Enhancing transglycosylation reaction by minimizing hydrolysis in oligosaccharide synthesis

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Abstract

Oligosaccharides are a class of carbohydrates which play important roles in several areas including therepeutics, food, phamaceuticals, animal feed and their use as prebiotics. Current commercial production of oligosaccharides involves lengthy multistep procedures with low yields. To improve oligosaccharide yield, a broad range of factors related to oligosaccharide synthesis by transglycosylation was evaluated. Three enzymes, β-galactosidase from *Aspergillus oryzae*, lactase from lactose intolerance supplements, and *Enterobacter* β-galactosidase were tested for their ability to catalyze a transglycosylation reaction. The enzymes were applied in both free and immobilized forms and the activities toward transglycosylation were compared. Immobilization of enzymes led to increased oligosaccharide synthesis and improved enzyme stability under different conditions. Lactose, which is a main milk carbohydrate, was used as the major substrate for synthesis of oligosaccharides. Factors like organic solvents, temperature, and pH were varied with the aim of obtaining the conditions with higher transglycosylation results. The analysis of products was done by thin layer chromatography. The yield of transglycosylation products was dependent on both temperature and pH. The preferred conditions for formation of oligosaccharides are pH 8 and 50 - 60 °C. High concentrations of glucose and galactose, the products of lactose hydrolysis, suppressed both transglycosylation and hydrolysis reactions. When sucrose and lactose were used together, an increase in the sucrose to lactose ratio led to an increase in transglycosylation products. Addition of alcohols resulted in formation of alkyl-glycosides instead of oligosaccharides. *Enterobacter* β-galactosidase was successfully overexpressed in *E. coli* cells and demonstrated transglycosylation and hydrolysis activity.

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List of abbreviations

BSA	Bovine serum albumin
CMP-Neu-5Ac	Cytidine monophosphate N-acetylneuraminic acid
ChtS	Chitosan-silica beads
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EtOH	Ethanol
Fru	Fructose
Glc	Glucose
Gal	Galactose
GNB	Galacto-N-bioses
GT-A	Glycotransferase-A
GT-B	Glycotransferase-B
GT-C	Glycotransferase-C
GT-D	Glycotransferase-D
GA	Glutaraldehyde
IMAC	Immobilized metal-ion affinity chromatography

IDA	Iminodiacetic acid
kDa	Kilo Dalton
KPi	Potassium phosphate
Lac	Lactose
LB	Luria-Bertani medium
Man-GlcNAc	Conjugate of N-acetylgalactosamine and mannose residues
NDO	Non-digestible oligosaccharides
*o*NPGal	*o*-nitrophenyl-β-D-galactopyranoside
*o*NP	*o*-nitrophenolate
OD_{600}	Optical density at 600 nm
SOB	Super optimal broth
SOC	Super optimal broth with catabolite repression
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris	Tris(hydroxymethyl)aminomethane
UDP-Gal	Uridine diphosphate galactose

CHAPTER 1. INTRODUCTION

1.1: Oligosaccharides

Oligosaccharides are complex sugars made of monosaccharides which are linked by α or β (1 - 4), (1-2), (1-3), and (1-6) glycosidic linkages. [1] Presence of different oligosaccharide linkages leads to innumerable oligosaccharide types.[4] Depending on the number of monosaccharide units, oligosaccharides can be termed as disaccharides, trisaccharides, tetrasaccharides, pentasaccharides, and so on.[5] In general, a degree of oligosaccharide polymerization is between two and ten monomeric units with an estimated molecular weight between 300 to 2000 Da.[2] The major oligosaccharides produced naturally include those that contain fructose, galactose, glucose, and xylose monosaccharides. [3] In addition to this, oligosaccharides can be either linear, branched, or cyclic.[5] By convention, the structure of oligosaccharide is represented with the reducing end on the right-hand side and the non-reducing end on the left-hand side.[5]

Study of oligosaccharides has gained a lot of attention due to their wide range of applications as well as structural complexity when compared to proteins and lipids.[1] Oligosaccharides are soluble in water and have a mild sweetness when compared to sucrose.[3] Crittenden and Playne concluded that the sweetness of oligosaccharides depends on the degree of polymerization, molecular weight and chemical structure.[3] In general, long chain oligosaccharides are less sweet as compared to short chain oligosaccharides.[3]

Based on physiological properties, oligosaccharides are classified as either digestible or non-digestible, and both play important roles. Digestible oligosaccharides can be easily broken down in the body by digestive enzymes. Hydrolysis of these oligosaccharides mostly occurs in the upper parts of the gastrointestinal tract. On the other hand, non-digestible oligosaccharides cannot undergo digestion by humans. This is because the anomeric carbons of the individual

monosaccharides are configured in a manner that their β-linkages cannot be cleaved by the hydrolytic enzymes in the upper gastrointestinal tract.[6] However, the colon microbiota can ferment non-digestible oligosaccharides forming compounds like short chain fatty acids which can be easily absorbed. This fermentation is important since it leads to a decrease in intestinal pH which has been found to suppress pathogens and increases beneficial bifidobacteria in the body. According to Delzenne and Roberfroid, fermentation of non-digestible oligosaccharides serves as an indirect energy source, improves lipid metabolism, regulates gastrointestinal function, prevents, and treats constipation, enhances nutrient production such as nicotinic and folic acids, and improves human immunity. [6]

1.2: N-linked or O-linked glycoproteins

Oligosaccharides can be conjugated to protein to form either N-linked or O-linked glycoproteins. Literature suggests that over half of the body proteins are conjugated to oligosaccharides. [10] Their association with proteins leads to functional and structural modification of the proteins. [11] In N-linked glycoproteins, oligosaccharides are added as core oligosaccharides where they are covalently linked to the nitrogen atom in the side chain of asparagine. For the linkage to be formed, the sugar must be attached to either an asparagine -X- serine or an asparagine -X-threonine polypeptide sequence.[11] N-linked glycoproteins are categorized into three classes depending on the type of oligosaccharide attached but all have the same core structure $(Man)_3$-$(GlcNAc)_2$. The first class is mannose chain N-linked glycoproteins with at least six α-mannoses bound to the core $(Man)_3$-$(GlcNAc)_2$. The second type includes the complex N-linked glycoproteins which have the core extended by a sequence of a trisaccharide composed of sialic acid - galactose - N-acetyl glucosamine. The third class is a hybrid type which consists of a mixture of mannose type and complex type N-linked oligosaccharides.[12] Panza stated that these types of N-linked

oligosaccharides are involved in mediating the onset of various diseases like cancer, Alzheimer's disease, and AIDS.[10]

In O-linked oligosaccharides, the sugar is attached to the protein via the oxygen atom of the side chain of serine or threonine. Examples of O-linked oligosaccharides include mucins, immunoglobulins, and caseins. As in the case of N-linked oligosaccharides, most O-oligosaccharides are characterized by a common core N-acetylgalactosamine (GalNAc). The core GalNAc is usually attached to Galβ1-3 to form galacto-N-biose (GNB) which serves as a building block for core 1 and core 2. Core 1 and core 2 can be further elongated to form larger chains.[64]Attachment of oligosaccharides to proteins makes them resistant to proteolysis. Also, these proteins act as signal molecules for cell secretion. Consumption of the O-linked oligosaccharides by the beneficial gut microbiota leads to growth of the next generation of probiotics like the butyrate producing bacteria.[64]

The roles of oligosaccharides are not as well-known as that of other macromolecules like proteins.[9] Many hypotheses have been developed to explain the biological role of oligosaccharide units in glycoconjugates. One of the hypotheses suggests that oligosaccharide units of glycoproteins play a part in protecting the polypeptide from protease action. Also, these oligosaccharides are important in initiating proper polypeptide folding in the rough endoplasmic reticulum and maintaining the protein conformation as well as increasing their solubility. [9]

1.3 Biological roles of oligosaccharides

Symbiotic relationships between host animals and microorganisms are found to be controlled by specific oligosaccharides. In this case, oligosaccharides act as receptors for symbiosis. For instance, binding of certain commensal bacteria to host cell surfaces is controlled by specific sugar

chains. In such cases the binding of bacteria and host via oligosaccharides benefits both organisms.[9]

In plants, some free oligosaccharides like oligosaccharins act as hormones where they regulate specific responses in a manner that is dependent on their structure.[9] Oligosaccharides that contain high levels of free mannose have been found to highly suppress the immune response in *in vitro* mammalian systems and this also depends on their specific structure.[9] However, the alleged receptors of oligosaccharides are yet to be identified, and their working mechanisms remain unknown.[9]

1.4: Use of oligosaccharides as prebiotics

Prebiotics are compounds that improve the host health through selective augmentation of growth and function of beneficial bacteria in the large intestine.[65] Although many oligosaccharides are still being evaluated for their prebiotic potential, fructo-oligosaccharides, lactulose, and galacto-oligosaccharides have been reported to play an important role as prebiotics.[7, 43] Studies indicate that their function as prebiotics is attributed to the type of glycosidic linkages and the degree of polymerization.[7, 43] Oligosaccharides with β (1-2), β (1-3), β (1-6) and those with three to seven monomeric units have higher selectivity for bifidobacteria compared to their respective polysaccharides.[7, 43] A low degree of polymerization permits these oligosaccharides to be metabolized by bacteria at a higher rate compared to polysaccharides which may or may not be metabolized.[8] The bifidobacteria use non-digestible oligosaccharides as substrates for fermentation and, as a result, boost human health.[6] As prebiotics, oligosaccharides cause a significant reduction in the formation of short-chain fatty acids, inhibit harmful clostridia, and reduce gas production. They also selectively stimulate growth of intestinal probiotics.[6]

1.5 Oligosaccharides in food industry

Study of oligosaccharides as food supplements started around 1970 in Japan. Oligosaccharides' health benefits made many food companies adopt their application as food ingredients in processing.[3, 8] Oligosaccharides have abilities to enhance food flavor, organoleptic characteristics, and food stability. Increasing interest in low sugar foods has caused the food industry to substitute oligosaccharides as a bulking agent in place those of high sweetness. The bulking agents are added to food as additives to increase weight or volume of food while maintaining its functionality and nutritional value. This, in turn, contributes to flavor of the food. In sugary foods, use of artificial sweeteners alone leads to production of foods with a strong aftertaste. To cover this up, oligosaccharides are incorporated in addition to artificial sweeteners as bulking agents.[3] Oligosaccharides have higher molecular weight compared to monosaccharides and disaccharides which enhances the food viscosity and improves the general characteristic of the produced foods.

Most sugars used in foods can cause tooth decay (cariogenic), thus non-digestible oligosaccharides (NDO) are used as low-cariogenic sugar substitutes in foods like confectioneries and soft drinks. This is possible since NDO breakdown cannot be achieved by microorganisms present in the mouth and production of cariogenic products will not occur.[3]

Studies show that oligosaccharides can be used to prolong the shelf life of foods by controlling the Maillard reaction and water activity, thereby reducing the overall microbial food contamination.[14] In heat processed foods, the Maillard reaction occurs which involves condensation of the reducing sugar and amino group of amino acids leading to browning of the product and quality deterioration.[3]

Inulin, a fructose oligosaccharide, is a low-calorie oligosaccharide with a mild to sweet taste. It has been used in low fat dairy products to replace fat, enhance mouthfeel and food texture.

Research done by Paseephol indicated that inulin's long chain highly enhances creaminess and mouthfeel in low fat yogurt.[15] In addition, inulin's hydroxyl groups interaction with water allows it to behave as a surfactant. This leads to the formation of gels with similar characteristics as fat thus it is used as a fat substitute [16] Inulin forms stable gels with water mostly at concentrations between 13 and 50%. Studies show that inulin's application in cheese production resulted in cheese with 63% less fat without any effect on the melting point. According to Hennelly and Cardarelli, the same effect was observed in ice cream, chocolate mousse, and custards.[17]

1.6: Oligosaccharides in pharmaceuticals

Chemical and physical properties of oligosaccharides have led to their use as stabilizers and for drug delivery in pharmaceuticals.[16, 18] Oligosaccharides are hydrophilic and have the ability to modify solutions by forming gels thus they are applied in drug delivery. The hydrophilic nature of oligosaccharides enhances their use as carriers of drugs with poor solubility.[18] Most of the orally administered drugs are insoluble and tend to dissolve slowly in the intestinal tract. Scientists came up with an idea of using a highly hydrophilic carrier which is dissolved first followed by dispersing the drug in the solution. As a result, the drug gets hydrated faster leading to its dissolution.[18] Low molecular weight oligosaccharides have high solubility and have been used as carriers. Drooge compared the dissolution behavior of the insoluble drug diazepam using disaccharides and fructo-oligosaccharides.[19] It was found that use of disaccharides as carriers led to poor dissolution of the drug since the drug precipitated and formed large crystals which are dangerous. This detrimental effect was not observed when fructo-oligosaccharides were used and thus the conclusion was made that oligosaccharides are superior as drug carriers compared to disaccharides.[19]

In some instances, drugs like Fenofibrate had poor dissolution rates with inulin as a carrier. Srinarong tried to enhance dissolution of this drug using inulin by incorporating agents that

promote disaggregation of drug into small particles (disintegrants) like sodium starch glycolate.[20] It was found that under these conditions the dissolution rates were increased. The study further concluded that to improved dissolution, the appropriate hydrophilic carrier for a lipophilic drug should be used.[20] Occasionally, increasing the concentration of an oligosaccharide improves the dissolution rates.[18] Oligosaccharides are used as a filler-binder for production of tablets prepared by direct compaction.[18] Direct compaction is a method of producing tablets in which dry powder mixtures are blended under pressure and then compressed to form tablets. Studies show that oligosaccharides like inulin, which is used in tablet production, have low crystallinity, small particle size, high stability under various storage conditions, inertness, and good blending properties which contribute to an efficient compaction process.[21] Also, long chain oligosaccharides are preferred over short chains since they produce tablets with better dissolution rates.[21]

Oligosaccharides are also used to modify the release of drugs in the body. A research on encapsulation and drug release was performed where inulin and inulin acetate were used to make microspheres with serine protease inhibitors. It was determined that inulin-associated drug release was prolonged up to four days.[22]

The ability of oligosaccharides to modify the release of drugs also prompted their application in drugs targeting specific systems. For instance, if the drug target is the colon, non-digestible oligosaccharides-based drugs are used since they can only be digested by the action of colon microbiota.[18] Since oligosaccharides form gels when dissolved, the drug will not be hydrolyzed until it reaches the colon where the oligosaccharide can be fermented and release the drug.[18] In this application, the hydrogel characteristic of an oligosaccharide is an important aspect that should be well controlled to avoid gel swelling and premature drug release.[18]

Apart from their application in drug delivery, oligosaccharides are also used as membrane and protein stabilizers in the pharmaceutical arena. Use of proteins as therapeutic agents to enhance immunogenicity has been increasing since insulin was first applied in therapeutics in the early 1920s.[24] The proteins are usually unstable in aqueous solutions which causes a reduction in shelf life of a product.[16] In order to avoid this effect, drying and rehydration procedures are being applied aiming to maintain the therapeutic activity of a protein. Removal of surrounding water has been done by procedures like lyophilization, dehydration, and freeze drying.[18] Though, lyophilization may alter the physical and chemical properties of proteins and lead to their misfolding and formation of aggregates which causes diminished immunogenicity.[23, 24] Hermeling compared the relationship between the protein structure and the immunogenicity effect and concluded that freeze drying, when done appropriately, can maintain the pharmacological effect and the immunogenic activity.[24] Oligosaccharides have been applied in formulation during the drying process where they act as protective agents reducing the loss of protein activity.[16] Two theories that explain how oligosaccharides interact with the membrane or protein are the vitrification theory and the water replacement theory.[18, 25]

In the vitrification process, the sugar acts as a matrix in which the protein becomes immobilized leading to the formation of a glassy solid which does not crystallize upon drying. This minimizes the molecular mobility of protein and thus prevents protein degradation in the dried product and extends its shelf life.[16, 25] For vitrification to be effective, the glassy substance needs to have high viscosity, and this is achieved by high molecular weight oligosaccharides. High viscosity ensures no diffusion of the immobilized protein as well as no or reduced mobility.[18] To achieve a better protein stabilization, the glass transition temperature has to be higher than the storage temperature.[18] Apart from the transition temperature, other required physicochemical properties of

oligosaccharides include low moisture absorption, inability to form crystals, and lack of reducing groups.[18] One of the oligosaccharides that acts as a good stabilizer at different conditions is inulin.[18] Amorij conducted a study using inulin as a stabilizer for influenza vaccine constituents and observed that inulin stabilized haemagglutinin during freezing and freeze-drying processes.[27]

Water replacement theory suggests the replacement of hydrogen bonds between water and proteins by hydroxyl groups of oligosaccharides. In solution, the protein conformation is maintained by hydrogen bonds between water and proteins and these bonds are lost during the drying process.[25] Oligosaccharides replace water in the hydrogen bonds thus ensuring the proteins in dry product persist for long periods, up to decades, in a state called anhydrobiosis. To achieve the stabilization, oligosaccharides need to be in an amorphous form instead of crystalline to ensure maximum interaction between the sugar molecules and the irregularly shaped proteins.[25]

1.7: Oligosaccharides in cosmetic industry

Oligosaccharides have also been applied in the cosmetic industry as stabilizers and bulking agents. The presence of oligosaccharides like cyclodextrins in deodorants and sprays facilitates spraying and fragrance, and controls volatility.[28] This helps mask various odors from clothes, giving a fresh smell. Adachi and Valle carried out research to evaluate the skin softening characteristic contributed by oligosaccharides extracted from brown seaweed.[28] They found that high molecular weight oligosaccharides can enhance the water retention ability that enables them to protect the skin against drying, cigarette smoke, pesticides, and conditions like hyperpigmentation. Low molecular weight oligosaccharides were found to have anti-inflammatory effects. Some oligosaccharides form complexes with other molecules and ions in solution and have been applied in shampoos and soap where they contribute to the antibacterial characteristic of the products.[28]

1.8: Oligosaccharides in animal feed

Oligosaccharides are applied as dietary supplements to promote animal health. [28] Mahious conducted a study on assessing the effect of three oligosaccharides on microbial changes in te digestive tract and growth of turbot, *Psetta maxima.*[29] It was found that the oligosaccharides increased daily dietary intake, facilitated efficient food digestion, led to an increase in serum proteins, and promoted positive gut microflora.[29] Oligosaccharides can be used as additives in livestock feed to substitute antibiotics since many pathogens have developed resistance to the antibiotics.[28] Dietary fructo-oligosaccharides and *trans*-galacto-oligosaccharides were applied in young pigs diets where they increased the daily dry matter intake with time and improved the growth rate compared to those pigs which were not fed these oligosaccharides.[30] In addition, inclusion of these oligosaccharides in the young pigs feed led to decreased diarrhea and loose feces.[3]

1.9: Chemical synthesis of oligosaccharides

Oligosaccharides can be synthesized by use of chemical methods which in many cases lead to formation of highly branched oligosaccharides.[34] In general, these methods involve glycosyl donor, glycosyl acceptor, and activator (**Figure 1**). Synthesis leads to the generation of a new stereo-genic center which is controlled by protecting groups. Protecting groups help in covering hydroxyl groups and prevent reactions with activated glycosyl donors and other reagents used.[34] Thus to achieve efficiency in synthesis, choosing a suitable protecting group is very crucial and the most challenging.[34] The specific glycosylation point on an acceptor molecule is controlled by selectively exposing a single hydroxyl group. Glycosidic linkages are formed when the activated donor is replaced by a hydroxyl group from the acceptor molecule.[34] Several chemical synthesis methods have been developed, each trying to solve the drawbacks of the other.

