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The feasibility of using Bifidobacteria bifidum (ATCC 700541) for the production of prebiotic oligosaccharides

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THE FEASIBILITY OF USING BIFIDOBACTERIA BIFIDUM (ATCC 700541) FOR THE PRODUCTION OF PREBIOTIC OLIGOSACCHARIDES

BY

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ABSTRACT

Galactooligosaccharides (GOS) are prebiotics that are being used to influence intestinal microbiota towards health promoting, beneficial bacteria like bifidobacteria. Natural sources of GOS are not found in suitable levels to achieve the desired health effects so economically viable commercial production methods are important. Enzymatic conversion of lactose to GOS through transgalactosylation is a preferred method since the starting material is inexpensive and readily available. Enzymes used in batch production are purified from fungal or bacterial sources or remain in whole cells cultured for GOS production. Purified or unbound enzymes are single use whereas enzymes retained within whole cells can potentially be used for multiple cycles. In the present study *Bifidobacteria bifidum* ATCC 700541 is investigated as an organism for GOS production. The strain was cultured using a previously successful medium for growing bifidobacteria and the β –galactosidase activity was measured and compared to levels in purified enzymes from *A. niger* and *E. coli.* We found that when compared to purified enzyme activity levels the costs of a single batch reaction using whole cells is the same as using purified enzymes. When the possibility for reusing the cells for multiple batches of GOS production is considered whole cells by far are a more economically feasible option for commercial production.

ACKNOWLEDGMENTS

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I wish to say thank you to my family for their unwavering support through this entire adventure. I thank especially my parents who support my dreams with fervent prayer and love no matter where they might lead me.

I thank God for granting me the ability to be able to complete this work and for the endurance to persist even when things become difficult. I praise Him for everything I have been given and thank Him for every opportunity from which I have been blessed to learn.

Lastly but not least, I would like to thank my loving, patient, understanding, supporting and faithful husband Marty Kofsky. Without his trust, patience, and love, I would not be as capable of dealing with what life brings our way.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

The human gastrointestinal tract is a dynamic array of diverse micro-organisms consisting of both potentially harmful and potentially beneficial bacterial species. A major function of the colonic microbiota is to act as scavengers, harvesting the energy stored in dietary residue that passes through the stomach and small intestine undigested. Members of the genus *Bifidobacterium*, genus *Lactobacilli* and other beneficial bacteria provide health benefits to their host by outcompeting potentially harmful microbiota cohabitating the colon. Bifidobacteria are the most abundant beneficial bacteria. Bifidobacteria utilize indigestible sugar molecules called oligosaccharides for growth and also ferment these sugars into vitamins and other nutrients which provide health benefits to the host. Recent research uses diet to influence microbial populations of the gut towards the beneficial microbiota, thereby increasing host health. Figure 1.1 is an overview of the major species found in the gut, their relative numbers, and potential health effects.

One way to influence gut microbiota is through the use of probiotics and prebiotics. Probiotics are live organisms added to foods, such as yogurt products like Activia©, and after they are ingested they remain in the gut and provide health benefit to the consumer. Prebiotics, on the other hand, are nutritional elements which pass the stomach undigested thus allowing the indigenous bacteria of the gut to utilize them for growth and the host receives the benefits. Oligosaccharides have been identified as prebiotics for the purpose of increasing microbial numbers of such beneficial bacteria. Oligosaccharides, such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and inulin are naturally occurring in foods such as bananas, tomatoes, soybeans, garlic, onions, and asparagus and also in human and bovine milk. Human milk, in particular, is rich with GOS and comparison of nursing infants to infants fed formula indicates GOS is essential to increasing the abundance of indigenous *Bifidobacteria* in the large intestine increasing the health benefit to the host while minimizing potential side effects. Natural sources of GOS are insufficient or inconvenient to extract for use as health enhancing additives. A way of producing synthetic GOS is necessary to meet demands and needs.

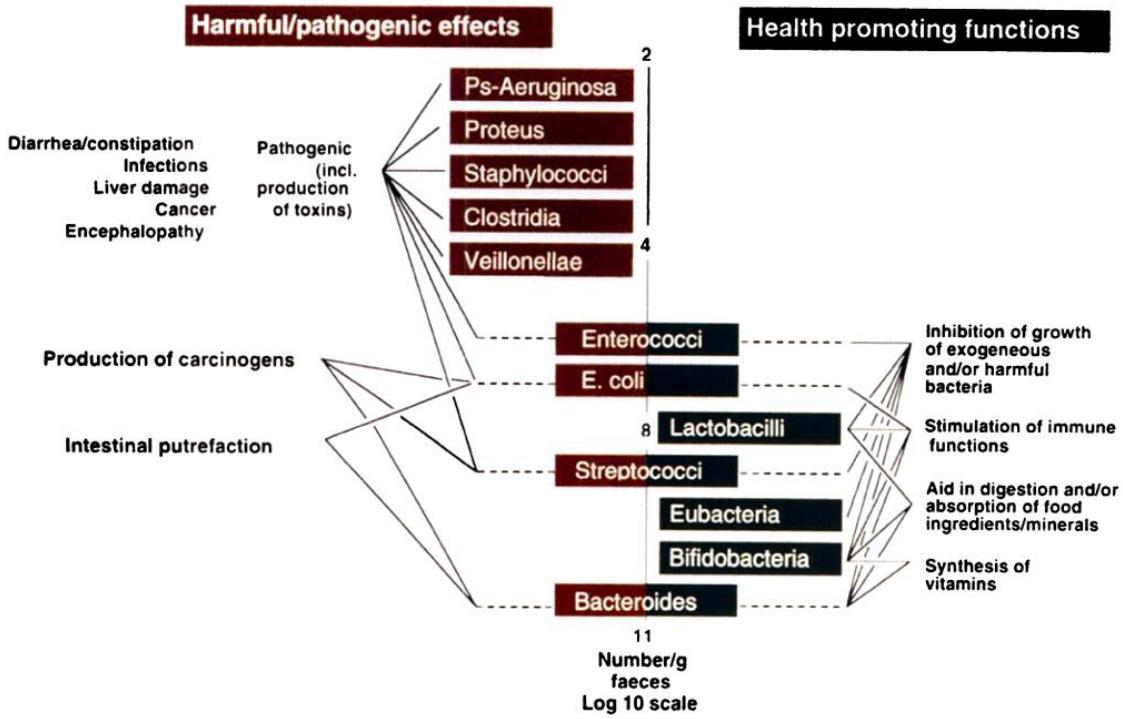


Figure 1.1 Generalized Scheme and Health Effects of Predominant Human Fecal Bacteria (Isolauri et al. 2004).

Current commercial production of synthetic GOS involves using a β-galactosidase enzyme to convert a high concentration lactose solution to a heterogeneous solution comprised of varying length GOS, galactose, and glucose. The feed stream for the conversion is usually lactose whey concentrate, a by-product considered waste of the dairy industry. Figure 1.2 illustrates an overview of a cheese making process and whey production. β-galactosidase enzymes used for production of GOS are typically isolated from yeasts, fungi, and other bacteria such as *E. coli*. Most species of *Bifidobacteria* produce β-galactosidase as well as α-galactosidase.

The main linkage structure present in GOS converted from lactose is β-gal$_n$-(1-4)-β-glc although other β-glycosidic linkages have been found including β-gal$_n$-(l-6)-β-gal, β-gal$_n$-(1-2)-β-glc, β-gal$_n$-(1-3)-β-glc, β-gal$_n$-(1-4)-β-glc, β-gal$_n$-(1-6)-β-glc, β-gal$_n$-(1-2)-β-gal and β-gal$_n$-(1-3)-β-gal. GOS not derived from lactose also include α-glycosidic

linkages such as α -gal $(1\text{-}6)_2$- α -glc and α -gal $(1\text{-}6)_2$- α –gal. While there is no direct evidence that α-linked or β-linked sugars provide different benefits it is believed that both linkage types within the GOS molecule provide health benefit to the host without the harmless, but unwanted, side effects when they are utilized by the gut bacteria.

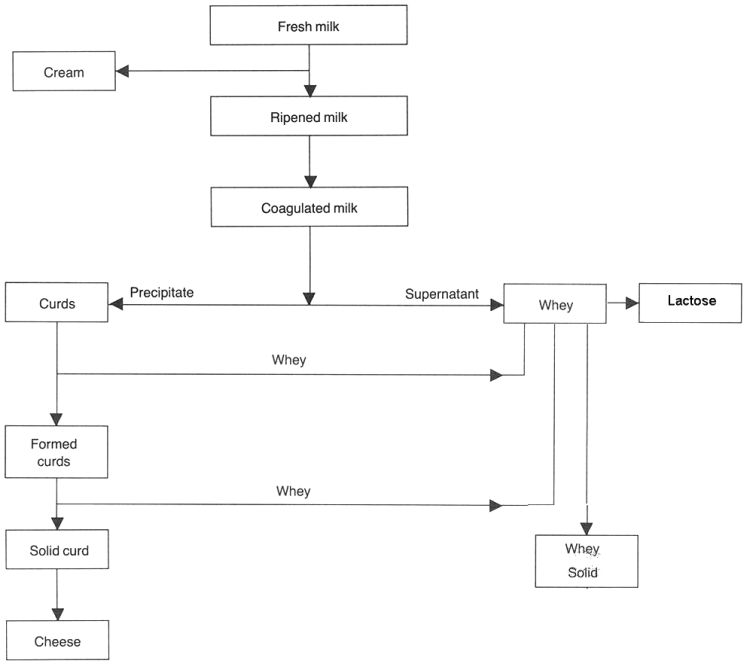


Figure 1.2 Overview Schematic of Cheese Processing with End Products as Cheese, Whey Solids and Lactose.

The percent conversion of lactose to GOS depends on time, temperature, initial lactose concentration and, as recent work has shown, enzyme source. Since reaction time is a key factor for maximum conversion of lactose to GOS, the use of extracted enzymes requires enzyme inactivation, usually by temperature denaturation, to end the reaction at the optimized time. Denaturation renders the enzyme unusable so fresh enzyme must be used for each GOS conversion batch. In order to create a more economical GOS

production process, promising work has been done using cells of a novel strain of *Bifidobacteria bifidum.* Since the enzymes are contained within the cell structure, using cells allows for physical removal of the enzyme source from the reaction mixture without deactivating the enzyme and allowing recycle of the enzymes for multiple conversions.

In order for the use of whole cells to be economical a strain should be grown on an inexpensive media using a readily available sugar as its carbohydrate source. The culture must also reach a high cell density with desirable enzyme kinetics. *Bifidobacteria* do not easily grow on a purely synthetic media. Much effort has been put towards determining what growth factors exist and how they can be incorporated into a synthetic media. So far, research has identified that human milk, bovine milk casein fractions and yeast extract contain growth enhancers but the mechanism of enhancement is not known. The biggest obstacle to determining the specifics to growth enhancement is that the growth patterns of each *Bifidobacteria*, including different strains within the same species, have drastically different results when grown under the same media conditions.

The scope and focus of this work is to investigate *B. bifidum* ATCC 700541 as a potential organism for GOS production as an alternative to purified enzymes. The organism must actively grow on a convenient, simple media and also produce significant quantities of the β-galactosidase enzyme to be useful for the production process. The α-galactosidase activity of the cells will also be evaluated as it has been shown that α-linked GOS may provide additional benefit as a prebiotic agent. The kinetics of the investigated strain of *B. bifidum* are compared to the kinetics of purified β-galactosidase from *E. coli* (Sigma Aldrich, MFCD00130623) and purified α-galactosidase from *Aspergillus niger* (Deerland Enzymes). The economics of culturing bacteria are evaluated against the purchase of purified enzymes for use in GOS production in a batch reaction system.

2. BACKGROUND

2.1. PROBIOTICS AND PREBIOTICS

Live *Lactobacilli* and *Bifidobacteria* cultures ingested in pill form or added to food products with the aim of increasing the intestinal numbers of beneficial bacteria are known as probiotics. In 1989, probiotics were defined as live microbial feed supplements which beneficially affect the host by improving its intestinal microbial balance (Fuller 1989). In 2001, the World Health Organization further defined a probiotic as "a live micro-organism which when administered in adequate amounts confer a health benefit on the host" (www.who.int/entity/foodsafety Nov 2010; Leahy et al. 2005). Establishing change in the gut microbiota through probiotics requires passing the bacteria through the stomach to the intestines in a viable form. In order for the probiotic organism to become established in the intestinal tract, the cultures must remain viable during processing and storage, resist the acidic environment of the stomach fluids, compete with existing gut bacteria for nutrients and adhere to the intestinal wall (Fuller 1991; Isolauri et al. 2004). Probiotic treatment requires repeated ingestion for sustained benefit since often the organism washes out of the host when it is discontinued as part of the diet (Gibson and Roberfroid 1995). To overcome the obstacles presented by probiotic therapy, researchers began looking at alternatives for influencing gut microbiota towards beneficial bacteria.

The alternative to ingesting the desired organisms is to stimulate the indigenous populations. Research shows that *Bifidobacteria* and *Lactobacilli* are capable of utilizing food elements for growth that many gut bacteria cannot digest. A nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth or metabolic activity of a single or limited number of bacteria imposing a health benefit upon the host is the definition of a prebiotic (Gibson and Roberfroid 1995; Leahy et al. 2005). Based on this definition the current criteria for classifying a food component as a prebiotic are it must: a) be able to pass through the upper gut undigested b) provide selective fermentation by a single or limited number of beneficial bacteria in the colon c) provoke the colonic microbiota towards a healthier composition and d) generate effects that are beneficial to the host health (Fooks and Gibson 2002). Oligosaccharides are non-digestible carbohydrates that possess these characteristics.

