Biochemical Characterization of *β-galactosidase* from *Enterobacter sp. YSU*

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Abstract

The enzyme β-galactosidase plays a role in the hydrolysis of lactose to galactose and glucose. Depending on the source, β-galactosidases can also carry out transglycosylation. This research was aimed at the biochemical characterization of β-galactosidase from *Enterobacter sp. YSU*. The enzyme is within the family of glycoside hydrolases. The *Enterobacter sp. YSU* β-galactosidase was overexpressed in *E. coli*. Subsequently, it was isolated using ammonium sulfate precipitation and a Q-Sepharose ion-exchange column. The single polypeptide chain protein contains 1056 amino acids with a molecular weight of 120 kDa. An in-gel activity test using 4-methylumbelliferyl-β-D-galactopyranoside established that the protein is active in its dimeric form. Dissociation of *β*-galactosidase into monomers in the presence of detergents like SDS results in the loss of enzymatic activity.

The enzyme shows its optimal activity at pH 7.4 and a temperature of 40 °C. It has a limited substrate specificity of *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPGal) and lactose. The catalytic parameters of the enzyme for *o*-NPGal were determined: K_M is 0.3 mM, and k_{cat} is 146 s^{-1}. With respect to lactose, K_M is 22 mM, and k_{cat} is 3.86×10^3 min^{-1}. Galactose competitively suppresses β-galactosidase activity, whereas glucose uncompetitively inhibits the enzyme. The enzymatic activity of *β*-galactosidase was affected by the presence of Mg^{2+} but not other divalent ions like calcium, zinc, or copper. Dimethyl sulfoxide caused a notable decrease in the activity of β-galactosidase while 2-mercaptoethanol had no effect on the activity of the enzyme. The β-galactosidase from *Enterobacter sp. YSU* showed a similar K_M for lactose with most β-galactosidases isolated from other organisms but a higher $k_{cat,}$ and, therefore, there is a need to explore its applications in the hydrolysis of lactose.

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I would be neglectful not to recognize my wife Patricia Makena, my daughters; Michelle Neema, Shanice Mwangaza, and Shanelle Penda, my parents Samuel Motari and Marcella Motari, and my siblings for their prayers for discernment, counsel, tranquility, and wholesomeness.

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BSA	Bovine serum albumin
DMSO	Dimethylsulfoxide
DTT	DL-dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
Fru	Fructose
Gal	Galactose
Glc	Glucose
GOD	Glucose oxidase
iPrOH	Isopropanol
kDa	Kilodalton
Lac	Lactose
LB	Luria-Bertani
MUGal	4-methyllumbelliferyl-β-*D*-galactopyranoside
NaPi	Sodium phosphate buffer
OD_{600}	Optical density at 600nm
o-NPGal	*o*-nitrophenyl-β-D-galactopyranoside
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sp.	Species
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	Thin Layer Chromatography

CHAPTER I: INTRODUCTION

The enzyme β-galactosidase is a glycoside hydrolase (EC 3.2.1.23) that hydrolyzes the β-D-galactosyl residues of oligosaccharides from a non-reducing end. The β-galactosidases also catalyze transglycosylation reactions when hydrolyzing lactose. They have been isolated from different organisms like fungi, yeast, plants, animals, and bacteria. Those isolated from bacteria like the *Kluyveromyces lactis* or fungi like *Aspergillus oryzae*, have been used in industrial applications[1]. The β-galactosidases are of great interest in studying, especially those that can thrive in extreme environmental conditions like high temperature, pH, and high saline concentrations. In addition, those that carry out transglycosylation to produce oligosaccharides are being explored to make the synthesis more efficient.[2] Understanding the β-galactosidases' structure and their biochemical properties helps to apply these enzymes in industrial processes.

1.1 β-galactosidases activities

The β-galactosidases (EC 3.2.1.23), also called lactases, are naturally found in animals, microorganisms, and plants.[3] They are glycoside hydrolase enzymes responsible for catalyzing the hydrolysis of a β-D-galactose residue within β-D-galactosides at the terminal non-reducing end. The β-galactosidases break the β-glycosidic bonds in lactose to form galactose and glucose. In *E. coli*, β-galactosidase's structural gene is the *lacZ* gene, an integral component of the adaptive *lac operon* system that is commonly activated in the presence of lactose and is repressed when there is an adequate glucose supply.[4]

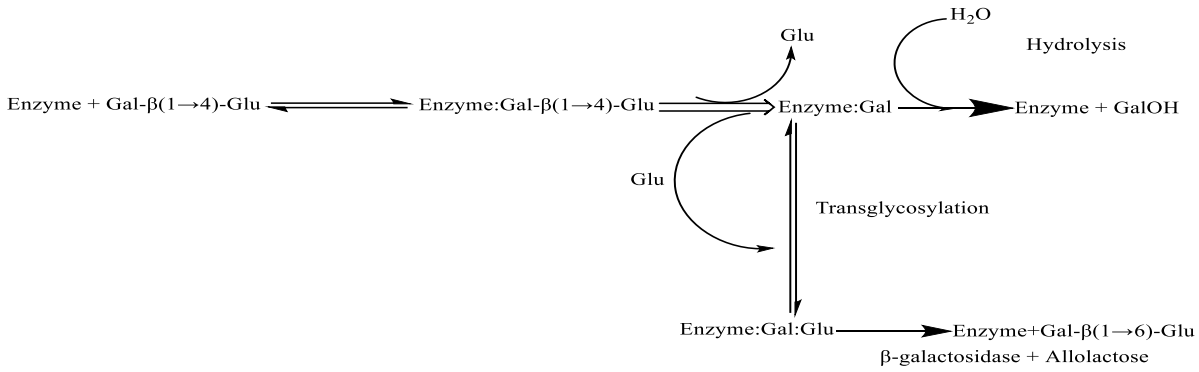


Figure 1: The reactions catalyzed by β-galactosidase enzyme; hydrolysis and transglycosylation.

Upon glycosidic bond cleavage, β-galactosidase transfers galactosyl residue from lactose to another molecule. In hydrolysis reaction, water is the galactosyl acceptor.[5] Lactose or glucose can act as the galactosyl acceptor in transglycosylation reactions. The extent to which transglycosylation and hydrolysis occur is dependent on the specific origin of β-galactosidase and the initial concentration of lactose.[5] For example, the enzyme obtained from *Lactobacillus sakei* Lb790 has demonstrated superior yields of 48% of the reactants converted to galactooligosaccharides compared to galactosidases obtained from other bacteria like *L. acidophilus, L. reuteri* and *L. plantarum* that showed lower yields of about 38% of the reactants converted to galactooligosaccharides.[6]

The β-galactosidase have been utilized in the synthesis of galactooligosaccharides. In industrial applications, the selected concentration of β-galactosidase, the reaction time, and the reaction conditions should provide stability to the enzyme during the reaction and be close to the ranges where the enzyme has the highest activity.[5,7] Different oligosaccharides have been synthesized from lactose, inulin, and sucrose. Lactose has been used in synthesizing the galactooligosaccharides lactulose and lactosucrose. Sucrose has also been utilized in the synthesis of lactosucrose using β-galactosidase from *Bacillus circulans*.[8,9] Lactosucrose is a trisaccharide

which consists of glucose, galactose, and fructose. The fructose in lactosucrose is attached to the glucose residue by a β (2↔1) glycosidic bond and a β (1→4) glycosidic bond is formed between galactose and glucose.

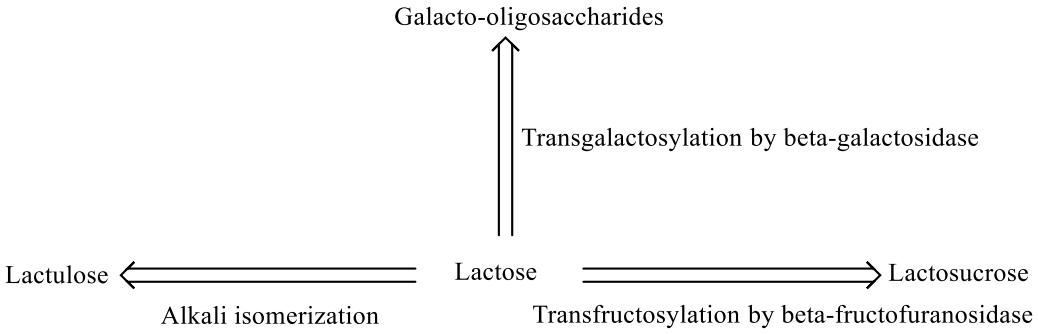


Figure 2: Transglycosylation of lactose to form different oligosaccharides.[9]

1.2 Structure of β-galactosidase

Some β-galactosidases, like those obtained from *Escherichia coli,* are homotetrameric with 1023 amino acids per subunit.[10] Each of the monomers has an active site, but for a functional site to be complete, it requires two monomers, and, therefore, they cannot work independently.[11] *In vitro*, the presence of magnesium ions and thiols like 2-mercaptoethanol and DTT maintains the protein in the stable dimeric form; otherwise, it will dissociate into monomers. The thiols reverse or prevent the formation of the disulfides or other oxidized forms like sulfenic and sulfonic acids that can lead to the disruption of the enzyme's structure.[10,11] Chemicals without sulfhydryl groups like ethanol and glycerol do not affect the enzyme's dimeric structure.[12]

Most β-galactosidases require metal ions for activity. The β-galactosidase from *E. coli* has several Mg^{2+} bound to each subunit. However, only the Mg^{2+} attached to the active site has profound impact on the activity of the enzyme, while the rest enhances β-galactosidase stability. However, the β-galactosidases do not entirely depend on these ions to catalyze their reactions as they have shown some activity in their absence. The cations with a charge of +1, sodium and potassium,

affect the enzyme's affinity for the substrate and determine how stable the transition states are during the reaction.[10,11]

1.3 Substrate specificity of β-galactosidase

The rate of hydrolysis of lactose and the affinity of the enzyme for lactose differs significantly among the β-galactosidases depending on their source. The β-galactosidase isolated from *Lactobacillus plantarum* HF571129 hydrolyzes lactose with a rate of 0.0111 min^{-1}.[13] The β-galactosidases extracted from other organisms have shown to have a wide range of K_M values. For example, *Enterobacter cloacae* B5, and *Bifidobacterium infantis* HL96 have been identified to hydrolyze lactose with K_M of 0.30 mM, and 73.2 mM, respectively.[5,14]

The β-galactosidase's ability to hydrolyze other galactosides led to the introduction of the substrates with chromogenic properties for monitoring the enzyme activity like X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) and *o*-NPGal. The *o*-NPGal compound is colorless but after being hydrolyzed, the reaction product ortho-nitrophenolate exhibits a yellow color while the X-gal gives a dark blue precipitate.

The experiments have shown that β-galactosidase hydrolyzes various substrates at different rates. The β-galactosidase from *Lactobacillus sakei* Lb790 has been shown to have a higher rate in hydrolyzing *o*-NPGal as a substrate than lactose. The enzyme has no activity with other substrates namely *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *o*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, and *p*-nitrophenyl-β-D-mannopyranoside, indicating limited substrate specificity. The enzyme from *Lactobacillus sakei* Lb790 is used as a starter culture in the fermentation of sausages and other meat products.[6]

1.4 Impact of temperature and pH on β-galactosidases

Temperature and pH affect the functioning of the enzymes, therefore, their effects on the activity of β-galactosidases have been studied extensively. The optimum pH and optimal temperature vary from one β-galactosidase to another depending on their source. The β-galactosidases have demonstrated differences in their thermal stability. Enzymes that are stable at high temperatures have a greater likelihood of catalyzing the transglycosylation reactions. Heat can cause the denaturation of the enzyme by supplying the kinetic energy that causes the protein molecules to rapidly vibrate. This disrupts the weak hydrogen bonds and dispersion forces.[15] Zhang et al. examined novel β-galactosidase (Gal308) that demonstrated an outstanding thermal stability for temperature ranges between 40 and 70 °C when incubated for 60 minutes.[16] However, the thermal stability of Gal308 was considerably reduced when temperatures exceeded 80 °C. The β-galactosidase extracted from *A. niger* had an optimum temperature between 55 °C and 60 °C. The β-galactosidase isolated from *Bacillus stearothermophilus* showed activity in a wide range of temperatures with the optimum temperature falling within 50 °C and 60 °C.[3] The enzyme isolated from *Lactobacillus sakei* has shown a range of activity between 25 °C and 55 °C with an optimum temperature of 55 °C for both *o*-NPGal and lactose.[6]

A change in pH leads to the alteration in ionization of the two glutamate residues important for enzyme catalysis. At low pH, the carboxyl groups in glutamate side chains are protonated and uncharged while at high pH they are deprotonated and, therefore, are charged. The β-galactosidases' activity ranges widely with respect to pH. Enzymes obtained from fungi function at pH between 2.5 and 5.4, whereas enzymes from bacteria are active in a pH range from 6.0 and 7.0.[17] A study on the thermostable β-galactosidase extracted from *Bacillus stearothermophilus* showed that the enzyme's optimum pH is 7.0, and the enzyme retains at least 80% of its activity

between pH 6.0 and 7.5.[18] This optimum pH range was an important property in relation to milk processing since normal bovine milk has pH of 6.7. The optimum pH for the β-galactosidase isolated from the *Aspergillus carbonarius* is 4.5.[19] The *Aspergillus lacticoffeatus* has an optimal pH between 3.5 and 4.5 with a notable decrease in activity when the pH is increased. However, its optimum pH differs for lactose and *o*-NPGal as 6.5 and 7.5, respectively.[20]

1.5 Product inhibition

Enzymes interact with inhibitors which decrease their efficiency in catalyzing reactions. Irreversible inhibitors covalently bind to an enzyme's active site leading to the permanent loss of activity. This is contrary to the reversible inhibitors. Galactose has been identified as a competitive inhibitor of most β-galactosidases as it competes for the same active site with the substrates. For example, it inhibits the activity of β-galactosidase from *Lactobacillus sakei* by 50% while glucose with similar concentrations inhibits it by only 14%.[6] Studies carried out by Godoy et al. showed that galactose, once formed after the hydrolysis reaction, competes for the active site with the substrate.[20] This leads to inhibition. Achieving the hydrolysis of high concentrations of lactose is difficult and can only be fixed by immobilization of the enzyme or by continuously removing the products.[21] Hung and Lee noted that 10 mM galactose inhibited the hydrolysis of 20 mM lactose when it was incubated for 10 minutes.[14] Interestingly, they did not find galactose as an inhibitor in the hydrolysis of *o*-NPGal.

1.6 Impact of solvents on β-galactosidases

Enzymes frequently face hostile environments when utilized in various industrial bioprocesses. Thus, the stability profile of β-galactosidase in various solvents with varying concentrations needs to be investigated. Some solvents and chemicals can denature proteins. Denaturation of a protein involves a change in its native conformation with the loss of the secondary, tertiary, and quaternary

structures. The change may or may not be reversible. The disulfide bridges may be cleaved, but the other covalent bonds are not. Research has been done to evaluate the effect of various chemicals like 2-mercaptoethanol (reducing agent), isopropanol, ethanol, methanol, and detergents, like SDS, on the activity of enzymes.

SDS has been found to form SDS-protein complexes.[22] These complexes possess a negative charge. SDS causes the disruption of the non-covalent interactions as it inserts its non-polar tail into the protein core affecting the hydrophobic interactions of non-polar amino acids. However, SDS has no effect on the disulfide bridges.[23] SDS has been found to reduce the activity of *Aspergillus carbonarius* to about 50% of its initial activity. This occurs due to the enzyme getting partially denatured.

An investigation conducted by Kamran et al.[24] using β-galactosidase obtained from *Aspergillus nidulans* revealed that organic solvents like ethanol, formaldehyde, methanol, and isopropanol, had a positive impact on the catalytic activity of β-galactosidase in a range of concentrations between 1.0 mM and 5.0 mM, nonetheless, the enzymatic activity declined above 10.0 mM. The β-galactosidase obtained from *Halorubrum lacisprofundi* was found to show increased catalytic activity in the solution of n-butanol, methanol, isoamyl alcohol with concentrations between 10 % and 20 %.[25] The alcohols alter the polarity of the media affecting the ionization state of the residues in the enzyme's active site.[26]

1.7 Effect of divalent ions on the activity of β-galactosidase

As stated earlier, many β-galactosidases require metal ions for their function, however, there are some differences in the effect of divalent ions on various β-galactosidases. Their activities are either reduced or increased depending on the source of the enzyme and the type of metal ion. On

the other hand, metal ions may not have any effect on the activity of the enzyme. Some β-galactosidases have shown to be affected by Mg^{2+}.[5] For example, Mg^{2+} increased the activity of the β-galactosidase isolated from *Bacillus sp* MTCC 3088 at concentrations between 2.5 mM and 10 mM by 42 %. The enzyme was inhibited by Cu^{2+} at very low concentrations while the Mn^{2+} inhibited the enzyme at higher concentrations of 20 mM. The Fe^{2+}, Zn^{2+}, and Ni^{2+} ions also showed enzyme inhibition at concentrations of 20 mM. Even at higher concentrations (up to 10 mM), the Ca^{2+} and Mo^{2+} did not show any effect on enzyme activity. The metal ions form strong ionic bonds with the anions of the amino acids' carboxylates. This causes the disruption of salt bridges and may affect the enzyme stability. [27]

The β-galactosidases' activity is affected by the presence of heavy metals. The β-galactosidase isolated from *Bacillus sp* MTCC 3088 was inactivated in the presence of Hg^{2+} and Ag^{+} ions at a concentration of 1 mM most likely due to their effect on enzyme's disulfide bonds.[27] The β-galactosidase isolated from *Enterobacter cloacae* B5 was completely inhibited by Cu^{2+}, Hg^{2+}, and Ag^{+} while the Co^{2+}, Mg^{2+}, Mn^{2+} did not affect its activity. The enzyme in the presence of Zn^{2+} was partially active.

