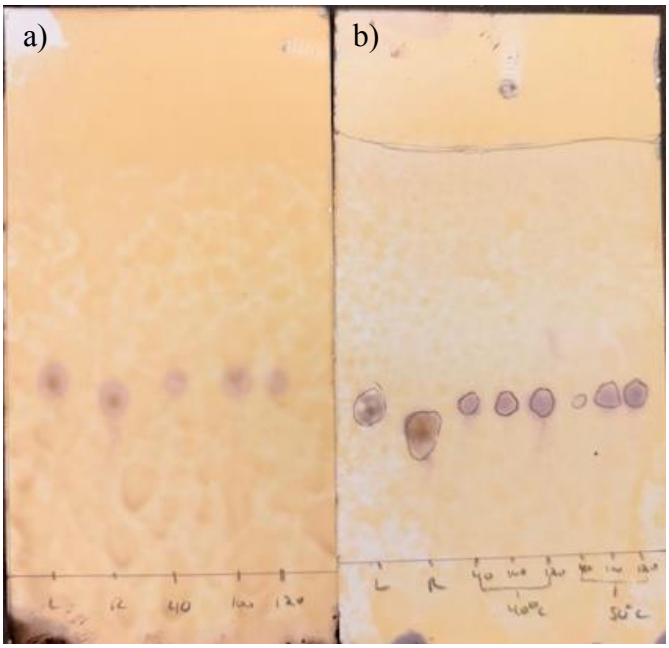


Figure 19. TLC plates spotted with 10-fold diluted reaction mixtures at intervals of interest (40, 100, and 120 minutes) to assess if oligosaccharides were synthesized. a) Transglycosylation reaction at 45 ºC, and b) transglycosylation reactions at 40 ºC and 50 ºC. L = 35 mM lactose standard, R = 30 mM raffinose standard.

3.13 Analysis of transglycosylation products produced by immobilized lactase

The lactase from over-the-counter lactose intolerant supplements immobilized on chitosan-silica beads cross-linked with glutaraldehyde was used for transglycosylation reactions. The beads were mixed with 40% (v/v) lactose solution and were incubated in a 40 ºC water bath for 2 hours to promote the synthesis of galacto-oligosaccharides. Aliquots of 100 µL were taken every 20 minutes and stored at 4 ºC. The aliquots were spotted on a TLC plate and checked for the presence of saccharides. From the aliquots tested on the TLC plate (**Figure 20a**), the ones showing the darker spots were combined into a single sample and directly injected into the ESI-MS with a 1:1 (v:v) water: methanol solvent. Using the optimum disaccharide tuning described

in the Materials and Methods section, a mass spectrum was obtained (**Figure 20b**). The larger peak at *m/z* = 365.1, the sodium adduct of lactose, is expected as a high concentration of lactose was used in the transglycosylation reaction and sodium adducts are common when using the positive ion mode in ESI-MS. The presence of the *m/z* peaks at 527.1 and 689.1 supported transglycosylation process. These peaks are representative of sodium adducts of a tri- and tetrasaccharide, respectively. As the sample was analyzed, the apparent ratio in the intensity of tri- to disaccharide signal seemed to be steady at a 1:2 ratio while the intensity ratio between tetra- and trisaccharide signals was steady at a 1:5 ratio.

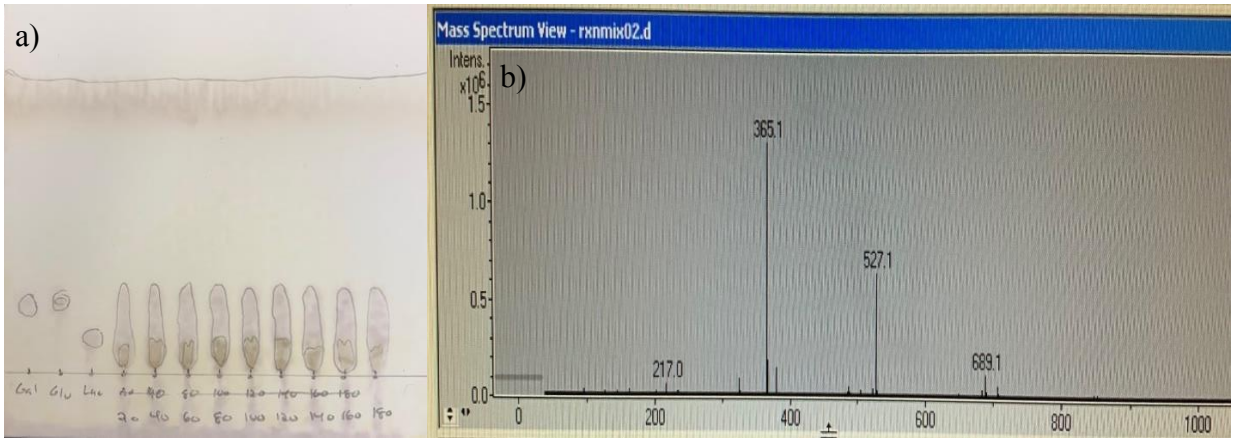


Figure 20. a) A TLC plate spotted with aliquots from the transglycosylation reaction and developed using 5% sulfuric acid in ethanol containing orcinol. b) A mass spectrum of a sample of the combined aliquots, 40-120 minutes, showing the $[M+Na]^+$ peaks of interest at a *m/z* = 365, 527, and 689.