In 1999 the value of functional food additives in the U.S. was estimated at $163 million and was expected to increase by 7% annually reaching $322 million by 2009 (Reisch 2001). With public awareness through major marketing campaigns of commercial products such as yogurts and other fermented dairy products the market has reached $16 billion and is expected to continue to increase at a rate of 12.6% a year the next 5 years reaching $32.6 billion (Markets and Markets, 2009). Oligosaccharides have emerged worldwide as the focus of a new health movement and in 2001 the U.S. Food and Drug Administration (FDA) approved Galacto-oligosaccharides (GOS) as a dietary ingredient (FDA, 2001). Oligosaccharides are obtained by extraction from natural sources or synthesized through glycosylation reactions, enzymatic hydrolysis of polysaccharides, or transgalactosylation reactions (e.g. GOS and FOS) (Espinosa-Martos and Ruperez 2006; Borman 2007; Hsu et al. 2007). GOS are proving to be promising food additives in that they are stable in high temperature acidic environments and can be added as sugar substitutes in a variety of foods including infant formulae, dairy products, sauces, soups, cereals, beverages, snack bars, ice cream, breads, and animal feeds (Macfarlane et al. 2008; Manning and Gibson 2004). Also, GOS has a convenient starting material in cheese whey. Whey contains lactose and is abundantly available as a byproduct of cheese making. After concentration, the lactose in the whey is converted to GOS through enzymatic transgalactosylation. Using whey to produce GOS adds value and eliminates a costly waste stream (Pruksasri 2007).

2.2. GALACTO-OLIGOSACCHARIDES (GOS)

Oligosaccharides are indigestible, multi-moiety carbohydrate molecules that pass undigested through the stomach and small intestines into the large intestines. GOS are oligosaccharides which contain two to five galactose molecules and a single glucose molecule linked through glycosidic bonds. GOS possess a molecular structure generalized as $(Galactose)_n$ – Glucose (Sako et al. 1999). GOS are found naturally in bananas, tomatoes, soybeans, garlic, onions, and asparagus and also human milk. The FDA has granted GOS the generally regarded as safe (GRAS) status allowing commercial uses for human consumption. GOS are used as additives to infant formula and as food sweeteners with a reported caloric value of 1.75 kcal/g compared to 4.2

kcal/g caloric value of sucrose (table sugar) (Macfarlane et al. 2008). The true value of GOS comes from the health benefits incurred by the host when they are consumed.

2.2.1. Health Benefits of GOS. Research indicates consuming 4-8 g/day of GOS can provide the desired health benefits. A gut with an already healthy microbial balance will likely see little or no benefit and can possibly have the ill effect of increasing discomfort, in particular gas and bloating (Manning and Gibson 2004). However for individuals who gain from GOS consumption the benefits experienced are directly related to the effect GOS has on the microbial composition in the intestine. Intestinal bacteria have nonspecific metabolic pathways and utilize an array of sugars as carbohydrate source for growth; however, amongst all the intestinal organisms *Bifidobacteria* grow at a faster rate on GOS and convert GOS to cell mass to a greater extent (Macfarlane et al. 2008; Rycroft et al. 2001). An increased presence of *Bifidobacteria* has an antagonistic effect on the pathogenic organisms in the gut by metabolic suppression and reduced toxin release (Macfarlane et al. 2008). Consuming GOS is a treatment for diarrhea related to food poisoning and traveler's sickness since it promotes the growth of indigenous healthy bacteria and in so doing makes it more difficult for pathogens to establish and survive. *Bifidobacteria* prevent colonization of new pathogens by using available nutrients keeping harmful bacteria from being able to establish a significant presence in the gut (Isolauri et al. 2004; Leahy et al. 2005).

Intestinal microbiota is established during the birth process. Studies on infants who are fed mother's milk vs. those fed infant formulae show that in those which are fed mother's milk *Bifidobacteria* dominate the bacterial numbers accounting for up to 95% of the bacterial population where those which are fed formula have bacterial profiles more similar to an adult (Macfarlane et al. 2008). Studies also show that the *Bifidobacteria* present in the breast fed infants play an important role in establishing the immune system, protects the infant from development of allergies, and prevents bouts of acute and chronic diarrhea. Infant formula is fortified with GOS in atempt to promote these same health effects in infants which are not breast-fed (Isolauri et al. 2004; Leahy et al. 2005).

In adults some benefits attributed to *Bifidobacteria* are stimulating immunological factors, lessening bloating caused by gas, improving digestion, aiding in absorption of essential nutrients, increasing calcium balance, producing vitamins and digestive

enzymes and releasing short chain fatty acids which are used for energy (Gibson and Roberfroid 1995; Leahy et al. 2005). *Bifidobacteria* inhibit growth of pathogenic bacteria by releasing acidic metabolites (e.g. lactate and acetate) that lower the pH of the gut making a harsher environment for survival. Some metabolites act as antimicrobial agents directly. Amines and ammonia released when proteins are digested are protonated by the acidic metabolites causing blood levels of toxic ammonia to decrease (Gibson and Roberfroid 1995). Consuming GOS helps promote *Bifidobacteria* growth restoring the intestinal microbial balance after the administration of antibiotics which, in elderly patients especially, can wipe out the indigenous *Bifidobacteria* and *Lactobacilli* in addition to the bacteria actually causing the infection. GOS are also used to correct bowel inconsistencies (Gibson and Roberfroid 1995; Fooks and Gibson 2002). Research is being conducted to define the role that *Bifidobacteria* play in the prevention of colon cancer, irritable bowel syndrome and other colon related diseases. The extent to which GOS consumption can benefit the consumer is an area of research that is receiving a great amount of attention and it will be likely many years before it is fully understood.

2.2.2. Methods of GOS Production. In an adult diet or for infants not feeding on mother's milk it is difficult to attain through natural food sources the recommended 4-8 grams/day of GOS needed for the desired health benefits. Commercially produced GOS are added as low calorie sweeteners to consumer goods such as infant formulas, dairy products, sauces, soups, cereals, beverages, snack bars, ice cream, and breads in order to supplement natural sources to reach the recommended daily dose (Macfarlane et al. 2008; Manning and Gibson 2004). Methods of production include synthesis reactions (Borman 2007), extraction from natural sources such as soybeans (Espinosa-Martos and Ruperez 2006) and enzymatic transgalactosylation reactions to convert the high concentration lactose solution into a mixture of GOS and other sugars. Enzymatic conversion reactions are carried out in numerous ways and are the most utilized method for GOS production (Sako et al. 1999). Lactose from cheese processing is the preferred and most convenient starting material. Figure 2.1 shows the overall production scheme using enzyme reactions for conversion of lactose to GOS.

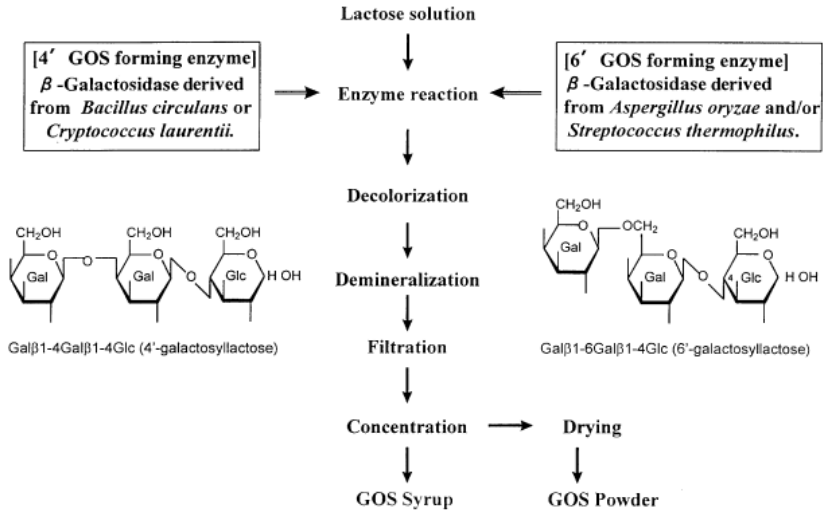


Figure 2.1 Industrial Production Process of GOS (Sako et al. 1999).

Enzymatic conversion can be applied as any of the following: a) Batch production, illustrated in Figure 2.2, in which enzymes purified from either a microbial or fungal source are added to a concentrated lactose solution, incubated, and then heated to terminate the conversion by denaturing the enzymes (Onishi and Tanaka 1995; Splechtna et al. 2006; Hsu et al. 2007) b) Biotransformation production, also illustrated in Figure 2.2, using whole cells containing the useful enzymes in a batch reactor. As with purified enzymes the whole cells are added to the lactose solution and incubated. After incubation the solution is not heated to denature the enzymes. Instead, the cells are removed from the solution by either filtration or centrifugation so they can be reused (Onishi et al. 1995; Goulas et al. 2005).

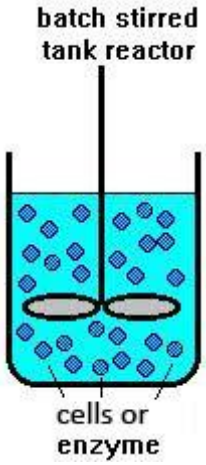


Figure 2.2 GOS Production Using a Batch Reaction with Whole Organisms or Purified Enzymes to Convert Lactose.

c) Continuous fed-batch reaction is shown in Figure 2.3. Enzymes or whole cells are added to a high concentration lactose solution and after incubation the mixture is circulated through a tangential flow membrane. The enzymes or cells are retained on the retentate side of the membrane and the sugar solution is removed as permeate. To drive the reaction towards continuous GOS production, a high concentration lactose solution is fed to the reactor at the same rate that permeate is leaving the system keeping the volume in the reactor constant (Chockchaisawasdee 2005) and d) Immobilization where enzymes or cells are entrapped in porous beads and packed in a column as shown in Figure 2.4. Concentrated lactose solution is passed over the column where the conversion reactions take place and the outlet stream is collected for further processing (Dey-Chyi et al. 1998; Yang and Bednarcik 2001).

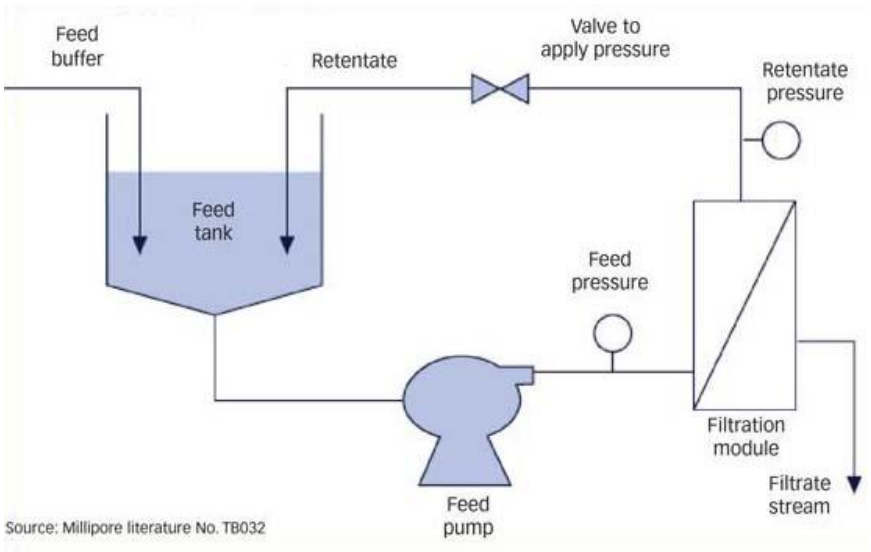


Figure 2.3 Continuous GOS Processing Using a Tangential Flow Filtration System.

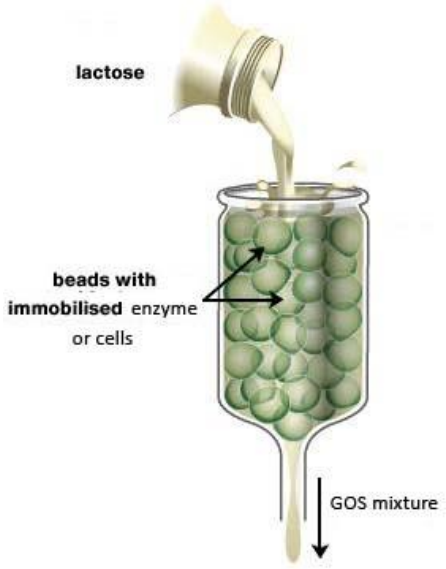


Figure 2.4 Enzymatic Conversion of Lactose to GOS Using Immobilized Enzymes or Cells.

In order to have an efficient process it is important to be able to convert the most amount of lactose at the least amount of cost. If whole cells can be grown so they contain a sufficient amount of enzyme activity then they provide opportunity for cost effectiveness based on potential for reuse. Slow diffusion kinetics and problems with fouling, plugging and pressure drop limit the potential for immobilization to be cost effective. Using pure enzyme is effective for constant dosing for conversion but is costly due to single use.

2.2.3. GOS Reaction Kinetics Using Lactose. GOS synthesis from lactose is characterized by an initially rapid decrease in lactose concentration and is accompanied by the formation of glucose and galactose. The final reaction product is a mixture of monosacharides (glucose and galactose), disaccharides (lactose and allolactose), trisaccharides and tetrasaccharides (GOS). Figure 2.5 is a chromatographic representation of the enzyme conversion products (Splechtna et al. 2006).