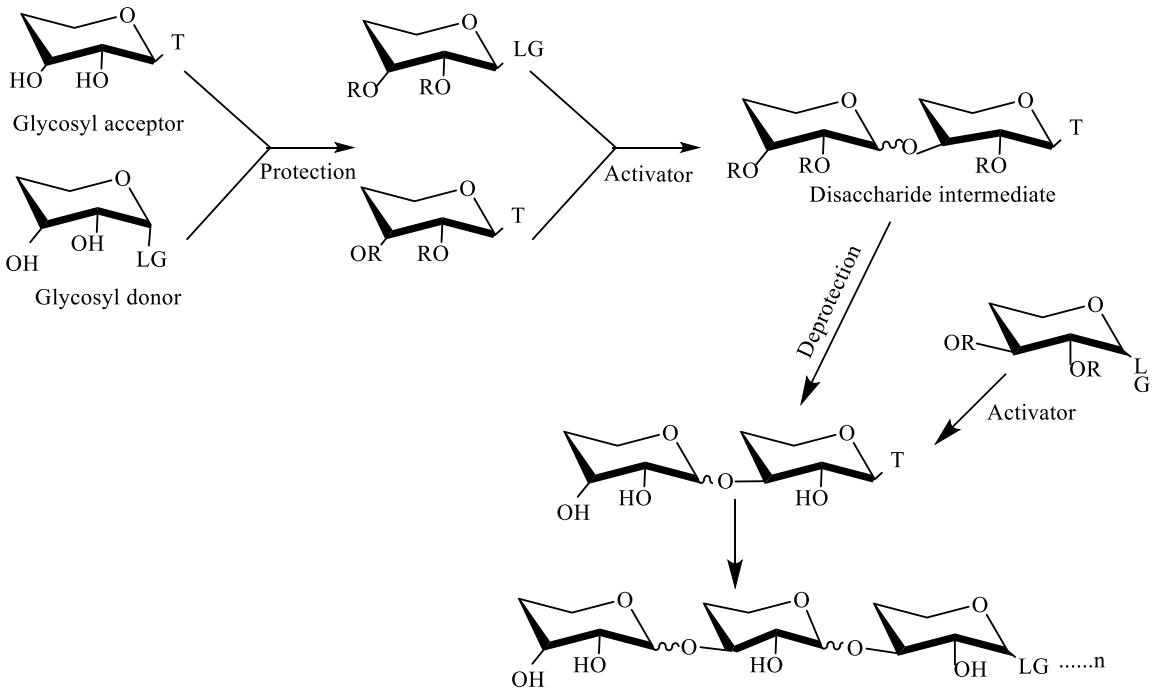


Figure 1: Typical chemical synthesis scheme for oligosaccharides where R represents the protecting groups, LG represents the leaving group, and T is a temporary group [38, 48]

The Koenings-Knorr method was one of the first methods applied for the chemical synthesis of oligosaccharides.[31, 34] In this method, O-acetylated glycosyl halides are used as glycosyl donors which react with an alcohol to form the corresponding glycosides, and silver carbonate or mercuric cyanide act as promoters.[31, 34] This method mostly led to the synthesis of β (1-2) *trans* glycosides in that the oxygen atom of the glycosidic bond formed lies in the opposite direction with the hydroxyl group on the anomeric carbon of the glycoside unit. This is unlike in the *cis* glycosides where the oxygen atom of the glycosidic bond lies in the same direction with the hydroxyl group on the anomeric carbon of the glycoside. Use of bromide-based or chloride-based halides in the synthesis has major drawbacks - bromide halides are unstable when used as donors while chloride halides are stable but less reactive. This makes the synthesis to be effective only when using primary alcohols. Even though this method produced oligosaccharides of high stereoselectivity,

the glycosylation yields were inadequate. Another drawback of this method is that the glycosylation either was restricted to a primary hydroxyl group at carbon-6 (C6) or more complex sugars were formed in moderate yields.

Conventional linear and convergent block syntheses are other traditional chemical synthesis methods that were developed. In the conventional linear approach, a disaccharide is first modified and then is used to generate a glycosyl donor or acceptor. The donor and acceptor are coupled to obtain a trisaccharide after which the reaction is repeated until the required sequence is obtained.[31, 47] In convergent block synthesis, fragments are first pre-synthesized after which they are linked together in glycosylation to form an oligosaccharide. Conventional linear and convergent block synthetic methods were advantageous because building blocks are first modified by protecting groups. Also, there are pre-activation steps which can be used with any protecting or leaving groups and the regioselectivity is controlled by differential reactivity of various acceptors. The overall glycosylation in this method is faster as oligomeric building blocks are used.[31] This process is economical since the same leaving group is used for all the building blocks of the oligosaccharide. However, this method affects the stereochemistry of the synthesized oligosaccharide and leads to formation of both *cis* and *trans* oligosaccharides which makes the final product impure and unsuitable for many applications.[31, 34] This method also requires multiple preparatory steps to make several building blocks before the glycosylation step. This requires application of several chemical reagents, some of which are toxic and pollute the environment.[34]

Another traditional synthesis method is one pot synthesis which involves all glycosylation steps in one flask. This method is advantageous since several purification steps are reduced, there is no manipulation of protecting groups and no product isolation at every step of glycosylation.[31, 48] One pot glycosylation has been performed in many approaches but three of them are most used. They

include the chemoselective approach, the orthogonal approach, and the pre-activation approach. In chemoselective one pot glycosylation, electronic properties of the protecting groups are varied to differentiate the reactivities of donor and acceptor **(Figure 2a).** It is based on an "arming" or "disarming" donors' strategy in which protection of the glycosyl donor by ether leads to armed donors while introduction of ester protecting groups leads to disarmed donors. In the presence of a disarmed donor or acceptor, armed donors become highly reactive. The donor with the highest reactivity is made to condense with a donor with less reactivity to form a glycoside. The formed glycoside can condense with the next donor with least reactivity.[48] The steps are repeated until a required sequence is obtained.

In orthogonal one pot glycosylation, one leaving group is selectively activated over the other. At least two different types of donors are used in glycosylation so the activation of the leaving group at the anomeric position of one donor does not affect leaving or protecting groups on other donors. The anomeric groups are used as protecting group or leaving groups because they are stable at various conditions used during activation process **(Figure 2b)**. In pre-activation one pot glycosylation, glycosyl donor is activated in the absence of glycosyl acceptor to form a reaction intermediate. It is then instantly reacted with a glycosyl acceptor which has the same type of leaving group at the anomeric center to form a saccharide with an aglycone that can be activated at the reducing end. This process is then repeated in the same flask to form a desired sequence **(Figure 2c)**. [31, 34, 47, 48]

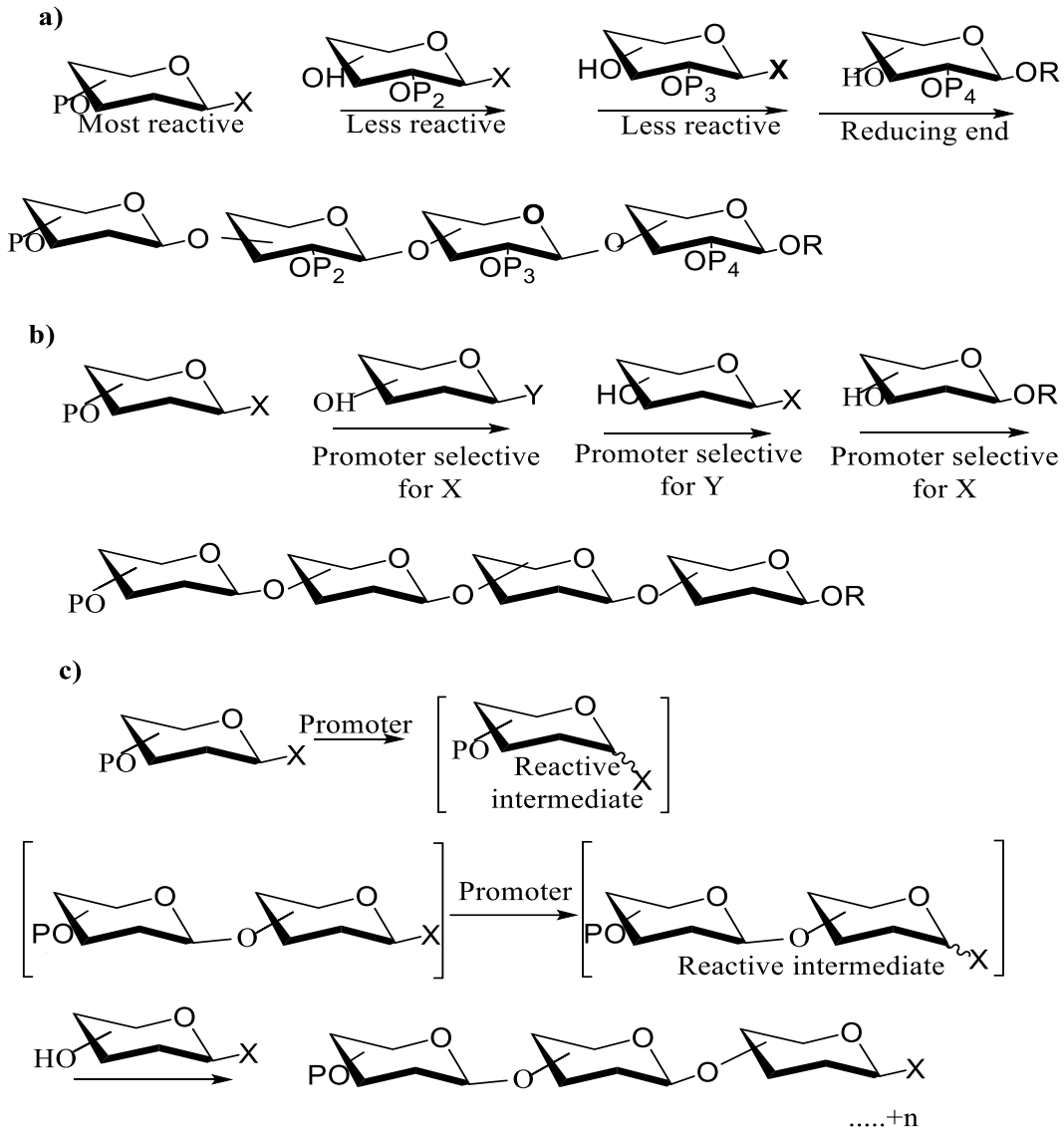


Figure 2: Example of one pot synthesis. PO, OH and O between C3 and C4 means they can be on either C3 or C4; P and R represent protecting groups. a) One pot synthesis based on chemo selective strategy; b) Orthogonal strategy; and c) Pre-activation strategy. [47 48]

One of the modern methods called the solid phase synthesis was developed to synthesize oligosaccharides rapidly on a solid support mainly using polymer beads or polystyrene-based resins.[31, 35] This method can be either a glycosyl donor-bound approach or a glycosyl acceptor-bound approach **(figure 3).** [35, 47] In the acceptor-bound method, a monosaccharide's reducing end

is attached to a support using a cleavable linker. Then, a hydroxyl group to be modified is exposed by a deprotection step. Glycosylation is initiated when a glycosyl donor and activator are added. In each cycle, the reaction is repeated by addition of the next sugar.[34] This method does not require purification steps and tends to minimize chemical reagents applied for synthesis. In the donor-bound approach, the glycosyl donor is bound to the support. However, this approach led to donor side reactions which prevent chain elongation and thus affects the oligosaccharide sequence.[34, 47]

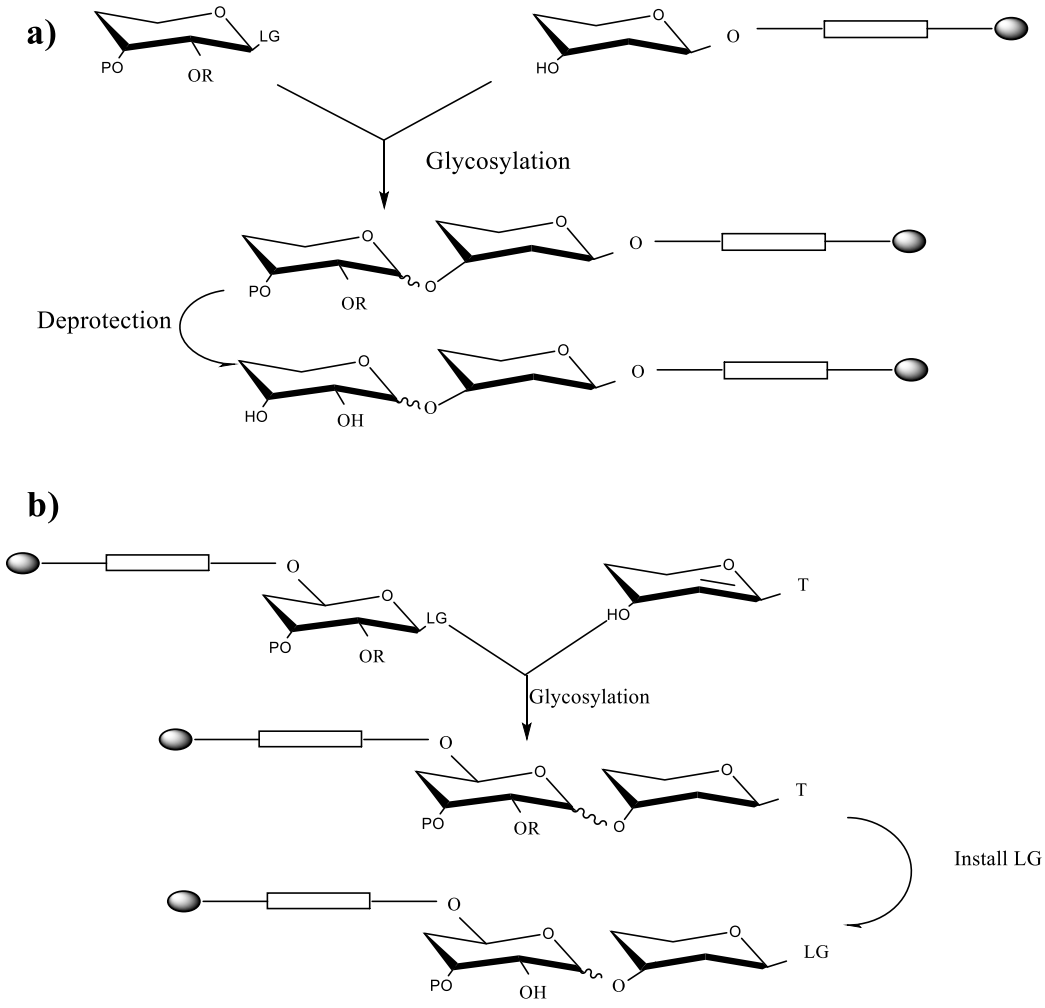


Figure 3: Solid support oligosaccharide synthesis. R and P - protecting groups, LG - leaving group, and T - temporary group. a) Solid support oligosaccharide synthesis based on acceptor-bound approach; b) Solid support oligosaccharide synthesis based on donor-bound approach.[47]

Automated oligosaccharide synthesis using computerized apparatus and liquid handling equipment improved synthesis in that most human errors were avoided. The reactions are well monitored thus decreasing the overall reaction time and reagents needed for synthesis. Wong developed a one pot automated method which is based on relative reactivity value of building blocks and high chemoselectivity.[35] The study used thioglycosides as donors which were activated in the presence of a promoter system to determine the relative reactivity value. To synthesize a required oligosaccharide sequence, a computer program called Optimer was used to select the best reactants based on the relative reactivity values. [35, 47]

Takahashi further introduced semi-automated synthesis methods in which the synthesis was done in one pot.[32] A manual synthesizer instrument was used, and it led to synthesis of both linear and branched oligosaccharides. This approach was successful resulting in a rapid synthesis of seventy-two trisaccharides using glucosides, galactosides, and mannosides. [32]

Seeberger and coworkers developed a fully automated synthesizer called Glyconeer. This was the most successful commercially available automatic system for oligosaccharide synthesis.[33] Since then, other researchers have built different synthesizers like a HPLC-assisted automated synthesizer and syringe pump based electrochemical synthesizer, among others. In Glyconeer, acceptor solid support-bound synthesis is applied. Monomeric building units are first prepared. First, the resin with an acceptor bound to it is added into the synthesizer's reaction vessel. The oligosaccharide building blocks and other required reagents are supplied through the valves. A series of coupling, protection, and deprotection steps then follows. Fractions are collected via filter

and pipes at the bottom of the container. After the synthesis cycle is completed, all reagents are removed and the resin is rinsed with a solvent. This system produced α(1-6) linked oligosaccharide which had 30 monosaccharides, and this was the largest oligosaccharide synthesized using solid phase to date.[33, 35]

Despite the diverse improvements in chemical synthesis by development of new asymmetric methods, it still poses crucial challenges.[36] Major challenges associated with this method include need for regioselective protection, need for protection and deprotection of poly-hydroxyl groups, complicated choice of a suitable protecting group, and lack of stereoselectivity in formation and assembling of glycosidic bonds. Controlling the anomeric configuration of the final product is still complicated since chemical reactions mostly occur via first or second order nucleophilic substitution reactions. This is because the anomeric configuration of the final product is dependent on the protecting groups used and not the activated sugars. [35] Use of solid supports has many disadvantages since it involves attachment of the molecules on one side thus causing the yields to drop drastically. Availability of compatible and cleavable linkers to solve this issue is rare.[33] Also, it is difficult to monitor the reaction progress and control the protecting groups on resins. [35] Selection of the appropriate protecting groups and their manipulation during synthesis remains the critical challenge in chemical synthesis.[34, 35] There is a requirement for additional modifications after every glycosylation cycle which is supposed to be repeated after every single cycle until the desired length is synthesized. Also, creation of a new stereocenter at every step of glycosylation complicates the whole process, thus there is a need for purification at each step of synthesis by chromatography which makes the process more laborious, time consuming and cumbersome. [33, 34]

1.10: Enzymatic synthesis

The enzymatic synthetic methods have been found to outdo most of the disadvantages of chemical synthesis. This method does not need any protection or deprotection of hydroxyl groups. It is also suitable for production of oligosaccharides for animal and human applications since there is no use of toxic chemicals. In addition to that, this method is environmentally friendly. [34] Two major categories of enzymes that have been applied in enzymatic synthesis include glycotransferases and glycosidases or glycosynthases.