Ethylenediaminetetraacetic acid (EDTA) is a chelating agent that removes divalent ions from a protein. It is used to chelate metal ions and study their effect on the enzyme's activity. When β-galactosidase from *Aspergillus lacticoffeatus* was incubated with EDTA, its activity was decreased by the addition of lithium, barium, potassium, and iron (II) ions. Other β-galactosidases from *Aspergillus alliacens* and *Penicillium multicolor* treated with EDTA have shown the same negative effect when exposed to divalent ions.[3] Furthermore, the authors revealed an inhibitory impact of metal ions (Ca^{2+}, Fe^{2+}, Co^{2+}, Hg^{2+}, K^{+}, Na^{+}, Mn^{2+}) at concentrations ranging between 1.0 and 10.0 mM on β-galactosidase activity, while, Mg^{2+}, Zn^{2+} and Ni^{2+} ions did not affect the enzyme's

activity. In another study, Ca^{2+}, Mg^{2+} and Mn^{2+} enhanced the catalytic property of the enzyme obtained from *Bacillus licheniformis* DSM 13 at concentrations of 1.0 mM.[10] Hung and Lee found that EDTA and urea had little effect on the enzyme after being incubated with β-galactosidase obtained from *Bifidobacterium infantis* HL96.[14] It was inhibited by most metal ions except Na^{+} and Mg^{2+} that activated the enzyme. The authors concluded that the enzyme was inhibited by interaction of the heavy metal ions with sulfhydryl groups of the enzyme.

EDTA has been shown to have little effect on the activity of β-galactosidase from *Aspergillus carbonarius.* The enzyme is not a metalloenzyme as it does not need metal cofactors for its activity. Contrarily, it showed that its activity was hindered in the presence of most of the divalent ions apart from Mg^{2+} and Ca^{2+}.[19] EDTA has minimal effect on the β-galactosidase isolated from *Aspergillus lacticoffeatus*.[3] This shows that the enzyme from this source also does not require metal ions for its function.

1.8 Applications of β-galactosidase

Immobilized and free β-galactosidases have been utilized in analytical, medical, biotechnological, and industrial uses. The β-galactosidase can be deployed in several industrial applications: food technology, environment, biology, health, and pharmaceuticals. In foodstuff manufacturing, the enzyme can be used to enhance the digestibility of dairy products and improve sweetness, flavor, and solubility.[28] Additionally, it is important for people suffering from lactose intolerance since it contributes to the production of lactose-free milk plus other dairy products. [29] The β-galactosidase is deployed in dairy like sour cream, cheeses, and yogurt to assist in breaking down any lactose before being consumed by humans. This is with the aim to reduce the effects of lactose intolerance on humans with that challenge.[9]

1.9 Immobilization of β-galactosidases

The usage of enzymes in industries calls for prolonging the shelf-life of enzymes, increasing structural stability, and reusability as well as for optimization of their features like the optimal pH and thermostability. Immobilization involves the chemical or physical confinement of enzymes within environments that facilitate reuse. [30,31] Immobilization enables the removal of enzymes from the products thus avoiding product contamination. The methods utilized in immobilization need to consider the impact it has on how the enzyme works.[32] Immobilization can be accomplished through entrapment, adsorption, covalent attachment, encapsulation, affinity binding, chemical aggregation, and other methods. To enhance the stability, use, and reusability in continuous reactors, β-galactosidase can be immobilized on inorganic and organic supports.[29]

The β-galactosidase immobilization through adsorption encompasses ionic interaction, making it effective since the conformation of the enzyme faces only minor changes.[33] Meanwhile, entrapment of the enzyme has been found to enhance temperature and pH stability. However, the supports deployed during the process of entrapment immobilization cannot be reused after the enzymatic activity is lost.[34] Table 1 presents various methods of β-galactosidase immobilization. The β-galactosidase can be immobilized in various solid supports like agarose, Sephadex, diethylamino ethyl agarose, alginate, polyvinyl alcohol polymers, nylon, porous glass, zeolite, polyurethane foams, chitosan, and others.

Table 1: Immobilization methods for β-galactosidase

Immobilization Approaches	Origin of the enzyme	Immobilizing support	Reference
Physical Adsorption	*E. coli*	Chromosorb-W	[35]
	K. fragilis and K. lactis	Chitosan	[36]
	A. oryzae	Polyvinyl chloride & silica gel membrane	[37]
	K. lactis	Agarose and CPC-silica	[38]
	B. circulans	Silica and polyvinyl chloride	[39]
	A. niger	Porous ceramic monolith	[40]
	Thermus sp. T2	DEAE-agarose	[41]
	Pisum sativum	Sephadex G-75 & chitosan beads	[42]
Entrapment	*E. coli*	Polyacrylamide	[43]
	Penicillium expansum F3	Calcium alginate	[19]
	K. bulgaricus	Alginate via BaCl2	[44]
	T. acquaticus YT-1	Agarose bead	[45]
	A. oryzae	Nylon-6 plus zeolite	[46]
Covalent Bonding	*E. coli*	Polyvinyl alcohol	[47]
	L. bulgaris	Egg shells	[48]
	A. oryzae	Carrageenan Gel beads	[49]
	S. anamensis	Calcium alginate	[50]
	K. lactis	Thiosulfinate	[51]

The immobilized enzymes have been utilized in synthesizing glycol-oligosaccharides and lactose hydrolysis.[45] In addition, immobilized enzymes have been used in therapeutic industries and the food sector.[52] The enzymes have been applied in the designing of biosensors which have been used to detect lactose in food. The β-galactosidases are co-immobilized on biosensors with glucose oxidase which is very sensitive to the presence of glucose. Glucose is formed during lactose hydrolysis. The glucose oxidase acts on the glucose producing gluconic acid and hydrogen peroxide. Horseradish peroxidase then acts on the hydrogen peroxide to generate oxygen which is detectable as a signal.[53]

The β-galactosidases have been immobilized to increase the production of bioethanol. The β-galactosidases are immobilized together with the yeast cells within calcium alginate beads. The β-galactosidase slowly releases glucose from the hydrolysis of lactose. The glucose is then fermented to produce bioethanol.[54] The enzyme can be immobilized with the lysozyme enzyme to avoid any contamination from the microbes as lysozyme is anti-bacterial. This facilitates continuous usage of β-galactosidase and the development of products that are free from contamination.[55]

1.10 *Enterobacter sp. YSU*

The β-galactosidase used for this research was obtained from *Enterobacter sp.* YSU. The *Enterobacter sp.* YSU is a strain of bacteria that was discovered in Oak Ridge, Tennessee at the Y-12 plant which was used as a site for developing nuclear munitions during the World War II and the Cold war.[56] Thus, Y-12 was deployed as a code name to denote an electromagnetic isotope separating plant that played a key role in processing massive quantities of uranium utilized in manufacturing nuclear weaponry. Due to a lack of proper containment, nearby East Ford Poplar Creek was exposed to heavy metals like silver, gold, mercury, copper, zinc, cadmium, chromium, arsenic, and selenium. Therefore, the organisms in this region were exposed to the heavy metals and only organisms that were able to resist the effects of these constituents survived and reproduced.[57]

1.11 Research Problem

Various studies have investigated the properties of β-galactosidases in bacteria species such as *Alicyclobacillus acidocaldarius* subsp. *rittmannii*[58], *Enterobacter agglumerans* B1[59], *Bifidobacterium longum* subsp. *longum*[60], *Bacillus licheniformis* KG9[61], *Bacillus subtilis*[62], *Streptococcus mitis*[63], *Escherichia coli*[64], *E. cloacae* B5[59], and *Anoxybacillus* sp. KP1[65]. There is substantial research regarding the regulation, reaction mechanisms,[66,67] structure [68], and biochemical properties of galactosidases.[69,70] The characterization of β-galactosidase from *Enterobacter sp. YSU* delivers the benefit of exploring its structural and biochemical characteristics and developing a model for comparison with other enzymes.

1.12 Objectives of the Research

The thesis is focused on studying the properties of β-galactosidase from *Enterobacter sp. YSU*. The study aimed to provide the biochemical characterization of the enzyme by examining its catalytic parameters, pH dependence, its thermal stability, the effect of divalent metal ions on its action, potential inhibitors, and substrate specificity. Experiments were done to determine whether the enzyme catalyzes transglycosylation that involves sugars and alcohols. The enzyme was immobilized using calcium alginate beads to evaluate how this affects its optimal pH and thermal stability. The inhibitory effects of galactose and glucose were investigated to determine their type of inhibition and the concentration at which they suppress the activity of the enzyme.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

DNA plasmid *pET20b-lacZ* was a kind gift of Dr. Caguiat, (YSU). Luria-Bertani (LB) medium, ampicillin sodium salt, sodium phosphate monobasic anhydrous, ammonium sulfate, streptomycin, sodium dodecyl sulfate, dithiothreitol (DTT), sodium chloride, glycerol, Tris hydrogen chloride, glycine, MES, sodium acetate, glucose oxidase, calcium chloride anhydrous, Blue BANDit^{TM} Protein Stain Ultra-Pure, coomassie brilliant blue, and EDTA were obtained from AMRESCO (Solon, Ohio). Bromophenol blue, L-rhamnose, bovine serum albumin (BSA), *o*-nitrophenol-β-D-galactopyranoside (*o*-NPGal), D-lactose, hydrogen peroxide, *p*-nitrophenyl-*β*-D-glucopyranoside, 4-methylumbelliferyl-β-D-galactopyranoside (MUGal), 4-amino antipyrine, ammonium persulfate, isopropanol, phenol crystals, TEMED, 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, and Millipore centrifugal units were purchased from Sigma (St. Louis, Missouri). Sepharose was purchased from Amersham Biosciences (Piscataway, New Jersey). Rotary shaker, and Protein marker were obtained from Thermo Scientific (Waltham, Massachusetts). Boric acid was purchased from Matheson Coleman and Bell (Norwood, Ohio). Sodium alginate was bought from Landor Trading Company (Montreal, Canada). Acetic acid was purchased from Pharmc*o*-AAPER (Brookfield, Connecticut). Laminarin was purchased from Spectrum Chemical Manufacturing Corporation (New Brunswick, New Jersey). Dimethylsulfoxide was purchased from Acros (Geel, Belgium). Copper (II) chloride, zinc chloride, manganese sulfate, magnesium chloride, 1-butanol, concentrated sulfuric acid, D-galactose, ethanol, sodium carbonate, and acrylamide were purchased from Fisher Chemical Company (Fair Lawn, New Jersey). D-Glucose was obtained from Mallinckrodt Inc. (Paris, KY). TLC plates were purchased from Merck KGaA (Darmstadt, Germany). UV light transilluminator, gel

electrophoresis equipment, and Thermal cycler were from Bio-Rad (Hercules, California). Centrifuge was from Beckman Avanti J251 (Brea, California). Spectrophotometric assays were carried out using the photodiode array Spectrophotometer Hewlett Packard 8453 (Agilent Technologies, Santa Clara, CA).

2.2 Methods

2.2.1 Expression, and purification of the β-galactosidase enzyme

The *E. coli* KRX cells harboring plasmid *pET20b-lacZ* were cultured in the Luria-Bertani (LB) medium with 100 µg/mL of ampicillin at 37 °C in a rotary shaker. The overnight culture was diluted 1:100 into two flasks of a larger volume (1.2 liters). After attaining the optical density (OD_{600}) of 0.6 at 600 nm, the cells were transferred into a water-shaker at 20 °C, and L-rhamnose was added to a final concentration of 0.01% (m/v) solution. Flasks were shaken overnight to allow protein expression.

The cell cultures were harvested by centrifugation at 6,000 × g for 10 minutes at 4 °C. The pellets were then stored at -25 °C. Subsequently, the pellets were suspended in 50 mL of 20 mM sodium phosphate buffer, pH 7.4. Sonication was done to disrupt the cell membranes in 10 cycles with the sample placed in an ice-water bath. Each cycle was done for 30 seconds with a minute break. Centrifugation was done at 11,000 × g for twenty minutes at 4 °C. Streptomycin sulfate was added to the supernatant to achieve a 1% (m/v) solution to precipitate the DNA. The mixture was centrifuged at 11,000 × g at 4 °C for 20 minutes. The supernatant was then mixed with ammonium sulfate to achieve 45% saturation. The ammonium sulfate was added slowly while stirring. Centrifugation was done for 20 minutes at 11,000 × g at 4 ^{0}C.

The pellets were isolated and redissolved in 20 mM sodium phosphate buffer, pH 7.4. Afterward, the protein sample was dialyzed against a 20 mM sodium phosphate buffer, pH 7.4, for 44 hours. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% resolving gel was used in every step of the isolation and purification to track the protein. These gels underwent staining using the Coomassie Blue stain for 45 minutes and then de-staining for 45 minutes. They were then washed in de-ionized water for 5 minutes after which they were then observed.

2.2.2 Ion-exchange chromatography

A dialyzed protein sample was loaded on the Q-Sepharose column (15 x 2.5 cm) equilibrated with 20 mM NaPi buffer, pH 7.4, and eluted by employing the stepwise sodium chloride gradient ranging from 100 mM to 1 M in 20 mM sodium phosphate buffer, pH 7.4. In each step, 100 mL of sodium chloride solution were used. SDS-PAGE was done to evaluate the presence of a protein band with a mass of 120 kDa. The fractions containing a protein band with the desired mass were pooled together.

2.2.3 Evaluation of the protein concentration

The protein sample was dialyzed against 20 mM sodium phosphate, pH 7.4 and then concentrated using the Millipore centrifugal unit by centrifugation at 6,000 × g for a period of 20 minutes for each 4 mL sample. To determine the protein concentration, 10 µL of the concentrated protein sample was added to 990 µL of the 20 mM sodium phosphate, pH 7.4. The absorbance of the protein sample was measured at 280 nm. The absorbance was used with the Beer-Lambert equation to determine the concentration of the protein using the extinction coefficient of 264,000 $M^{-1}cm^{-1}$ calculated from a protein sequence.

The Bradford assay was done using bovine serum albumin (BSA) with a concentration of 0.5 mg/mL as a standard to construct a calibration curve. The protein was diluted by a factor of 50 in 50 mM NaPi buffer, pH 7.4, and 100 µL of the diluted sample was used for the Bradford assay by mixing it with the Bradford reagent and determining the amount of protein using the calibration curve.

2.2.4 Determination of the hydrolytic activity of β-galactosidase using o-NPGal

Enzymatic activity was determined using the initial rate of hydrolysis of *o*-nitrophenol-β-D-galactopyranoside (*o*-NPGal) as a substrate. For every assay, 890 µL of the 50 mM NaPi buffer, pH 7.4, were added to a cuvette followed by 100 µL of *o*-NPGal prepared in 50 mM NaPi buffer, pH 7.4, and finally 10 µL of the protein were added to start the reaction. The concentration of protein in the reaction mixture was 0.025 µM per monomer. The reactions were monitored using a spectrophotometer that measured the absorbance at 405 nm every 10 seconds for a total of 90 seconds. The concentrations of *o*-NPGal in the reaction mixture varied between 0.2 mM and 6 mM. The experiment was repeated three times for each concentration of *o*-NPGal and the change in absorbance was averaged. The initial rate of the reaction was calculated from a change of absorbance at 405 due to the formation of *ortho*-nitrophenolate using an extinction coefficient of 3055 $M^{-1}cm^{-1}$. A graph for initial velocity vs *o*-NPGal concentration was constructed from which K_M, V_{max}, and k_{cat} were determined.

2.2.4.1 Inhibition of β-galactosidase by galactose

The solution of 1 M galactose prepared in 50 mM NaPi buffer, pH 7.4, was used to test whether galactose inhibits β-galactosidase. Initially, 800 µL of the 50 mM NaPi buffer, pH 7.4, was placed in a cuvette followed by 90 µL of 1 M galactose to make a 90 mM concentration in the reaction mixture. To the mixture outlined above, 10 µL of the enzyme were added to achieve 0.025 µM

and allowed to stand for one minute to enable the galactose to bind to the enzyme. Then, 100 μL of 30 mM *o*-NPGal was added to start the reaction. The change in absorbance of the reaction mixture was monitored using the spectrophotometer at 405 nm for a duration of 90 seconds. The rate of the reaction was calculated from the change of absorbance and compared to the one without galactose. The concentration of *o*-NPGal remained constant at 3 mM in the reaction mixture as the concentration of galactose was varied to decrease the rate of the reaction to 75% and 50% of the rate of reaction of the uninhibited enzyme. The concentration of galactose that produced a 75% reaction rate for 3 mM of *o*-NPGal was determined to be 100 mM and the concentration of galactose that resulted in 50% of the reaction rate for 3 mM of *o*-NPGal was determined to be 250 mM.

The following experiment was done to determine the mode of galactose inhibition. Into a cuvette, 790 μL of 50 mM NaPi buffer, pH 7.4 was added followed by galactose to achieve a 100 mM concentration of galactose in the reaction mixture. Then, 10 μL of 2.5 μM β-galactosidase were added and the mixture was allowed to stand for one minute to allow the galactose to bind to the enzyme. The reaction was initiated by the addition of 100 μL of *o*-NPGal and the cuvette was placed in the spectrophotometer set at 405 nm. The experiment was done in triplicate for each concentration of *o*-NPGal varying between 0.2 and 3 mM. The change in absorbance was recorded and averaged to determine the rate of the reaction. The experiment was repeated using 250 mM of galactose in the reaction mixture while varying the concentration of *o*-NPGal. The results were plotted as Lineweaver-Burk plots to determine the mode of inhibition.