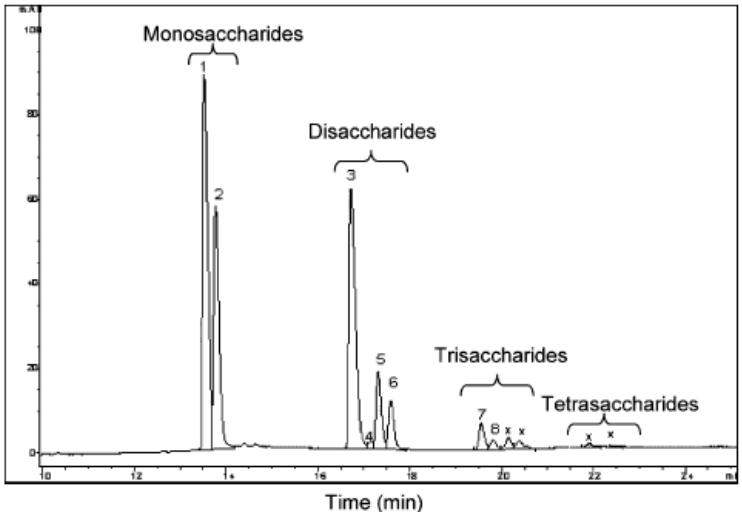


Figure 2.5 Separation by Capillary Electrophoresis of Individual GOS Products During Lactose Conversion Catalyzed by β-Galactosidase. (1) glucose (2) galactose (3) lactose (5) allolactose (6) Gal-Gal (7-8) Gal-Lac (x) products not identified (Splechtna et al. 2006).

As the conversion of lactose progresses towards 100% conversion the concentrations of glucose and galactose continue to increase and the concentration of GOS decreases. This reaction scheme suggests that in transgalactosylation GOS are formed as intermediates and are subject to hydrolysis thus serving as substrates as lactose becomes depleted. Figure 2.6 shows the reaction kinetics of lactose conversion by β-galactosidase from *Bifidobacterial longum* BCRC 15708 carried out at 45 °C and pH 6.8 with an initial lactose concentration of 40%. GOS concentrations increase steadily until lactose conversion reaches about 50% then there is a sharp drop in GOS concentration and if allowed to continue, the GOS would completely hydrolyze into galactose and glucose (Hsu et al. 2007).

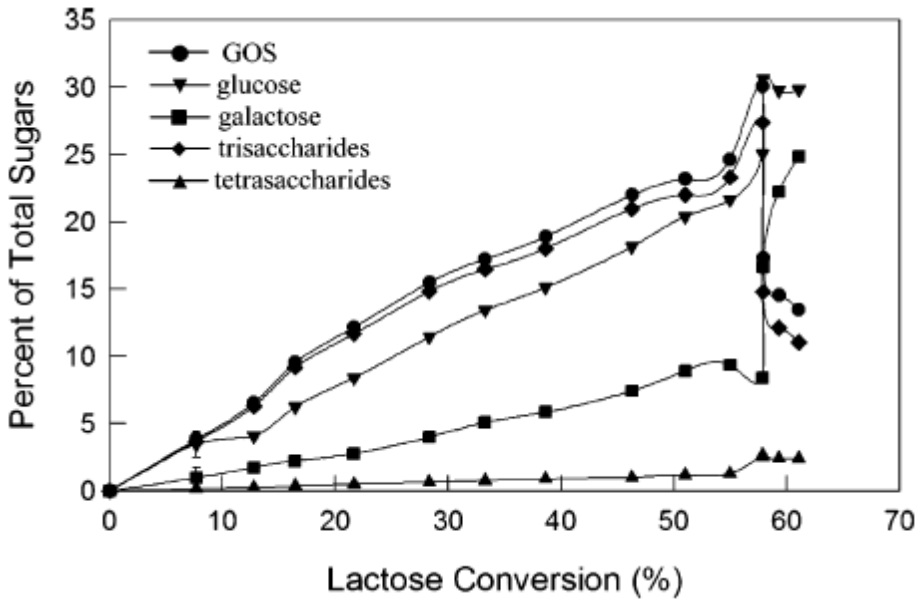


Figure 2.6 Lactose Conversion by β-Galactosidase from *Bifidobacterial longum* BCRC 15708. Carried Out at 45 °C and pH 6.8 with an Initial Lactose Concentration of 40% (Hsu et al. 2007).

Much work has been done to find conditions which maximize GOS production. Research has shown that enzyme source, temperature, pH, hydrolysis product concentrations and most importantly initial lactose concentration dictate GOS concentrations during processing. Enzyme source will determine what pH and temperature are optimal for conversion. For example, in a study using β-galactosidase from *E. coli*, the optimal pH was between 7 and 7.5 (Huber et al. 1976) whereas in a study using β-galactosidase from *Bifidobacterium longum* the optimal pH was 6.8 (Hsu et al. 2007). Most conversion reactions are run at 40 - 45 °C but some enzymes purified from thermophilic organisms are capable of operating at higher temperatures. In general both glucose and galactose have an inhibitory effect and decrease total GOS formation (Hsu et al. 2007; Chockchaisawasdee et al. 2005; Onishi et al. 1995). In a study using β-galactosidase from *Kluyveromyces lactis* it was demonstrated that the conversion of lactose to GOS is independent of enzyme concentration. The results of this study, as presented in Figure 2.7 and Table 2.1, show that increasing enzyme concentration causes the reaction to progress to the end point faster but did not impact the overall amount of GOS present in the mixture.

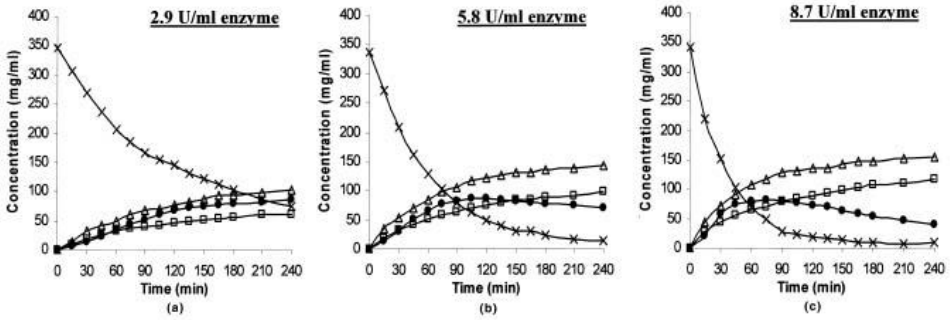


Figure 2.7 Time Course of GOS Synthesis From 340 mg/mL Lactose Using Differing Concentrations of Enzyme ((a), 2.9 U/mL, (b), 5.8 U/mL, (c), 8.7 U/mL) in a Batch Reactor at 40 °C, pH 7 (x, lactose; Δ, glucose; □, galactose; ●, GOS) (Chockchaisawasdee et al. 2005).

No matter the enzyme source it is evident that at lower lactose concentrations the hydrolysis reaction is the dominant reaction. Initial lactose determinations made using *B. longum*, *K. lactis*, and *B. bifidum* all demonstrate that initial concentrations of lactose between 40% and 50% are necessary to engage the transgalactosylation reaction of the enzymes in order to produce GOS. The dramatic effect that changes in initial lactose concentration has on GOS production in contrast to fluctuations in pH or temperature are shown in Figure 2.8 (Hsu et al. 2007; Chockchaisawasdee et al. 2005; Tzortis et al. 2005).

Table 2.1 Observed GOS Concentration in a Batch Reactor Over 4 h at 40 °C at pH 7 (Chockchaisawasdee et al. 2005).

	GOS Concentrations (mg/mL)			
Enzyme Concentrations	*220 mg/mL lactose	*280 mg/mL lactose	*340 mg/mL lactose	*400 mg/mL lactose
2.9 U/mL	53	68	88	96
5.8 U/mL	49	64	85	99
8.7 U/mL	45	62	81	96

* Initial lactose concentrations

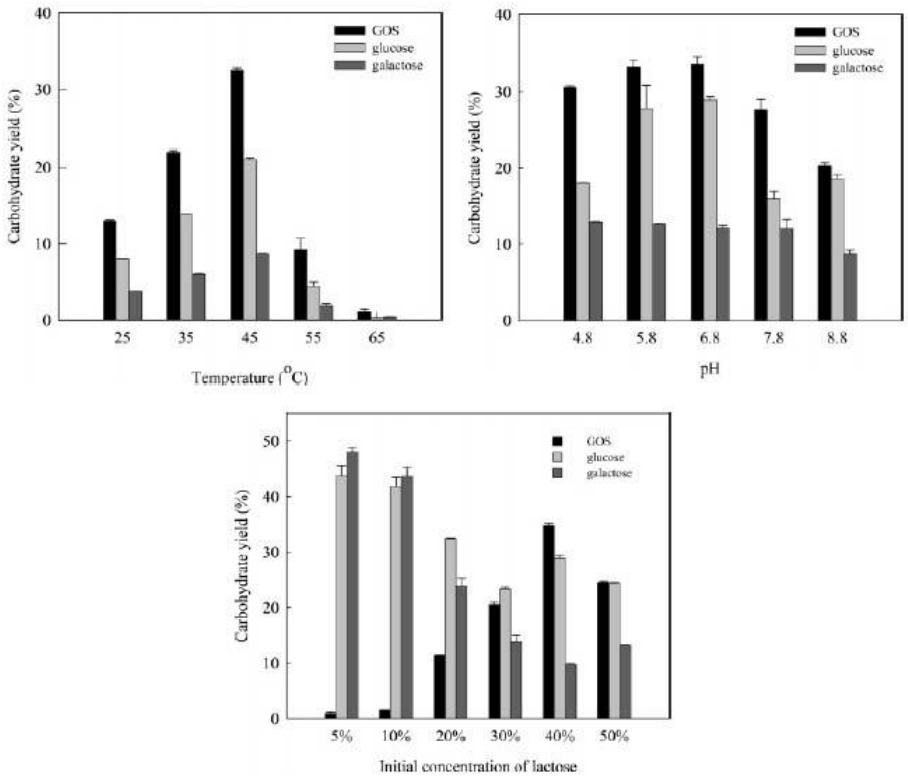


Figure 2.8 Effects of Temperature (pH 6.8, 40% lactose), pH (45 °C, 40% lactose) and Initial Lactose Concentration (pH 6.8, 45 °C) on GOS Production Catalyzed by β-Galactosidase from *B. longum*. (Hsu et al. 2007).

2.3. ENZYME ACTION

In the early 1950's oligosaccharides were discovered during lactose hydrolysis reaction studies using lactase. Figure 2.9 shows a lactose hydrolysis reaction by β-galactosidase where galactose and glucose are the hydrolysis products.

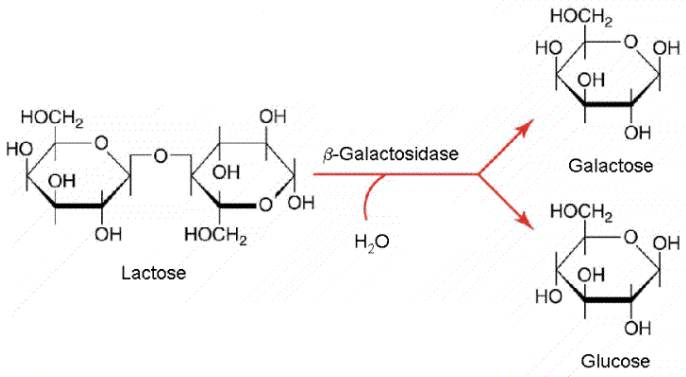


Figure 2.9 Lactose Hydrolysis Reaction by β-Galactosidase.

In commercial production, galactosidase enzymes are the functional enzymes in lactose conversion to GOS. In 1953 John Pazur proposed a two step process as the mechanism for the enzymatic synthesis of GOS by β-galactosidase transgalactosylation. The first step in Pazur's proposed two step process is the formation of a galactose-enzyme complex and the liberation of glucose. The second step in his process is the transfer of the galactose molecule from the galactose-enzyme complex to a cosubstrate in the system to form the GOS molecule. Figure 2.10 illustrates the mechanism proposed by Pazur. In his system, if the cosubstrate is glucose then complex I, allolactose, is formed. If the cosubstrate is galactose then complex II is formed. If the cosubstrate is lactose or allolactose then either complex III or IV, GOS, are formed. Complex III consists of glucose β(1-6) galactose β(1-4) galactose and complex IV consists of glucose β(1-4) galactose β(1-4) galactose (Pazur 1954). This mechanism can be extended to continue building GOS consisting of more than three moieties.

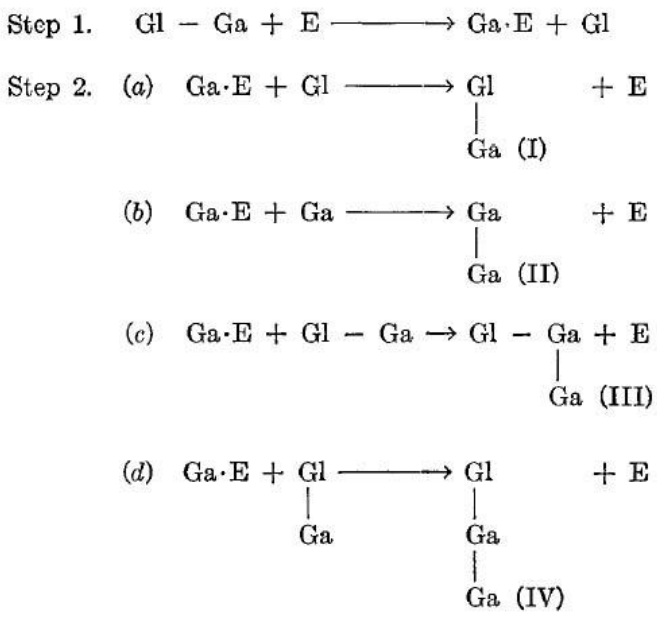


Figure 2.10 GOS Formation Mechanism as Proposed by Pazur (Pazur 1954).