1.10.1: Glycotransferases

Glycotransferases are a group of enzymes that catalyze transfer of a glycosyl from a specific activated donor to an acceptor molecule.[53] Enzyme catalysis enhances stereo- and regioselectivity and improves the synthesis efficiency. [38] For instance, the transferase will catalyze formation of only one type of a linkage making purification easy. [38] Two commonly known classes of glycotransferases are the glycotransferase-A (GT-A) and the glycotransferase-B (GT-B) families. Also, recently two other super families - glycotransferase-C (GT-C) and glycotransferase-D (GT-D) have been identified which are yet to be accepted by the glycobiological community. [34] GT-As require metal ions for their activity and have a Rossman fold motif while GT-Bs do not require metal ions but have double Rossman folds which face each other. [34] Glycotransferases are advantageous in glycosylation applications since they can work under mild conditions and require short reaction times to construct large oligosaccharides. [34] The major drawback of these enzymes, however, is that they are highly unstable in solutions.[41] Besides that, they are only found in small concentrations and they require the use of expensive glycosyl nucleotide donors like uridine diphosphate galactose (UDP-Gal) and cytidine monophosphate N-acetylneuraminic acid (CMP-

Neu-5Ac) [38] Multiple studies for developing stable recombinant glycotransferases have been done, however, none has been a success due to purification issues.[38, 41]

1.10.2 Glycosidases

Glycosidases have been employed in synthesis where they transfer glycosyl group from donor to an acceptor other than water. [34] These enzymes can be either exoglycosidases which catalyze formation of glycosidic linkages on the non-reducing end of a sugar or endoglycosidases which catalyze the reaction within the chains of sugars. [38] Glycosidases can catalyze synthesis using one donor and different acceptors resulting in formation of a mixture of (1-6), (1-4), (1-3), and (1-2) glycosidic bonds. These prompt the need for purification thus making the reaction less selective when compared to glycotransferases. [38] Activity of this class of enzymes allows synthesizing shorter oligosaccharides in most cases. Unlike glycotransferases, glycosidases do not require cofactors for activity and can use simple and easily available substrates.[38] Overall, glycosidases can catalyze both cleavage of glycosidic bonds as well as synthesis depending on the reaction conditions. [34]

Oligosaccharide synthesis can be achieved in several ways. One is reverse hydrolysis (condensation reaction) in which the same enzymes used for hydrolysis of disaccharides are used to synthesize oligosaccharides by reacting two sugars in the presence of the enzyme. In this process, the enzyme does not form complex with the sugar as in the case of transglycosylation. Thermodynamics of reaction conditions in this case dictate the position of the final equilibrium.[39] Shifting the reaction from hydrolysis to synthesis is controlled by high reactant concentrations, decreasing water activity, and product removal from the medium.[39] Since the equilibrium of the reaction is normally in the hydrolytic direction, synthesis of oligosaccharides is still not practical.[40]

Transglycosylation is another method that is a based on kinetic control because it is caused by two competing reactions which are hydrolysis and synthesis. [44] It involves transfer of a glycosyl moiety from a glycoside donor to a hydroxyl-containing acceptor.[42] In this process, after the substrate has occupied the active site of the enzyme, the anomeric center of the glycosyl moiety of the donor is attacked leading to formation of covalent bond between the glycosyl moiety and the enzyme. The fate of the formed intermediate depends on the type of acceptor available. If water is present as the acceptor, the reaction shifts to hydrolysis and monosaccharides are formed. If a saccharide acceptor is present, the reaction will shift towards transglycosylation (**Figure 4**).[44] This approach was first introduced in 1935 by Rabete when β-glucosidase was employed to catalyze the transfer of a glucose moiety from aryl-β-glucosides to alcohols. [38] It was concluded that the product yield is dependent on the relevant reactivities of donor substrate and acceptor molecule as well as reaction conditions.[38] This method requires a substrate that is more reactive than the product as well as shorter reaction times. In some instances, high product yield is obtained when there is a higher concentration of donor. However, this process is hindered by the competing hydrolysis reaction.[38] The amount of oligosaccharide recovery is dependent on the relation between product formation and the hydrolysis rate.[38] Since hydrolysis is the competing reaction, the enzyme's mechanism for transglycosylation is believed to be based on a hydrolysis mechanism but the enzyme mechanism for transglycosylation is not well understood.[45]

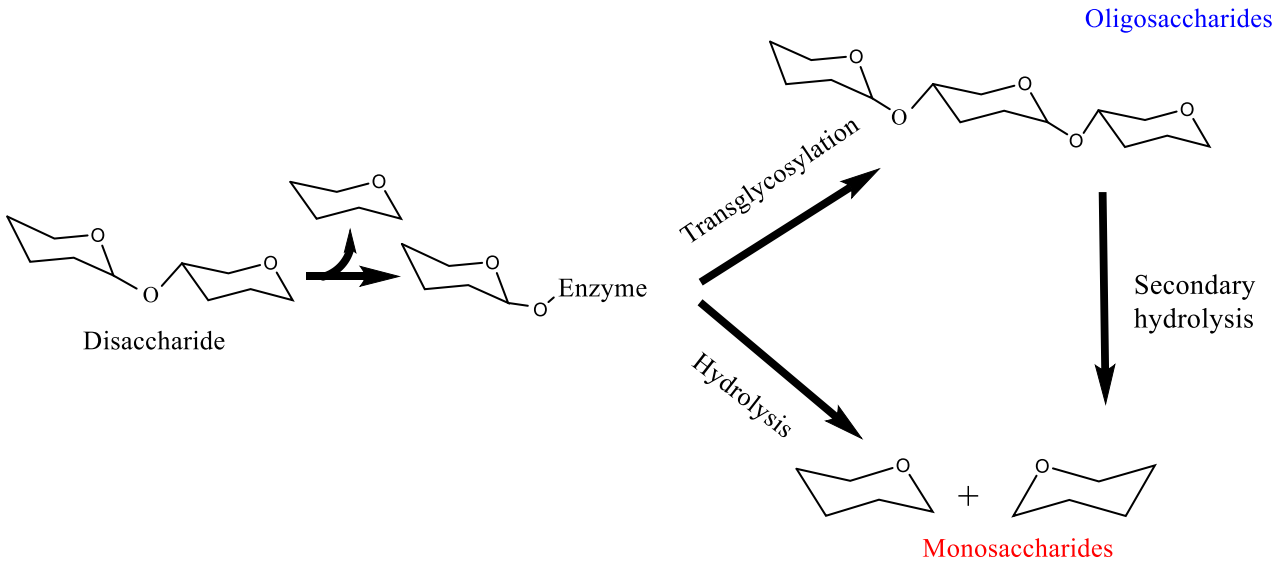


Figure 4: General scheme for transglycosylation and hydrolysis of disaccharides

1.11 Statement of problem

The objective of this project is to enhance enzymatic synthesis of oligosaccharides by transglycosylation. Three glycosidase enzymes, lactase from lactase supplements, β- galactosidase from *Enterobacter* species, and β-galactosidase from *Aspergillus oryzae* will be applied. These enzymes are known to catalyze hydrolysis of lactose which is a major milk carbohydrate but can also be utilized for the synthesis of oligosaccharides. First, transglycosylation and hydrolysis of lactose using free enzymes will be tested. Each enzyme will then be immobilized on a chitosan-silica support and used in the transglycosylation/hydrolysis reaction to identify the one with the highest transglycosylation activity. Lactose will be utilized as the major substrate for synthesis of oligosaccharides. Besides that, sucrose, glucose, and galactose will also be applied as substrates. Different reaction times, hydroxyl containing acceptors, and organic solvents will be used with an aim of obtaining the best conditions for transglycosylation. Reaction progress will be monitored by thin layer chromatography (TLC).

Once the suitable reaction conditions are obtained, these conditions will be applied to test for immobilized enzyme reusability and the reaction will be scaled-up to make enough products for structural analysis.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

Ampicillin sodium salt, agar bacteriological, ammonium sulfate, Acryl/Bis-acrylamide (40%), Luria Bertani (LB) broth, nutrient broth, super optimal broth (SOB), coomassie brilliant blue G-250, dithiothreitol (DTT), potassium phosphate monobasic anhydrous, PIPES sodium salt, streptomycin sulfate, sucrose, sodium acetate trihydrate, sodium chloride, sodium dodecyl sulfate, TEMED and Tris were purchased from Amresco (Solon, Ohio). Bovine serum albumin (BSA), beta-galactosidase from *Aspergillus oryzae,* D-lactose, glutaraldehyde solution (Grade II, 25% in H_2O), silica gel HF, and imidazole were purchased from Sigma Aldrich (St. Louis, MO). Acetic acid, Bradford reagent, D-galactose, D-fructose, 99% ethanol, *o*-nitrophenyl-β-D-galactopyranoside (oNPGal), single step KRX competent cells, sodium hydroxide, 1-butanol, 1-propanol, 2-propanol, methanol, 4-nitrophenol (98%), and PageRuler unstained protein ladder were purchased from Fisher Scientific (Fairlawn, N.J). D-Glucose was purchased from Mallinckrodt Inc (Paris, KY). Chitosan powder was purchased from Bulk Supplements (Henderson, NV). Lactase enzyme tablets were from Rite Aid (Camp Hill, PA). Boric acid was purchased from J.T Baker Chemical Co. (Phillipsburg, N.J). *pET-20b(+)lacZ* DNA plasmid was a kind gift from Dr. Caguiat (Youngstown State University). Coomassie brilliant blue R250 staining solution were purchased from Bio-Rad (Hercules, California). Spectrophotometric assays were performed using Hewlett Packard Agilent 8453 photodiode array spectrophotometer. All incubation steps were done using New Brunswick Scientific Class Series C76 water bath shaker from (Edison, N.J) and 200 series ISO Temp incubator from Fisher Scientific (Fairlawn, N.J)

2.2 Methods

2.2.1 Preparation of β. galactosidase from Aspergillus oryzae solution

To prepare a solution of β-galactosidase from *Aspergillus oryzae*, 0.70 g of β-galactosidase powder was added into 10 mL of 0.1 M KPi buffer at pH 6.8. The solution was mixed until the powder was dissolved. The solution was stored in the refrigerator at 4 °C.

2.2.2 Preparation of lactase solution

Two tablets of Fast Acting Dairy Relief lactase enzyme supplement were crushed using a pestle and mortar. The powder was mixed with 10 mL of 0.1 M KPi buffer, pH 6.8. The solution was centrifuged for 10 minutes at 4000 rpm using Sorvall ® RT6000B Refrigerated Centrifuge. The supernatant was saved as lactase solution and the pellet was discarded. The solution was stored in the refrigerator at 4 °C

2.2.3 Preparation of chitosan - silica beads

Chitosan mixture was prepared by mixing 0.10 g chitosan with 10 mL 1 M acetic acid using a magnetic stirrer until it was completely mixed. When all chitosan was solubilized, 0.10 g of silica HF powder was added and left to moderately stir on a magnetic stirrer overnight. Beads were made by adding dropwise 200 µL of chitosan - silica mixture to 1 mL of 12% NaOH - 20% ethanol solution in Eppendorf tubes. The beads were left to cure for 15 minutes and then rinsed with distilled water to neutral pH. The beads were stored in the refrigerator in distilled water.

2.2.4 Crosslinking chitosan - silica beads with 0.1% glutaraldehyde

Chitosan - silica beads were crosslinked using glutaraldehyde. Water was removed from the beads and replaced with 200 µL of 1% (v/v) glutaraldehyde solution. The beads were incubated in a shaker in the cold room for 90 minutes and the solution was removed. The beads were rinsed with distilled water and stored in refrigerator

2.2.5 Immobilization of lactase and β-galactosidase from Aspergillus oryzae on chitosan - silica support

Into each of the six Eppendorf tubes with chitosan - silica beads, 900 µL of 0.1 M KPi at pH 6.8 and 100 µL lactase solution were added. The samples were gently inverted to expose beads to the solution. From each tube, 100 µL samples were taken before incubation into new Eppendorf tubes and stored at 4 ºC. Remaining samples were incubated for at least 16 hours with continuous shaking in the cold room. After incubation, lactase solution was removed and 100 µL of this solution was saved in Eppendorf tubes for protein quantification. Beads were rinsed with distilled water and stored in a refrigerator at 4 °C

2.2.6 Determination of concentrations of lactase and β-galactosidase

"Before incubation" and "after incubation" samples were quantified by Bradford assay. Solutions were prepared in 16 × 150 mm test tubes according to **Table 2.2.6** and incubated at room temperature for 10 minutes. Absorbance at 595 nm was measured using a spectrophotometer. The spectrophotometer was blanked using a solution of 100 µL distilled water and 1000 µL Bradford reagent.

Table 1: Table of sample preparation for Bradford assay

	Bradford reagent (mL)	0.5 mg/mL BSA (µL)	Distilled water (µL)	Enzyme solution (µL)
Standard 1	1	10	90	0
Standard 2	1	30	70	0
Standard 3	1	50	50	0
Standard 4	1	70	30	0

Protein sample	1	0	50	50

2.2.7 Immobilized lactase and β-galactosidase enzyme activity assay with chromogenic substrate

The enzyme activity test was done using a spectrophotometric assay that is based on monitoring the hydrolysis of model substrate *o*-nitrophenyl-β-D-galactopyranoside (*o*NPGal). Water was removed from Eppendorf tubes with immobilized enzyme and replaced with 900 µL of 0.1 M KPi buffer at pH 6.8 and 100 µL of 30 mM *o*NPGal. The tubes were gently inverted to mix the solution and then placed on a shaker at room temperature. Timing was started immediately. Aliquots (100 µL) were taken at 10, 20, and minutes from each of the six Eppendorf tubes and mixed with 900 µL 0.1 M KPi buffer, pH 6.8. Absorbance of the samples was measured at 405 nm using a spectrophotometer. The spectrophotometer was blanked using 100 µL of 30 mM oNPGal and 900 µL of 0.1 M KPi buffer, pH 6.8.

2.2.8 Transglycosylation versus hydrolysis using free enzyme β-galactosidase from Aspergillus oryzae

To set up the reaction mixture, 900 µL of 400 mM lactose in sodium acetate buffer, pH 4.8 was added into an Eppendorf tube. Then, 100 µL of β-galactosidase from *Aspergillus oryzae* was added to the above solution. The solution was gently mixed and incubated in a 50 °C water bath for 72 hours. To monitor the reaction progress, 100 µL aliquots were taken after 20 minutes, 1 hour, 24 hours, 48 hours, and 72 hours. The aliquots were stored at 4 °C before analysis by TLC.

2.2.9 Transglycosylation versus hydrolysis using immobilized lactase and β-galactosidase enzyme

Two tubes, one with immobilized lactase and another one with immobilized β-galactosidase enzyme were used. Into each tube, 800 µL sodium acetate buffer at pH 4.8 was added followed by 200 µL of 400 mM lactose solution. The mixture was incubated in a water bath at 50 °C while taking 100 µL aliquots after 20 mins, 30 mins, 1 hour, 2 hour, 6 hours, 12 hours, and 24 hours. Aliquots were stored at 4 °C before analysis by TLC.

2.2.10 pH dependence of transglycosylation and hydrolysis

Three reactions were set at pH 4.8, 6, and 8, respectively. To prepare 400 mM lactose solution, 1.5 g of lactose powder was dissolved in 10 mL of corresponding buffer. For pH 4.8, 0.1 M sodium acetate buffer was used, for pH 6 - 0.1 M PIPES buffer, and for pH 8, 0.1 M Tris buffer was used. The reactions were set up by adding 1000 µL of the lactose solution at respective pH to three different Eppendorf tubes with immobilized lactase enzyme. The mixtures were incubated at 50 °C for 2 hours while taking 100 µL aliquots at 20 minutes, 30 minutes, 60 minutes, and 120 minutes time intervals. Aliquots were stored at 4 °C before analysis by TLC.

2.2.11 Effect of temperature on hydrolysis and transglycosylation

Four tubes with immobilized lactase enzyme were used to analyze the effect of temperature on transglycosylation and hydrolysis. To each tube, 1000 µL of 400 mM lactose in 0.1 M Tris buffer, pH 8 was added and gently mixed to expose the beads to the solution. The tubes with reaction mixtures were then incubated at 25 °C, 50 °C, 60 °C, and 75 °C, respectively, for 120 minutes, while taking 100 µL aliquots after 20 minutes, 30 minutes, 60 minutes, and 120 minutes. The aliquots were stored at 4 °C before analysis by TLC.

2.2.12 Effect of hydrolysis products of lactose on transglycosylation and hydrolysis reactions

Solutions of 15% (w/v) glucose and 15% (w/v) galactose were made by dissolving 1.5 g of the respective sugar in 10 mL 0.1 M Tris buffer, pH 8. Three Eppendorf tubes with immobilized lactase enzyme were used. To the first tube, 1000 µL of 15% (w/v) lactose was added. To the second and third tube 500 µL of 15% (w/v) lactose was mixed with 500 µL of 15% (w/v) glucose and 15% (w/v) galactose, respectively. The solutions were then incubated in a water bath shaker for 120 minutes, at 50 °C, pH 8 while taking 100 µL aliquots for analysis after 20 minutes, 30 minutes, 60 minutes, and 120 minutes. Samples were then stored at 4 °C before analysis by TLC.