2.2.4.2 Determining the inhibitory effects of glucose on β-galactosidase.

A solution of 1 M glucose in 50 mM NaPi buffer, pH 7.4 was used to determine the inhibitory effects of glucose. Using a 1-mL cuvette, 790 µL of the 50 mM NaPi buffer, pH 7.4 was added followed by 100 µL of glucose to achieve 100 mM of glucose in the reaction mixture. To the mixture above, 10 µL of the 2.5 µM enzyme were added and given one minute to allow the glucose to attach to the enzyme. To start the reaction, 100 µL of *o*-NPGal was added to make 3 mM in the reaction mixture. The change in absorbance was assessed using the spectrophotometer at 405 nm for a duration of 90 seconds. The experiment was done in triplicate and the results were averaged. The above experiment was done to determine the quantity of glucose needed to achieve 75% of the reaction rate for the uninhibited enzyme at 3 mM of *o*-NPGal. The glucose concentration was found to be 150 mM. Then, the concentration of glucose was maintained constant as the *o*-NPGal concentration was varied between 0.2 and 3 mM. The results were plotted as Lineweaver-Burk plots to determine the mode of inhibition.

2.2.5 Thermal stability of β-galactosidase.

Three tubes with 100 µL of the protein solution in each tube were incubated at 36 °C in the Bio-Rad thermal cycler for 20 minutes. The tubes were allowed to cool to room temperature. The activity assay was done using the *o*-NPGal substrate. The mixture's pH was maintained at 7.4 using 50 mM NaPi buffer. The assay was done by adding 100 µL of 30 mM *o*-NPGal to 890 µL of 50 mM NaPi buffer, pH 7.4 in the cuvette. To start the reaction, 10 µL of the 2.5 µM protein solution was added. The reactions were monitored using a spectrophotometer that measured the absorbance at 405 nm every 10 seconds for a total of 90 seconds. All the assays were done in triplicate for the enzyme incubated at 40 ^{o}C, 45 ^{o}C, and 50 ^{o}C.

2.2.6 Impact of pH on β-galactosidase's activity

The impact of pH on the activity of β-galactosidase was assessed in the ranges of pH 5.0 to 9.0. The following buffers were used at a concentration of 50 mM: sodium acetate at pH 5.0, MES at pH 6.0, sodium phosphate at pH 7.4, Tris at pH 8.0, and borate at pH 9.0.

A blank for spectrophotometer was comprised of 100 µL of 30 mM *o*-NPGal solution, 2 mL of 0.5 M sodium carbonate, and 900 µL of selected buffer. The absorbance was measured at 405 nm. The assay was carried out by placing 890 µL of a specific buffer into a 3 mL cuvette. Subsequently, 100 µL of 30 mM *o*-NPGal was added and then 10 µL of 2.5 µM *β*-galactosidase were added to start the reaction. The reaction was allowed to proceed for 90 seconds before 2 mL of 0.5 M sodium carbonate was added to the mixture to halt the reaction. The absorbance was measured at 405 nm. Assays using the buffered solutions listed above were studied separately at room temperature and the concentration of the substrate was kept the same. The assays were done in triplicate for each pH and the results were averaged.

2.2.7 Immobilization of β-galactosidase

2.2.7.1 Preparation of the calcium alginate beads.

Sodium alginate (3.0 g) was dissolved in 100 mL of 50 mM NaPi buffer, pH 7.4, and 10 mL of this solution was mixed with 200 µL of the 50 µM protein solution. The solution was mixed by gentle inversion to achieve a uniform distribution of the protein. The solution was given time to sit overnight at 4 °C to allow any bubbles to escape. Next, the solution was pipetted into 0.02 M $CaCl_2$ solution in de-ionized water with gentle mixing and was incubated for 30 minutes at a temperature of 32 °C to produce the calcium alginate beads. Subsequently, the beads were washed with 20 mL of distilled water five times to get rid of the excess calcium chloride and enzyme that was not entrapped. The wash solution was mixed with *o*-NPGal to check if there was any free

enzyme present. This was repeated until there was no color change observed in the presence of *o*-NPGal.

2.2.7.2 Activity of the immobilized enzyme at various pH

The activity of immobilized β-galactosidase was measured at 405 nm to determine the concentration of *o*-nitrophenolate which was produced during the hydrolysis of *o*-NPGal. A sample of 100 µL of 30 mM *o*-NPGal in 50 mM NaPi, pH 7.4 was added to an Eppendorf tube with seven calcium alginate-enzyme beads and 900 µL of 50 mM NaPi buffer, pH 7.4. The tube was placed in a shaker to ensure that the beads were exposed to the solution for 10 minutes. To stop the reaction, 700 µL of the mixture was added to 300 µL of 0.5 M Na_2CO_3 solution in a cuvette. The absorbance was measured at 405 nm. The experiment was repeated using 50 mM sodium acetate buffer at pH 5.0, 50 mM MES at pH 6.0, 50 mM Tris at pH 8.0, and borate 50 mM pH 9.0.

2.2.8 Stability of β-galactosidase in high temperatures

To set up the control, seven calcium alginate beads with β-galactosidase were placed in an Eppendorf tube and 900 µL of 50 mM NaPi buffer, pH 7.4 were added. Into the same Eppendorf tube, 100 µL of *o*-NPGal was added to a final concentration of 3 mM and the tube was placed in a shaker for 10 minutes. The spectrophotometer was blanked at 405 nm using 900 µL of 50 mM NaPi buffer, pH 7.4, and 100 µL of 30 mM *o*-NPGal solution. After 10 minutes, the solution from the Eppendorf tube was transferred to a 1-mL cuvette and the absorbance was measured at 405 nm. The experiment was repeated three times and the results were averaged.

Seven calcium alginate beads with β-galactosidase were placed in an Eppendorf tube. Into the same tube, 900 µL of 50 mM NaPi buffer, pH 7.4 was added. The Eppendorf tube was placed in a water bath set at 45 °C. It was allowed five minutes to equilibrate and the incubated for 10 minutes

at 45 ºC. The beads were cooled in a 25 ºC water bath for 20 minutes. After that, 100 µL of *o*-NPGal solution were added to the beads to a final concentration of 3 mM and placed in a shaker for 10 minutes. After 10 minutes, the solution from the Eppendorf tube was transferred to the 1 mL cuvette and the absorbance was measured at 405 nm. The experiment was repeated for temperatures of 50 ºC, 60 ºC, and 70 ºC.

2.2.9 Effect of divalent ions on the activity of β-galactosidase.

A control experiment was first set up. In a cuvette, 890 µL of the 50 mM NaPi buffer, pH 7.4 was mixed with 100 µL of *o*-NPGal solution to a final concentration of 3 mM in the reaction mixture. Subsequently, 10 µL of 2.5 µM protein solution were pipetted into the same cuvette, and the change of absorbance was measured at 405 nm for 90 seconds.

The solution of protein with EDTA was prepared by placing 750 µL of the 50 mM NaPi buffer, pH 7.4 and 50 µL of 50 µM protein solution into an Eppendorf tube and adding 200 µL of 0.25 M EDTA prepared in 50 mM NaPi buffer, pH 7.4. The solution was left to rest for 35 minutes to allow the divalent ions present to bind to the EDTA. Into a 1-mL cuvette, 890 µL of 50 mM NaPi buffer, pH 7.4 was added followed by 100 µL of *o*-NPGal solution to make a final concentration 3 mM *o*-NPGal in the reaction mixture. Ten microliters of the protein - EDTA mixture was added and the change in absorbance was measured at 405 nm using the spectrophotometer.

2.2.9.1 Use of different divalent ions to restore the activity of the enzyme after adding EDTA.

Into an Eppendorf tube, 690 µL of 50 mM NaPi buffer, pH 7.4, was added, followed by 10 µL of the protein with EDTA yielding 0.025 µM of the protein in the reaction mixture. Subsequently, 200 µL of the magnesium chloride in 50 mM NaPi buffer, pH 7.4 was added to achieve 200 mM in the reaction mixture and incubated for 35 minutes. The mixture was transferred into a cuvette, 100 µL of *o*-NPGal solution was added to give a final concentration 3 mM *o*-NPGal in the reaction

mixture and the change of absorbance at 405 nm was observed for 90 seconds. The experiment was done in triplicate and the results were averaged. This experiment was repeated for other metal ions using calcium chloride, copper (II) chloride, zinc chloride, and manganese sulfate solutions prepared in 50 mM NaPi, pH 7.4.

2.2.10 Transglycosylation

2.2.10.1 Transglycosylation by β-galactosidase using lactose at 40 °C, pH 7.4.

Into an Eppendorf tube, 900 µL of 600 mM lactose in 50 mM NaPi buffer, pH 7.4 was mixed with 100 µL of β-galactosidase to reach 0.25 µM enzyme concentration in the reaction mixture. The tube was incubated in a water bath at 40 °C for 2 hours. The experiment was repeated at 37 °C as well as at 45 °C. The progress of the reaction was monitored by taking 100 µL aliquots after 20 minutes, 30 minutes, 1 hour, and 2 hours. The aliquots were kept at 4 °C prior to undergoing analysis by TLC.

2.2.10.2 Analysis of reaction by TLC

Reaction mixtures were evaluated using TLC. Standard mixtures of 1.5% (w/v) lactose, 1.5% (w/v) glucose, and 1.5% (w/v) galactose were spotted on the plate along with the aliquots from the reaction mixture. TLC plates were developed in a system consisting of 1-butanol: acetic acid: water in a ratio of 2:1:1. The plates were allowed to dry and sprayed with 5% (v/v) sulfuric acid in ethanol. The TLC plate was then placed on a hot plate until dark spots appeared.

2.2.10.3 Transglycosylation using β-galactosidase using lactose at 40 °C, pH 8.0.

Into an Eppendorf tube, 900 µL of 600 mM lactose prepared in 50 mM TRIS buffer, pH 8.0 followed by 100 µL of β-galactosidase to a final concentration of 0.25 µM in the reaction mixture. The solution was carefully mixed and incubated at 40 °C water bath for a period of 24 hours. The progression of the reaction was monitored by taking 100 µL aliquots after twenty minutes, thirty

minutes, 1 hour, 2 hours, and 24 hours. The aliquots were kept at 4 °C before being analyzed using TLC as described in 2.2.10.2.

2.2.10.4 Transglycosylation using lactose in isopropanol at 45 °C, pH 7.4.

A hundred microliters of lactose with a concentration of 600 mM prepared in 50 mM NaPi buffer pH 7.4 were added into an Eppendorf tube followed by 800 µL of 15% isopropanol prepared in the same buffer. Then, 100 µL of β-galactosidase was added to a final concentration of 0.25 µM in the reaction mixture. Subsequently, the mixture was incubated in a 45 °C water bath for 2 hours. To monitor the progress of the reaction, 100 µL aliquots were taken after 20 minutes, 30 minutes, 1 hour, and 2 hours. The aliquots were stored at 4 °C before analysis by TLC as described above.

2.2.10.5 Transglycosylation using β-galactosidase using lactose at 50 °C with immobilized enzyme beads.

The process entailed adding 900 µL of 400 mM lactose in 50 mM MES buffer, pH 6.0 into an Eppendorf tube containing seven beads of the immobilized β-galactosidase. The combination was slowly stirred and a water bath at 50 °C was used to incubate it for 18 hours. The samples were removed at different time points and kept at 4 °C prior to undergoing analysis using TLC as described above.

2.2.11 Hydrolysis of laminarin using β-galactosidase

Into an Eppendorf tube, 990 µL of 1% (m/v) of laminarin in 50 mM NaPi buffer, pH 7.4, were added followed by 10 µL of β-galactosidase to make a concentration of 0.025 µM in the reaction mixture. They were gently mixed and incubated in a 40 °C water bath for 2 hours and analyzed by TLC.

2.2.11.1 Analysis by TLC

The reaction with laminarin on a TLC plate was developed using a system containing ethyl acetate, acetic acid, and water in a ratio of 2:2:1 (v:v:v). Standards containing 1% (m/v) of glucose and 1% (m/v) laminarin were used in this process. The plate was allowed to dry, sprayed with 5% sulfuric acid (v/v) in ethanol, and then heated on a hot plate to produce the spots.

2.2.12 Effect of various chemicals on the activity of β-galactosidase

The enzyme β-galactosidase was exposed to ethanol, DMSO, 2-mercaptoethanol, isopropanol, and SDS to investigate their effect on its activity.

2.2.12.1 The effect of ethanol on β-galactosidase.

Ethanol was used in the range of concentrations between 0.5% and 35% (v/v) prepared in 50 mM NaPi buffer, pH 7.4. Subsequently, 890 µL of the ethanol solution was pipetted into a cuvette, then 10 µL of the 2.5 µM protein solution were added and allowed to stand for five minutes. Then, 100 µL of *o*-NPGal solution was added to a final concentration of 3 mM *o*-NPGal in the reaction mixture. Changes in absorbance were measured for 90 seconds at 405 nm. The experiment was repeated three times for each concentration of ethanol.

2.2.12.2 Experiment on the effect of isopropanol on β-galactosidase

The same procedure was followed in the preparation of isopropanol solutions in the range of concentrations between 0.5% and 35% (v/v) in 50 mM NaPi buffer, pH 7.4. Then, 890 µL of the isopropanol mixture was pipetted into the cuvette, 10 µL of the protein were added into the tube to a final concentration of 0.025 µM and the mixture was incubated at room temperature for five minutes. After that, 100 µL of *o*-NPGal solution prepared in 50 mM NaPi buffer, pH 7.4 was added to a final concentration of 3 mM *o*-NPGal in the reaction mixture and the change in absorbance

was measured for 90 seconds at 405 nm. This procedure was done in duplicate for each isopropanol concentration.

2.2.12.3 Experiment on the effect of SDS on β-galactosidase.

Solutions of SDS in concentrations ranging between 0.5% and 15% (m/v) were prepared in 50 mM NaPi buffer, pH 7.4. Then, 890 µL of the SDS solution was pipetted into a cuvette, 10 µL of 2.5 µM protein solution were added into the cuvette and allowed to rest for five minutes. Finally, 100 µL of *o*-NPGal solution were added to a final concentration of 3 mM *o*-NPGal in the reaction mixture and the change in absorbance was measured for 90 seconds at 405 nm.

2.2.12.4 Experiment on the effect of 2-mercaptoethanol on β-galactosidase

To assess the effect of 2-mercaptoethanol on β-galactosidase, solutions of 2-mercaptoethanol with concentrations ranging from 1.25 mM to 10.0 mM were prepared in 50 mM NaPi buffer, pH 7.4. Into a cuvette, 890 µL of the 2-mercaptoethanol mixture was added. This was followed with the addition of 10 µL of the protein to a final concentration of 0.025 µM in the reaction mixture. The tube was incubated at room temperature for five minutes. Then, 100 µL of *o*-NPGal solution were added to a final concentration of 3 mM *o*-NPGal and the change in absorbance was measured for 90 seconds at 405 nm.

2.2.12.5 Experiment on the effect of DMSO on β-galactosidase.

DMSO solutions in the range of concentrations between 0.5% and 60% (v/v) in 50 mM NaPi buffer, pH 7.4 were prepared. To a cuvette, 890 µL of the DMSO mixture was pipetted, then 10 µL of the 2.5 µM protein solution was added into a cuvette and allowed to rest for five minutes. Subsequently, 100 µL of *o*-NPGal solution was added to a final concentration of 3 mM *o*-NPGal and changes in absorbance were measured for 90 seconds at 405 nm.

2.2.13 Investigation of β-galactosidase quaternary structure using MUGal

Native polyacrylamide gels with 10% resolving gel and 4.5 % stacking gel were prepared. Two samples were prepared for loading on the gel. The first sample contained 20 µL of the protein mixed with 20 µL of the SDS/BME loading buffer and the tube was placed on the heat block for 10 minutes at 98 ^{o}C. In the second Eppendorf tube, 20 µL of the native loading dye was mixed with 20 µL of the protein. Subsequently, 15 µL of each of the above mixtures were loaded into the wells in duplicate. The gel was run at 200 V for about 1 hour. The resulting gel was cut in half. One side was developed using the coomassie stain for protein detection while the other was used to test for enzyme activity using fluorometric analysis through 4-methylumbelliferyl-β-D-galactopyranoside (MUGal). This portion of the gel was soaked in 100 mM sodium acetate buffer, pH of 5.0 for 15 minutes with a gentle shaking. It was then incubated for one hour at 37 ^{o}C with 10 mM of MUGal which was prepared in the same buffer. The zymogram was analyzed under UV light to detect the enzymatic activity due to the release of methylumbelliferone which fluoresces under UV light.

The experiment was repeated using β-galactosidase in the SDS/BME loading dye, β-galactosidase in the native loading dye, BSA in the native loading dye, and the protein marker. Half of the gel was stained with coomassie stain for protein detection while the other was incubated with MUGal and observed under UV light.

2.2.14 Hydrolysis of lactose

2.2.14.1 Calibration curve for the assay of different concentrations of glucose

The range of glucose concentrations used to prepare a calibration curve was 1 mM to 10 mM. The 10 mM solution was used to make the rest of the solutions using serial dilution. A cocktail of the reagents was prepared by mixing 1750 µL of the 50 mM NaPi buffer, pH 7.4, 1 mL of phenol/4-

aminoantipyrine, 100 µL of glucose oxidase (5 mg of glucose oxidase dissolved in 5 mL of 50 mM NaPi buffer, pH 7.4), and 100 µL of peroxidase (5 mg of peroxidase dissolved in 5 mL of 50 mM NaPi buffer, pH 7.4.). A similar mixture was used as a blank at 510 nm. The reaction was started by adding 50 µL of 10 mM glucose. The samples were placed in a water bath at 37 ^{o}C for 40 minutes. After 40 minutes, the absorbance was measured at 510 nm. The experiment was done in triplicate and the absorbances were averaged. The same was done with the glucose concentrations of 5 mM, 2.5 mM, and 1 mM. A calibration curve was prepared by plotting absorbances of solutions vs glucose concentration.