In 1976 R.E. Huber et al. further characterized the reaction mechanisms involved in GOS synthesis. They showed that β-galactosidase has two transgalactosylase functions and a hydrolysis function. The transgalactosylase functions are described as "indirect" transgalactosylase activity and "direct" transgalactosylase activity. Indirect transgalactosylase activity happens as Pazur describes where lactose is bound by the enzyme, glucose is released, and the galactose releases to an acceptor molecule. Direct transgalactosylase activity involves the binding of the lactose by the enzyme, the cleavage of the β-(1-6) bond and the formation of a β-(1-4) bond with the same glucose molecule forming allolactose (Huber et al. 1976). Figure 2.11 shows the linkage structures of lactose and allolactose.

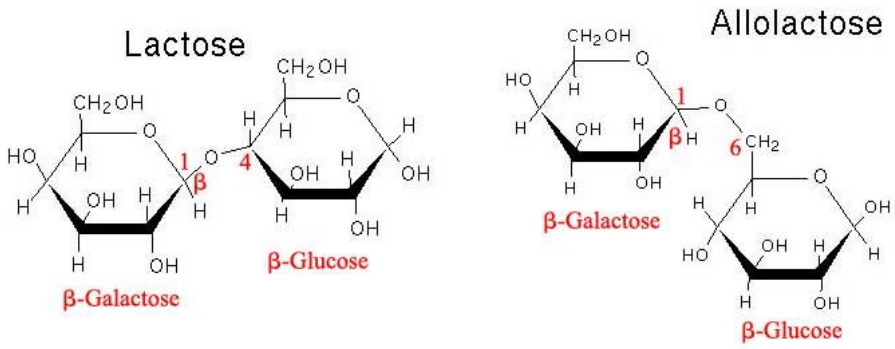


Figure 2.11 Lactose and Allolactose Molecules.

Juers et al. further explains the action of β-galactosidase. They suggest that β-galactosidase has two binding positions, shallow and deep binding. β-galactosidase hydrolyzes the substrate in a double displacement reaction with protonation of the leaving group (glucose) being a rate limiting step. During the formation of the galactose-enzyme intermediate complex the substrate first binds in the shallow binding position then moves into the deep binding position of the active site and the glycosidic bond is cleaved. The second step of the reaction is an acceptor molecule performs a nucleophilic attack on the bound galactose forming the reaction products. In hydrolysis the acceptor molecule is water. In transgalactosylation the acceptor molecule is either glucose, forming allolactose, or a di- or tri- saccharide forming GOS. Both free glucose and galactose effect the GOS formation reaction by binding the enzyme and competitively inhibiting the initial hydrolysis reaction (Juers et al. 2001). The extent to which the reaction is inhibited depends on the enzyme source and other reaction conditions (Hsu et al. 2007; Tzortis et al. 2005).

The thermodynamics of the catalysis favor hydrolysis; however, the available alcohol groups on the carbohydrates in solution, which allow them to act as acceptor molecules, cause GOS yield and compositions to change dramatically with reaction time. Figure 2.12 graphs the formation and degradation of individual GOS products over time from a lactose conversion reaction using β-galactosidase derived from *Lactobacillus reuteri*. In addition to time, the composition of GOS is also highly dependent on the source of β-galactosidase. Comparing GOS products using β-galactosidase from

Lactobacillus reuteri, *Pyrococcus* and *Sofolobus solfataricus* shows the composition variation between the enzyme sources and also the variation of linkages within the GOS molecules. Typically the linkages between the monomers are β-(1-4) glycosidic bonds; however, Table 2.2 compares the variety of glycosidic linkages in GOS in research results and in a commercially available product.

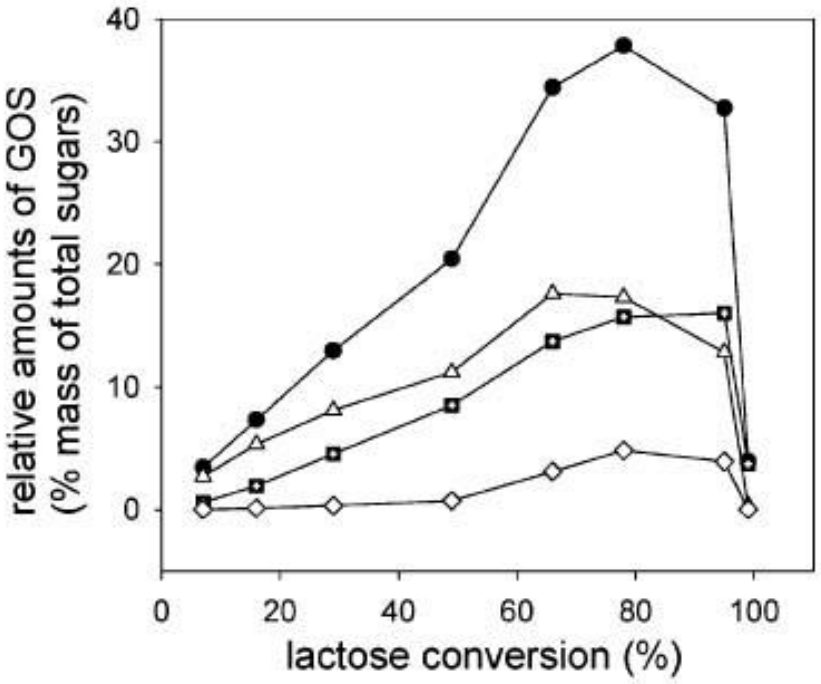


Figure 2.12 Formation and Degradation of Individual GOS During Lactose Conversion by β-galactosidase from *L. reuteri* (Initial Lactose Concentration , 205g/L). (●) total GOS, (◘) disaccharides, (Δ) trisaccharides, and (◊) tetrasaccharides. (Splechtna et al. 2006).

Table 2.2 Observed Glycosidic Linkages and GOS Compositions (Splechtna et al. 2006; Petzelbauer 2001; USFDA 2001).

	GOS Source and Structure*			
Carbohydrate	***Pyrococcus furiosus***	***Sulfolobus solfataricus***	***Lactobacillus reuteri***	Commercial GOS
Disaccharides	gal (1-3) glc gal (1-6) glc	gal (1-3) glc gal (1-6) glc	gal (1-6) glc gal (1-6) gal gal (1-3) glc gal (1-3) gal	gal (1-3) glc gal (1-6) glc gal (1-4) gal
Trisaccharides	gal (1-3) gal (1-4) glc gal (1-6) gal (1-4) glc	gal (1-3) gal (1-4) glc gal (1-6) gal (1-4) glc	gal (1-3) gal (1-4) glc gal (1-6) gal (1-4) glc	gal (1-4) gal (1-4) glc gal (1-6) gal (1-4) glc gal (1-3) gal (1-4) glc
Tetrasaccharides and higher oligomers	gal (1-3) gal (1-3) gal (1-4) glc	NA	NA	gal [(1-6) gal (1-4)]$_n$ gal (1-4) glc

*All linkages are β-glycosidic bonds. Gal (1-6) Glc is allolactose.

While the health benefits directly related to GOS with α-galactosidic bonds have not been fully investigated, an organism with α-galactosidase activity is able to utilize α-linked sugars such as raffinose and stachyose which are naturally occurring in soybeans. The known mechanism of α-galactosidase is primarily in the hydrolysis of α-linked oligosaccharides. However, in their investigation of α-galactosidase hydrolysis kinetics, Konstantin et al. observed transgalactosylation activity using melibiose, an α-linked disaccharide of glucose and galactose, resulting in the formation of GOS (Shabalin et al. 2002). Research efforts are being directed towards finding α-galactosidases capable of synthesizing GOS for the prebiotic production. Studies using *Bifidobacteria adolescentis*, *Bifidobacteria breve*, and *Bifidobacteria bifidum* are yielding promising results (Goulas et al. 2009a).

The kinetic properties of both enzymes are dependent upon pH and temperature. As described earlier, depending on the source of the enzyme the optimum conditions and final product structure and composition vary. Huber et al. confirm the optimal pH for β-galactosidase to be between 6.5 and 7.0 (Huber et al. 1976). Jurado et al. agree and show ideal temperatures range from 40°C to 50°C. They also establish a relationship between the activity of β-galactosidase and the ionic concentration of the solution (Jurado et al. 2004).

2.4. BIFIDOBACTERIA

GOS and other prebiotics modify intestinal health by influencing beneficial, health promoting bacteria. The primary targets of prebiotics are members of the *Bifidobacterium* genus and *Lactobacillus* genus (Macfarlane et al. 2008). Studies have shown bifidobacteria to be the dominant players in the health and development of the lower digestive tract microbiota in infants (Niittynen et al. 2007). Bifidobacteria have also been shown to be key in the health of the elderly with particular attention paid to intestinal microbiota recovery after the administration of antibiotics (Hamilton-Miller 2004; Macfarlane et al. 2008). Since bifidobacteria play such an important role in the health of the human gastrointestinal tract and have other health related benefits much effort is being made to characterize and understand what influences their growth.

Until recently, *Bifidobacterium* were known as *Lactobacillus bifidus* since they demonstrated many of the same properties as members of the *Lactobacillus* genus. However, bifidobacteria were found to involve the use of the enzyme fructose-6-phosphate phosphoketolase in their carbohydrate metabolic pathway which is not used in other species of *Lactobacillus*. In addition closer study of cell morphology, DNA and nutritional requirements for growth revealed *Bifidobacterium* as its own genus. Members of *Bifidobacterium* genus are gram positive, anaerobic bacteria. *Bifiobacterium* are a diverse group of species which are not nutritionally homogeneous. The nutritional requirements and aerotolerance of each species are strain dependent. One commonality amongst species of *Bifidobacterium* is they are not readily cultured on a purely synthetic media but require a complex nutritional component, such as casein or yeast extract, for culturing (Poupard et al. 1973; Poch and Bezkorovainy 1988). One species of

bifidobacteria that has been used as a probiotic and has received significant attention for prebiotic targeting is *Bifidobacteria bifidum*. The interest in these strains is directly linked to their β-galactosidase and α-galactosidase activities and how they respond to GOS and other prebiotics *in vitro* (Kneifel, and Kulbe 2000).

2.4.1. Bifidobacteria Utilization of GOS. Bifidobacteria have been selected as targets for prebiotic gut manipulation due to the potential health benefits that come from having increased numbers in the gut. Researchers have spent time examining how different prebiotic carbohydrates affect *Bifidobacteria* in vitro in order to gain understanding of the behavior in the gut. In the digestive tract a majority of simple carbohydrates that promote *Bifidobacteria* enumeration are absorbed in the stomach and small intestines. Selecting GOS as a suitable prebiotic is the result of research showing that *Bifidobacteria* readily utilize GOS and other oligosaccharides whereas *Enterobacteria* and other harmful organisms do not. Hopkins et al. (1993,1985) and Minami et al. (1998), show evidence that *Streptococcus, Lactobacillus, and Escherichia* readily utilize reducing sugars (glucose, fructose, maltose and lactose) but poorly utilize non-reducing sugars (GOS, FOS, etc) and *Bifidobacteria* generally utilize mono to tetrasaccharides, particularly those containing galactose, without requiring an adaptation period. They also conclude that as a general rule sugars containing galactose, glucose and fructose are fully utilized by *Bifidobacteria* (Minami et al. 1983, 1985; Hopkins et al. 1998). Rabiu et al. provide evidence that species show preference for GOS synthesized using cells of the same species. Table 2.3 lists the growth rates of selected gut bacteria on synthesized GOS (Rabiu et al. 2001). Evidence of improved growth of *Bifidobacteria* species on GOS made using β-galactosidase from the same organism adds to the rationale for using whole-celled organisms of indigenous gut bacteria for GOS production.

Table 2.3 Growth Rates of Selected Gut Bacteria on GOS. 1) Control – Oligomate 2) *B. bifidum* BB-12 oligosacharide 3) *B. infantis* DSM-20088 oligosaccharide 4) *B. pseudolongum* DSM-20099 oligosaccharide 5) *B. adolescentis* ANB-7 oligosaccharide 6) *B. angulatum* oligosaccharide (Rabiu et al. 2001).