2.2.13 Attempt to synthesize trisaccharide lactosucrose from sucrose and lactose

For 408 mM sucrose solution preparation, 1.40 g of sucrose powder was added to 10 mL of 0.1 M Tris buffer, pH 8. Water was removed from Eppendorf tube with immobilized lactase enzyme and replaced with 500 µL of sucrose solution. This was followed by addition of 500 µL of 400 mM lactose solution. The mixture was incubated in a water bath shaker at 60 °C for 2 hours while taking 100 µL aliquots at 20 minutes, 30 minutes, 60 minutes, and 120 minutes. The aliquots were then stored at 4 °C. For analysis by TLC, 2% boric acid: 60% (v/v) acetic acid: ethanol: acetone: ethyl acetate (5:7.5:10:30:30) was used as developing solvent.

2. 2.14 Effects of phenolic compounds on transglycosylation reaction

Phenol and 4-nitrophenol were used as the phenolic compounds in the hydrolysis/transglycosylation reactions. To prepare 0.1 M 4-nitrophenol solution, 0.07 g of 4-nitrophenol was dissolved in 5 mL of distilled water. For 0.1 M phenol preparation, 0.05 g of phenol was dissolved in 5 mL of distilled water.

Two tubes with immobilized lactase were used. Water was removed from beads and replaced with 800 µL of 400 mM lactose in 0.1 M Tris buffer, pH 8. This was followed by addition of 200 µL

of 0.1 M 4-nitrophenol solution to one tube and 0.1 M phenol solution to the other tube. The solutions were gently mixed and incubated in a water bath at 60 °C while taking 100 µL aliquots at 20 minutes, 30 minutes, 60 minutes, and 120 minutes. The aliquots were then stored at 4 °C.

2. 2.15 Effect of organic solvents on transglycosylation reaction

The effect of organic solvents on transglycosylation and hydrolysis was tested using three different organic solvents: 1-butanol, 2-propanol, and 1-hexanol. For each of the organic solvents, three Eppendorf tubes with immobilized lactase enzyme were used. Each reaction contained 400 mM lactose in 0.1 M Tris buffer, pH 8 and the respective organic solvent in concentrations 30%, 50%, or 100% (v/v) in 1:1 ratio.

Tubes with reaction mixture were gently inverted to expose the solution to beads and incubated in a water bath shaker at 60 °C for 2 hours while taking 100 µL aliquots at 20 minutes, 30 minutes, 60 minutes, and 120 minutes. Aliquots were stored at 4 °C.

2.2.16 Reusability assay of immobilized enzyme at high temperatures

Immobilized lactase enzyme was incubated with 1000 µL of 400 mM lactose solution (pH 8) at 60 °C for 60 minutes while taking aliquots at 30 minutes and 1 hour. Beads were rinsed twice with distilled water and then supplemented with fresh 1000 µL of 400 mM lactose solution (pH 8) to initiate another cycle at the same temperature. Using the same procedure, a total of four cycles were executed. Enzyme activity test using *o*-nitrophenyl-β-D-galactopyranoside was performed after each cycle and compared to the first cycle (activity defined as 100%). All aliquots were saved at 4 °C. The same procedure was repeated at 50 °C.

2.2.17 Analysis of reaction by TLC

Qualitative analysis of the sugars was done by thin layer chromatography (TLC) using high grade 10 x 10 cm aluminum-backed silica plates. Solutions of 1.5% (w/v) lactose, 1.5% (w/v) glucose, 1.5% (w/v) galactose, and reaction aliquots were spotted by capillary tube on the TLC plate. TLC plates were incubated in a closed chamber containing 1-butanol: acetic acid: water in ratio 2:1:1 (v/v/v), respectively. The plate was removed, allowed to air dry, and sprayed with 95% ethanol: concentrated sulfuric acid in ratio 19:1(v/v), respectively. The TLC plate was then placed on a hot plate until the dark spots appear.

2.2.18 Transformation and expression β-galactosidase from Enterobacter species

pET20b(+)lacZ plasmid was a gift from YSU professor Dr. Jonathan Caguiat. Single step KRX competent cells were obtained and 50 µL was added to the bottom of a chilled sterile culture tube on ice. Then, 2 µL of the *pET20b(+)lacZ* plasmid was added to the cells and gently mixed with the end of the tip. The mixture was incubated on ice for 20 minutes after which the cells were subjected to heat shock at 42 °C for 45 seconds. The culture tube was placed on ice for 2 minutes. Then, 950 µL of room temperature SOC media was added. The tubes were placed in an incubator at 37 °C for 45 minutes while shaking at 175 rpm. The culture (150 µL) was spread into 100 mm×15 mm LB agar plates containing ampicillin and placed in an incubator at 37 °C overnight.

To express *Enterobacter* β-galactosidase a single colony was inoculated into 50 mL of LB media containing 100 µg/mL of ampicillin. The culture was grown overnight in the incubator at 37 °C with shaking at 200 rpm. Then, 10 mL of overnight culture was diluted into 1 L of LB media containing 100 µg/mL of ampicillin. The diluted culture was placed in an incubator at 37 °C while shaking at 200 rpm until an optical density (OD_{600}) of 0.4 was reached. Once the cells reach the required OD_{600}, the culture was transferred to a water bath shaker at 20 °C and 200 rpm for one

hour. This was followed by saving 2 mL of the sample in the Eppendorf tubes as the induction of sample. The rest of the culture was induced with 0.01 % rhamnose solution and grown overnight at 20 °C with shaking at 200 rpm. The cells were then harvested by centrifugation at 6000 × g at 4 °C for 10 minutes and stored at -20 °C.

2.2.19 Protein purification with streptomycin sulfate treatment and precipitation by 75% ammonium sulfate

All initial purification steps were done on ice with stirring on a magnetic stir plate. Centrifugation of the solutions was done at 11,000 × g and 4 °C.

Cells were obtained from the freezer and suspended in 50 mL of 50 mM NaPi buffer, pH 7 containing1 mM DTT. The mixture was subjected to sonication for 30 seconds and allowed to cool for 1 minute for a total of ten cycles to lyse the cells. The lysate was then divided equally into two centrifuge bottles and centrifuged. The pellets were saved at -20 °C and the supernatant poured into a beaker on ice. The supernatant was treated with 1% (w/v) streptomycin sulfate which was added dropwise as a solution of streptomycin in 2 mL 50 mM NaPi buffer, pH 7. The solution was left to stir for 10 minutes and then centrifuged for 25 minutes. The pellet was saved at -20 °C and to the supernatant, ammonium sulfate was added up to 75% saturation. The mixture was stirred for 10 minutes and then centrifuged for 25 minutes. The pellet and the supernatant were stored at 4 °C. After each centrifugation, 20 µL of samples and trace amounts of the pellets were collected and saved. The small pellet from the samples was dissolved in 20 µL distilled water. These samples were mixed with 2X SDS loading dye in 1:1 ratio and mixed. The samples were heated at 100 °C for 5 minutes, cooled, and analyzed by SDS-PAGE.

2.2.20 Protein purification by immobilized metal-ion affinity chromatography (IMAC)

Immobilized metal-ion affinity chromatography was tested for protein purification. The pellet obtained from 75% ammonium sulfate precipitation was dissolved in 50 mM NaPi buffer pH 7 and dialyzed against 1 L of the same buffer overnight at 4 °C. Immobilized metal-ion affinity chromatography column was washed with 20 mL 0.02 M Tris, 0.5 M NaCl buffer, pH 7, then 0.1 M $CuSO_4$ (1 mL) was added to the column. The column was re-equilibrated again with 20 mL of 0.02 M Tris, 0.5 M NaCl buffer, pH 7. A portion (1 mL) of the dialyzed protein was loaded into the column and the flow through collected. The column was washed with 10 mL of 0.02 M Tris, 0.5 M NaCl buffer, pH 7 while collecting 2 mL fractions. The collected fractions were analyzed using diode array spectrophotometer at 280 nm to determine fractions with high absorbance. The proteins bound to the column were eluted with 25 mL of 0.02 M Tris, 0.5 M NaCl, 0.02 M imidazole buffer, pH 7, and 2 mL fractions were collected. Absorbance of the fractions was measured using diode array spectrophotometer at 280 nm. All fractions were stored at 4 °C. Aliquot (20 µL) from samples that had high absorbance at 280 nm were mixed with 20 µL of 2X SDS loading dye. The samples were heated at 100 °C and used for SDS-PAGE analysis.

2.2.21. Enterobacter β-galactosidase enzymatic activity test

Enzyme activity test of *Enterobacter β*-galactosidase was done using a spectrophotometric assay that is based on monitoring the hydrolysis of a model substrate, *o*NPGal. The spectrophotometer was blanked with 900 µL of 50 mM NaPi buffer, pH 7 and 100 µL of 30 mM *o*NPGal. In the cuvette, 850 µL of 50 mM NaPi buffer, pH 7 and 100 µL of *o*NPGal were added. This was followed by addition of 50 µL of 20-fold diluted dialyzed pellet. The mixture was quickly inverted, and absorbance of the samples was measured at 405 nm for 5 minutes using a spectrophotometer.

2.2.22. Transglycosylation and hydrolysis activity using free Enterobacter β-galactosidase

To set up the reaction mixture, 900 µL of 400 mM lactose in 0.1 M Tris buffer, pH 8 was added into an Eppendorf tube. Then, 100 µL of dialyzed sample of *Enterobacter* β-galactosidase was added to the above solution. The solution was gently mixed and incubated in a 50 °C water bath for 2 hours. To monitor the reaction progress, 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.

CHAPTER 3. RESULTS AND DISCUSSION

Transglycosylation and hydrolysis reactions are often catalyzed by the same enzyme and these two reactions compete. Yield of transglycosylation products is dependent on the balance between synthesis, primary hydrolysis, and secondary hydrolysis.[55] The purpose of this project was to improve transglycosylation by evaluating at which conditions glycosidic bond formation will be favored more than bond breaking. Since transglycosylation and hydrolysis activities vary based on the origin of the enzyme, three enzymes known to hydrolyze lactose were used to test if they have transglycosylation activity.[63] The enzymes were used both in free and immobilized forms. When using free enzyme, optimal conditions for transglycosylation by β-galactosidase from *Aspergillus oryzae* as determined earlier (pH 4.8, 50 ºC, 15% lactose and a 72-hour incubation time) were used. [49] To immobilize the enzyme, chitosan was used as a support. The structural properties of chitosan were enhanced using silica as a fortifying agent and cross-linking with the bifunctional reagent glutaraldehyde.[44] The amount of enzyme bound to the chitosan - silica support was determined by Bradford assay using bovine serum albumin as a standard. Enzymatic activity was assessed spectrophotometrically at 405 nm following hydrolysis of model substrate *o*-nitrophenyl-β-D-galactopyranoside (*o*NPGal) which leads to release of *o*-nitrophenolate (*o*NP).[56]

To improve transglycosylation, high and low temperatures were tested for the most favorable temperature. Also, pH was tested. Hydrolysis usually occurs due to the presence of water in the medium. Organic solvents are known to enhance the selectivity of the enzymes for the competing nucleophiles (acceptor and water molecule) by reducing water activity in the reaction media.[55] For this reason, three alcohols (2-propanol, 1-butanol, and 1-hexanol) were applied to evaluate the effect towards transglycosylation and hydrolysis. Lactose, sucrose, glucose, and galactose were

used as substrates. During the reaction, samples were saved at timed intervals and monitored by TLC to evaluate the products.

At the end, the best transglycosylation conditions were used to run a reaction and the enzyme was re-used at 50 °C and 60 °C to evaluate how many times the immobilized enzyme can be reused at high temperatures.

3.1 Formation of chitosan beads, fortification with inert material, and crosslinking with glutaraldehyde

Choice of chitosan as a support in enzyme immobilization is advantageous because it is inert, inexpensive, non-toxic, biodegradable, and has been found to have good immobilization efficiency.[50, 57] It has reactive amino and hydroxyl groups which makes it flexible to chemical modifications. Chitosan is not soluble in water but because of its amino groups it is soluble in acidic media.[50] In this study, chitosan-silica beads (ChtS) were made by mixing chitosan powder with acetic acid to form a viscous solution. Silica was then added to the mixture as a fortifying agent and the solution was dispersed dropwise in a sodium hydroxide - ethanol solution (NaOH/ EtOH) **(Figure 5a)**. Once chitosan is dissolved in acidic media, its viscous solution forms water insoluble aggregates when in contact with anionic polyelectrolytes **(Figure 5b)**.[50] The chitosan-silica beads were washed with distilled water until the pH was neutral and stored in distilled water at 4 °C until further use. These beads were fragile and were breaking easily. To make the beads more compact and rigid, chemical crosslinking of the beads with the bifunctional reagent glutaraldehyde (GA) was done. Crosslinking is achieved when glutaraldehyde reacts with the amino groups on chitosan's backbone to form a Schiff base. This makes the beads more defined and compact compared to unlinked beads. Also, the beads become more stable under different external forces. During incubation with glutaraldehyde, chromophore groups (-C=N-) are formed

which make the cross-linked chitosan-silica beads to appear darker in color compared to un-linked beads **(Figure 5c)**. [58]

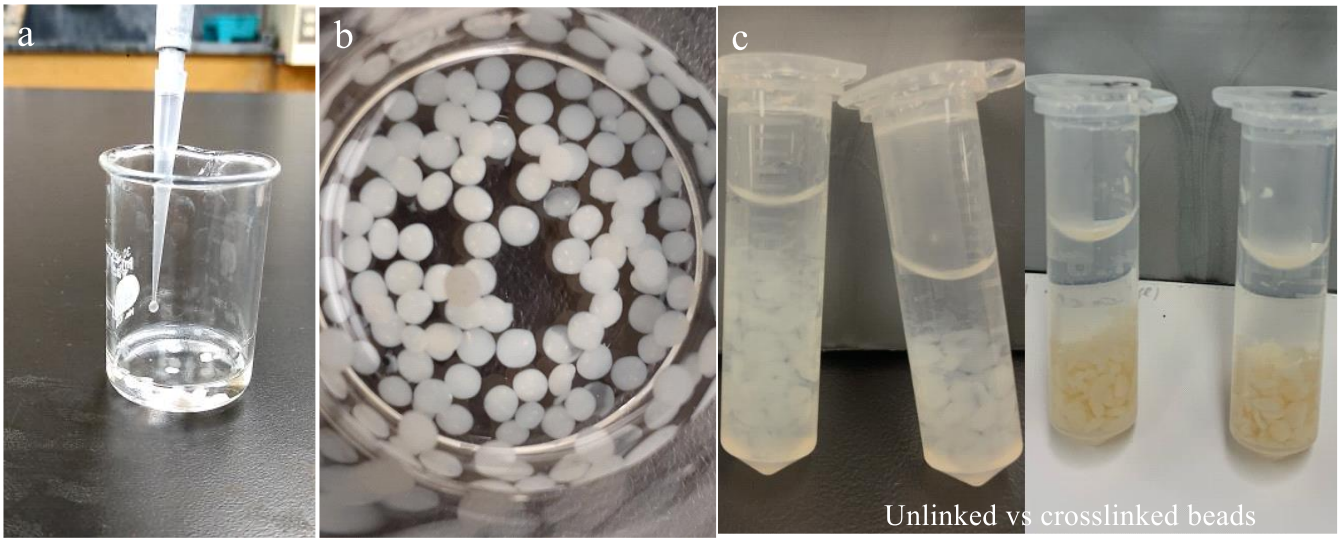


Figure 5: Chitosan-silica beads (ChtS) **a)** making ChtS beads, **b)** ChtS beads **c)** un-linked ChtS beads (left) verses ChtS beads cross-linked with glutaraldehyde (right).

3.2 Immobilization of lactase and β-galactosidase enzymes on chitosan-silica beads and protein quantification

Both β-galactosidase and lactase were immobilized by incubating the enzymes with the same amount of chitosan-silica beads at 4 °C for at least 16 hours. The samples were taken before and after incubation and the amount of enzyme immobilized was quantified by Bradford assay. Bradford assay was done using 0.5 mg/mL BSA as a standard. Absorbance of four standards with known amounts of BSA was measured at 595 nm. This was used to plot a standard curve of absorbance at 595 nm against micrograms of protein **(Figure 6)**. The equation from the calibration curve and the correlation coefficient (R^2) were obtained. The line equation ($y = 0.0227x + 0.0158$) was used to calculate the amount of protein (µg) in the samples. Using the

formula, $\frac{amount\ immobilized\ (\mu g)}{protein\ load\ (\mu g)} \times 100\%$, the percent of protein bound was calculated for each sample. For each enzyme, six samples were analyzed, and the results were averaged. **Table 2** indicates the relative amounts of the enzyme bound to the chitosan-silica beads and the percent immobilization. Despite the same amount of beads used for immobilizing the enzymes, the amount of lactase immobilized was found to be 28.00 µg which is higher when compared to β-galactosidase (13.17 µg).

Table 2: Average amount of lactase and β-galactosidase bound on chitosan-silica beads

Enzyme	**Initial amount of enzyme (µg)**	**Final amount of enzyme (µg)**	**Amount of enzyme bound (µg)**	**Immobilization (%)**
Lactase	33.32 ± 6.5	5.32 ± 3.9	28.00 ± 5.8	84.22 ± 6.9
β-galactosidase	31.85 ± 15.3	18.67 ± 3.1	13.17 ± 3.4	41.17 ± 9.0

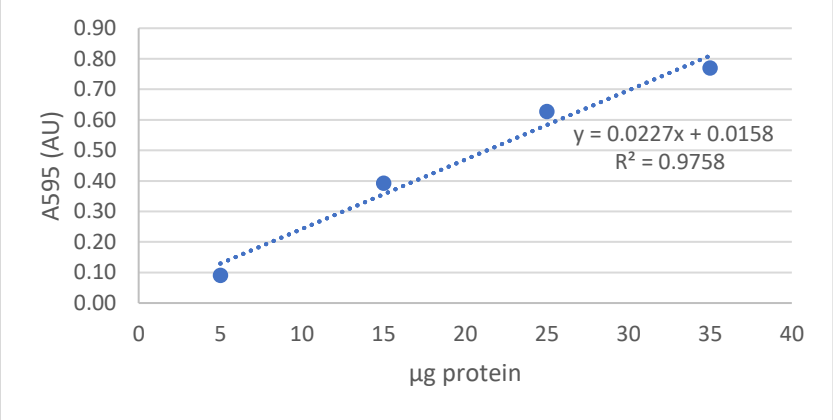


Figure 6: Bradford assay standard curve of absorbance at 595 nm verses micrograms of protein (BSA).