2.2.14.2 Hydrolysis of lactose to determine the enzyme parameters of β-galactosidase.

The stock solution of 480 mM lactose was prepared in 50 mM NaPi buffer, pH 7.4. To an Eppendorf tube, 775 µL of lactose is mixed with 200 µL of 50 mM NaPi buffer, pH 7.4. Twenty-five microliters of the enzyme solution were added to a final concentration of 0.625 µM in the reaction mixture. The reaction was allowed to take place for 20 minutes. Glucose released was then quantified using the glucose oxidase/peroxidase method. The mixture contained 50 µL of the reaction mixture and 2950 µL of the cocktail reagent. This reagent contains 100 µL of glucose oxidase (5 mg of glucose oxidase dissolved in 5 mL of 50 mM NaPi buffer, pH 7.4), 1750 µL of 50 mM NaPi buffer pH 7.4, 100 µL of peroxidase (5 mg peroxidase dissolved in 5 mL of 50 mM NaPi buffer, pH 7.4), and 1 mL of phenol/4-aminoantipyrine. The same volume of the cocktail reagent was used in a blank at 510 nm. The color is developed at 37 ^{o}C after 40 minutes and the absorbance was determined at 510 nm. The experiment was repeated for the serial dilutions of the 480 mM lactose solution. The concentration of glucose released during the reaction was determined from the calibration curve and used to construct a plot of rate of the reaction vs lactose concentration.

CHAPTER 3: RESULTS AND DISCUSSION

The research on β-galactosidase from the *Enterobacter sp. YSU* was done to elucidate the biochemical properties of the enzyme. It was expressed in *E. coli* cells and then purified for carrying out the study. The enzyme activity was monitored using the chromogenic substrate *o*-NPGal. The enzyme's behavior in various conditions, e.g., varying pH and temperature was investigated. The β-galactosidase's activity towards lactose hydrolysis was studied. Kinetic parameters for both lactose and *o*-NPGal were obtained from the graphs using Michaelis-Menten and Lineweaver-Burk plots. The Lineweaver-Burk plot was used to find the type of inhibition demonstrated by galactose and glucose. The enzyme was also tested for transglycosylation activity using thin layer chromatography. The enzyme's active oligomeric form was determined with the use of 4-methlylumbelliferyl-β-D-galactopyranoside as the substrate.

3.1 Bacterial culture and the conditions, expression, and purification of the β-galactosidase enzyme

The *E. coli KRX* cells with the plasmid containing the *lacZ* gene encoding the *Enterobacter sp. YSU* β-galactosidase were cultured in Luria-Bertani medium containing ampicillin, incubated at 37 ^{o}C overnight then diluted 100 times. L-rhamnose was added for protein expression. The *E. coli KRX cells* have their $rhaP_{BAD}$ genes replaced with the T7 RNA polymerase gene. This allows the $rhaP_{BAD}$ promoter to control expression of T7 RNA polymerase. The promoter is repressed in the presence of glucose and activated in the presence of *L*-rhamnose allowing the expression of T7 RNA polymerase. This then initiates the transcription of the *lacZ* gene from the plasmid which is translated into the β-galactosidase protein in the bacterial cells. The cells were grown overnight at 20 ^{o}C and harvested by centrifugation.

The cells were frozen at -25 ^{o}C. This was done to avoid the degradation of the proteins before sonication. Sonication releases the cells' content by disrupting the cell membranes. The DNA was precipitated using streptomycin sulfate. After removing the streptomycin precipitate, the supernatant was mixed with ammonium sulfate starting with 45% ammonium sulfate saturation followed by 70% ammonium sulfate saturation. The protein was observed in the precipitate obtained at the 45% ammonium sulfate saturation as shown in figure 3.

3.2 Ion-exchange chromatography

After dialyzing out the ammonium sulfate, the protein sample was loaded on the Q-Sepharose column that was equilibrated with 20 mM NaPi buffer, pH 7.4. Q-Sepharose is a strong anion exchanger and, therefore, allows the negatively charged proteins to bind to it. The β-galactosidase enzyme is negatively charged at a pH of 7.4 as this pH is above its isoelectric point of 5.2. The column was washed with 20 mM NaPi buffer, pH 7.4 and the protein was eluted using sodium chloride gradient (range 100 mM to 1 M). The salt solution was used to break the β-galactosidase - Q-Sepharose interactions which releases the enzyme. SDS-PAGE was done to identify the fractions containing β-galactosidase using a 10% resolving gel and a 4.5 % stacking gel. The protein subunit has a molecular weight of 120 kDa which was calculated from its amino acid sequence.[57] The bands of 120 kDa appeared in the fractions collected with 400 mM sodium chloride. The fractions with salt concentrations above 400 mM did not show strong protein bands at 120 kDa as depicted in figure 3.

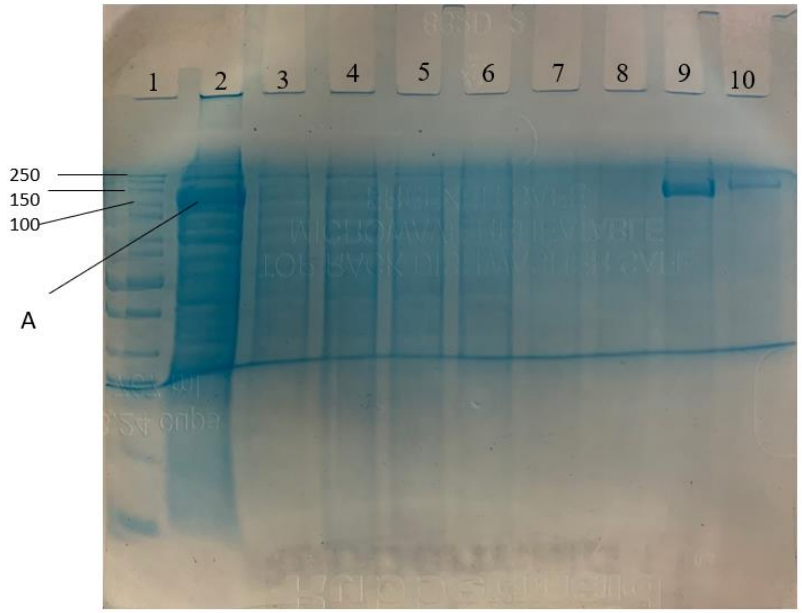


Figure 3: SDS-PAGE gel with the fractions obtained upon elution of the protein from Q-Sepharose ion-exchange column using NaCl gradient. Letter A shows the protein band.

Lane 1- Protein Marker; lane 2- Load dialyzed 45% ammonium sulfate precipitate; lane 3- Flow through; lane 4- Wash 1; lane 5- Wash 2; lane 6- 100 mM NaCl; lane 7- 200 mM NaCl; lane 8- 300 mM NaCl; lane 9- 400 mM NaCl; lane 10 - 1 M NaCl.

3.3 Evaluation of the protein concentration

Millipore centrifugal units were used to concentrate the protein sample. The protein's absorbance was measured at 280 nm and the concentration was calculated using the Beer-Lambert equation and the extinction coefficient of 264,000 $M^{-1}cm^{-1}$. The concentration was determined as 50 μM *per* monomer. The concentration per monomer was used throughout the research. The β-galactosidase concentration was also measured using the Bradford assay with bovine serum albumin (BSA) as a standard. The absorbance was recorded at 595 nm. A calibration curve was plotted as shown in figure 4. The linear equation $y= 0.0074x + 0.0243$ was obtained. The equation was used to determine the amount of protein. The concentration was found to be 7.44 mg/mL. To convert to moles, the molar mass of the monomer 120,000 g/mol was used, to give a concentration of 60 μM per monomer.

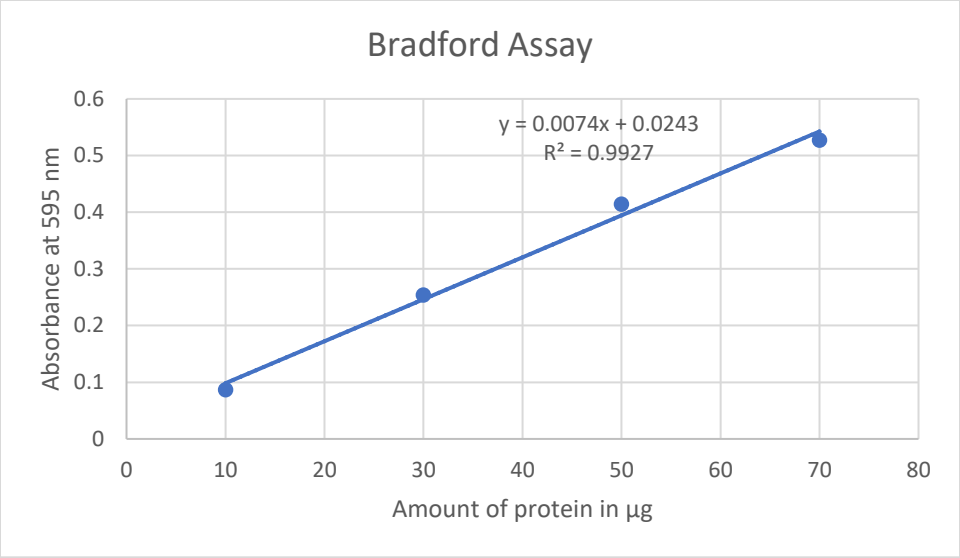
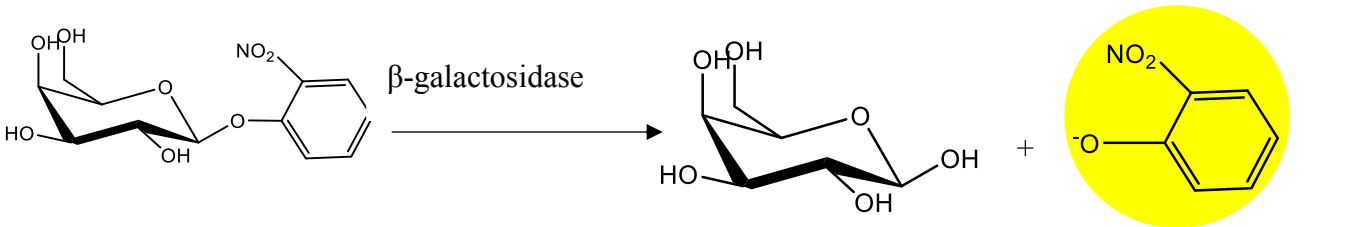


Figure 4: Bradford Assay Calibration Curve

3.4 The activity of β-galactosidase

The hydrolytic activity of β-galactosidase was monitored using *o*-NPGal as a chromogenic substrate. The concentration of the enzyme was maintained at 0.025 µM in the reaction mixture while the concentration of *o*-NPGal was varied. The *o*-NPGal consists of D-galactose and *o*-nitrophenol which are bound by a β-glycosidic bond. The cleavage of the bond leads to the formation of its two constituents, galactose and *o*-nitrophenolate. Galactose is not easy to detect, however *o*-nitrophenolate has a yellow color. The change of absorbance at 405 nm was measured using the spectrophotometer every 10 seconds for 90 seconds and used to calculate the initial rate of *o*-nitrophenolate formation.



o-NPGal Galactose

Figure 5: The hydrolysis of *o*-NPGal.

From the measured change of absorbance, the rate of the reaction was calculated using the Beer-Lambert equation. The concentration of *o*-nitrophenolate was calculated using the extinction coefficient of 3055 $M^{-1}cm^{-1}$ from which the reaction rate was calculated as shown in table 2.

Table 2: The concentration of *o*-NPGal in mM and the rate of its hydrolysis in µM/s

The concentration of *o*-NPGal in mM	**Rate of the reaction in µM/s**
6.0	3.52
4.5	3.62
3.0	3.65
1.5	3.48
0.80	3.03
0.40	2.25
0.20	1.41

The results demonstrated that the enzyme showed maximal activity at about 3.0 mM *o*-NPGal in the reaction mixture as shown in figure 6. This corresponded to a rate of reaction of 3.65 µM/s which was considered as the V_{max} of β-galactosidase with *o*-NPGal. The K_M for *o*-NPGal was determined from a concentration of substrate at ½V_{max} as 0.35 mM. The k_{cat} for the enzyme is 146 s^{-1}. Figure 7 shows the Lineweaver-Burk plot which was used to determine the K_M and V_{max} as 0.35 mM and 4.11 µM/s. The k_{cat} of β-galactosidase using the Lineweaver-Burk plot is 164 s^{-1}. There was a decrease in activity at higher concentrations of the substrate. This was likely due to inhibition by either substrate or by the products, galactose or *o*-nitrophenol.

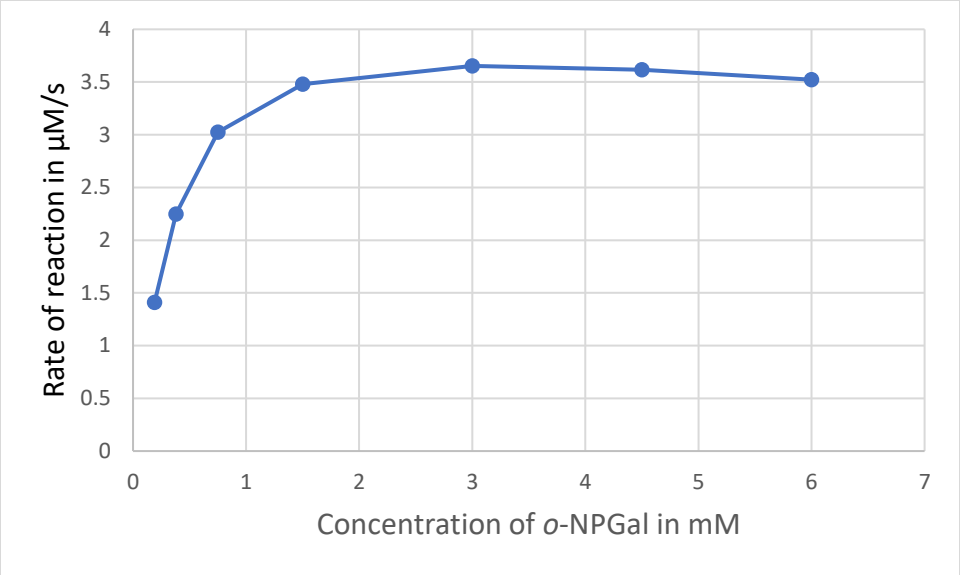


Figure 6: Michaelis-Menten plot for hydrolysis of *o*-NPGal catalyzed by β-galactosidase.

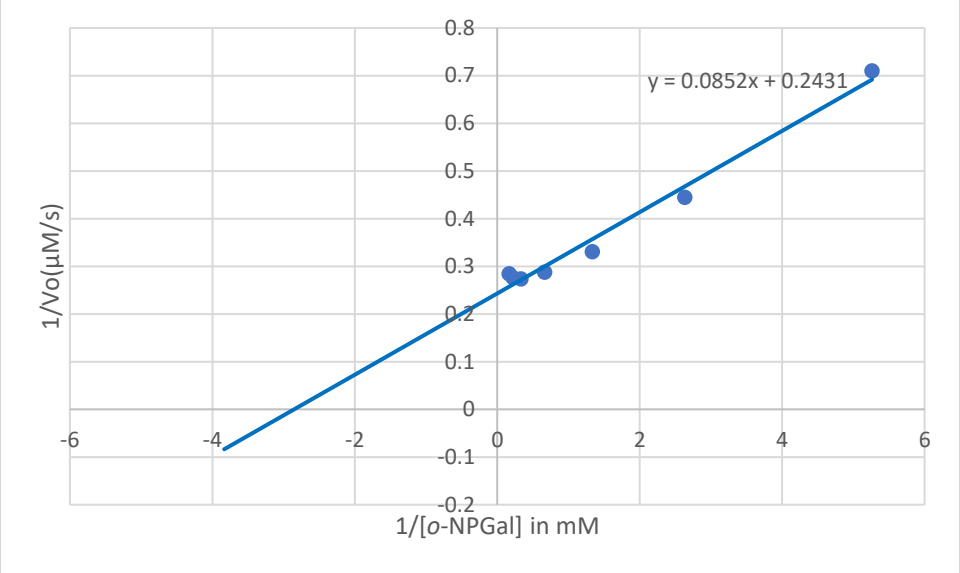


Figure 7: Lineweaver-Burk plot for hydrolysis of *o*-NPGal catalyzed by β-galactosidase.

3.4.1 The inhibitory effects of galactose and glucose on β-galactosidase

To test the effect of galactose on the enzyme activity, the hydrolysis of *o*-NPGal was conducted in the presence of high concentrations of galactose. The amount of galactose required to reduce the rate of the reaction was determined by keeping the concentrations of enzyme and *o*-NPGal constant and varying the amount of galactose added to the reaction mixture. The galactose concentration to

achieve 75% of the reaction rate at 3 mM *o*-NPGal was determined as 100 mM, while 250 mM galactose decreased the reaction rate by half. Then, 100 mM galactose concentration was kept constant as the concentration of *o*-NPGal in the reaction mixture was varied from 0.2 mM to 3 mM. The same was done for 250 mM galactose. Lineweaver-Burk plots were constructed and plotted together for reaction without galactose and in the presence of 100 mM and 250 mM galactose (figure 8).

The same experiment was repeated for glucose. It was determined that to achieve 75% of the reaction rate of the 3 mM *o*-NPGal mixture, the glucose concentration in the reaction mixture should be 150 mM. Then, 150 mM glucose concentration was kept constant as the concentration of *o*-NPGal in the reaction mixture varied from 0.2 mM to 3 mM. The experiments were conducted in triplicate and the outcomes were averaged. The rate of the reaction was determined from the absorbances measured using the spectrophotometer and calculated using the Beer-Lambert law. The reciprocal of velocity was plotted against the reciprocal of substrate concentration for reactions in the presence and the absence of 150 mM glucose as shown in figure 9.