Microorganism	Mean growth rate (μ h^{-1}) ± SD on oligosaccharide prepn[a]:					
	1	2	3	4	5	6
L. acidophilus ANR-1	0.69 ± 0.03	1.16 ± 0.06	0.93 ± 0.10	0.57 ± 0.04	1.30 ± 0.03	0.72 ± 0.05
B. ovatus ANGNI[b]	0.50 ± 0.04	0.26 ± 0.04	0.25 ± 0.03	0.25 ± 0.03	0.35 ± 0.05	0.22 ± 0.02
B. pseudolongum DSM-20099	0.56 ± 0.02	0.69 ± 0.02	0.66 ± 0.01	**0.99 ± 0.05**	0.64 ± 0.02	0.69 ± 0.03
B. longum ANB-2	0.52 ± 0.05	0.58 ± 0.00	0.67 ± 0.03	0.64 ± 0.01	0.52 ± 0.01	0.84 ± 0.02
B. infantis DSM-20088	0.95 ± 0.01	0.98 ± 0.00	**1.20 ± 0.04**	0.73 ± 0.03	0.99 ± 0.05	0.95 ± 0.01
B. bifidum BB-12	0.95 ± 0.03	**1.05 ± 0.01**	0.76 ± 0.02	0.79 ± 0.03	0.73 ± 0.02	1.05 ± 0.01
B. angulatum	0.87 ± 0.05	0.89 ± 0.00	0.96 ± 0.02	0.71 ± 0.00	0.91 ± 0.04	**1.27 ± 0.09**
B. adolescentis ANB-7	1.02 ± 0.06	0.48 ± 0.02	0.48 ± 0.01	0.81 ± 0.05	0.83 ± 0.01	1.01 ± 0.03

2.4.2. Carbohydrate Source for Culturing and β-Galactosidase Production. In the gut Bifidobacteria have been shown to have a preference for oligosaccharides while other less beneficial bacteria cannot process these sugars. Since the hydrolysis from the enzymes in the Bifidobacteria is desired for GOS production it is convenient to culture these bacteria. A media that will support the growth of the Bifidobacteria and allow them to produce the desired enzyme activity level is necessary. *Bifidobacteria* can grow on a variety of carbohydrate substrates; preference for a particular substrate is strain dependent (Hopkins et al. 1998; Mlobeli 1998). Glucose lends itself as a simple carbohydrate that allows high cell yields for most strains of *Bifidobacteria* however glucose does not induce the expression of the α – galactosidase and β – galactosidase enzymes which are needed for GOS production or utilization in the gut. Studies showed that sucrose and fructose are carbohydrates that promote high cell yields but also do not initiate production of the desired enzymes. Galactose, lactose and arabinose each produced some measure of enzyme activity. For *Bifidobacterium bifidum*, culture media containing lactose yields the highest growth rate with the highest specific enzyme activity (Astapovich and Ryabaya 2006). Figure 2.13 is a graph of the β – galactosidase and cell growth of two strains of *B. bifidum* cultured on media containing various carbohydrates as the carbon source.

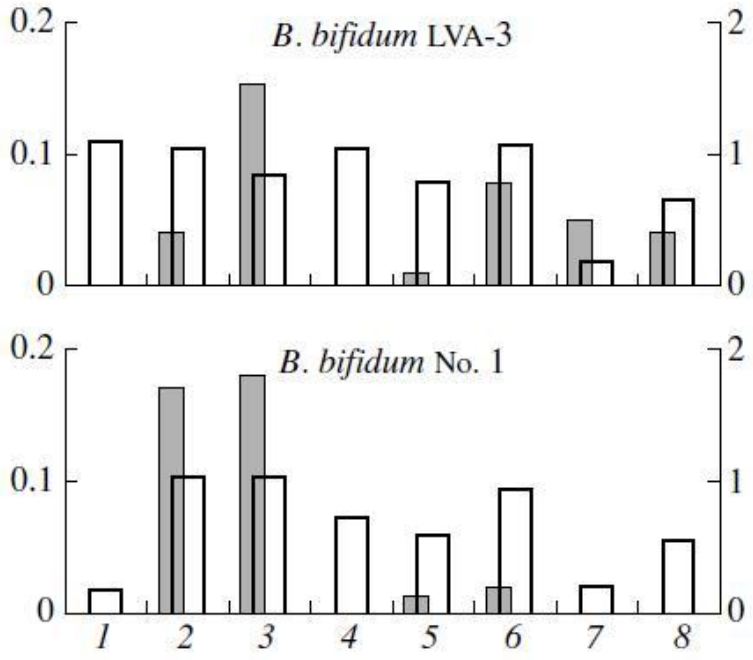


Figure 2.13 β–Galactosidase Activity (Dark Columns, U/ml) and Growth (Non Shaded Columns, mg/ml) of Bifidobacteria on Media with Various Carbon Sources. 1) Sucrose 2) Lactose 3) Galactose 4) Glucose 5) Fructose 6) Maltose 7) Arabinose, 8) Cellobiose (Astapovich and Ryabaya 2006).

Among the tested carbon sources that demonstrate production of β – galactosidase during cell culturing, lactose is the highest yielding sugar. Figure 2.14 is a graph demonstrating the differences between lactose, glucose and galactose as carbohydrate sources as measured by cell growth and enzyme activity. As can be seen in the graph the three sugars perform comparatively in terms of cell growth but lactose yields the highest enzyme activity (Hsu et al. 2005). Since *Bifidobacteria* can grow on a lactose substrate, whey provides a convenient feed stream for culturing species, such as *Bifidobacterium bifidum,* as a GOS producing organism.

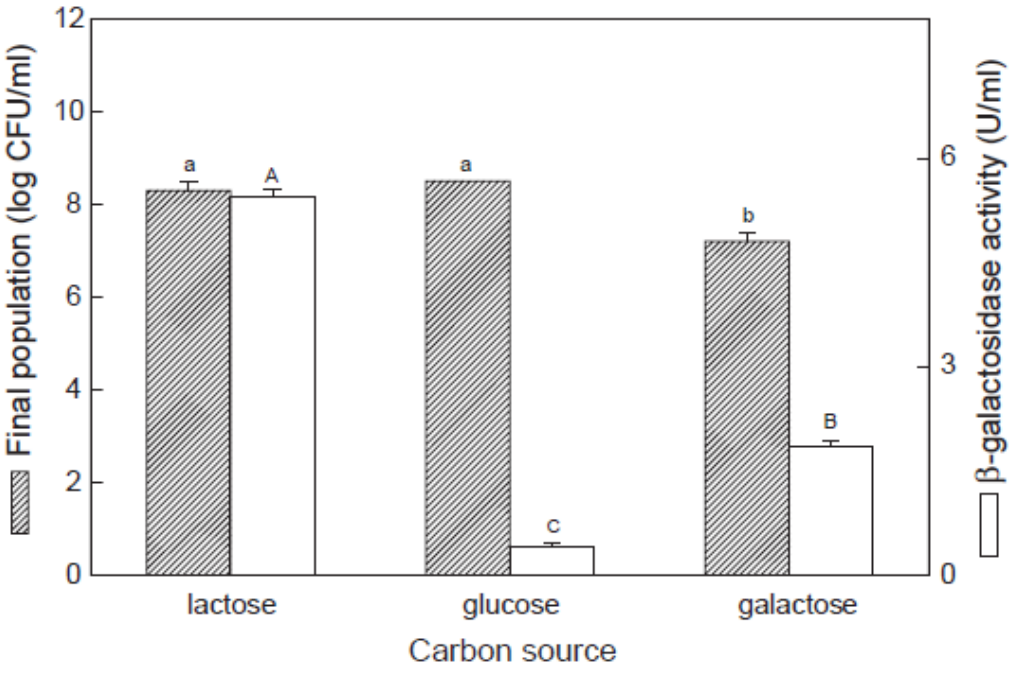


Figure 2.14 Effect of Carbon Source on Growth and β – Galactosidase Production (Hsu et al. 2005).

2.4.3. Nitrogen Source for Culturing and β-Galactosidase Production. With a vast variety of species and differences between strains even within the same species of *Bifidobacteria* specific growth requirements for each has not been thoroughly investigated; however, much work has been done to find media that supports growth for all types. Early investigations focused on developing a defined media capable of selectively culturing *Bifidobacteria* species. First attempts at developing a synthetic medium included a carbohydrate, usually glucose or lactose or both, an assortment of vitamins, salts, amino acids and a nitrogen source generally called "bifid factors". Based on the "bifid factor" chosen different levels of growth activity were observed. Initial media contained peptone which was later substituted by cow's milk and then later human milk (Bezkorovainy and Miller-Catchpole 1989). Modern media are comprised of combinations of tryptone, peptone, meat extract, yeast extract, ammonia salts, potassium salts, magnesium salts, calcium salts, cysteine and Tween 80®.

The observation of increased activity with the addition of cow and human milk to the culture media led to further characterization of the growth promoting factors in the milk as well as investigation into other growth promoting agents. Closer study of both types of milk revealed that there is a complex mix of protein and non-protein growth factors (Petschow and Talbott 1991). When separated into casein and whey there is a significantly greater amount of growth activity associated with casein fractions than whey fractions for both human and cow milk (Petschow and Talbott 1990). Other growth promoters were studied as well including yeast extract, gelatin, tryptone, peptone, hog gastric mucin, bovine serum albumin digest and beef extract (Poch and Bezkorovainy 1988, 1991, Hsu et al. 2005). Research indicates that for strains of *Bifidobacteria bifidum* employing yeast extract as the nitrogen growth-promoting source yields the greatest level of activity (Poch and Bezkorovainy 1988). When yeast extract is used in conjunction with casamino acids or ascorbic acid in a whey stream, culture cell mass is almost 100-fold greater than with yeast extract and whey alone and is comparable to MRS medium (Difco, Detroit MI, USA) which is one of a few complex prepared media used for culture maintenance (Corre et al. 1992).

While the nitrogen source has a significant impact on the growth kinetics of the organisms it also appears to impact the β – galactosidase synthesis by the cells. Figure 2.15 shows the effect of nitrogen source on growth and β – galactosidase activity using *B. longum*. The growth achieved by each of the nitrogen sources is comparable however yeast extract as the nitrogen source generates the greatest level of β – galactosidase activity. Table 2.4 shows the effect of yeast extract concentration on growth and β – galactosidase production (Hsu et al. 2005).

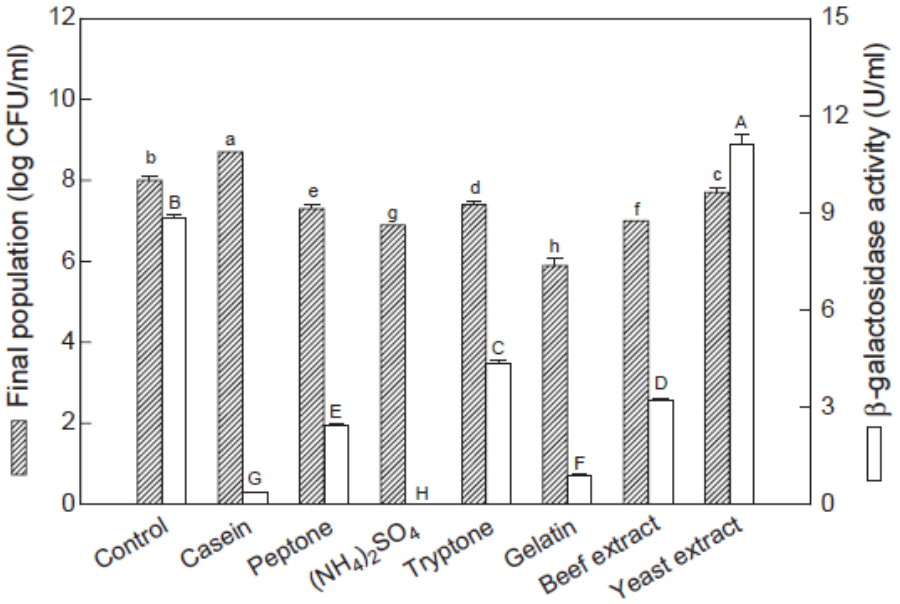


Figure 2.15 Effect of Nitrogen Source on the Growth and β–Galactosidase Production by *B. longum* CCRC 15708. Medium Contained 4% lactose, 0.3% K2HPO4, 0.1% KH2PO4, 0.05% MgSO4d 7H2O, 0.03% l-Cysteine and Various Nitrogen Sources. Determinations Were Made After a 12-h Cultivation at 37 °C (Hsu et al. 2005).

Table 2.4 Effect of Yeast Extract Concentration on the Growth and β – Galactosidase Production by *B. longum* CCRC 15708. Fermentation Was Conducted in a Medium Containing 4% Lactose, 0.3% K2HPO4, 0.1% KH2PO4, 0.05% MgSO4d 7H2O, 0.03% l-Cysteine and Different Concentrations of Yeast Extract at 37 °C for 12 h (Hsu et al. 2005).

Yeast extract (%)	Final pH	Final population (log CFU/ml)	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)
0	6.7 ± 0.0ab**	7.3 ± 0.4e	0.00 ± 0.00i	0.00 ± 0.00f	0.00 ± 0.00e
1.5	4.5 ± 0.3d	8.0 ± 0.3d	6.03 ± 0.61e	0.59 ± 0.08d	10.27 ± 0.60b
3.5	4.4 ± 0.2d	8.5 ± 0.1ab	13.40 ± 0.55d	1.26 ± 0.17c	10.73 ± 1.20b
4.5	4.4 ± 0.1d	8.6 ± 0.0a	15.71 ± 0.56c	1.50 ± 0.19b	10.61 ± 1.04b
5.5	4.4 ± 0.4d	8.5 ± 0.1ab	17.32 ± 0.44b	1.62 ± 0.04a	10.70 ± 0.37b
10	5.3 ± 0.1c	8.2 ± 0.1c	18.80 ± 0.78a	1.53 ± 0.12ab	12.32 ± 0.45a
12	6.5 ± 0.1b	7.2 ± 0.1b	2.78 ± 0.18f	0.55 ± 0.01e	10.20 ± 0.97bc
14	6.5 ± 0.1b	7.0 ± 0.1f	2.21 ± 0.02g	0.47 ± 0.01e	9.36 ± 0.39c
16	6.8 ± 0.0a	6.9 ± 0.2f	0.62 ± 0.05h	0.18 ± 0.01f	6.93 ± 0.69d

2.4.4. Temperature and pH Effect on Growth and β-Galactosidase Production.

Temperature and pH are important environmental factors to optimize when culturing any micro-organism. Some organisms are tolerant of a wide range of both temperature and pH while other organisms require a more controlled environment in order to grow. *Bifidobacteria* are organisms which will grow in a range of pHs and at various temperatures. Hsu, et al. use *B. longum* to demonstrate the versatility of the *Bifidobacteria* species over a range of pHs and temperatures. Their work also shows the effect that changes in initial media pH and culture temperatures has on β – galactosidase activity. Figure 2.16 shows growth and β – galactosidase of *B. longum* cultures with varying initial pHs and Figure 2.17 shows growth and β – galactosidase of *B. longum* cultures with varying growth temperatures.