3.3 Analysis of hydrolysis versus transglycosylation using free enzyme β-galactosidase from Aspergillus oryzae

This experiment was performed to evaluate the transglycosylation capacity of commercial enzyme β-galactosidase from *Aspergillus oryzae*. The reaction was set using 400 mM lactose substrate which was incubated with free enzyme β-galactosidase from *Aspergillus oryzae* under conditions that were suggested as optimal transglycosylation conditions (pH 4.8, 50 ºC) for 72 hours.[49] To monitor the rate of product formation, aliquots were taken at various time intervals up to 72 hours and later analyzed using thin layer chromatography (TLC). **Figure 7** shows the TLC plate of the reaction mixture obtained at different time intervals. R_t and R_h indicate transglycosylation and hydrolysis products, respectively. Glucose, galactose, and lactose were spotted on the plate as standards. The transglycosylation products formed were mainly trisaccharides since they appeared below the lactose spot. Intensity of transglycosylation products for the two-hour period was found to be higher when compared to hydrolysis products. β-galactosidase from *Aspergillus oryzae* has been known to produce three major trisaccharides namely, Gal-β(1-6)-Gal-β(1-4)-Glc, Gal-β(1-3)-Gal-β(1-4)-Glc, and Gal-β-(1-4)-Gal-β-(1-6)-Glc.[59] Thus, the trisaccharides formed in this experiment could be either one of those or a mixture. Despite formation of transglycosylation and hydrolysis products, there was a significant amount of lactose that did not undergo the reaction. This effect might be due to the presence of reaction products glucose and galactose which are potential inhibitors of β-galactosidase. [54] However, some new disaccharides could also form and thus the spot representing lactose (substrate) could have a mixture of disaccharides. β-galactosidase from *Aspergillus oryzae* is known to form a disaccharide containing a β(1-6) linkage. [59] According to **Figure 7**, at 30 minutes, the hydrolysis rate was low and increased after 1 hour. As indicated by the TLC plate, there were more transglycosylation products than hydrolysis

products because the spots for transglycosylation products are darker compared to that of hydrolysis.

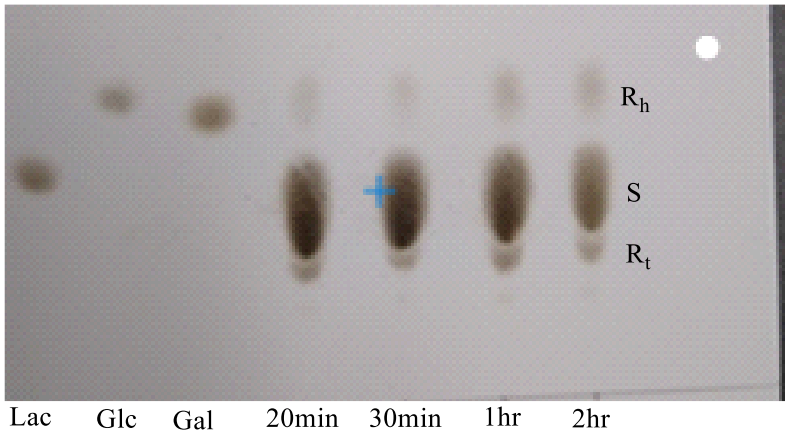


Figure 7: TLC analysis for products formed from 400 mM lactose using free enzyme β-galactosidase from *Aspergillus oryzae*. S - substrate, Glc - glucose, Gal – galactose, Lac – lactose, R_h - hydrolysis products, and R_t - transglycosylation products**.**

3.4 Transglycosylation versus hydrolysis using immobilized lactase and β-galactosidase from Aspergillus oryzae

β-galactosidase from *Aspergillus oryzae* and lactase were immobilized on chitosan -silica beads, then the beads were washed with distilled water to remove any residual protein that was not trapped by the matrix. The immobilized enzymes were used in synthesis of oligosaccharides by incubating with lactose substrate at 50 °C and pH 4.8. The rate of product formation was monitored by taking aliquots at time intervals and analyzing by TLC. Lactose, glucose, and galactose were used as standards. The oligosaccharide formation for two enzymes was compared. **Figure 8** shows TLC plates for reactions catalyzed by immobilized β-galactosidase from *Aspergillus oryzae* and lactase, respectively. The inverse relation between hydrolysis and transglycosylation was expected which means when transglycosylation rate (R_t) is high, hydrolysis rate (R_h) is low.[55] This statement,

however, did not apply to immobilized enzyme in this study, because use of immobilized enzyme led to an improved enzyme activity in both hydrolysis and transglycosylation. **Figure 8** shows a significant amount of hydrolysis as well as transglycosylation products as compared to free enzyme in **Figure 7.** In **Figure 8a,** when immobilized β-galactosidase was used, significant increase in hydrolysis and a reduction in transglycosylation activity was observed over time. After 2-hour incubation, all substrate and transglycosylation products were used up and only hydrolysis products appeared. This is an indicative that both primary hydrolysis and secondary hydrolysis took place. Secondary hydrolysis occurs when the same enzyme hydrolyzes the transglycosylation products.[55] For immobilized lactase enzyme, there was a significant amount of trisaccharides and tetrasaccharides formation as compared to immobilized β-galactosidase **(Figure 8b).** The highest concentration of transglycosylation products is shown at 20 minutes and 30 minutes. After 1 hour, the tetrasaccharides begin to disappear and after two hours only trisaccharides were present. At this time, the tetrasaccharides could possibly be undergoing secondary hydrolysis[55] Despite significant difference in immobilization, both enzymes had similarities in product formation. Both formed trisaccharides up to two hours. This means low amounts of immobilized enzymes can be used to yield products in kinetically controlled transglycosylation compared to reverse hydrolysis.[38]

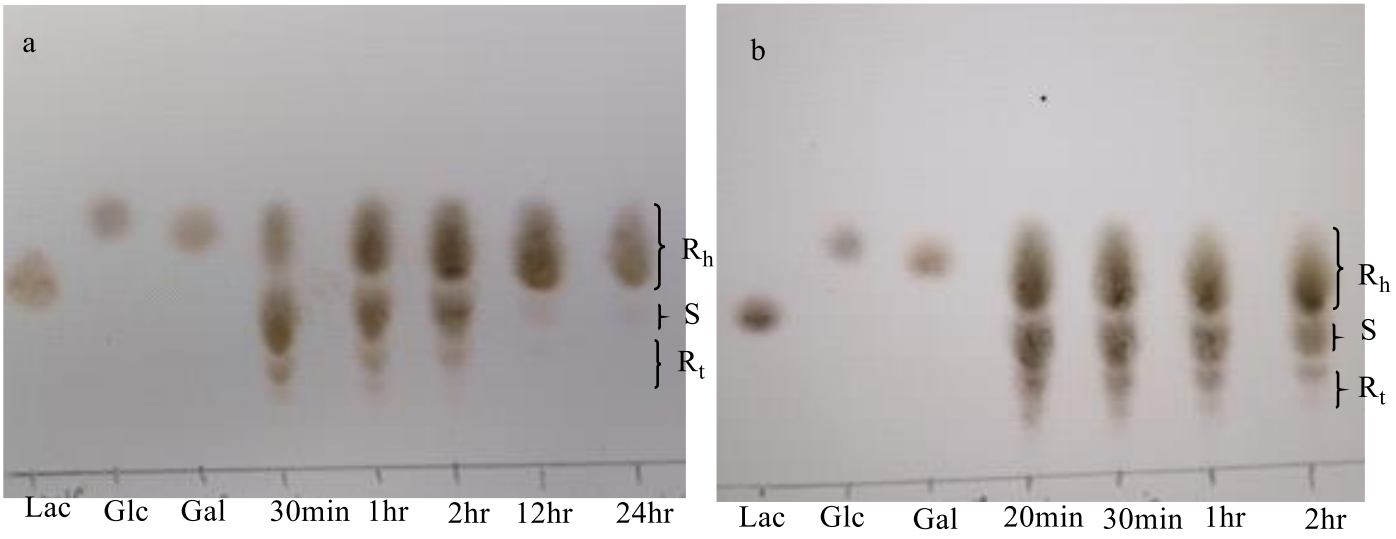


Figure 8: TLC analysis of the reactions using enzymes immobilized on a silica-chitosan support. R_h - hydrolysis products, R_t - transglycosylation products, S - substrate, Lac - lactose, Glc - glucose, and Gal - galactose. a) immobilized β-galactosidase enzyme from *Aspergillus oryzae;* b) immobilized lactase

3.5. pH dependence of transglycosylation activity

Evaluation of the effect of pH on the yield of transglycosylation and hydrolysis products was done using a range of 0.1 M buffers (sodium acetate buffer, pH 4.8, PIPES buffer, pH 6, and Tris buffer, pH 8). Reactions were set up using 400 mM lactose at respective pH and immobilized lactase. Reaction mixtures were incubated for two hours at 50 °C while taking aliquots at time intervals and product formation was analyzed by TLC. The immobilized lactase remained active at all pH tested. As shown in **Figure 9**, transglycosylation and hydrolysis reactions were found to depend on pH. Different product distributions were observed at different pH values. At pH 8, there was a significant amount of oligosaccharide formed **(Figure 9c)**. Both trisaccharides and tetrasaccharides were observed during the first 30 minutes. At 30 minutes and 1 hour, the hydrolysis rate is minimal compared to the transglycosylation rate. At 1 hour and 2 hours samples

had very low levels of hydrolysis products. At pH 4.8 and pH 6 **(Figure 9a & 9b)**, there is no significant difference in the reaction rates since in both transglycosylation was suppressed. Also, by 1 hour lactose was already hydrolyzed. The effect of pH on transglycosylation and hydrolysis indicated that immobilized lactase yielded the most oligosaccharide products at pH 8.

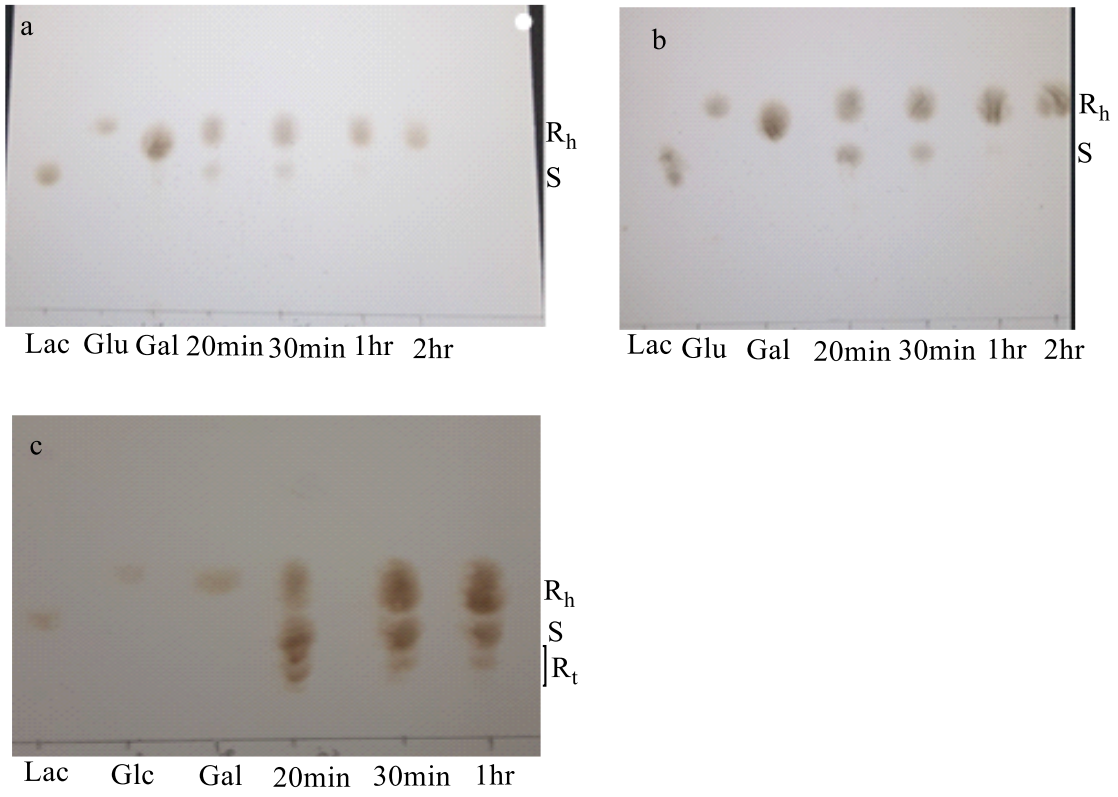


Figure 9: pH dependence of transglycosylation and hydrolysis reactions using lactase enzyme, Rh - hydrolysis products, Rt - transglycosylation products, S - substrate, Lac - lactose, Glc - glucose, and Gal - galactose. a) The reaction in 0.1 M sodium acetate buffer, pH 4.8, b) the reaction in 0.1 M PIPES buffer, pH 6, and c) the reaction in 0.1 M TRIS buffer, pH 8.

3.6. Effect of temperature on transglycosylation

Figure 10 shows the effect of temperature on transglycosylation and hydrolysis catalyzed by immobilized lactase. Reactions were set up at respective temperatures using 400 mM lactose at pH 8 and immobilized lactase. The highest production of oligosaccharides was recorded at 60 °C when the produced a mixture of trisaccharides, tetrasaccharides and small amounts of oligosaccharides with a higher degree of polymerization **(Figure 10c)**. However, at higher temperature (75 °C) neither transglycosylation nor hydrolysis products were detected **(Figure 10d)**. This is most likely the indication of denaturation of the enzyme. At 50 °C, reaction mixture had a significant amount of trisaccharides being formed at 30 minutes while hydrolysis was reduced. After 1 hour, the hydrolysis rate is observed to be higher than that of transglycosylation **(Figure 10d)**. There was no significant amount of transglycosylation products detected at room temperature while the hydrolysis reaction was observed, although at slow rate. At 20 minutes, 30 minutes and 1 hour, there was no observable reaction taking place and lactose appears to start being hydrolyzed after 2 hours **(Figure 10a)**. The reason for this is that room temperature is below the optimal temperature for the enzyme thus the enzyme is active, but the reaction rate is slow. The optimal temperature for immobilized lactase to exhibit a high production of transglycosylation products was 50 °C- 60 °C.

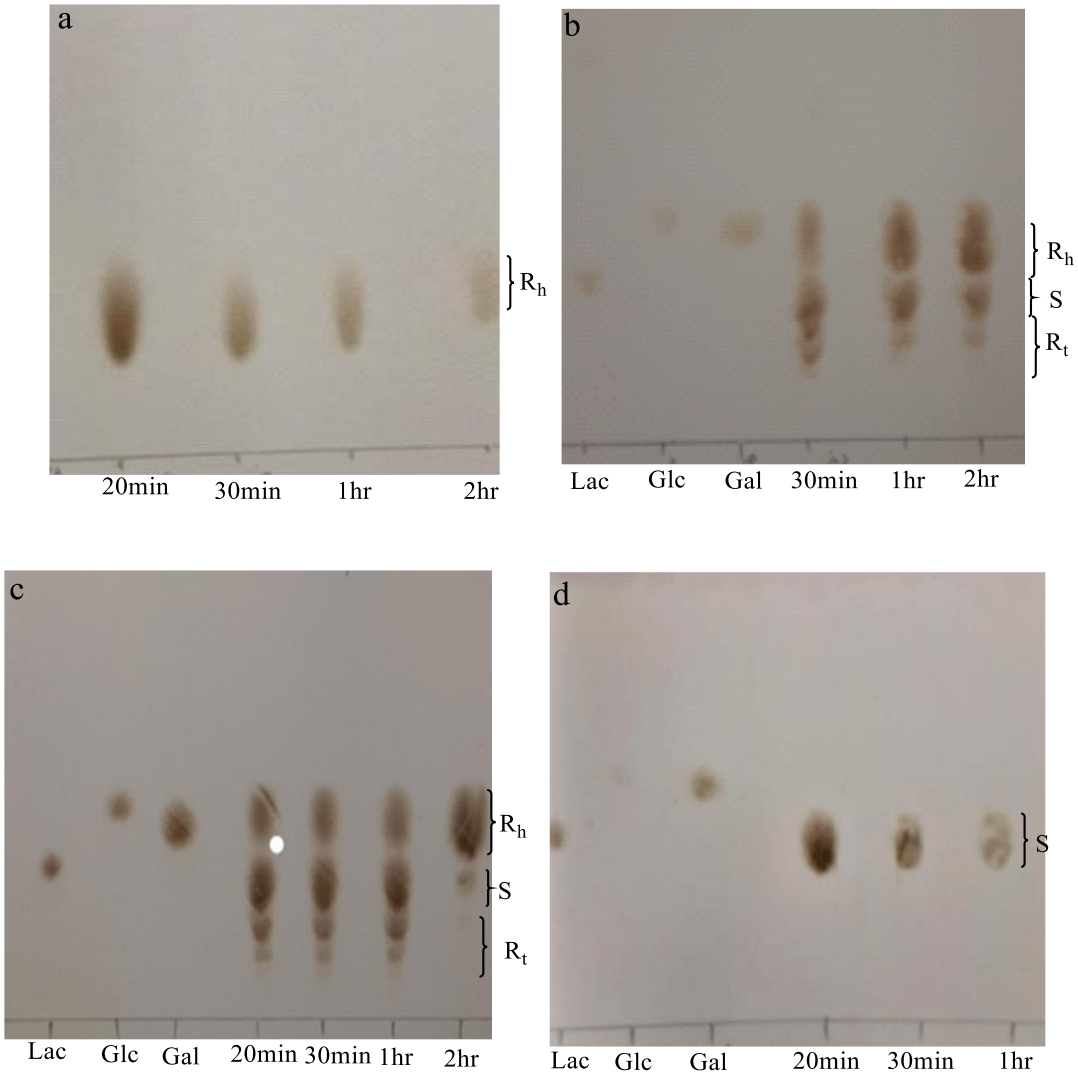


Figure 10: Effect of temperature on transglycosylation and hydrolysis reactions using immobilized lactase. Rh - hydrolysis products, Rt - transglycosylation products, S - substrate, Lac - lactose, Glc - glucose, and Gal - galactose. a) 25 °C, b) 50 °C, c) 60 °C , d) 75 °C.