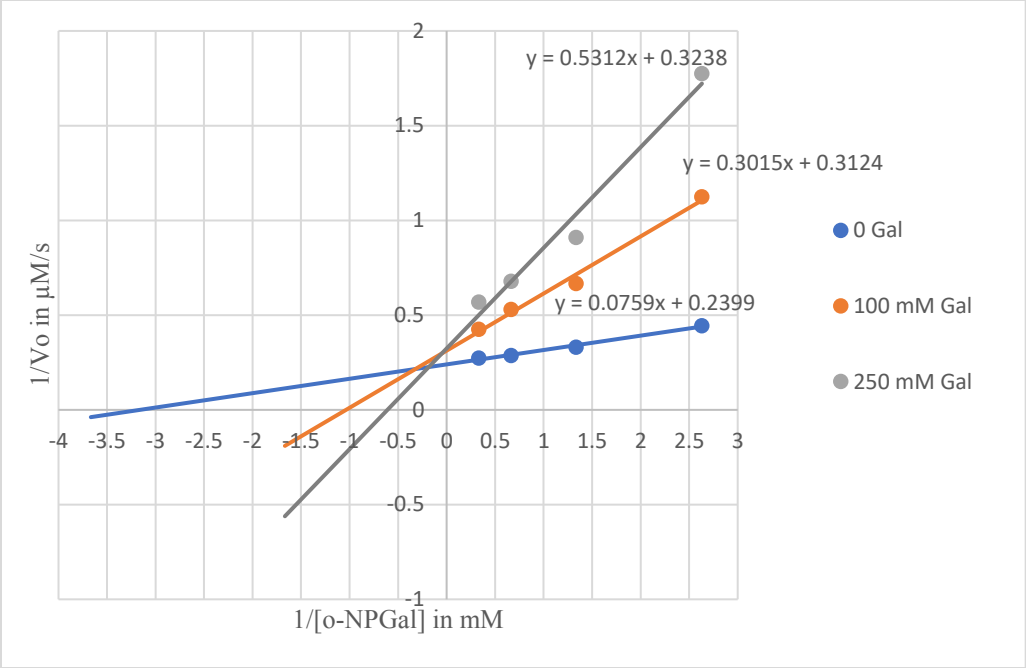


Figure 8: Lineweaver Burk plot for hydrolysis of *o*-NPGal in the presence of galactose as an inhibitor.

The graph in figure 8 demonstrates the inhibitory effects of galactose on β-galactosidase. It shows that galactose acts as a competitive inhibitor. The three lines intersect on the y-axis. On the Lineweaver Burk plot the y-intercept represents $1/V_{max}$ while the x-intercept represents $1/-K_M$. When the concentration of the substrate is high enough, the galactose has no effect, and the V_{max} remains unchanged. V_{max} was the same as shown in figure 8 within the margin of error of the experiment. At low substrate concentrations, galactose competes with the *o*-NPGal for the enzyme's active sites. The apparent K_M values are affected in the presence of galactose. The K_M increased from 0.3 mM without galactose to 1.0 mM and 1.6 mM for the 100 mM galactose and 250 mM galactose, respectively.

Glucose demonstrated uncompetitive inhibition as shown in figure 9. The lines representing the reciprocal for the rate of reaction in the presence and absence of the inhibitor on the graph were parallel to each other. Glucose lowered both the K_M and V_{max} for β-galactosidase by about the same

fold. The K_M was lowered from 0.3 mM without glucose to 0.2 mM in the presence of 150 mM glucose while the V_{max} was lowered from 4.2 µM/s for zero glucose to 2.6 µM/s. This shows that the glucose demonstrated uncompetitive inhibition as both parameters decrease proportionally.

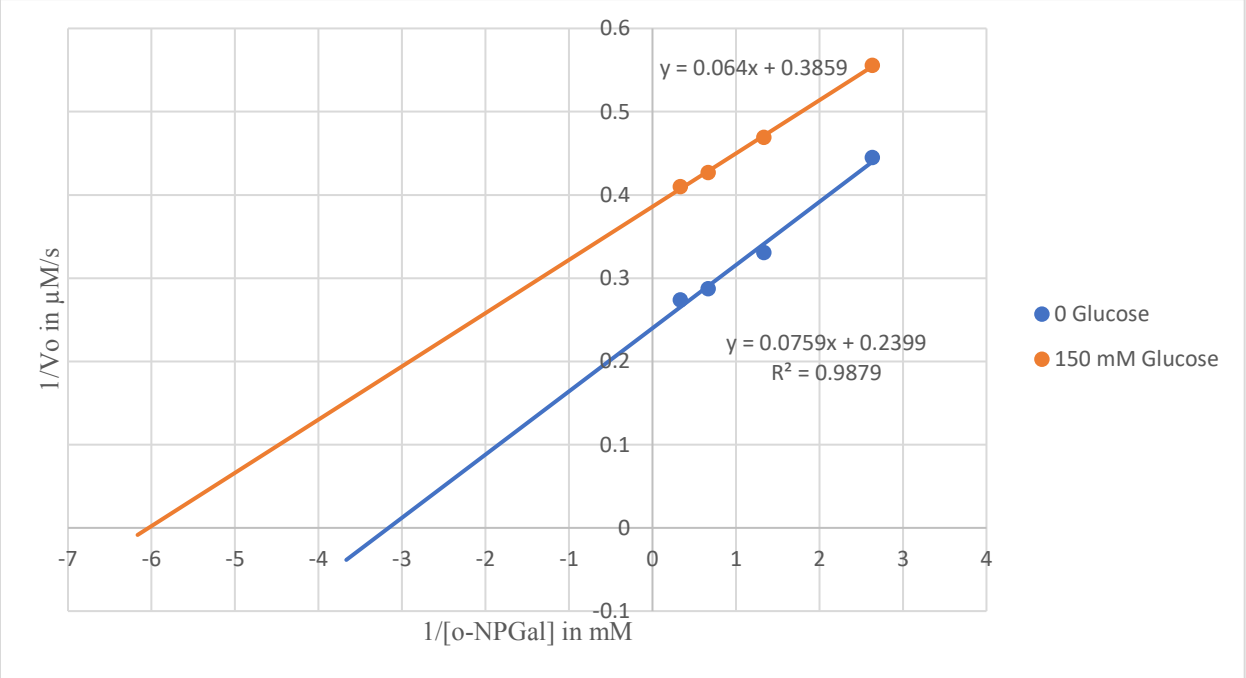


Figure 9: The results showed that the glucose displayed uncompetitive inhibition.

According to these results, the enzyme was less affected by glucose as compared to galactose. To decrease the rate of the reaction at 3 mM of *o*-NPGal to 75%, 100 mM galactose was required as compared to 150 mM glucose.

Galactose has been identified as a competitive inhibitor of β-galactosidases by previous studies. The β-galactosidase enzyme is used for hydrolyzed-lactose milk production as well as whey hydrolysis.[71] Nonetheless, complete hydrolysis with high lactose concentrations can be challenging because of the inhibitory role of galactose that slows the progression of hydrolysis and even stops the entire process.[72]

3.5 Thermal stability of β-galactosidase

The enzyme's thermal stability was investigated at temperatures ranging from 36 ˚C to 50 ˚C. The experiment was done in triplicate where the enzyme was incubated at different temperatures for 20 minutes and allowed to cool to room temperature before the assay was done using *o*-NPGal as a substrate. The enzyme's catalytic activity was measured for each incubation temperature to determine the stability of β-galactosidase up to the point at which its hydrolytic activity was lost. The rate of the reaction was calculated using the Beer-Lambert Law. The graph in figure 10 was drawn to show the thermal stability of the enzyme.

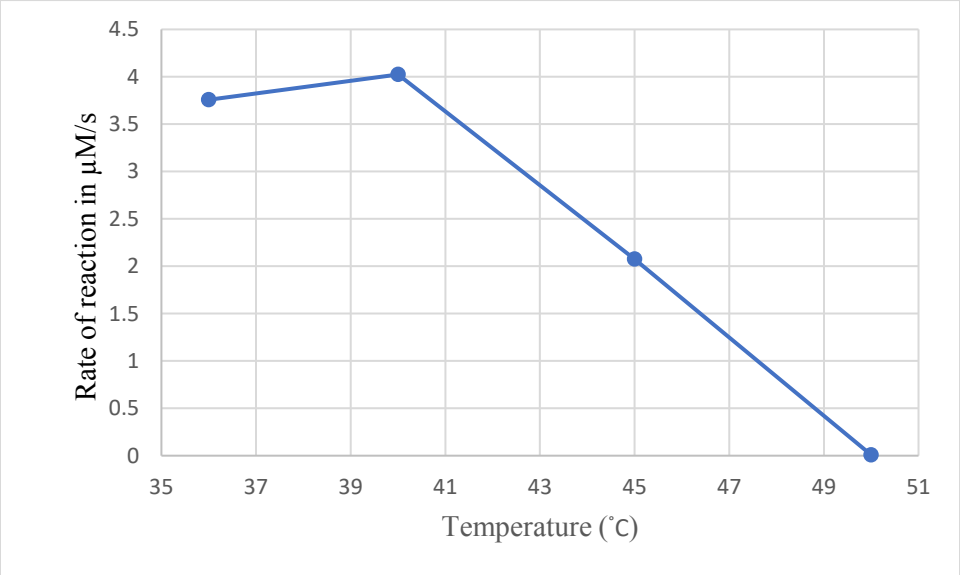


Figure 10: Thermal stability of β-galactosidase

The enzyme retained its activity only up to 40 ˚C. There was a rapid loss in activity when the temperature was increased to 45 ˚C, and the enzyme was fully inactivated at 50 ˚C. The thermal stability of this enzyme was low but comparable to some other galactosidases. For example, β-galactosidases extracted from *Kluyveromyces lactis* have exhibited the need for milder conditions to catalyze their reactions. These enzymes require temperature of 40 ºC to have high rates of lactose hydrolysis .[73]

The low thermal stability is a hindrance to the transgalactosylation reactions carried out by the galactosidases as these processes require higher temperatures to synthesize the oligosaccharides. The synthesis of oligosaccharides at high temperatures had been conducted by genetically modified β-galactosidase from *Kluyveromyces lactis* which is more stable. Meanwhile, the β-galactosidase obtained from *Bacillus circulans* was stable when incubated for 24 hours at 25 °C, there was a loss of 84 percent of its activity after a day of incubation at a temperature of 40 °C, and it was entirely inactivated at a temperature of 60 ºC.[74,75] Warmerdam et al. investigated the stability of β-galactosidase extracted from *Bacillus circulans* and showed that galactooligosaccharides are generated using the enzyme at high temperatures coupled with high substrate concentration.[75] High temperatures are advantageous since they improve substrate solubility, and this allows for greater substrate concentration.

3.6 The impact of pH on the activity of β-galactosidase

The effect of pH on the β-galactosidase activity was investigated in the pH range from 5.0 to 9.0. All buffers had a concentration of 50 mM and they included sodium acetate (pH 5.0), MES (pH 6.0), sodium phosphate (pH 7.4), Tris (pH 8.0), and borate (pH 9.0). The assay was carried out just like in the hydrolysis of *o*-NPGal except for the use of sodium carbonate to stop the reaction. At a pH greater than 8.0, the hydroxyl group of *o*-nitrophenol will deprotonate to form an *o*-nitrophenolate anion which has an intense deeper yellow color, while at a lower pH the o-nitrophenol will be protonated reducing the yellow color even though the *o*-NPGal is hydrolyzed. The β-galactosidase showed maximum activity at pH 7.4, however, the activity decreased at a pH below and above that. The loss of the enzyme's activity at low and high pH values is often due to the change in ionization state of the amino acids in the active site. The graph in figure 11 shows the results obtained from the experiment.

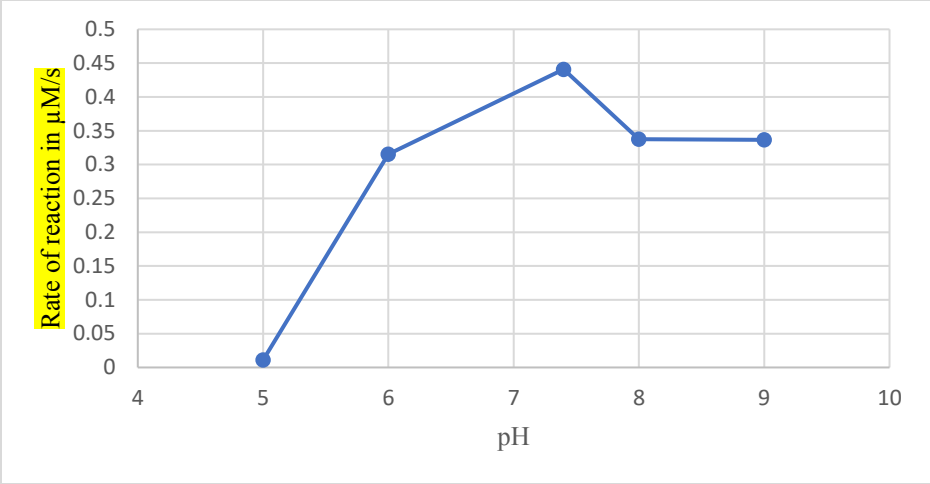


Figure 11: The rate of hydrolysis of *o*-NPGal at different pH.

The range of pH where the enzyme is active becomes important in the hydrolysis of lactose in milk or whey. This is because the pH of milk is between pH 5.0 and pH 6.5 while for acidic whey, it ranges from pH 4.5 to pH 5.0.[76] Other studies have revealed β-galactosidases with almost similar effect of pH on their activity. Chen et al. documented findings involving β-galactosidase from *Bacillus stearothermophilus,* the enzyme was estimated to have an optimum pH of 7.0 but retained activity between pH 6.0 and pH 7.5.[77] Fischer et al. examined the impact of pH on β-galactosidase from *Thermomyces lanuginosus* and found that its optimum pH is between 6.7 and 7.2 while the enzyme exhibited stability in a pH range of 6 to 9.[18] Therefore, β-galactosidase from *Enterobacter sp. YSU* has an optimum pH comparable with enzymes from other organisms.

3.7 Enzyme immobilization

As mentioned earlier, immobilization of β-galactosidase is an important process from an economic perspective because it makes the enzyme reusable and improves its continuous operation while eliminating the requirement of separating the enzyme from the reactants and products. It also improves the stability of the enzyme to changes in pH and temperature.[78]

3.7.1 Calcium alginate beads with the immobilized enzyme

The calcium alginate beads were prepared making sure they were the same size. Sodium alginate was dissolved in sodium phosphate buffer and then mixed with the protein to achieve a homogenous mixture. This was done to avoid any differences in the amount of enzyme trapped in each bead. The beads were formed while pipetting the alginate-protein solution into the calcium chloride solution in a petri dish. The beads were then cleaned in water to get rid of any calcium chloride and the enzyme that was not entrapped by the beads. The petri dish in figure 12 shows the beads that were prepared with the immobilized enzyme.

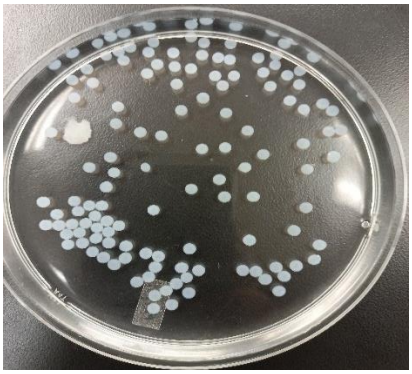


Figure 12: The calcium alginate beads with the immobilized enzyme in a petri dish.

The entrapment of the enzyme in calcium alginate beads is easy and the most used approach in the immobilization of enzymes in processing reactors.[79] The low concentrations of the sodium alginate and calcium chloride were used in bead formation for larger pore size to allow the reactants and products of the reaction to diffuse easily within the beads.[80]

3.7.2 Thermal stability of immobilized β-galactosidase.

The experiment was performed to determine the effect of enzyme immobilization on its thermal stability at temperatures ranging between 36 ^{o}C and 55 ^{o}C. For consistency, each experiment was done using seven calcium alginate - β-galactosidase beads. After incubation, the beads were cooled

to room temperature. The immobilized enzyme maintained its activity up to 50 ºC (figure 13), unlike the free enzyme which exhibited loss of activity at 50 ºC as shown in figure 10.

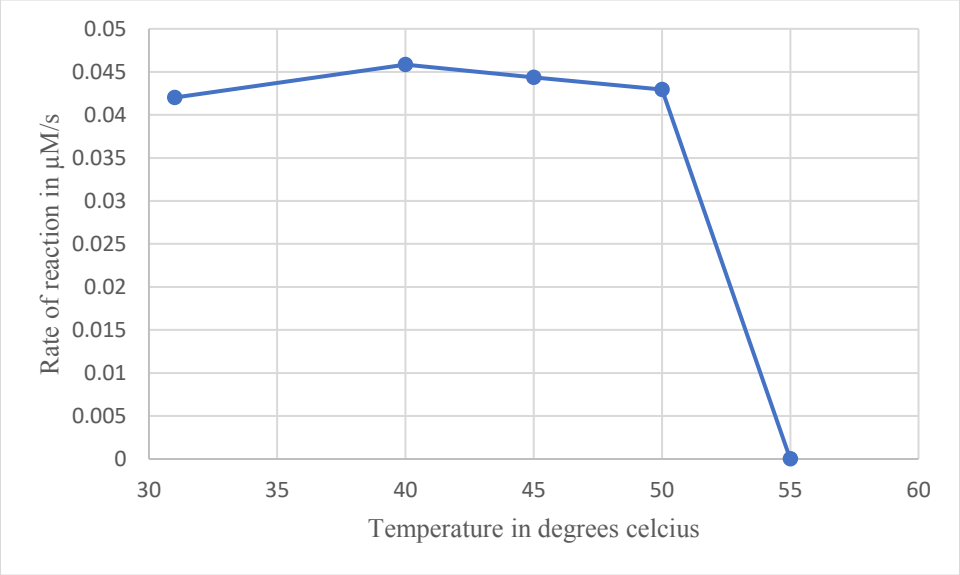


Figure 13: Thermostability of the immobilized β-galactosidase enzyme

Figure 13 shows that the enzyme retained activity at higher temperatures after immobilization compared to when it was in a free state. Similarly, Ansari et al. found that β-galactosidase from *Aspergillus oryzae* immobilized on bio-affinity support achieved thermal stability up to 60 °C while the free form had stability up to 55 °C.[81]

3.7.3 The pH dependence activity of immobilized β-galactosidase.

Seven calcium alginate beads were used in the activity tests at each pH for consistency. To stop the reactions, sodium carbonate was added. The absorbance was measured at 405 nm, and the Beer-Lambert equation was used to calculate the concentration of *o*-nitrophenolate which was plotted against pH in figure 14.