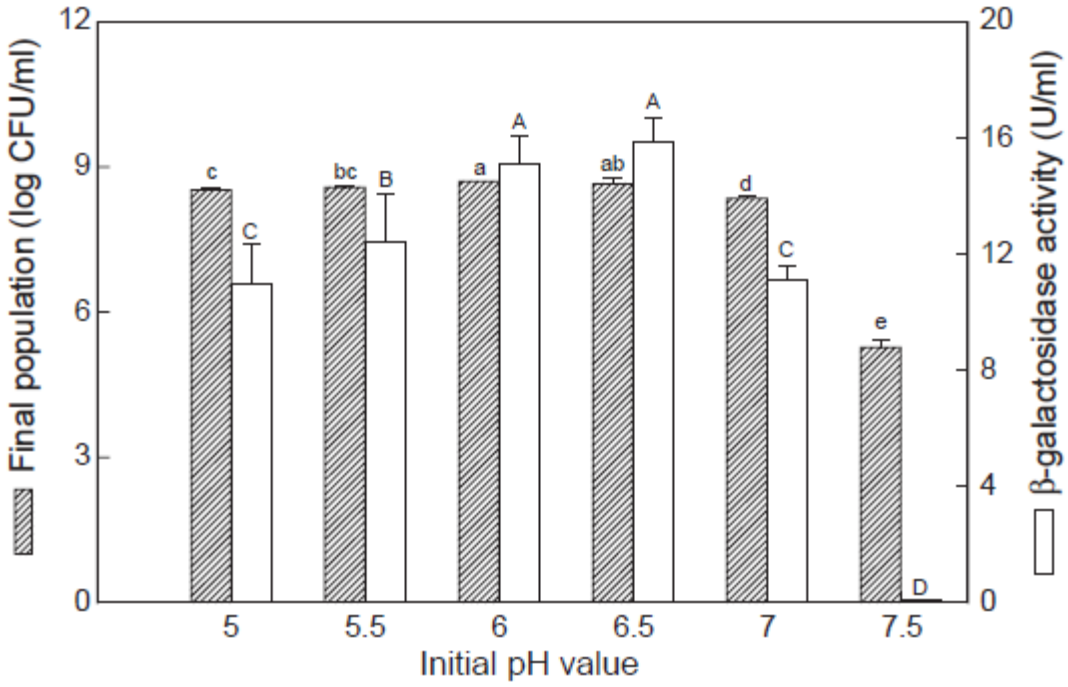


Figure 2.16 Effect of Initial pH on the Growth and β–Galactosidase Production by *B. longum*. Determinations Were Made After a 12-h Cultivation at 37 °C (Hsu et al. 2005).

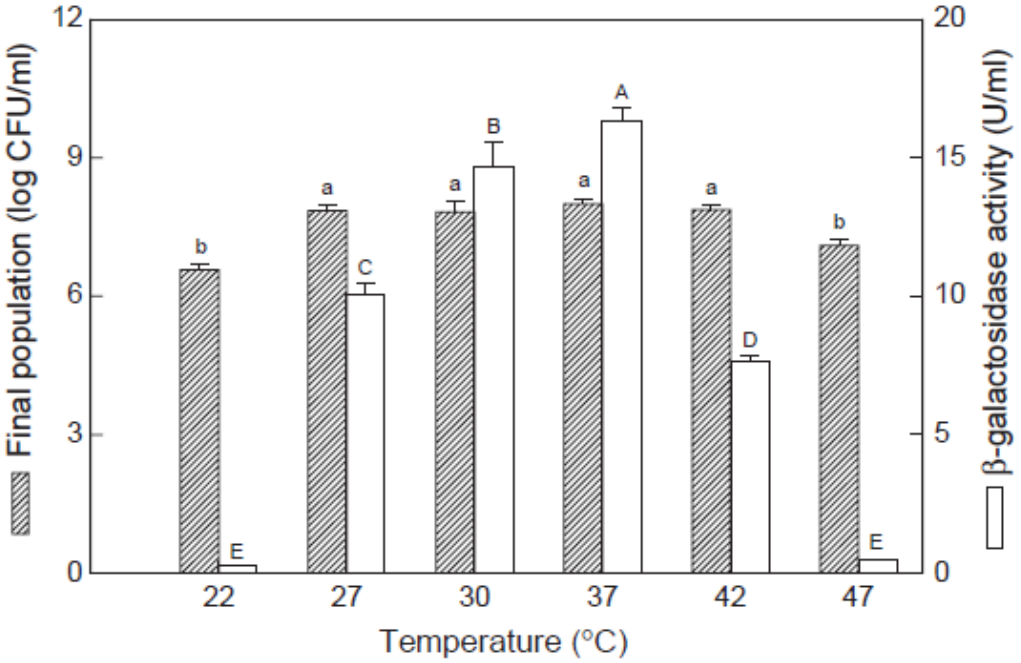


Figure 2.17 Effect of Temperature on the Growth and β–Galactosidase Production by *B. longum*. Medium With an Initial pH 6.5 for 12-h Cultivation (Hsu et al. 2005).

As is demonstrated there is not a single factor that determines growth and β – galactosidase activity of a *Bifidobacteria* culture. Even within the same species the enzyme and growth kinetics differ from strain to strain so that carbohydrate source, nitrogen source with growth factors, pH and temperature should all be investigated in order to optimize cell growth and β – galactosidase production.

3. MATERIALS AND METHODS

3.1. REAGENTS AND CHEMICALS

2-Nitrophenyl β–D-galactopyranoside (ONPG), 2-nitrophenyl (ONP), 4-nitrophenyl α-D-galactopyranoside (PNPG), and 4-nitrophenyl (PNP) were purchased from Sigma (St. Louis, MO). β –Galactosidase was purchased from Sigma (St. Louis, MO). α-Galactosidase was purchased from Deerland Enzymes (Kennesaw, GA). *Bifidobacteria bifidum* NCIMB41171 was provided by Milk Specialties Global (Carpentersville, IL). *Bifidobacteria bifidum* 700541 was purchased from American Type Culture Company (ATCC, Manassas, VA). Difco™ dehydrated media components were purchased from Becton, Dickinson and Company (BD Diagnostics, Franklin Lakes, NJ). All other chemicals were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

3.2. DETERMINATION OF ENZYME ACTIVITY

Reagent Preparation

Stock solution of 100mM sodium phosphate buffer pH 6.8 (phosphate buffer) was prepared by dissolving the following in deionized (DI) water to make one liter. The solution was filtered through a 0.2μm filter to remove dust and microbial contaminants. The resulting buffer is pH 6.8±0.2

8.725 grams $Na_2HPO_4 \bullet 2H_2O$ (MW 177.99 grams/mole)

7.038 grams $NaH_2PO_4 \bullet H_2O$ (MW 137.99 grams/mole)

Stock solution of 120mM sodium tetraborate stop reagent was prepared by dissolving 45.76 grams $Na_2B_4O_7 \bullet 10H_2O$ (MW 381.37 grams/mole) in one liter DI water then filtering the solution through a 0.2μm filter to remove dust and microbial contaminants. Both reagents are stored at room temperature.

3.2.1. β – Galactosidase Endpoint Assay. A colorimetric assay is used to determine the enzymatic rate which β – Galactosidase cleaves the glycosidic bond of ONPG leaving free galactose and ONP. In dilute concentrations, ONP is yellow in color and is detected by a spectrophotometer at 415nm (A_{415}). An increase in color intensity indicates hydrolysis. The assay is carried out in 100mM sodium phosphate buffer pH 6.8 at 40°C and stopped using 120mM sodium tetraborate solution.

A. Standard Curve Preparation

1. Standard solution of 10mM ONP was prepared using volumetric glassware by dissolving 139 milligrams of ONP (MW 139.1 grams/mole) in enough phosphate buffer to make 100 milliliters (mL).
2. Standard solution of 1mM ONP was prepared by adding five mL of 10mM ONP to 45mL phosphate buffer.
3. Standards for measurement were prepared by serial dilution of 1mM ONP standard in phosphate buffer to 1.35mL of the desired concentration according to Table 3.1. Each standard was prepared in triplicate.
4. To mimic assay conditions, 0.15 mL phosphate buffer and 3 mL sodium tetraborate stop reagent were added to each standard. The zero standard was used to blank the spectrophotometer and the absorbance of each standard was measured at 415nm using 1cm quartz cuvettes. Figure 3.1 shows the standard curve with trend line analysis for ONP. Raw data is given in Appendix A, Table A.1.

The data was analyzed using ANOVA statistics and linear regression. Statistical results are presented in Table 3.2 and Appendix B. A P-value of <0.0001 indicates that the linear model for ONP concentration is significant. The standard error can be used as the standard deviation as indicated by the R Square value.

Table 3.1 Serial Dilutions of ONP Standard Solutions for Standard Curve.

[ONP] mM	Volume ONP solution (mL)	Volume phosphate buffer (mL)
1	1.350	0
0.9	1.215	0.135
0.8	1.080	0.270
0.7	0.945	0.405
0.6	0.810	0.540
0.5	0.675	0.675
0.4	0.540	0.810
0.3	0.405	0.945
0.2	0.270	1.080
0.1	0.135	1.215
Zero standard	0	1.35

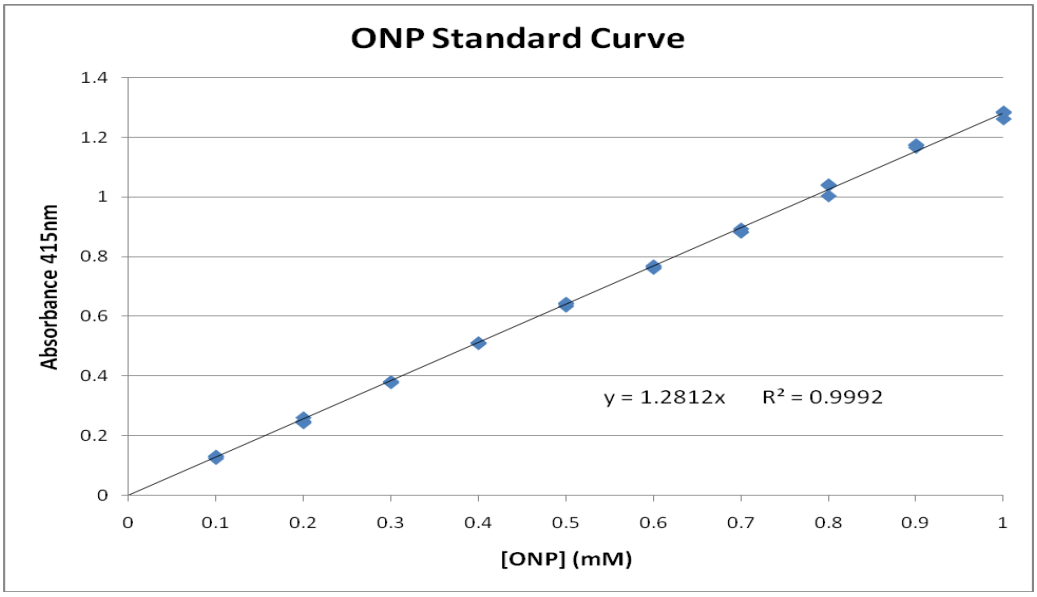


Figure 3.1 ONP Standard Curve for β – Galactosidase Assay.

Table 3.2 ANOVA Statistical Analysis and Regression Output for ONP Standard Curve.

	Degrees of Freedom	*Sum of Squares*	*Mean Squares*	*F-Value*	*P-value*
Model	1	18.95929753	18.95929753	157408.5	<0.0001
Residual	29	0.003492948	0.000120446		
Total	30	18.96279048			

	Coefficients	*Standard Error*	*Lower 95%*	*Upper 95%*
Absorbance 415	1.281209437	0.003229283	1.274604812	1.287814062

Regression Statistics	
Multiple R	0.999907896
R Square	0.9998158
Standard Error	0.010974812

The extinction coefficient (ε) for ONP was calculated from the standard curve regression using Equation (1) where A_{415} is the absorbance measurement, ε is the slope of the linear regression divided by dilution factor (ONP volume (mL)/total assay volume (mL)), and *l* is the path length (1cm). The experimentally determined value of the extinction coefficient for ONP is 4.27 $mM^{-1}cm^{-1}$. The published literature value of the extinction coefficient for ONP is 4.5 $mM^{-1}cm^{-1}$ lies outside the 95% confidence interval. The experimentally determined coefficient was used for the rate calculations.

$$A_{415} = \varepsilon\,[ONP]\,l \qquad (1)$$

B. Enzyme Activity Determination

1. Stock substrate solution of 10mM ONPG was prepared by dissolving 301.3 milligrams of ONPG (MW 301.3 grams/mole) in 100mL of phosphate buffer. Buffer was stored cold in a light protective container.
2. Cell samples were prepared at 1mg/mL dry cell weight in phosphate buffer. Samples were prepared in duplicate or triplicate depending on the availability of sample.