3.7 Effect of hydrolysis products of lactose on transglycosylation

Possible effect of monosaccharides on transglycosylation was evaluated adding glucose or galactose to the reaction mixture that was run with immobilized lactase at pH 8 and 60 °C. Each monosaccharide was added to the reaction in equal amount with lactose and incubated for two hours while aliquots were taken at time intervals and analyzed using TLC. Addition of galactose

(Figure 11b) resulted in no hydrolysis or transglycosylation products observed. The intensity of lactose, glucose, and galactose spots within the reaction mixture on the TLC plate remained the same throughout the 2-hour period. It is documented that galactose is a competitive inhibitor of lactase [54, 60] According to the results, there is a clear indication that galactose is a strong inhibitor of both transglycosylation and hydrolysis reactions.[60] On the other hand, high concentration of glucose led to minute spots (marked in red) forming below lactose which indicated the presence of transglycosylation products **(Figure 11a)**. The possible reason why some products formed when glucose was used in the reaction media is because unlike galactose, glucose is a non-competitive inhibitor of lactase.[54, 60] The results show, both glucose and galactose have a negative effect on hydrolysis of lactose and do not stimulate synthesis of oligosaccharides. Another approach to increase the rate of transglycosylation is to remove these monosaccharides from the reaction media. For example, glucose can be removed from the reaction medium by using glucose oxidase which will oxidize glucose to gluconic acid.

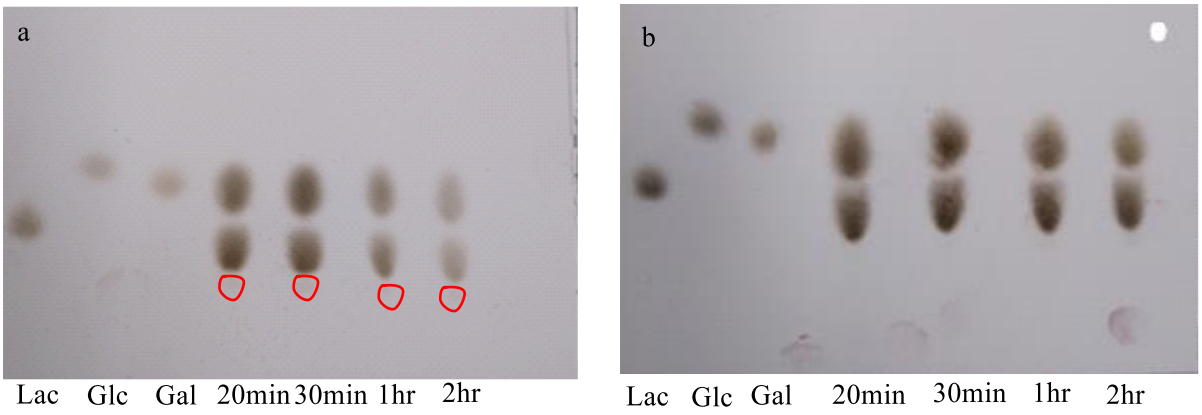


Figure 11: Effect of high concentrations of glucose and galactose on transglycosylation and hydrolysis. Glc - glucose, Gal - galactose, Lac – lactose. Both reactions were set using immobilized lactase enzyme, pH 8 at 60 °C. a) 15% w/v glucose and 15% w/v lactose as substrates at the ratio 1:1, b) 15% w/v galactose and 15% w/v lactose as substrates at the ratio 1: 1.

3.8. Attempt to synthesize trisaccharide lactosucrose from sucrose and lactose using immobilized lactase

Lactosucrose is a trisaccharide (Gal-β(1-4)-Glc-(α1-2)-β -Fru) obtained by enzymatic synthesis where lactose and sucrose are used as substrates.[62] In most cases, the enzyme β-fructofuranosidase has been used in lactosucrose production where fructose is transferred to lactose. However, this enzyme is costly, has poor stability and has low transfructosylation efficiency.[62] In this experiment, an attempt to synthesize lactosucrose from lactose and sucrose using immobilized lactase was conducted. Lactase cannot hydrolyze sucrose; therefore, the reaction should involve transfer of galactose to sucrose. Two reaction mixtures were incubated at 60 °C for 2 hours while taking aliquots at timed intervals. One mixture had a lactose to sucrose ratio of 1:1 **(Figure 12a)** and another had a lactose to sucrose ratio of 1:4 **(Figure 12b)**. Due to the presence of sucrose, 2% boric acid: 60% (v/v) acetic acid: ethanol: acetone: ethyl acetate (5:7.5:10:30:30) was used as a developing solvent when analyzing the products by TLC. The system butanol: acetic acid: water (2:1:1) did not provide clear separation of reactants and products. With an equal ratio of lactose to sucrose, trisaccharides formed throughout the 2 hours. However, when the lactose to sucrose ratio was 1:4, trisaccharides and tetrasaccharides were formed. The intensity of the spots formed by trisaccharides was much higher for the ratio 1:4 than for 1:1. According to our results, the ratio of two substrates in the reaction mixture greatly affects the synthesis of lactosucrose. Changing the ratio of lactose to sucrose from 1:1 to 1:4 reduced hydrolysis and increased the overall amount of oligosaccharides formed including trisaccharides which could be lactosucrose. This could be due to the reduction of glucose concentration from hydrolysis since there is less lactose used. Further analysis of the chemical identity of the produced trisaccharide is necessary to confirm formation of lactosucrose.

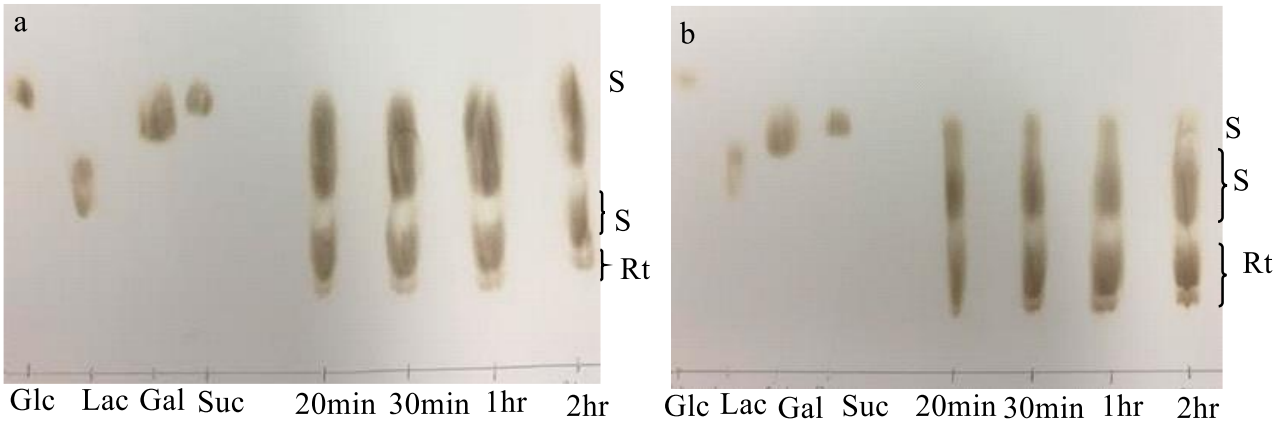


Figure 12: TLC for synthesis of lactosucrose by immobilized lactase, R_t- transglycosylation products, and S - substrate, Lac - lactose, Glc - glucose, and Gal galactose. a) lactose: sucrose 1:1 and b) lactose: sucrose 1:4.

3.9. Effect of solvents on transglycosylation

Presence of water in a reaction media leads to undesirable primary and secondary hydrolysis and a decrease in transglycosylation. [49] Thus decreasing water activity will shift the synthesis – hydrolysis equilibrium towards synthesis. This experiment was done to study possible ways to decrease hydrolysis and increase the transglycosylation reaction. Two miscible organic solvents 2-propanol, 1- butanol (15% to 50% v/v), and immiscible 1-hexanol were selected and used in the reaction medium. Reactions were done at pH 8 and 60 °C with 400 mM lactose and a2-hour incubation. Aliquots were taken at timed intervals and then used for analysis by thin layer chromatography. Contrary to the expectations, all alcohols used had lower yields of transglycosylation products and in some cases transglycosylation was totally suppressed.

When 15% and 25% 2-propanol were used in the reaction mixture **(Figure 13a&b)**, the transglycosylation reaction was completely suppressed. The hydrolysis reaction was also slow over the 2-hour incubation period. In this case, most lactose did not undergo any reaction. In 50%

2-propanol **(Figure 13c),** however, some oligosaccharides had formed at 1 hour and the intensity increases at 2 hours. This means, the longer incubation period for 50% 2-propanol can lead to formation of more transglycosylation products. At the same time, the hydrolysis reaction was also found to increase from one hour and at 2-hours the hydrolysis rate is high compared to transglycosylation.

In **Figure 14a**, 15% (v/v) 1-butanol was used in the reaction media. In this case, the rate of lactose hydrolysis and oligosaccharide synthesis increased with time. For the first 20 and 30 minutes trisaccharides and hydrolysis products were being formed. Increasing the concentration of 1-butanol to 25% (v/v) **(Figure 14b)** led to a reduction of transglycosylation activity. Very few trisaccharides (marked in red) were formed throughout the 2-hour incubation. Hydrolysis rate was high at 20 and 30 minutes and started to reduce with time. At a higher concentration of 1- butanol (50% (v/v)), there was no transglycosylation products formed at 20 minutes and 30 minutes. At 1-hour, some trisaccharides started forming. The hydrolysis reaction was favored, and it was highest at 1 and 2 hours. Comparing the concentrations of 1- butanol used, 15% (v/v) showed the maximum transglycosylation activity.

Figure 15 represents the effect of a two-phase reaction system (1-hexanol/buffer) on transglycosylation and hydrolysis activity of immobilized lactase. As in the case of 1-butanol and 2-propanol, 1-hexanol led to formation of additional products **(**marked X on **Figure 15)**. The transglycosylation activity in this case was low and was constant up to 1 hour. Hydrolytic activity was also high up to 1 hour.

Presence of alcohols in the reaction mixtures led to formation of additional products **(**marked X on **Figures 13, 14 and 15).** In all cases, the intensity of these products increased with time up to 2 hours. Transglycosylation of lactose involves the formation of a β-D-galactosyl-enzyme

intermediate. This intermediate is either attacked by a water molecule to form hydrolysis products or by a glycosyl acceptor (hydroxyl containing acceptor) to form transglycosylation products.[55] Since alcohols contain hydroxyl functional group, the additional products (X) are most likely to be alkyl glycosides, which are the products of the reaction between a β-D-galactosyl intermediate and alcohols. Formation of alkyl glycosides is an acid-catalyzed process; therefore, at pH 8 the reaction should involve participation of enzyme. This was confirmed by setting the reactions between lactose and hexanol as well as glucose and hexanol without enzyme. **Figure 15b** indicates no X products were formed when enzyme was not present. Thus, the additional products X are formed when alcohols accept a glycosyl group from the enzyme. In all solvents used, most lactose remained unreacted after a two-hour incubation. This could be due to the stripping effect caused by alcohols in which they form a layer around the enzyme thus reducing accessibility of the substrate to the active site of the enzyme. [49] In addition, lactose precipitates were observed in the Eppendorf tubes as the presence of alcohols decreases lactose solubility.[52]

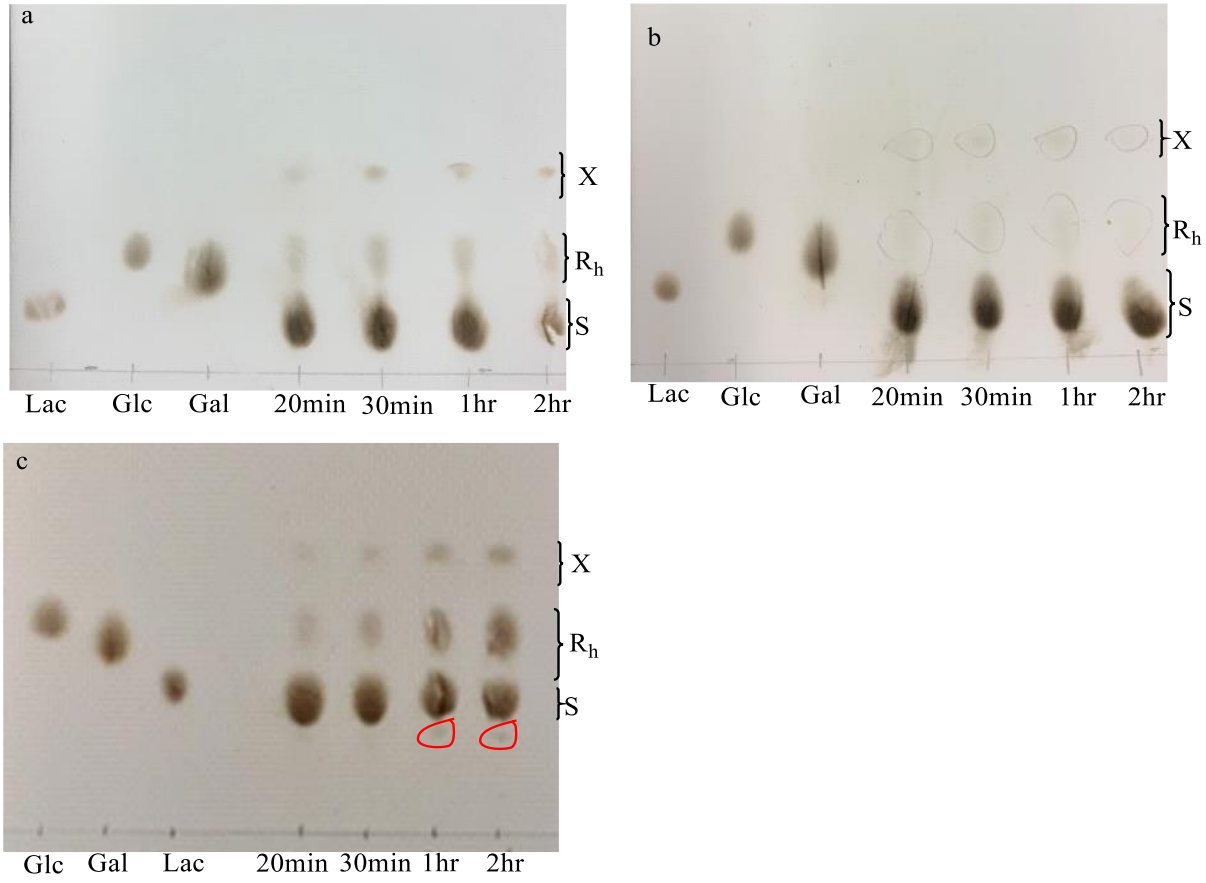


Figure 13: Effect of 2-propanol on transglycosylation activity of immobilized lactase R_h - hydrolysis products, X - unknown products, R_S - transglycosylation products, S - substrate, Lac - lactose, Glc - glucose, and Gal - galactose. a) 15% v/v 2-propanol in the reaction, b) 25% v/v 2-propanol in the reaction, c) 50% v/v 2-propanol in the reaction mixture

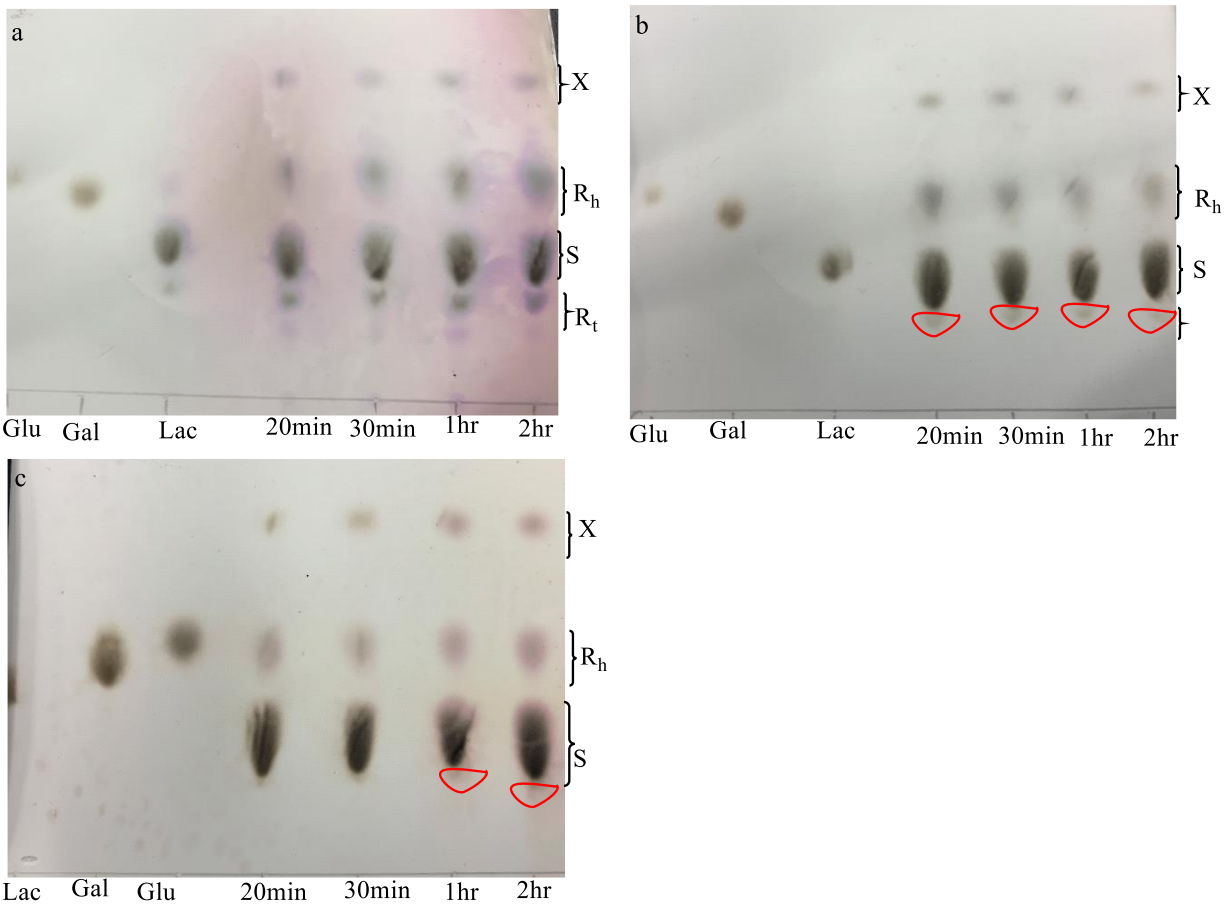


Figure 14: Effect of 1-butanol on transglycosylation and hydrolysis activity of immobilized lactase, R_h - hydrolysis products, X- unknown products, R_t - transglycosylation products, S- substrate, Lac - lactose, Glc - glucose, and Gal - galactose. a) the reaction in 15 % v/v 1-butanol, b) the reaction in 25% v/v 1-butanol, c) the reaction in 50% 1-butanol