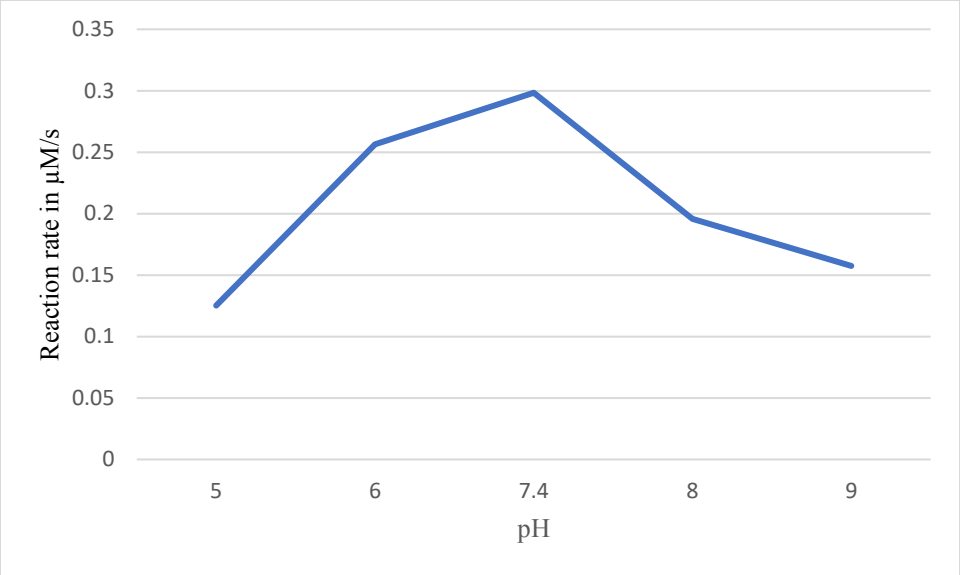


Figure 14: The activity of the immobilized enzyme at various pH

The optimal pH of immobilized β-galactosidase was found to be 7.4 as was shown in the earlier experiments with free enzyme. This demonstrated that the immobilization of the enzyme did not change its optimum pH. Conversely, Zhou et al. found that immobilization of β-galactosidase from *K. lactis* within a graphite surface using cross-linking technique increased the optimum pH of the enzyme by 1.1 pH unit.[82]

3.8 Effect of divalent ions on the activity of β-galactosidase.

Many β-galactosidases require metal ions for their activity. However, some β-galactosidases are inhibited by the presence of metal ions. For example, the β-galactosidase extracted from *Bacillus sp* MTCC 3088 is inhibited by the presence of Hg^{2+}, Cu^{2+}, and Ag^{+} in the concentrations between 1 mM to 2.5 mM.[27] *Enterobacter sp. YSU* β-galactosidase activity was investigated in the presence of selected ions to determine how they affect its activity.

EDTA is a chelating agent. It strongly binds Pb^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, and Co^{2+}.[83] To remove potential metal ions from the protein, β-galactosidase was allowed to incubate for 30

minutes with EDTA. The activity of the enzyme was tested upon EDTA exposure. Addition of EDTA affected the activity of β-galactosidase. There was a decline in activity to about 33% after incubation of enzyme with EDTA. The selected divalent ions were added to determine if they could restore the enzyme activity after EDTA exposure. The ions were added in excess compared to the EDTA concentration to overcome any free EDTA left in the solution. After the addition of each divalent ion, the mixture was incubated for 30 minutes to allow the divalent ions to bind to the enzyme. The rate of the reaction in the presence of each divalent ion was calculated and the data sets were plotted as a bar graph (figure 15) using the reaction rates expressed as a percentage of the control where the reaction was done using the enzyme before EDTA was added.

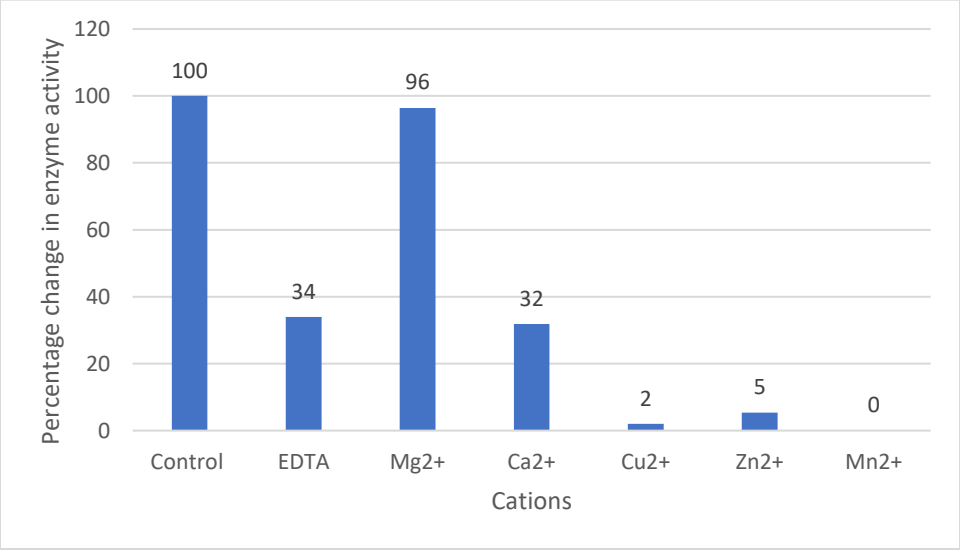


Figure 15: The effect of the divalent ions in restoring the activity of the enzyme lost after the addition of EDTA.

In this research, the addition of Mg^{2+} to the enzyme-EDTA solution restored the activity to 96% of the control. The Ca^{2+} did not show any significant restoration of activity. When the Cu^{2+} or Zn^{2+} were added, the enzyme activity drastically decreased to 2% and 5% of the control, respectively. The enzyme activity was much lower than the decrease caused by the addition of EDTA. The effect of manganese ions was not conclusive as the color of the manganese solution interfered with

the absorbance of the product and the rate of the reaction was difficult to determine. The inhibition of the enzyme in the presence of EDTA and the restoration of its activity by the addition of Mg^{2+} indicates that this enzyme requires Mg^{2+} for its activity and, probably, its stability. Therefore, it can be concluded that it's a metalloenzyme. According to Atwood, metalloenzymes involve a group of enzymes that utilize metal cations as the cofactor at the enzymes' active sites.[84] Such enzymes catalyze a wide range of reactions, including reduction-oxidation and hydrolysis. It has been noticed that the presence of Mg^{2+} increases the catalytic activity of many β-galactosidase enzymes.[85] Magnesium ions have been shown to increase activity of β-galactosidase isolated from *Lactobacillus pentosus* by interacting with amino acids in the active site and, at the same time, by providing stability to the enzyme.[85] Contrarily, it has been shown that EDTA in concentrations up to 25 mM had no effect on β-galactosidase isolated from *Bacillus sp.* MTCC 3088. Divalent ions like Cu^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, and Ni^{2+} showed an inhibitory effect on this enzyme. Consequently, this demonstrated that some β-galactosidases do not require the divalent ions for its activity.[27]

3.9 Transglycosylation

3.9.1 Transglycosylation by β-galactosidase using lactose.

Oligosaccharides are classified according to the number of monomeric units present. They can be disaccharides, trisaccharides, tetrasaccharides, etc. The synthesis of the oligosaccharides can occur by transglycosylation. This involves the transfer of a glycosyl moiety from a donor which is a glycoside to a sugar acceptor which contains a hydroxyl group. During the β-galactosidase reaction, a covalent bond is formed between the enzyme and the glycosyl moiety. The intermediate formed may undergo hydrolysis forming monosaccharides if the acceptor is water. It may also

undergo transglycosylation if the acceptor is a saccharide. The oligosaccharides produced via transglycosylation reactions catalyzed by β-galactosidase using lactose reach a maximum concentration within a given period and, if not carefully monitored, undergo hydrolysis to form the reactants. This occurs when the lactose is used up or the concentration of products has increased.[6] This research was done to determine if β-galactosidase from *Enterobacter sp. YSU* was capable of carrying out transglycosylation by varying the conditions of the reaction like the pH, temperature, and the composition of the solvent.

The pH of the reaction was varied using buffers with pH 6.3, 7.4, and 8.0 to determine the conditions necessary for transglycosylation. The temperatures varied from 37 ^{o}C to 50 ^{o}C. The results obtained from the study using lactose as a substrate at various pH, temperature, and the solvents are illustrated in figure 16. The TLC plates indicate that some lactose was hydrolyzed to glucose and galactose, but no oligosaccharides were formed in any of reactions conducted. In figure 16**e**, the transglycosylation of lactose at pH 8.0 at 45 ^{o}C was tested. The spots on the TLC indicate that no oligosaccharides were formed in the process and only hydrolysis took place. The lactose was hydrolyzed to glucose and galactose.

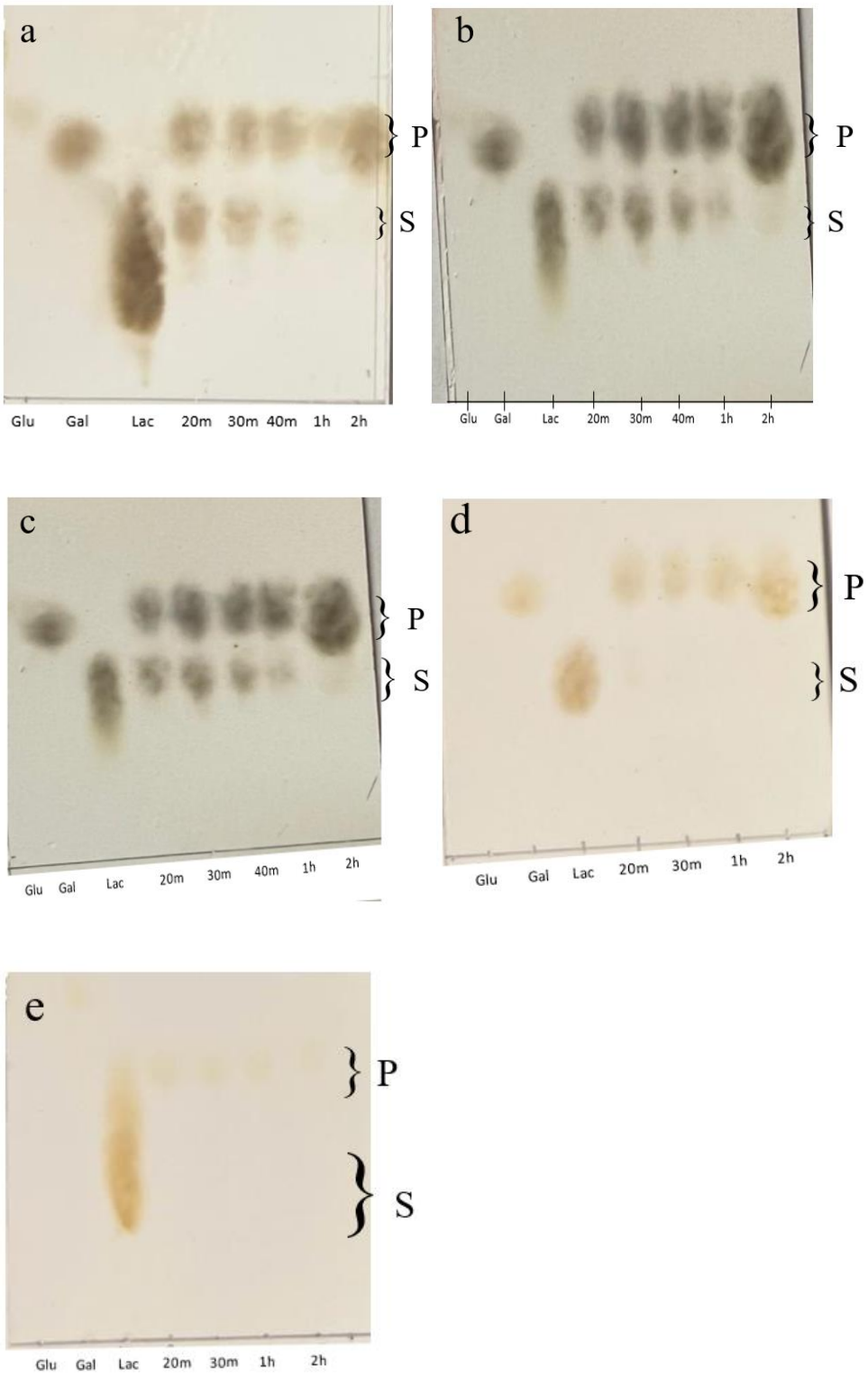


Figure 16: TLC plates for investigating transglycosylation of lactose. **a.** pH 7.4 at 40 ^{o}C, **b**. pH 7.4 at 37 ^{o}C, **c**. pH 7.4 at 45 ^{o}C, **d**. pH 6.3 at 45 ^{o}C, **e.** pH 8.0 at 45 ^{o}C; P-the products of hydrolysis, S- the substrates.

The experiment was done with the intention to determine whether higher temperatures could lead to transglycosylation. The immobilized enzyme was used in the reactions carried out at pH values of 6.3 and 7.4 at 50 ºC for 2 hours. The results analyzed by TLC did not show any transglycosylation activity. The spots on the TLC plates in figure 17 indicate that the lactose was not hydrolyzed to glucose and galactose as the enzyme likely lost activity over time due to the lack of stability at high temperature for long periods.

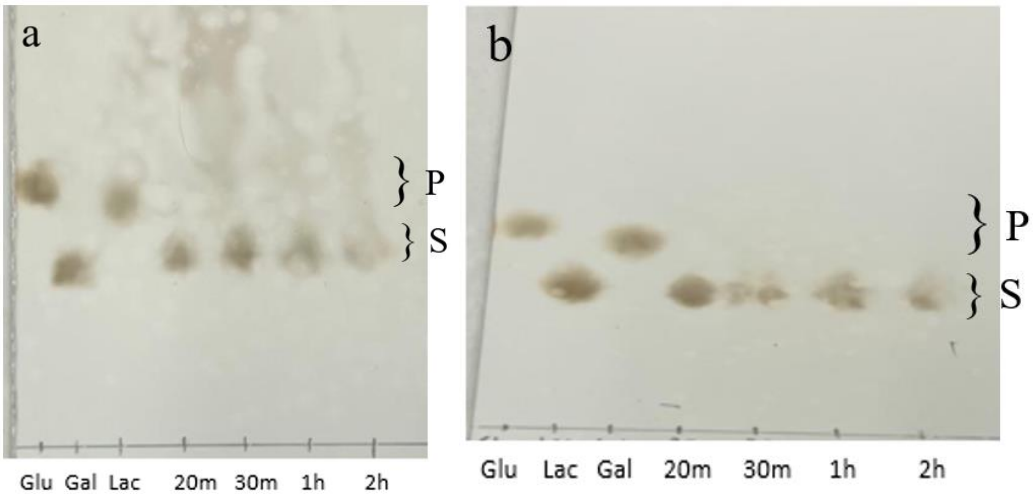


Figure 17: Transglycosylation of lactose at 50 ºC using immobilized β-galactosidase.

a. pH 6.3, 50 ˚C; **b.** pH 7.4, 50 ˚C for 2 hours, P – the products of hydrolysis, S- the substrates.

3.9.2 Transglycosylation in the presence of 5% isopropanol and 15 % ethanol.

The buffer was replaced with solutions of either isopropanol or ethanol prepared in 50 mM NaPi buffer, pH 7.4. In the presence of ethanol and isopropanol, there was an increase in the rate of hydrolysis of lactose but transglycosylation did not take place as shown in figure 18a and 18b.

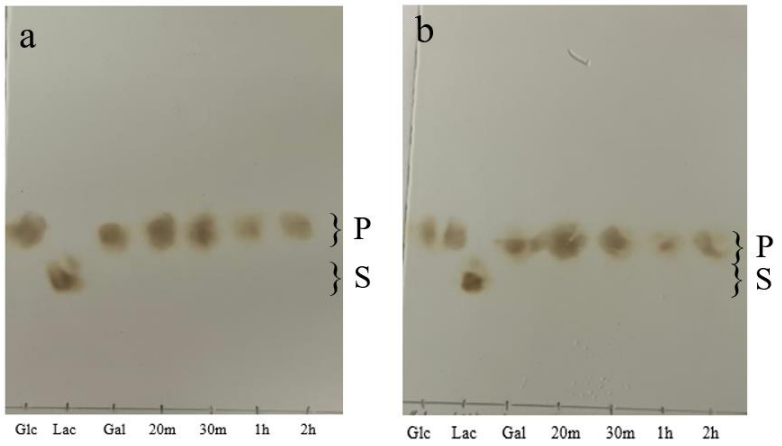


Figure 18: Transglycosylation in the presence of ethanol and isopropanol.

a. 15% ethanol, pH 7.4, 40 °C; **b.** 5% isopropanol, pH 7.4, 40 °C: P – the products of hydrolysis, S- the substrates.

3.9.3 Reactions to determine if laminarin is a substrate for β-galactosidase.

Laminarin from brown algae contains β (1→3) glucans and β (1→6) branch linkages.