3. 1.35 mL of 10mM ONPG was dispensed into clean glass tubes and equilibrated to 40°C in a circulating water bath.
4. 0.15 mL of 1 mg/mL cell sample was dispensed into the glass tubes to initiate the reaction. The reaction was carried out at 40°C for 10 – 20 minutes.
5. After the appropriate amount of time had elapsed, 3 mL of sodium tetraborate was dispensed into each tube. The tubes were removed from the water bath and the ONP concentration was determined by measuring the absorbance values at 415nm using the spectrophotometer.
6. A blank was prepared in the same fashion as the samples except the cell sample was added after the stop reagent to account for turbidity without the reaction being carried out.
7. Enzyme activity was calculated as enzyme units (EU)/gram cells/min using Equation (2). One EU will hydrolyze 1.0 μmole of ONPG to ONP and galactose per minute.

$$\frac{EU}{g/\min} = \frac{A_{415}(TotalVolume)(DilutionFactor)}{(Time)(\varepsilon)(SampleVolume)(100mL/g)} \quad (2)$$

3.2.2. α – Galactosidase Endpoint Assay. A colorimetric assay is used to measure the enzymatic rate α – Galactosidase cleaves the glycosidic bond of PNPG leaving free galactose and PNP. In dilute concentrations, PNP is yellow in color and is detected by a spectrophotometer at 400nm (A_{400}). Increase in absorbance indicates hydrolysis. The assay is carried out in 100mM sodium phosphate buffer pH 6.8 at 40°C and stopped using 120mM sodium tetraborate solution.

A. Standard Curve Preparation

1. Standard solution of 10mM PNP was prepared using volumetric glassware by dissolving 139 milligrams of PNP (MW 139.1 grams/mole) in enough phosphate buffer to make 100 milliliters (mL).
2. Standard solution of 1mM PNP was prepared by adding 5 mL of 10mM PNP to 45mL phosphate buffer.

3. Standards for measurement were prepared by serial dilution of 1mM PNP standard in phosphate buffer to make 1.35mL of the desired concentration according to Table 3.3. Each standard was prepared in triplicate.
4. To mimic assay conditions 0.15 mL phosphate buffer and 3 mL sodium tetraborate stop reagent were added to each standard. The zero standard was used to blank the spectrophotometer and absorbance was measured at 400nm using 1cm quartz cuvettes. Figure 3.2 shows the standard curve with trend line analysis for PNP. Raw data is given in Appendix A, Table A.2.

The data was analyzed using ANOVA statistics and linear regression. Statistical results are presented in Table 3.4 and Appendix B. A P-value of <0.0001 indicates that the linear model for ONP concentration is significant. The standard error can be used as the standard deviation as indicated by the R Square value.

Table 3.3 Serial Dilutions of PNP Standard Solutions For Standard Curve.

[PNP] mM	Volume PNP solution (mL)	Volume phosphate buffer (mL)
0.5	0.675	0.675
0.4	0.540	0.810
0.3	0.405	0.945
0.2	0.270	1.08
0.1	0.135	1.215
BLANK (zero standard)	0	1.35

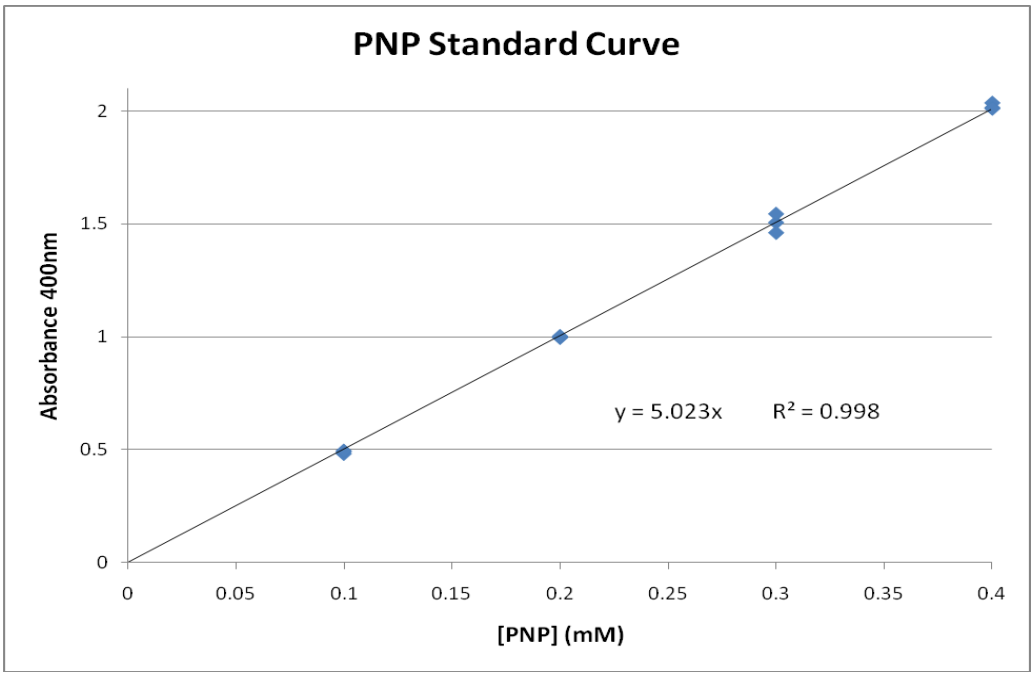


Figure 3.2 PNP Standard Curve for α–Galactosidase Assay.

Table 3.4 ANOVA Statistical and Regression Analysis Output for PNP Standard Curve.

	Degrees of Freedom	*Sum of Squares*	*Mean Squares*	*F-Value*	*P-value*
Model	1	22.71102	22.71102	48904.85	<0.0001
Residual	11	0.005108	0.000464		
Total	12	22.71613			

	Coefficients	*Standard Error*	*Lower 95%*	*Upper 95%*
Absorbance 400	5.023392222	0.022715438	4.973395881	5.073388564

Regression Statistics	
Multiple R	0.999887556
R Square	0.999775124
Standard Error	0.021549756

The extinction coefficient (ε) for PNP was calculated from the standard curve regression using Equation (3) where A_{400} is the absorbance measurement, ε is the slope of the linear regression divided by dilution factor (PNP volume (mL)/total assay volume (mL)), and *l* is the path length (1cm). The experimentally determined value of the extinction coefficient for PNP is 16.74 $mM^{-1}cm^{-1}$. The published literature value of the extinction coefficient for PNP is 18.5 $mM^{-1}cm^{-1}$ is outside the 95% confidence interval so the experimentally determined value is used for rate calculations.

$$A_{400} = \varepsilon\,[PNP]\,l \qquad (3)$$

B. Enzyme Activity Determination

1. Stock substrate solution of 10mM PNPG was prepared by dissolving 301.3 milligrams of PNPG (MW 301.3 grams/mole) in 100mL of phosphate buffer. Buffer was stored cold in a light protective container.
2. Cell samples were prepared at 1mg/mL dry cell weight in phosphate buffer. Samples were prepared in duplicate or triplicate depending on the availability of sample.
3. 1.35 mL of 10mM PNPG was dispensed into clean glass tubes and equilibrated to 40°C in a circulating water bath.
4. 0.15 mL of 1 mg/mL cell sample was dispensed into the glass tubes to initiate the reaction. The reaction was carried out at 40°C for 20 – 40 minutes.
5. After the appropriate amount of time had elapsed, 3 mL of sodium tetraborate was dispensed into each tube. The tubes were removed from the water bath and the ONP concentration was determined by measuring the absorbance values at 415nm using a spectrophotometer.
6. A blank was prepared in the same fashion as the samples except the cell sample was added after the stop reagent to account for turbidity without the reaction being carried out.
7. Enzyme activity was calculated as enzyme units (EU)/gram cells/min using Equation (4). One EU will hydrolyze 1.0 μmole of PNPG to PNP and galactose per minute.

$$\frac{EU}{g/\min} = \frac{A_{400}(TotalVolume)(DilutionFactor)}{(Time)(\varepsilon)(SampleVolume)(100mL/g)} \quad (4)$$

3.2.3. β - Galactosidase Kinetic Assay. Using ONPG as a substrate, purified enzyme activity was determined using the kinetic colorimetric assay adapted from Sigma Corporation (St. Louis, MO) protocol SPONPG01 "Enzymatic Assay of β - Galactosidase". Enzyme and substrate solutions were made by adding crystals to phosphate buffer adjusted to make a known concentration stock solutions. Stock solutions were made fresh for each experiment. Test samples were made by serial dilution of the stock solution to the desired concentration. Cuvette volume was 3mL. With the exception of data gathered on the effect of temperature on reaction rate, all experimental runs were controlled at 37°C by a circulating water bath.

A. Reagent Preparation

1. Stock solution of 100mM sodium phosphate buffer pH 7.2 (phosphate buffer) was prepared by dissolving the following in deionized (DI) water to make one liter then filtering the solution through a 0.2μm filter to remove dust and microbial contaminants. The resulting buffer is pH 7.2±0.2

   3.338 grams $Na_2HPO_4 \bullet 2H_2O$ (MW 177.99 grams/mole)
   13.146 grams $NaH_2PO_4 \bullet H_2O$ (MW 137.99 grams/mole)

2. Stock solution of 30mM magnesium chloride reagent was prepared by dissolving 0.608 grams $MgCl_2 \bullet 6H_2O$ (MW 203.31 grams/mole) in 100mL phosphate buffer pH 7.2. Both reagents are stored at room temperature.

B. Computer Software Interface

The Agilent 8543 diode array spectrophotometer with UV/VIS Chemstation software version A.09.01 with the Kinetics module Biochem Analysis Software was used to collect and record data. A method was created using the following conditions:

Wavelength – 415nm

Background Correction – Subtract Average 550-650nm

Run Time – 600s (run time changed for tests requiring less or more time)

Cycle Time – 20s (how often the detector gathers data from the test cells)

Start Time – 0s (When the detector starts collecting data)

Kinetic Calculation – Initial Rate Calculation

The initial rate calculation is determined from the linear portion of the data curve. All rate calculations were made using the time point immediately preceding the reaching or exceeding the limit of detection as the last data in the linear portion of the curve.

Before adding samples to the cuvettes a "zero cells" function was performed to remove any variations among the quartz cuvettes. The cuvette tray holds up to eight cells. The software was configured for the number of samples to be run with a blank run with each test condition.

C. Enzyme Activity Determination

1. Stock substrate solution of 68mM ONPG was prepared by dissolving 0.204 grams of ONPG (MW 301.3 grams/mole) in 10mL of phosphate buffer. Buffer was stored cold in a light protective container.
2. Enzyme stock solution was prepared at 2-4 U/mL in phosphate buffer by diluting a stock solution at 3000 U/mL by 1/1000.
3. Prepare test cuvettes per Table 3.5.

Table 3.5 Sample Preparations for Kinetic Experiments of β –Galactosidase (Analysis performed by L. Jorgenson, A. Sutterer, and J. Roam).

		Substrate Concentration (mM)				
	Blank	**1.13**	**2.27**	**4.53**	**9.07**	**18.13**
Phospate Buffer	2.70 ml	2.65 ml	2. 60 ml	2.50 ml	2.30 ml	1.90 ml
$MgCl_2$	0.10 ml	0.10 ml	0.10 ml	0.10 ml	0.10 ml	0.10 ml
Enzyme	0.10 ml	0.10 ml	0.10 ml	0.10 ml	0.10 ml	0.10 ml
Substrate	-	0.05 ml	0.10 ml	0.20 ml	0.40 ml	0.80 ml
Inhibitor	0.10 ml	0.10 ml	0.10 ml	0.10 ml	0.10 ml	0.10 ml

4. Immediately before adding the enzyme solution to the cuvettes, start the data collection on the software. Add the enzyme solution to each of the cuvettes before the first data point was collected.
5. Allow the method to run to completion then adjust the calculation range for the initial rate based on the data.
6. Enzyme activity was calculated as enzyme units (EU)/gram cells/min using Equation (5). One EU will hydrolyze 1.0 μmole of ONPG to ONP and galactose per minute.

$$\frac{EU}{mL/\min} = \frac{Rate(TotalVolume)(DilutionFactor)}{(\varepsilon)(SampleVolume)} \tag{5}$$

3.2.4. α – Galactosidase Kinetic Assay. Using PNPG as a substrate, purified enzyme activity was determined using a kinetic colorimetric assay adapted from Bioassay Systems protocol POPN006 "pNPP Phosphatase Assay". Enzyme and substrate solutions were made by adding crystals to phosphate buffer to make a known concentration stock solutions. Stock solutions were made fresh for each experiment. Test samples were made by serial dilution of the stock solution to the desired concentration. Cuvette volume was 3mL. For each data point a blank was made consisting of 1.5mL of the PNPG solution and 1.5mL of phosphate buffer in place of enzyme solution. With the exception of data gathered on the effect of temperature on reaction rate, all experimental runs were controlled at 25°C by a circulating water bath.