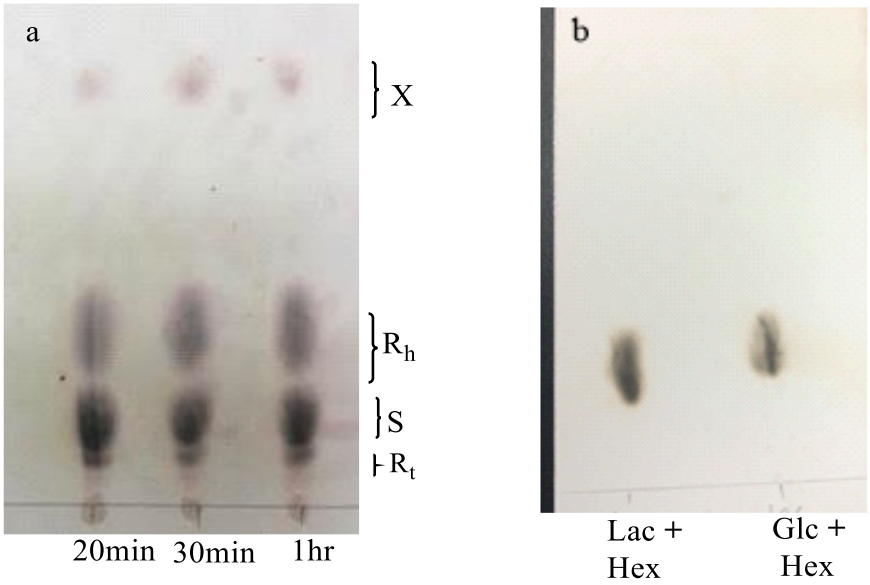


Figure 15: a) Transglycosylation and hydrolysis activity of immobilized lactase run in a hexanol/buffer two-phase system. R_h - hydrolysis products, X - unknown products, R_t - transglycosylation products, S - substrate, Lac - lactose, Glc - glucose, and Gal – galactose, b) Reaction between 1-hexanol and sugar (lactose or glucose).

3.10. Effect of phenol and 4-nirophenol on transglycosylation

Phenol and 4-nitrophenol were used in the reaction to reduce water activity in the media in the synthesis of oligosaccharides by transglycosylation. Each phenol was incubated with 400 mM lactose in the molar ratio 1:4 at pH 8, 60 °C, and immobilized lactase was used. The reaction mixture was incubated for 1 hour while taking aliquots at timed intervals. The analysis of products was done using thin layer chromatography. An increase in transglycosylation products was observed in both cases with a maximum attained at 1 hour **(Figure 16)**. When 4-nitrophenol **(Figure 16a)** was used, the amount of both hydrolysis and transglycosylation products increased with time. The same effect was not observed in the case of phenol **(Figure 16b).** Phenol led to a significant decrease in the hydrolysis reaction and increased synthesis of trisaccharides.

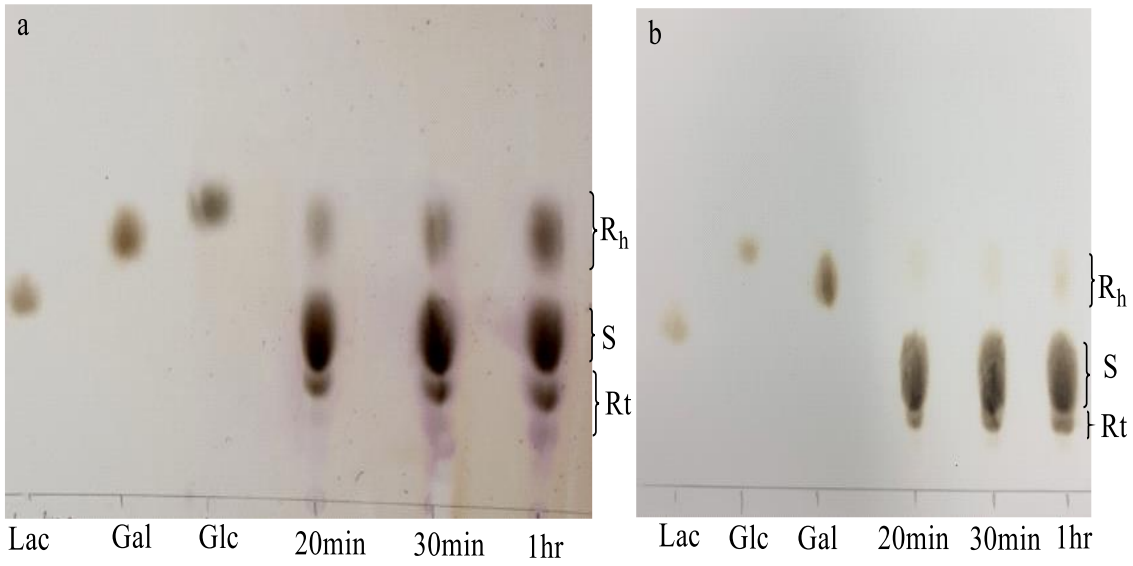
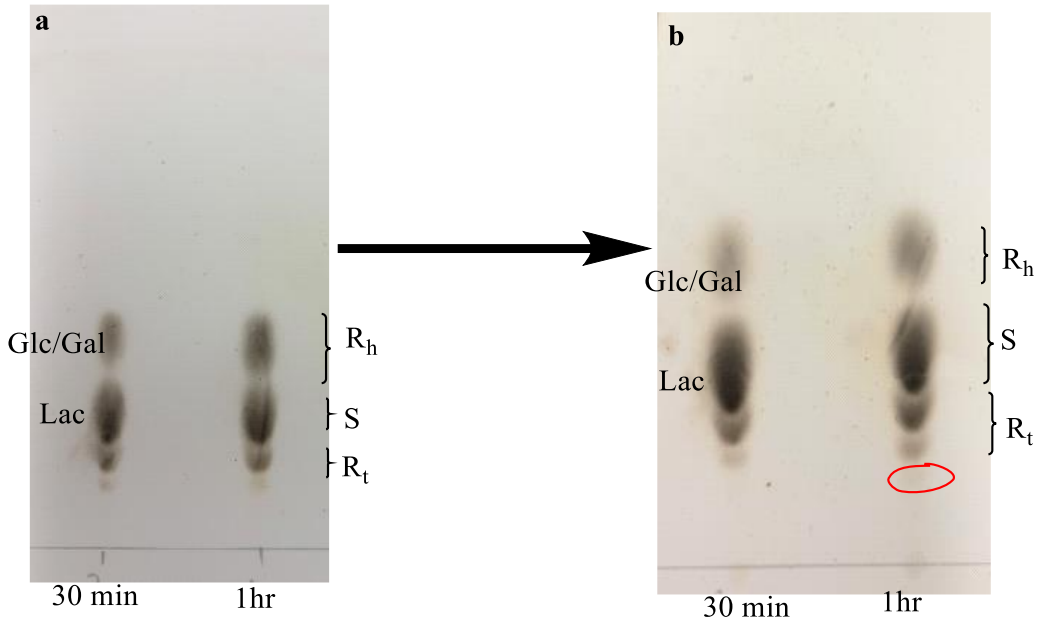


Figure 16: Time course of transglycosylation and hydrolysis catalyzed by immobilized lactase in presence of phenols in the reaction media, R_h - hydrolysis products, R_t - transglycosylation products, S - substrate, Lac - lactose, Glc - glucose, and Gal - galactose. a) 0.1 M 4-nitrophenol and b) 0.1 M phenol**.**

3.11. Reusability assay of chitosan - silica immobilized enzyme lactase at high temperature

To investigate the reusability of the immobilized lactase enzyme at high temperature, a series of transglycosylation reactions was repeated at 50 °C and 60 °C using the beads at pH 8, 15% (w/v) lactose and taking aliquots at 30 minutes and 1hour. For 50 °C, a total of four experiments were done. Analysis by TLC indicated that both trisaccharides and tetrasaccharides were formed through the four trials **(Figure 17 a&b)**. After being used successfully for four cycles there were small changes in product distribution between the first trial and the fourth trial **(Fig 17a&b)**. Surprisingly, in trial 4 **(Figure 17b)** oligosaccharides of a higher degree of polymerization were forming at 1 hour (marked in red). Before and after all trials were done, an activity test for the

immobilized enzyme was also done using model substrate *o*NPGal. **Figure 17c** illustrates the activity of lactase before and after four trials. The immobilized enzyme was still active at the end of the trials since it had the ability to hydrolyze model substrate *o*NPGal to *o*NP; however, there was some loss in overall activity as indicated by a decrease in *o*NP absorbance at 405 nm between trial 1 and trial 4. The reusability test at 60 °C was, however, different from that of 50 °C. The first trial had tetrasaccharides and trisaccharides forming during the first hour **(Fig 18a)**. The same immobilized enzyme in the second trial yielded only trisaccharides **(Fig 18b)**. For the third and fourth cycles, both hydrolysis and transglycosylation activities of the enzyme were suppressed **(Fig 18 c&d)**. This could be due to enzyme denaturation by its continuous use at high temperatures. Another possible reason is the chitosan-silica beads would have become loose due to high temperature exposure and thus the enzyme leaked from the support and was washed away between the trails. The results of the reusability tests suggest that enzyme is quite stable when used in immobilized form compared to free form. An ability to be reused at least four times at 50 °C and twice at 60 °C can be beneficial in industrial application as this will reduce operational cost.[42]



c

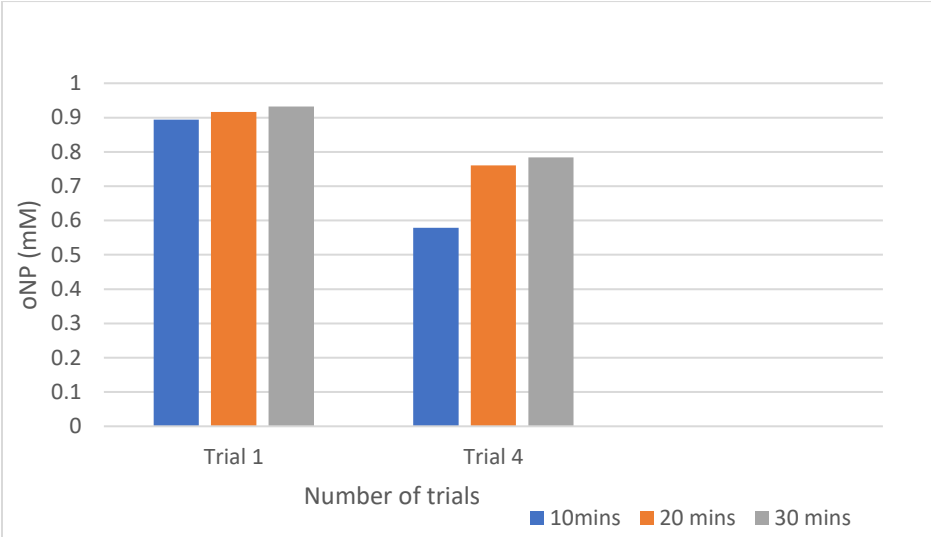


Figure 17: Reusability test of immobilized lactase at 50 °C. The immobilized enzyme lactase was used consecutively for four cycles at 50 °C: R_h - hydrolysis products, R_t - transglycosylation products, S - enzyme substrate, Lac - lactose, Glc - glucose, and Gal - galactose. a) trial 1, b) trial 4, c) Activity test before the reactions and the remaining activity after using immobilized lactase in four reaction cycles.

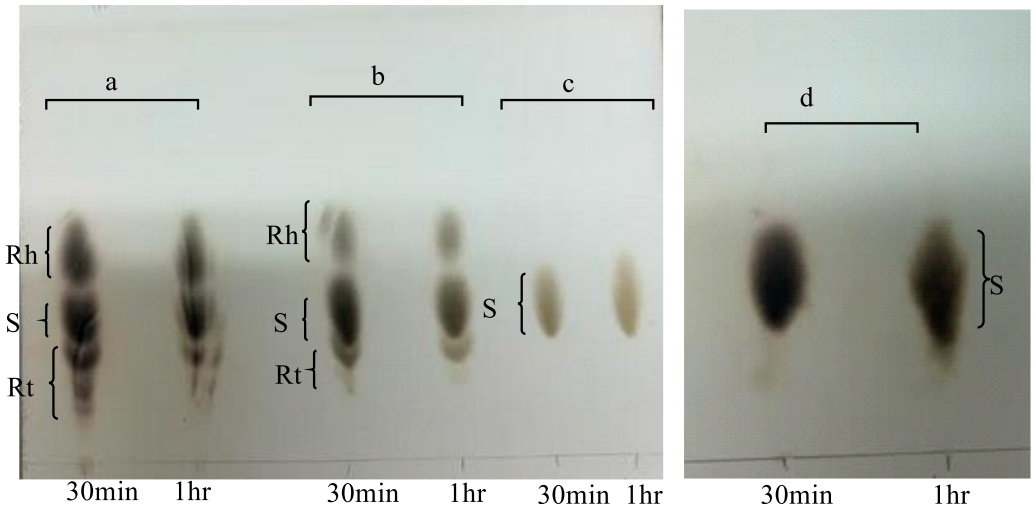


Figure 18: Reusability test of immobilized lactase at 60 °C. The immobilized enzyme lactase was used consecutively for four cycles at 60 °C: R_h - hydrolysis products, R_t - transglycosylation products, S - substrate, Lac - lactose, Glc - glucose, and Gal - galactose. a) trial 1, b) trial 2, c) trial 3, and d) trial 4.

3.12. Transformation and expression of β-galactosidase from Enterobacter species

Enterobacter β-galactosidase was the third enzyme in this study to be tested for its hydrolysis and transglycosylation activity. A *pET20b(+)lacZ* plasmid containing the *Enterobacter* β-galactosidase gene was a gift from Dr. Caguiat of YSU. Single step (KRX) competent cells were transformed with *pET20b(+)lacZ* plasmid using heat-shock. KRX strain which is an *E. coli* K12 derivative has an engineered feature that ensures controlled protein expression. The bacteria culture was spread into LB agar plates containing ampicillin for selection of transformed cells. LB agar plates were then incubated at 37 °C overnight for bacterial colonies to grow. **Figure 19** shows LB agar plates with bacterial colonies formed on the plate.

Expression of *Enterobacter* β-galactosidase was examined using colonies of KRX cells containing the β-galactosidase plasmid. Successful expression of *Enterobacter* β-galactosidase as a soluble

protein in *E. coli K*12 (KRX) cells was achieved. Analysis of protein expression was done by SDS-PAGE before **(Figure 20 lane 3&5)** and after **(Figure 20 lane 7&9)** induction with 0.01% rhamnose. Lanes 7 and 9 shows a better expression of soluble proteins after induction with rhamnose. An arrow indicates the band corresponding to the position of *Enterobacter* β-galactosidase which is 120 kDa.

Figure 19: LB/ampicillin plates with colonies of transformed *E. coli* KRX2 cells.

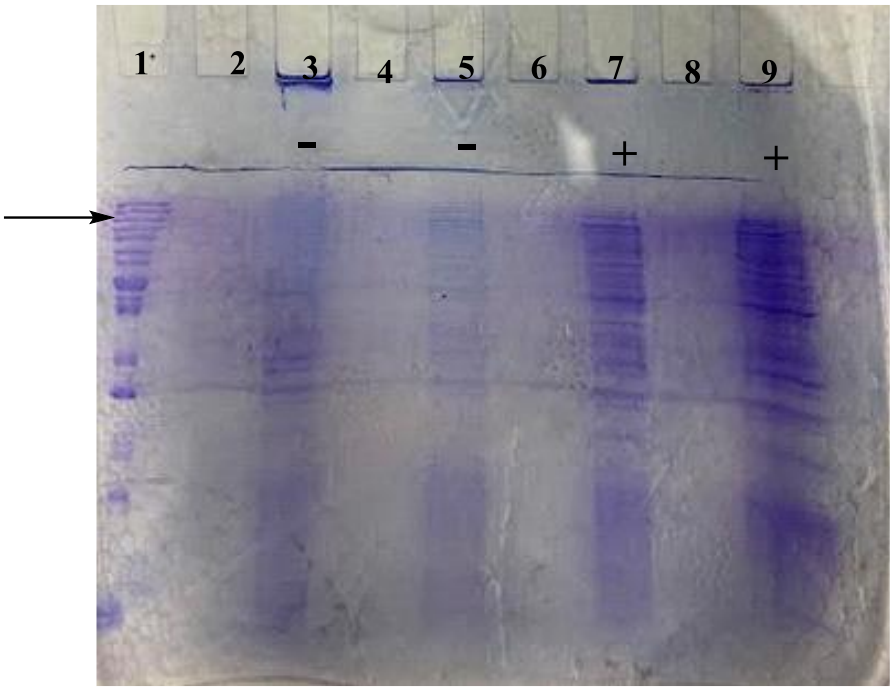


Figure 20: SDS-PAGE of single step KRX cells transformed with *pET-20(+) lacZ* before (-) and after (+) induction with 0.01% rhamnose. The arrow shows 120 kDa position which corresponds to *Enterobacter* β-galactosidase

3.13. Initial protein purification with streptomycin sulfate treatment and precipitation by 75% ammonium sulfate

To purify *Enterobacter* β-galactosidase, the cells were lysed by sonication followed by streptomycin treatment, and precipitation with ammonium sulfate. After each step of purification, samples were collected and analyzed by SDS-PAGE. The KRX*(pET20b(+)lacZ)* cells were harvested by centrifugation and the pellet was resuspended in 50 mM NaPi buffer pH 7 containing 1 mM DTT which reduces disulfide bonds and prevents oxidation damage. To disrupt the cells, the resuspended cells were sonicated and centrifuged. The supernatant obtained after sonication **(Figure 21, lane 3)** was treated with 1% streptomycin sulfate to remove DNA by precipitation and centrifugation **(Figure 21, lane 5**). The supernatant from streptomycin treatment was fed with

ammonium sulfate at 75% saturation to precipitate proteins including protein of interest from the solution. During ammonium sulfate precipitation, most proteins were precipitated **(Figure 21, lane 6)**. Lane 7, the pellet obtained from 75 % ammonium sulfate saturation, indicates that most proteins including the protein of interest were in the pellet. The pellet was thus used for further purification.