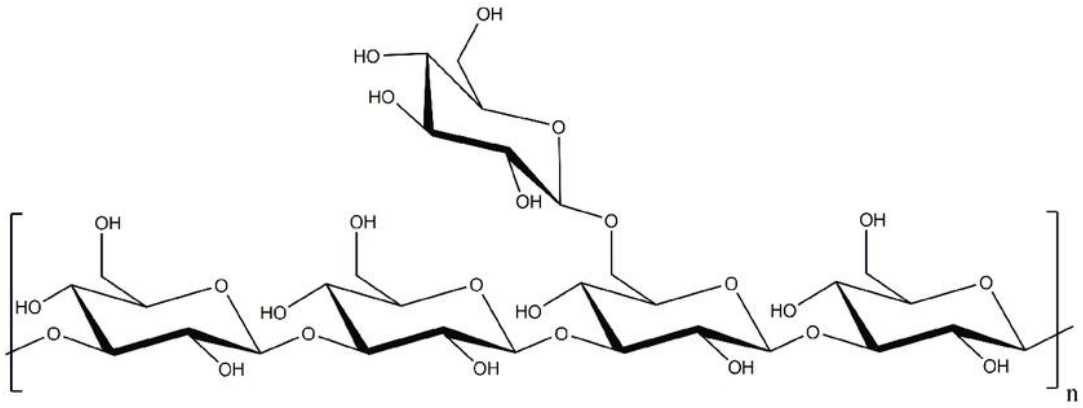


Figure 19: Laminarin structure

According to Rocher et al. laminarin can be hydrolyzed to glucose by using a laminarinase that splits the β-1,3 bonds of the D-glucan, forming oligosaccharides comprising between 2 to 10 residues.[86] The enzymes from the glycoside hydrolase (GH) family such as keratan-sulfate end*o*-1,4-β-galactosidase and end*o*-β-1,4-galactosidase (EC 3.2.1.103) demonstrate hydrolase activity on marine and plant polysaccharides.[87]

Laminarin was prepared as a 1% (m/v) solution and then was mixed with protein. The reaction was conducted at 40 ^{o}C for 2 hours and aliquots were taken and analyzed using TLC. The results in figure 20 showed that laminarin was not broken as there were no spots representing the lower mass saccharides in the mixture of the protein and the laminarin. The β-galactosidase did not hydrolyze the laminarin. Therefore, the enzyme cannot break the β (1→3) or β (1→6) linkages in laminarin.

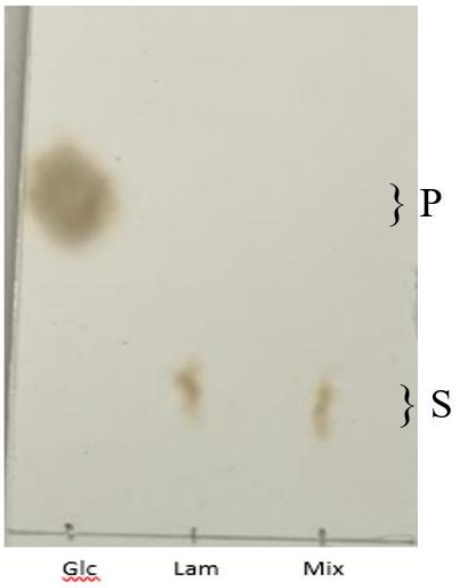


Figure 20: Investigation on laminarin hydrolysis

Spots of laminarin, glucose and the mixture containing the enzyme and laminarin, P – the products of hydrolysis, S- the substrate.

3.10 The effects of solvents and other chemicals on β-galactosidase

3.10.1 The effect of ethanol and isopropanol on β-galactosidase

Some substances can change the activity of an enzyme by attaching to the specific sites on the enzyme preventing the substrates from binding. Alternatively, some compounds can bind to allosteric sites and distort or slightly change the profile of the active site affecting the substrate-enzyme interactions. Alcohol can act as chaotropic agents that disrupt hydrogen bonding. This can

change the protein conformation. Alcohol alters the solvent polarity. Their presence also can affect the ionization state of the amino acids in the active site.[26]

In this study, solutions of ethanol with concentrations between 0.5% and 35% (v/v) were prepared in 50 mM NaPi buffer, pH 7.4. They were used as a buffer in reactions of the hydrolysis of *o*-NPGal. The results indicate that in concentrations up to 15% (v/v), ethanol increases the activity of the enzyme and thereafter the activity starts decreasing as depicted in figure 21.

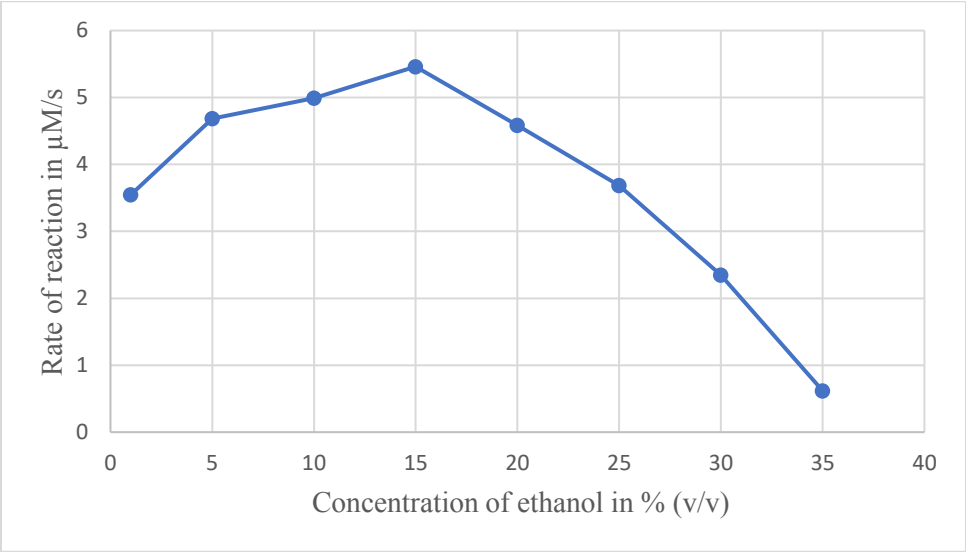


Figure 21: The effect of ethanol on the activity of β-galactosidase.

Solutions of isopropanol were prepared in 50 mM NaPi buffer, pH 7.4. The assays were carried out just like with ethanol. The effect of isopropanol on β-galactosidase's activity is shown in figure 22. The enzyme in 2.5% (v/v) isopropanol showed no significant change in its activity. There was an increase in enzyme activity in 5% (v/v) of isopropanol followed by a decrease in activity. Isopropanol produces a decrease in enzyme activity at lower concentrations as compared to ethanol. Alcohols like *n*-propyl alcohol are known to dissociate dimers to inactive subunits of the protein by disrupting hydrophobic interactions.[26,88] The alcohols have been identified to increase

the activity of the enzyme by altering the solvent polarity and causing the ionization of the two glutamate residues.[26]

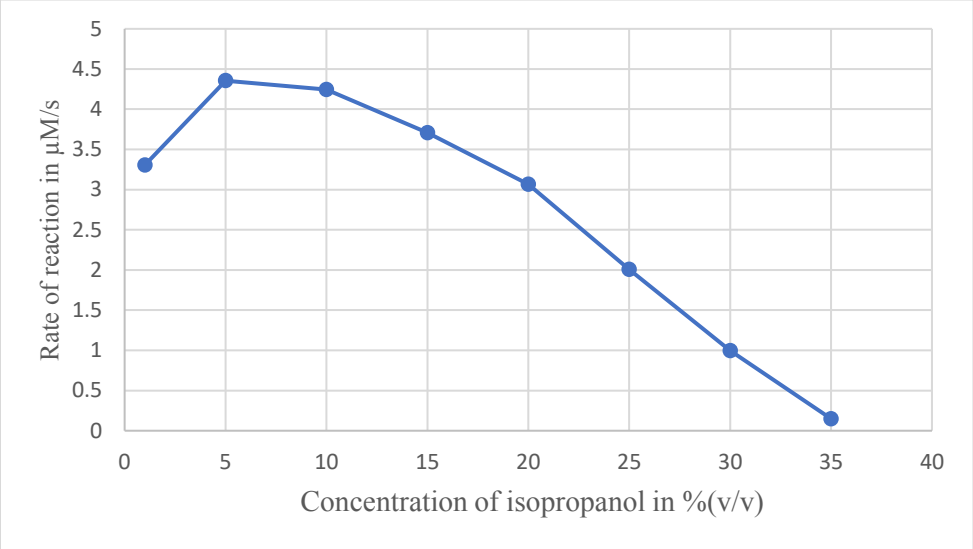


Figure 22: The effect of isopropanol on the activity of β-galactosidase.

3.10.2 Effect of SDS, DMSO, and 2-mercaptoethanol (BME) on β-galactosidase activity

The experiments were conducted to determine the effect of SDS, DMSO, and 2-mercaptoethanol on the activity of the enzyme. SDS is a detergent that consists of an anionic hydrophilic head and a long hydrophobic tail. SDS leads to the disruption of hydrophobic interactions when the concentrations are high. Solutions of SDS with concentrations ranging from 0.5% to 15% (m/v) were prepared in 50 mM NaPi buffer, pH 7.4. The assay was carried out using 0.025 µM protein and 3 mM *o*-NPGal as the substrate. The β-galactosidase showed high activity at 2.5 % (m/v) of SDS above which the activity started decreasing. Figure 23 depicts the impact of different concentrations of SDS on β-galactosidase activity.

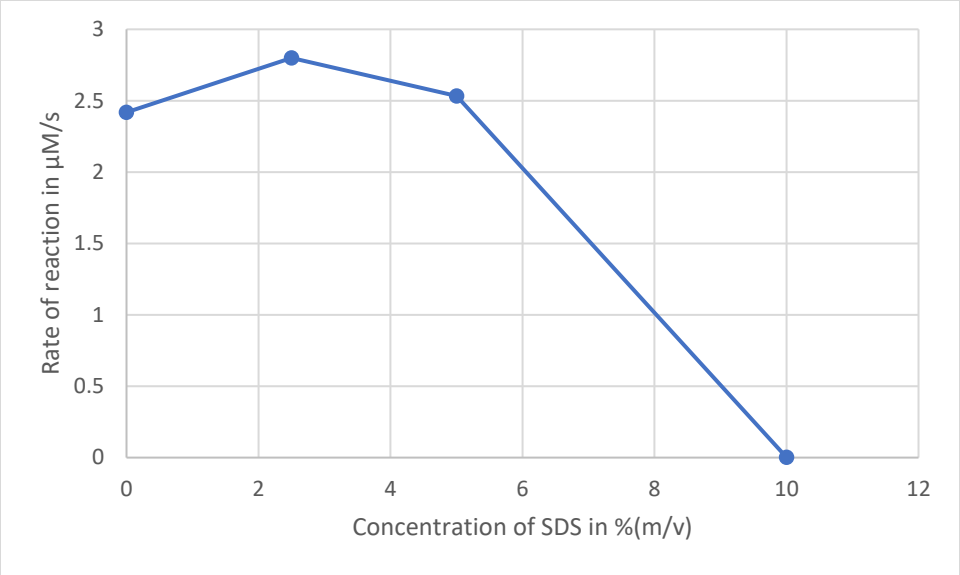


Figure 23: Effect of varying concentrations of SDS on the β-galactosidase

The impact of 2-mercaptoethanol (BME) on β-galactosidase was also investigated. BME is a reducing agent that reduces disulfide bonds. It may cause the disruption of the tertiary and quaternary structure resulting in unfolding of the protein. Solutions of 2-mercaptoethanol were prepared in 50 mM NaPi buffer, pH 7.4 and used as the assay buffer in concentrations ranging between 1.25 mM to 10 mM during the hydrolysis of *o*-NPGal. The reaction mixture contained the protein at concentration 0.025 µM and 3 mM *o*-NPGal. The study established that 2-mercaptoethanol had no significant effect on the enzyme activity as depicted by figure 24.

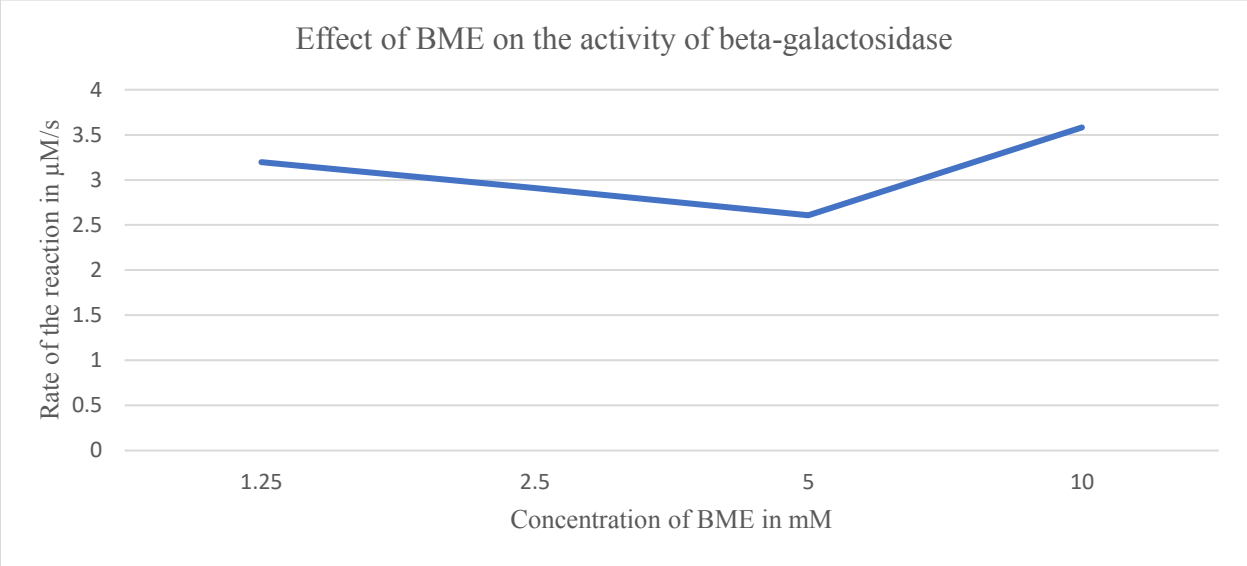


Figure 24: Effect of 2-mercaptoethanol on β-galactosidase activity.

Dimethylsulfoxide (DMSO) is amphiphilic and it binds to both polar and non-polar residues. It interacts with amino acids by preferentially binding to the hydrophobic aromatic groups. DMSO in high concentrations causes conformational changes in proteins as it binds to the hydrophobic and aromatic side chains affecting protein folding. Such binding significantly increases after unfolding due to the exposure of the hydrophobic residues.[89] Solutions of DMSO were prepared in concentrations ranging from 0.5% to 60% (v/v) in 50 mM NaPi buffer, pH 7.4. In this study, the assay was carried out using 0.025 µM protein and 3 mM of *o*-NPGal as the substrate. DMSO did not cause significant changes in the activity of the enzyme till the concentration reached 25% (v/v) and then the activity of β-galactosidase decreased at higher concentrations. Figure 25 shows the effect of various concentrations of DMSO on β-galactosidase activity.

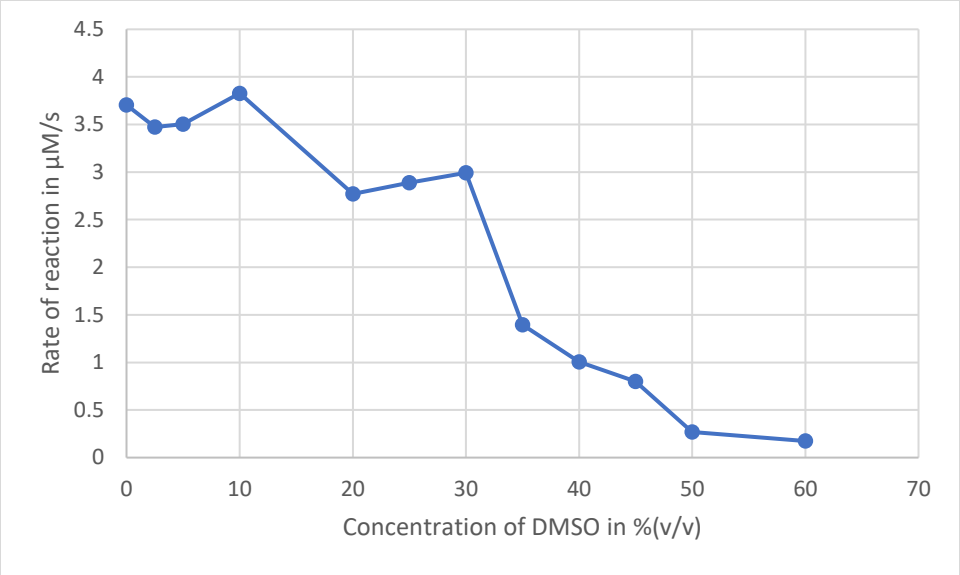


Figure 25: Effect of DMSO on β-galactosidase activity.

3.10.3 Investigating the quaternary structure of β-galactosidase using 4-methlylumbelliferyl-β-D-galactopyranoside.

To understand the quaternary structure of β-galactosidase, 4-methlylumbelliferyl-β-D-galactopyranoside was used as a substrate. When the enzyme cleaves the glycosidic bond, the methylumbelliferone formed fluoresces under UV-light. The experiment was done using 10% resolving gel without SDS. Two β-galactosidase samples were prepared to run on the native gel. One sample contained the protein sample prepared in denaturing conditions by mixing protein with SDS, BME and heating the mixture. Another sample was prepared by mixing the protein with the native loading buffer under non-denaturing conditions. The protein samples were loaded in duplicate. One-half of the resulting gel was developed with the coomassie stain while the other half was used to determine the activity of the enzyme using the fluorometric analysis using the 4-methylumbelliferyl-β-D-galactopyranoside.