3.14 Column chromatography of oligosaccharides

Of the nine aliquots collected from the transglycosylation reaction with immobilized lactase, aliquots collected between 100 and 160 minutes were combined. From the combined aliquots, 100 µL was added to a silica gel column with 4:1 (v/v) acetonitrile:water as the mobile phase. The first 10 mL were collected as wash since the desired compounds were expected to elute after that volume. After the wash, forty 0.5 mL-fractions were collected. These fractions were spotted on a TLC plate, sprayed with 5% sulfuric acid in ethanol mixed with orcinol, and heated on a hot plate to identify the fraction containing saccharides. Several fractions were then analyzed by TLC using 2:1:1 (v/v/v) 1-butanol:acetic acid:water as the elution solvent. As shown in **Figure 21**, the separation of oligosaccharides was not fully achieved as none of the fractions contained a single spot which would demonstrate one type of saccharide.

Figure 21. A developed TLC plate spotted with fractions collected from a silica gel column loaded with an aliquot from the transglycosylation reaction with immobilized lactase and 40% (v/v) lactose in 50 mM sodium phosphate buffer, pH 7.4. Raffinose (far left) was used as a standard for trisaccharides (The upper two spots in the raffinose sample are the hydrolysis products upon exposure to water).

Chapter 4: Conclusion

The novel enzyme, GalB2 from *Enterobacter* sp. YSU, is in the enzyme family of β-galactosidases, which hydrolyze β-glycosidic bonds formed by the monosaccharide galactose. The aim of this project was to purify and characterize this enzyme with respect to its thermal stability, optimal pH, substrate specificity, metal ion dependency, inhibitors, catalytic parameters, and the ability to catalyze a transglycosylation reaction. Some attempts were made to separate and characterize oligosaccharides, the products of transglycosylation reactions catalyzed by commercially available lactase.

The enzyme GalB2 was determined to have a Michaelis constant, K_M of 0.18 mM, and a catalytic constant, k_{cat} of 44 s^{-1} with respect to *o*-NPGal as the substrate. When lactose was used as a substrate the K_M was determined to be 2.5 mM, and k_{cat} was calculated as 51 min^{-1}. With regards to substrate specificity, *o*-NPGal and lactose were the most evident substrates, laminarin is a potential additional substrate but its hydrolysis was remarkably slow. Galactose demonstrated competitive inhibition while glucose showed uncompetitive inhibition of GalB2. When transglycosylation was evaluated with immobilized GalB2, the transglycosylation products were not observed.

Regarding general characteristics of GalB2, the optimal pH of the enzyme was determined to be 7.4 when the activity of the enzyme was assessed from pH 5.0 to 9.0. The thermal stability of the free enzyme was evaluated in the temperature range of 37 ºC to 55 ºC. GalB2 remained active after incubation at 37 ºC and 40 ºC, but when incubated at temperatures of 45 ºC and above, the enzymatic activity greatly diminished. The thermal stability of the immobilized GalB2 followed the same trend as the free enzyme, though residual enzyme activity was retained after incubation at temperatures of up to 60 ºC compared to the free enzyme.

Removal of divalent ions with EDTA resulted in an 88% loss of activity by GalB2. When magnesium ions were reintroduced to the EDTA-treated GalB2, activity was restored to 63% relative to the native enzyme, meaning that magnesium is crucial to the function of this β-galactosidase. A native gel activity test with 4-MUGal revealed that GalB2 was active as a dimer but was inactive in its monomeric form.

Column silica-gel chromatography with acetonitrile:water elution solvent was examined in an attempt to purify oligosaccharides. This method showed some promise for isolating oligosaccharides by nonpolar interactions where monosaccharides eluted first, and oligosaccharides eluted later. However, the complete separation of saccharides was not achieved.

The aliquots taken from the transglycosylation reactions with immobilized lactase were analyzed by LC/ESI-MS via direct injection in positive ion mode. It was found that peaks corresponding to the sodium adducts, $[M+Na]^+$, were observed for di-, tri-, and tetrasaccharides (*m/z* ratio = 365, 527, and 689, respectively). The ratio of relative abundance for tri- to disaccharides was observed to be 1:2, while the ratio of tetra- to trisaccharides was 1:5.

Although this study has shown GalB2 lacks transglycosylation activity, a substrate hydrolysis assay using a wide range of galacto-oligosaccharides containing β-glycosidic bonds could be done to gain insight on the active site pocket and how site-directed mutagenesis could aid in improving the transglycosylation capabilities of the enzyme. Further studies can be conducted involving the column chromatography system described to improve separation of oligosaccharides. Lastly, LC/ESI-MS could be continued to establish the types of glycosidic bonds formed in the transglycosylation products.

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