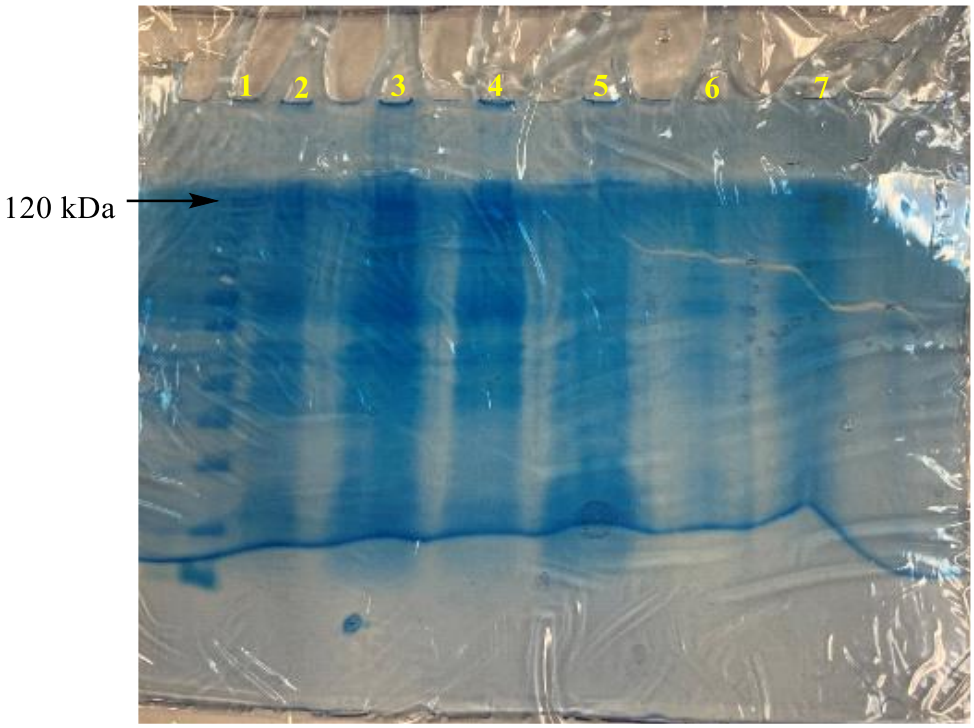


Figure 21: SDS-PAGE for initial purification of β-galactosidase from *Enterobacter sp* using treatment with streptomycin and precipitation by ammonium sulfate. The arrow corresponds to band position of β-galactosidase from *Enterobacter sp* which has a molecular weight of about 120 kDa. Lane 1: protein molecular marker; lane 2: supernatant from sonication; lane 3: pellet from first centrifugation; lane 4: streptomycin treatment supernatant; lane 5: streptomycin pellet; lane 6: 75% ammonium sulfate saturation supernatant and lane 7: 75% ammonium sulfate saturation pellet.

3.14 Protein purification by immobilized metal-ion affinity chromatography (IMAC)

The attempt of protein purification by immobilized metal-ion affinity chromatography (IMAC) was conducted. The stationary phase contained iminodiacetic acid (IDA) covalently linked to agarose. Solution of 1 M $CuSO_4$ was used as the metal ion source so that when the protein applied on the column it will bind to the copper (II) ion because the expressed β-galactosidase carries a His-tag. When a protein mixture is added to the column, histidine-tagged proteins bind to the metal ions. [61] The pellet obtained at 75% ammonium sulfate saturation was resuspended in 50 mM NaPi buffer, pH 7 buffer and dialyzed against the same buffer overnight at 4 °C. A portion of a dialyzed sample was loaded onto the affinity column and the proteins that did not bind to the column were eluted using 0.02 M Tris, 0.5 M NaCl buffer pH 7. A buffer containing imidazole (0.02 M Tris, 0.5 M NaCl, and 0.02 M imidazole buffer pH 7) was then used to elute the histidine-tagged proteins from the column as imidazole competes with histidine-tagged proteins for the copper ions leading to elution. **Figure 22** shows a plot of absorbance at 280 nm against fractions collected using both buffers. The fractions with high absorbance at 280 nm were analyzed by SDS-PAGE **(Figure 23).** SDS-PAGE results indicate that most of the *Enterobacter* β-galactosidase did not bind to the matrix and was washed off the column. **Figure 23** lane 4 indicates the fraction that contained the *Enterobacter* β-galactosidase with a molecular weight of 120 kDa.

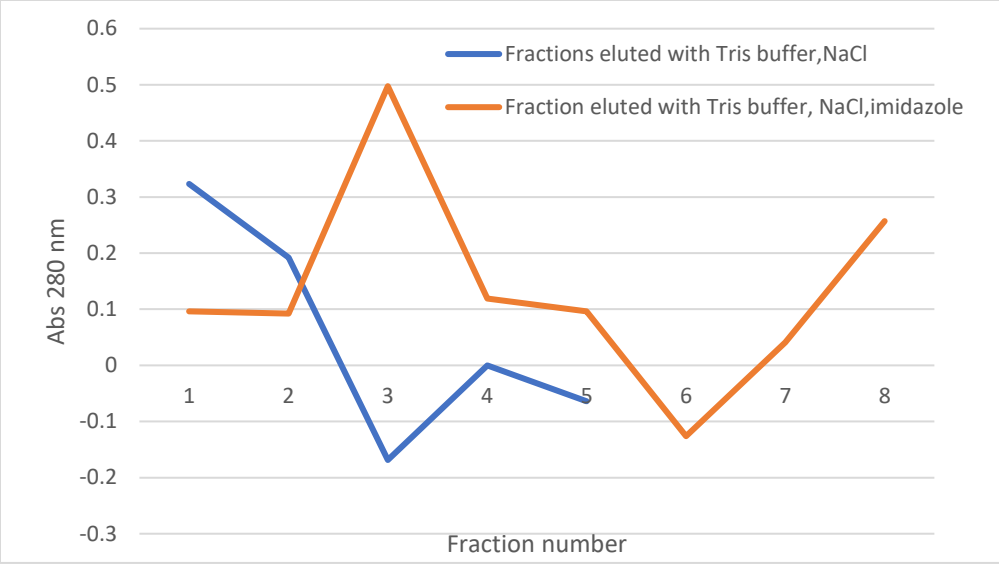


Figure 22: Graph of absorbance at 280 nm against fractions collected from immobilized metal affinity chromatography column.

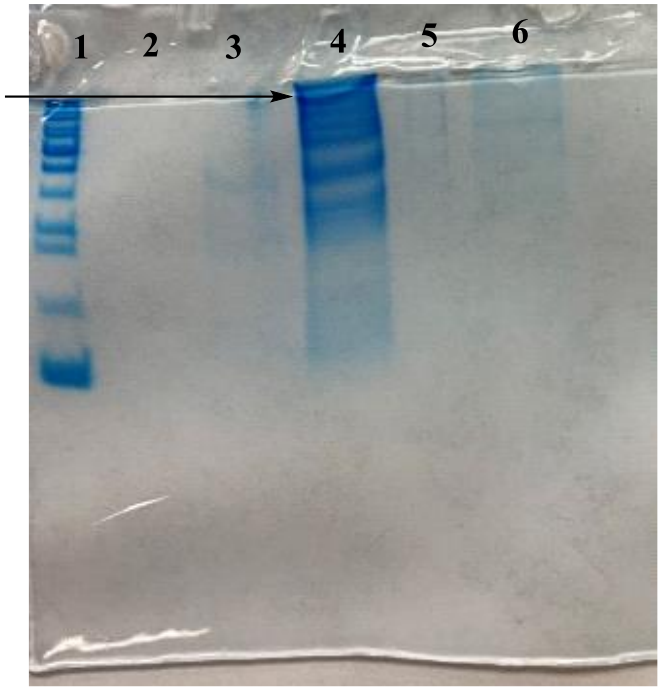


Figure 23: *Enterobacter* β-galactosidase purification by immobilized metal-ion affinity chromatography (IMAC). Lane 1: Protein molecular weight marker; lane 3: flow through; lane 4:

fraction 1; lane 6: fraction 3; lane 7: fraction 4; lane 8: fraction 8. The arrow points to lane 4 indicating the band for *Enterobacter* β-galactosidase (120 kDa).

3.15. Spectrophotometric assay of dialyzed sample of Enterobacter β-galactosidase

The pellet obtained from 75% ammonium sulfate saturation was resuspended in 50 mM NaPi buffer, pH 7 and dialyzed against the same buffer overnight at 4 °C. The catalytic activity of dialyzed sample was tested using the model chromogenic substrate *o*-NPGal. β-galactosidase should be able to cleave the glycosidic bond of the model substrate *o*-NPGal to form the yellow-colored *o*-NP which absorbs light at 405 nm **(Figure 24)**. **Figure 25** indicates a time trace of *o*-nitrophenolate anion formation. The increase of absorbance at 405 nm given by the slope of the curve confirms the activity of *Enterobacter* β-galactosidase.

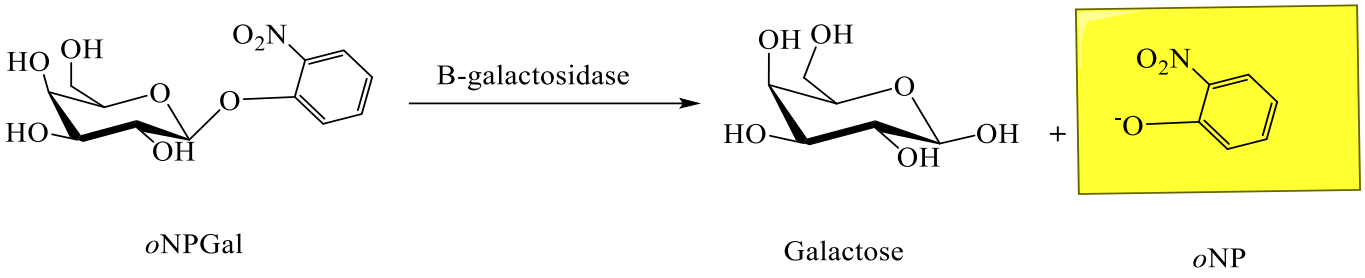


Figure 24: Enzymatic hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside to D-galactose and *o*-nitrophenolate.

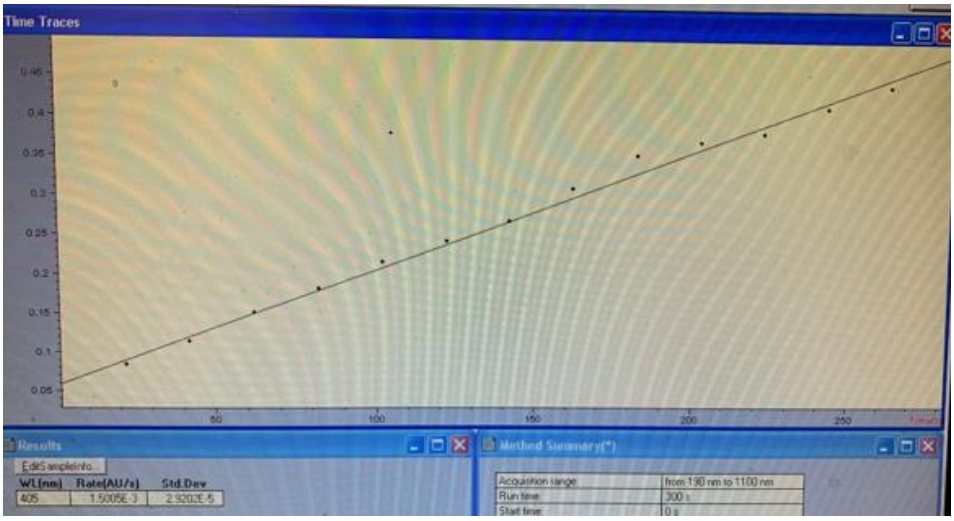


Figure 25: Spectrophotometric assay of *Enterobacter* β-galactosidase from dialyzed sample.

3.16. Transglycosylation and hydrolysis activity using free Enterobacter β-galactosidase

Since *Enterobacter* β-galactosidase demonstrated hydrolytic activity toward model substrate *o*-NPGal, its transglycosylation and hydrolysis activities were tested with lactose as a substrate. This was done by incubating 400 mM lactose in Tris buffer, pH 8 with a dialyzed sample of *Enterobacter* β-galactosidase at 50 ºC for 2 hours. To monitor the rate of product formation, aliquots were taken at various timed intervals up to 2 hours and analyzed using TLC. **Figure 26** shows a TLC plate demonstrating the transglycosylation and hydrolysis activities of *Enterobacter* β-galactosidase. The intensity of transglycosylation products was found to increase with time; however, the hydrolysis rate was the same throughout the incubation period.

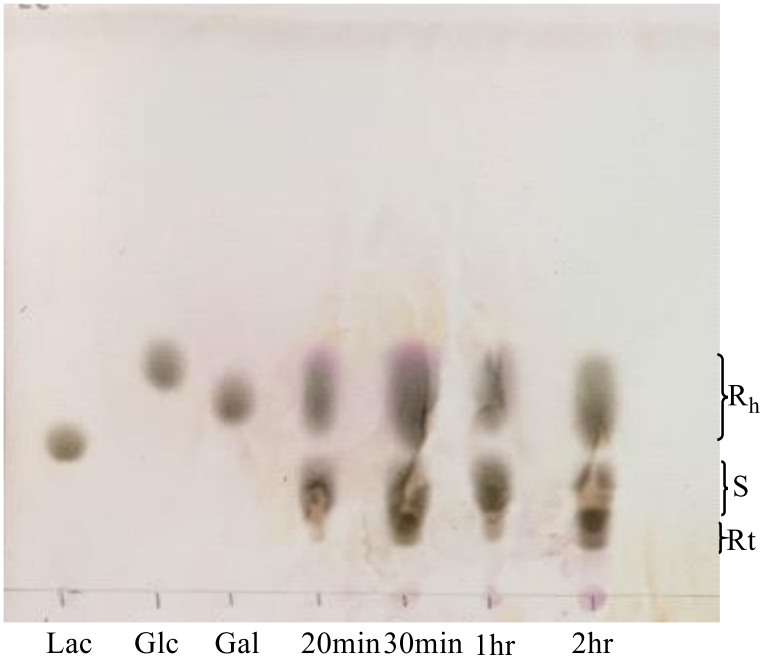


Figure 26: TLC analysis for products formed from 400 mM lactose using free *Enterobacter β*-galactosidase. S- substrate, Glc - glucose, Gal – galactose, Lac – lactose; R_h - hydrolysis products; and R_t - transglycosylation products**.**

CHAPTER 4: CONCLUSION

The goal of this study was to improve the transglycosylation reaction by minimizing hydrolysis in oligosaccharide synthesis. A comparative study of transglycosylation and hydrolysis activities of lactase, β-galactosidase from *Aspergillus oryzae,* and *Enterobacter* β-galactosidase at different conditions was done. The results show that all three enzymes can be used for synthesis of oligosaccharides. Both free form and immobilized enzymes have transglycosylation activity. Immobilization was successfully done using a chitosan-silica support which was crosslinked with the bifunctional reagent glutaraldehyde. Lactase had a better immobilization capacity compared to β-galactosidase from *Aspergillus oryzae.* Transglycosylation using immobilized enzyme produced trisaccharides, tetrasaccharides and in some cases oligosaccharides with a higher degree of polymerization.

Transglycosylation yields were found to be dependent on pH and temperature. pH 8 and 50-60 °C were the most appropriate for synthesis of oligosaccharides via transglycosylation of lactose. High concentrations of lactose hydrolysis products from the synthesis of oligosaccharides led to inhibition of both transglycosylation and hydrolysis activities. Use of high sucrose concentrations in media proved that high substrate concentration is necessary for the generation of transglycosylation products. Also, the recommended incubation time for transglycosylation was less than two hours because longer reaction times did not result in higher oligosaccharide yields due to secondary hydrolysis.

Immobilized enzyme can be used in the synthesis of oligosaccharides not only in water media but also in media containing alcohols and phenols. The results also indicated that use of immobilized enzyme was advantageous in that the enzyme can be reused multiple times with retention of the catalytic activity.

Enterobacter β-galactosidase is a suitable target for production of oligosaccharides by transglycosylation. *Enterobacter* β-galactosidase was successfully overexpressed in *E. coli* cells. The protein fraction that contained β-galactosidase showed high catalytic activity for the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside and had the ability to catalyze the transglycosylation reaction using lactose. This project has proved the potential of glycoside hydrolase enzymes to act as transglycosylation enzymes for oligosaccharide production under mild reaction conditions thus avoiding tedious protection/deprotection chemistry.

Future work

Future work for this study will include analysis of transglycosylation products by nuclear magnetic resonance (NMR), mass spectrum (MS) and development of chromatography methods which will allow obtaining oligosaccharides in large quantity.

Also, a purification procedure for *Enterobacter* β-galactosidase should be developed. Once a purified enzyme is obtained, the enzymatic activity of *Enterobacter* β-galactosidase should be analyzed. The enzyme will then be immobilized using chitosan-silica beads and its immobilization efficiency determined by Bradford assay. After immobilization, the enzyme should be applied in oligosaccharide synthesis.

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