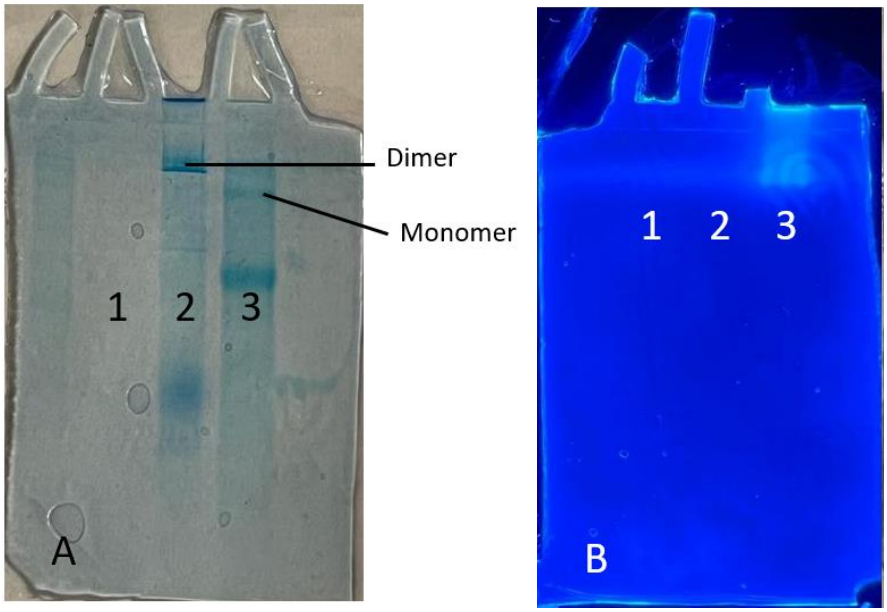


Figure 26: Investigation of the active oligomeric form of β-galactosidase

Gel A. Portion of the gel developed with coomassie blue stain. Lane 1: the BSA protein, lane 2: the protein in the native loading dye; lane 3: the protein in SDS/BME loading dye. **Gel B**. Portion of the gel that was incubated with 4-methylumbelliferyl-β-D-galactopyranoside and visualized under UV, lane 1: BSA protein, lane 2: the protein in SDS/BME loading dye, lane 3: the protein in the native loading dye.

Lane 3 on gel A (figure 26) shows that the SDS caused the dissociation of the enzyme into its monomers. Lane 2 in gel A shows the main band corresponding to a protein dimer. The bands in gel B showed that the dimer of the protein is active as the lane corresponding to the position of the dimer fluoresces while the monomer in lane 2 is not active as there is no fluorescence under UV light. It also showed that the BSA protein that was used as a control did not fluoresce under UV light as it does not cleave the glycosidic bond in 4-methylumbelliferyl-β-D-galactopyranoside. The gels show that the dimer of the enzyme with mass about 240 kDa is active while the monomer is not.

3.11 Hydrolysis of lactose using β-galactosidase.

The β-galactosidases hydrolyze lactose to galactose and glucose. To determine the rate of hydrolysis, the amount of glucose produced is quantified using the glucose oxidase – peroxidase assay. The D-glucose is oxidized by glucose oxidase to D-gluconic acid and hydrogen peroxide as shown in figure 27A. The phenol/4-aminoantipyrine is oxidized by hydrogen peroxide in the presence of peroxidase to form the pink *p*-quinoneimide as shown in figure 27B. First, the assay was carried out with known concentrations of glucose to draw the glucose calibration curve.

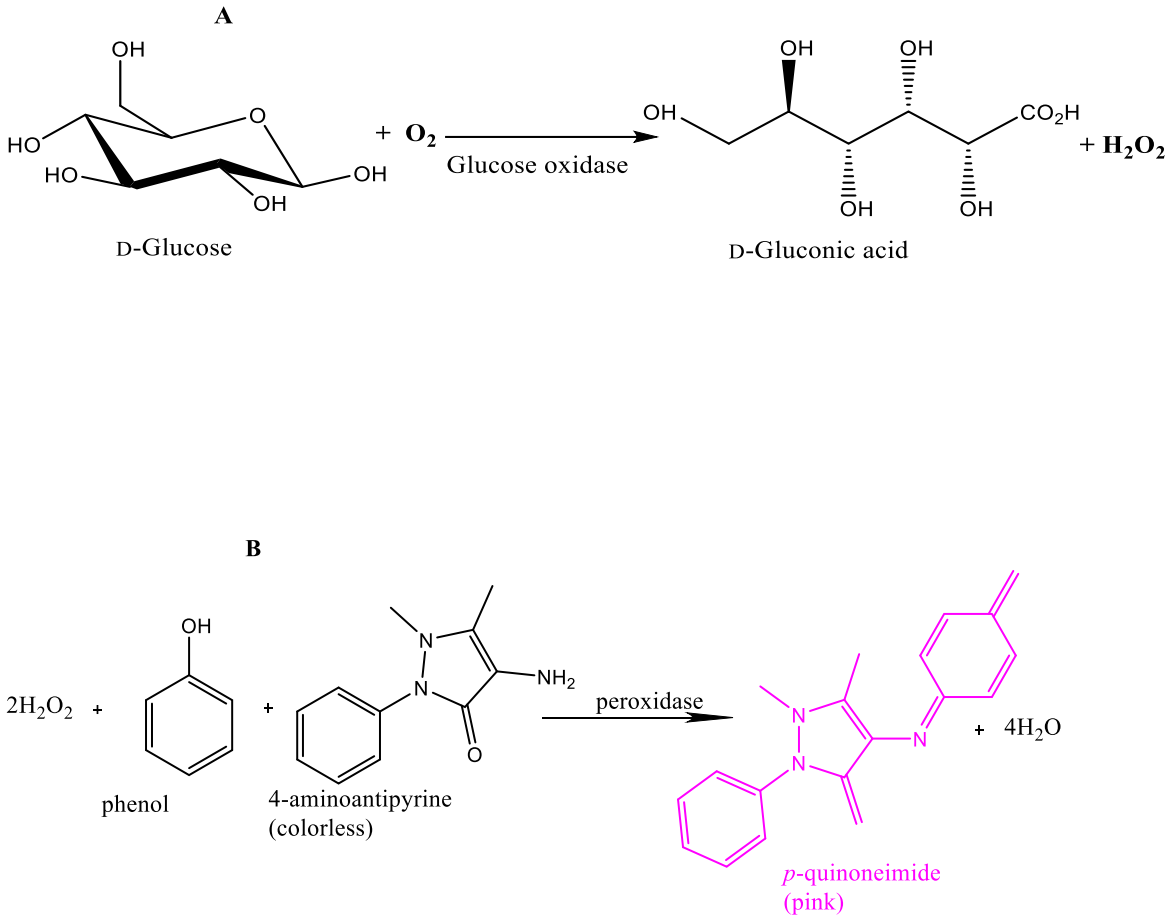


Figure 27: The glucose oxidase – peroxidase assay.

A. The oxidation of glucose to gluconic acid with release of hydrogen peroxide; **B.** The formation of *p*-quinoneimide.

The absorbance of *p*-quinoneimide was measured at 510 nm and the data was used to construct the glucose calibration curve shown in figure 28.

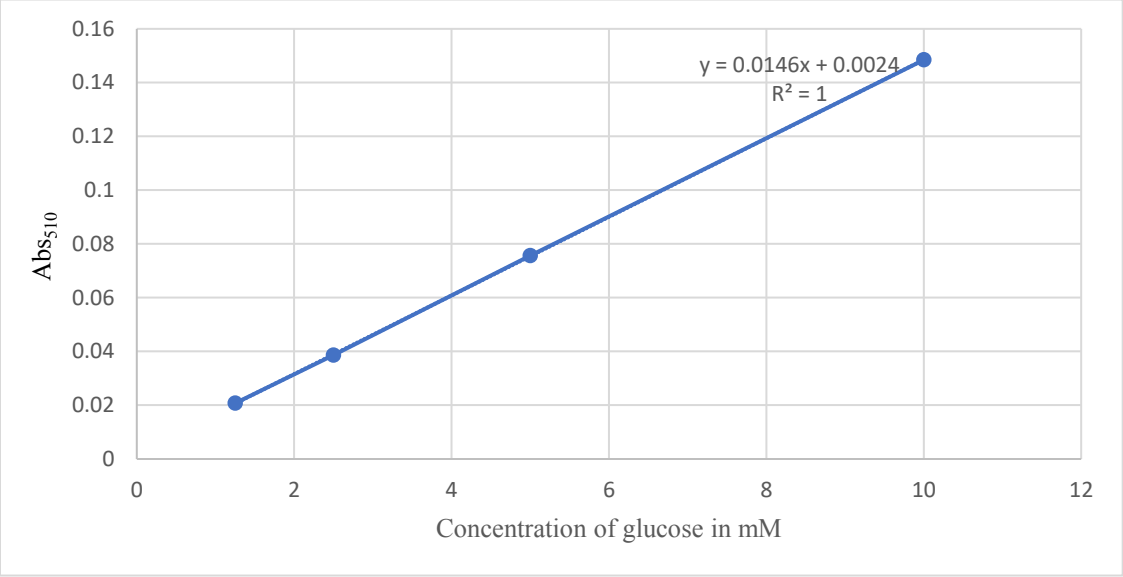


Figure 28: Calibration curve for the quantitation of glucose.

The glucose calibration curve was used to determine the amount of glucose formed in the hydrolysis of lactose. From the Michaelis-Menten plot in figure 29, the enzyme has a V_{max} of 2.38 mM/min in lactose and a calculated K_M of 22 mM. The k_{cat} of β-galactosidase with lactose is 3.81×10^3 per min. The Lineweaver-Burk plot in figure 30 showed the V_{max} and K_M to be 3.43 mM/minute and 63 mM, respectively. The k_{cat} is 5.49×10^3 per minute. The Michaelis-Menten plot provides more accurate parameters, and therefore its V_{max}, K_M, and k_{cat} will be used for further discussion.

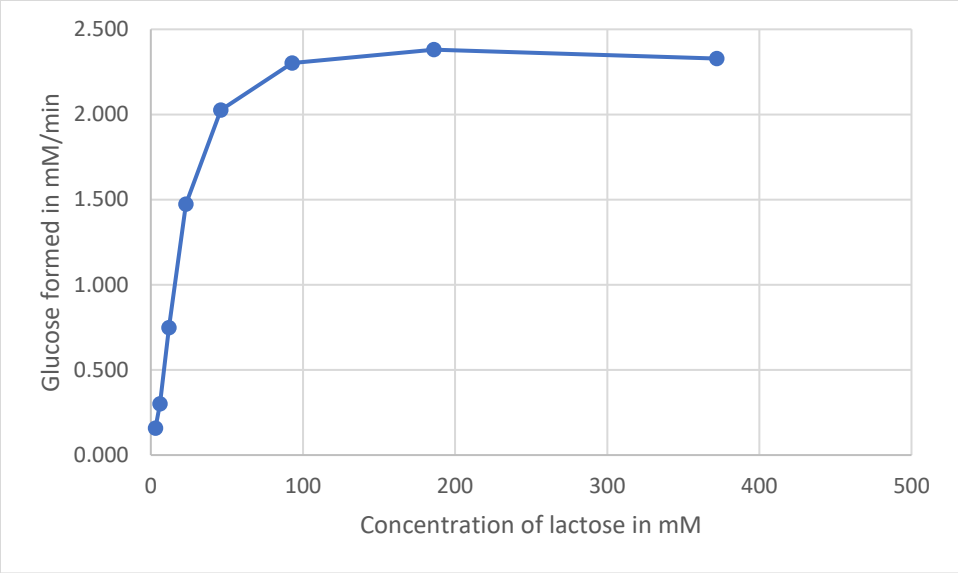


Figure 29: Michaelis-Menten plot for the rate of hydrolysis of lactose

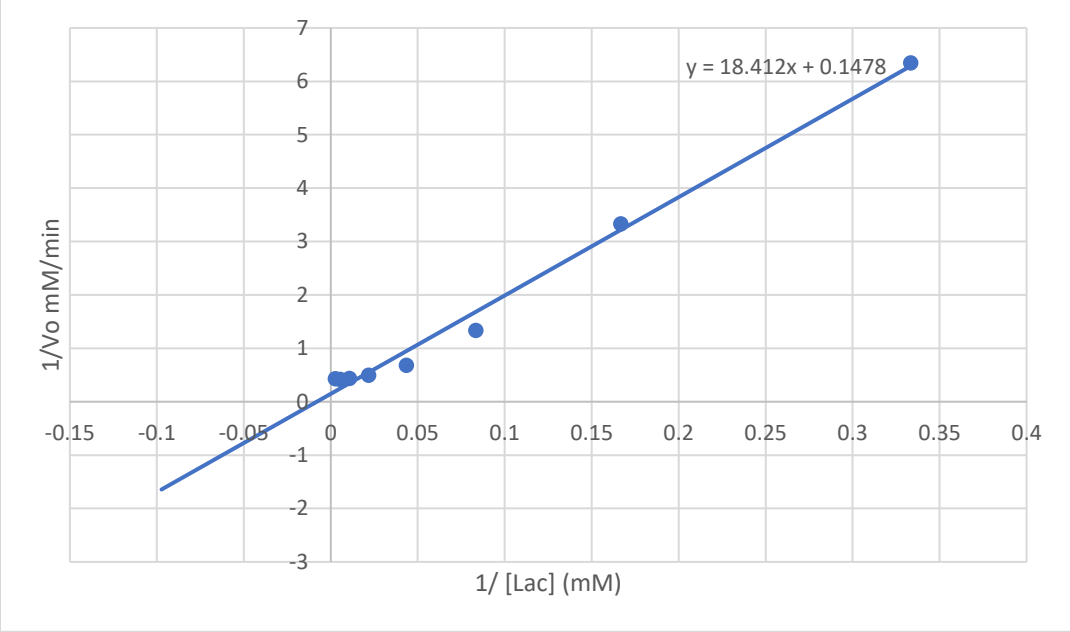


Figure 30: Lineweaver-Burk plot for the hydrolysis of lactose

The β-galactosidases hydrolyze the β-glycosidic bond in lactose at different rates depending on the enzyme source. The enzyme extracted from *Lactobacillus plantarum* HF571129 shows a K_M of 23.28 mM and k_{cat} of 0.0111 min^{-1} for lactose. The K_M values of the two enzymes are very close. These enzymes have similar affinity for lactose, but the catalytic constant of β-galactosidase

extracted from *Enterobacter sp. YSU* is much higher.[13] The β-galactosidase extracted from the *Lactobacillus sakei* Lb790 showed a K_M of 20 mM and k_{cat} of 43 s^{-1}.[6] The k_{cat} of this enzyme is lower than that for β-galactosidase extracted from *Enterobacter sp. YSU*. This enzyme requires more research to be done on it so that it can be exploited in industrial applications as it has a higher catalytic efficiency than most β-galactosidases.

CHAPTER 4: CONCLUSION

The β-galactosidases are enzymes that hydrolyze the disaccharide lactose to generate glucose and galactose both of which can be metabolized in glycolysis. The present study is focused on the purification and characterization of β-galactosidase from *Enterobacter sp.* YSU with a specific focus on the enzyme's substrate specificity, catalytic parameters, inhibitors, pH dependence, thermal stability, and the impact of divalent metal ions on its action.

The β-galactosidase has a limited substrate specificity of *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPGal) and lactose. The study on β-galactosidase's activity showed the enzyme had a K_M of 0.35 mM, V_{max} of 3.65 μM/s (for β-galactosidase concentration of 0.025 μM) and a k_{cat} of 146 s^{-1} for *o*-NPGal. The enzyme has a K_M of 22 mM at a V_{max} of 2.38 mM/min for lactose when the concentration of the protein is 0.625 μM. The k_{cat} of β-galactosidase with lactose is 3.81×10^3 per min. Furthermore, our findings indicated that laminarin was not hydrolyzed by the enzyme and, therefore, it is not a substrate for this β-galactosidase. The enzyme was tested for transglycosylation activity at different conditions, but it was not successful.

The inhibitory effects of glucose and galactose on β-galactosidase were examined and experiments revealed that the enzyme is significantly affected by D-galactose and only slightly by D-glucose. Galactose is a competitive inhibitor with respect to *o*-NPGal while glucose is an uncompetitive inhibitor. Moreover, the thermal stability and the effect of pH on the enzyme activity were scrutinized. The β-galactosidase retained its activity when it was incubated at 40 ^oC, but there was a reduction in the enzymatic activity when the temperatures surpassed 40 ^oC. Examination of the effect of pH revealed that β-galactosidase activity was low at pH 5.0 but increased considerably with increasing pH values. At a pH of 7.4, the enzyme exhibited maximum activity, but activity

dropped above pH 8.0. Entrapment of β-galactosidase within calcium alginate beads increased thermal stability up to 50 ^{o}C compared to the free enzyme which lost its activity at 50 ^{o}C. Nonetheless, immobilization did not have any significant effect on the optimal pH.

The enzyme's interaction with divalent ions was studied using EDTA as a chelating agent. Incubation of enzyme with EDTA resulted in a decrease in the activity of the enzyme whereas the addition of Mg^{2+} fully restored β-galactosidase's activity. However, calcium ions demonstrated no significant change in the enzyme's activity, while the cupric and zinc ions significantly decreased the enzyme's activity. As a metalloenzyme, β-galactosidase utilizes metal cations as cofactors and requires Mg^{2+} to facilitate activity and stability.

The impact of solvents and other chemicals on β-galactosidase was examined. SDS and DMSO showed a decrease in the enzyme's activity while the 2-mercaptoethanol does not affect the activity of the enzyme. Examination of the interaction of the active subunits of the enzyme using 4-methylumbelliferyl-β-D-galactopyranoside revealed that the dimer was active while the monomer was not.

Further research on the β-galactosidase from *Enterobacter sp. YSU* can be done to decipher the structure of the enzyme. This will help in understanding how the subunits of the enzyme interact and how the active site folds.

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