A. Computer Software Interface

The Agilent 8543 diode array spectrophotometer with UV/VIS Chemstation software version A.09.01 with the Kinetics module Biochem Analysis Software was used to collect and record data. A method was created using the following conditions:

Wavelength – 400nm

Background Correction – Subtract Average 550-650nm

Run Time – 1800s (run time changed for tests requiring less or more time)

Cycle Time – 30s (how often the detector gathers data from the test cells)

Start Time – 0s (When the detector starts collecting data)

Kinetic Calculation – Initial Rate Calculation

The initial rate calculation is determined from the linear portion of the data curve. All rate calculations were made using the time point immediately preceding the reaching or exceeding the limit of detection as the last data in the linear portion of the curve.

Before adding samples to the cuvettes a "zero cells" function was performed to standardize the lamp against any noise that may be introduced by the quartz cuvettes. The cuvette tray holds up to eight cells. The software was configured for the number of samples to be run with a blank run with each test conditon.

B. Enzyme Activity Determination

1. Prepare the appropriate dilutions of the enzyme and substrate solutions.
2. Dispense 1.5 mL of the non variable solution into each of the cuvettes (ie if testing changing enzyme concentrations then substrate concentration is the non variable). When testing temperature effects add either substrate or enzyme but do not mix the solutions until ready to begin measurements.
3. Immediately before adding the variable solution to the cuvettes start the data collection on the software. Add 1.5 mL of the variable solution to each of the cuvettes before the first data point is collected.
4. Allow the method to run to completion then adjust the calculation range for the initial rate, based on the data. A typical data plot is shown in Figure 3.3.
5. Enzyme activity was calculated as enzyme units (EU)/mg enzyme/min using Equation (6). One EU will hydrolyze 1.0 μmole of PNPG to PNP and galactose per minute.

$$\frac{EU}{mg/\min} = \frac{Rate(TotalVolume)(DilutionFactor)}{(\varepsilon)(SampleVolume)(EnzymeConcentration)} \qquad (6)$$

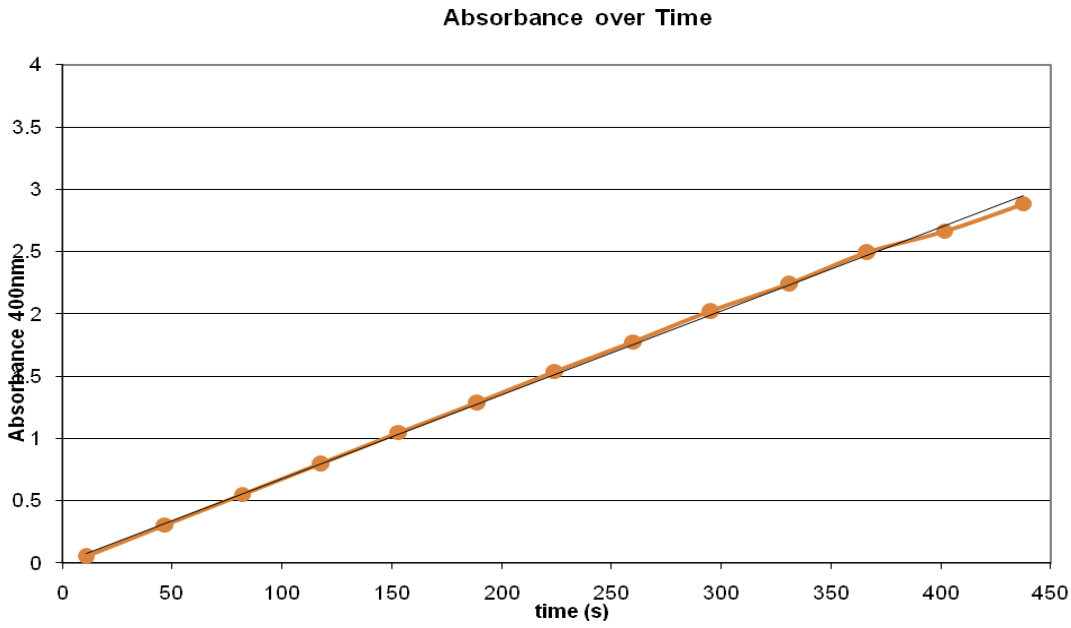


Figure 3.3 Example of a Typical Kinetic Assay Data Plot Used for Initial Rate Determination.

3.3. DRY-CELL WEIGHT DETERMINATION

Cell growth was determined by measuring the turbidity of the culture broth with time. The optical density (OD) probe was a Fundalux II detecting at wavelength 910nm used in conjunction with a Biostat® B Plus two-liter bioreactor with a 1.5 liter working volume. Micro DCU system software was used to control the bioreactor and record time point data of the cultures. An increase in turbidity indicated an increase in the number of cells present in the reactor. A set of standards were made using *Bifidobacteria bifidum* NCIMB41171. A 10 gram/liter cell slurry was made by weighing out three grams of freeze dried cells and mixing with 300 milliliters sodium phosphate buffer. The cell slurry was allowed to stir for 30 minutes to ensure a homogenous mixture. Serial dilutions of the 10 gram/liter solution were made as shown in Table 3.6 for the standard curve. A total of 20mL was needed for each measurement. All standards were made in triplicate. Raw data is given in Appendix A, Table A.3.

Table 3.6 Serial Dilutions of 10g/L NCIMB41171 Cell Slurry.

Cell Density (g/L)	Volume Cell Slurry (mL)	Volume phosphate buffer (mL)
7.5	15	5
5	10	10
4	8	12
3	6	14
2.5	5	15
2	4	16
1.5	3	17
1	2	18
0.5	1	19
0.25	0.5	19.5
0.1	0.2	19.8
Blank	0	20

The data follows a nearly exponential curve in the form shown in equation 7 however this model does not hold true at cell densities below 1gram/liter. Cell densities at or below 1 gram/liter are analyzed using linear regression (8) while cell densities greater than 1 gram per liter are analyzed using the exponential fit (7) where C and M are constants found through regression and OD is the optical density. Figure 3.4 shows the standard curve used for determining dry cell weight.

$$\text{Cell Density} > 1 \text{ gram/liter:} \qquad e^{OD910} = C + M * \text{Cell Density} \tag{7}$$

$$\text{Cell Density} \leq 1 \text{ gram/liter:} \qquad OD_{910} = C + M * \text{Cell Density} \tag{8}$$

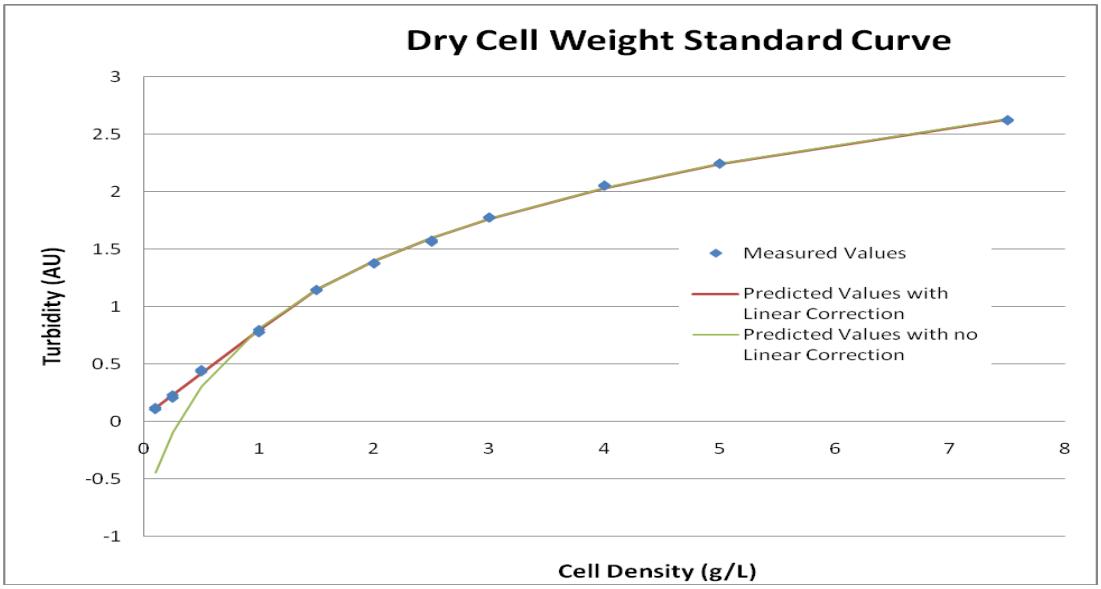


Figure 3.4 Dry Cell Weight Standard Curve. Linear Fit (Turbidity = 0.037635 + 0.7546631*Cell Density); Exponential Fit (Exp(OD910) = 0.4622332 + 1.7887159*Cell Density).

The data was analyzed using ANOVA statistics and linear and nonlinear regression. Statistical results are presented in Table 3.7, Table 3.8 and Appendix B. A P-value of <0.0001 indicates that the models for cell density are significant. The standard error can be used as the standard deviation as indicated by the R Square value.

Table 3.7 ANOVA Statistical Analysis Output for Dry Cell Weight Standard Curve (Linear).

	Degrees of Freedom	*Sum of Squares*	*Mean Squares*	*F-Value*	*P-value*
Model	1	2.5858120	2.58581	2847.803	<.0001
Residual	11	0.0099880	0.00091		
Total	12	2.5958000			

Regression Statistics	
R Square	0.999004
Standard Error	0.114113

Table 3.8 ANOVA Statistical Analysis Output for Dry Cell Weight Standard Curve (Exponential).

	Degrees of Freedom	*Sum of Squares*	*Mean Squares*	*F-Value*	*P-value*
Model	1	248.19015	248.190	19059.70	<.0001
Residual	19	0.24741	0.013		
Total	20	248.43756			

Regression Statistics	
R Square	0.995026
Standard Error	0.019969

3.4. SUGAR ANALYSIS BY HPLC

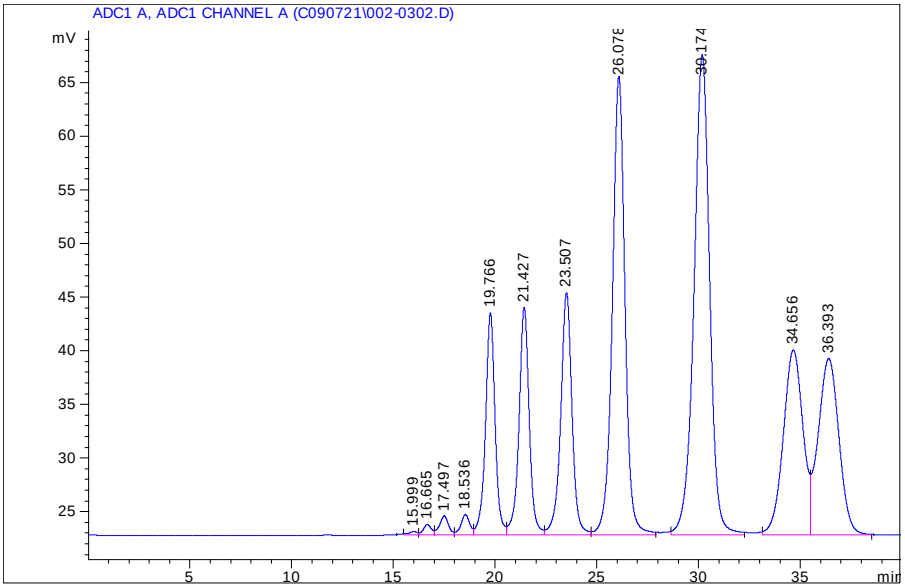
Sugars were analyzed using a Phenomenex Rezex-RCM ion exchange column in calcium form on an Agilent HP 1090 high performance liquid chromatograph (HPLC) using Chemstation A.03 software. The mobile phase was Milli-Q® water (18 mega-ohm resistivity) at 0.6 mL/min flow rate. The column temperature was controlled at 85^0C. The detector was an Agilent 1037 refractive index detector operated at 40^0C. The injection size was 10 µL for all samples and each sample is injected in duplicate. The Chemstation software calculates the area under the curve and generates calculated concentrations for each mixuture.

Standard Curve Preparation

Standard solutions A and B were prepared per Table 3.9 by O. Sitton. Standard A is a mixture of four oligosaccharides: maltohexaose (DP6), maltopentaose (DP5), maltotetraose (DP4) and maltotriose (DP3). Standard B is a mixture of lactose, glucose, and galactose. Injection samples are 500 microliter volumes made up of differing ratios of A and B with known concentrations of each sugar. Figure 3.5 is an example chromatograph showing typical retention times for each sugar. The known concentrations are the expected values which the HPLC results are being compared to for accuracy. Table 3.10 shows the mixture ratios and the expected sugar concentration for each sample.

Table 3.9 Standard Solution Sugar Concentration.

	Sugar Concentration (gram/Liter)						
Standard	**DP6**	**DP5**	**DP4**	**DP3**	**Lac**	**Glc**	**Gal**
A	9.91	9.45	9.82	20.4	-	-	-
B	-	-	-	-	102.08	51.33	52.96



Sugar	**Retention Time(min)**
DP6	19.766
DP5	21.427
DP4	23.507
DP3	26.078
Lactose	30.174
Glucose	34.656
Galactose	36.393

Figure 3.5 Sample HPLC Chromatagraph with Rention Time for Each Sugar.

Table 3.10 Expected Sugar Concentrations for Samples.

	Expected Sugar Concentration (gram/Liter)						
Mixture Ratio % (A:B)	**DP6**	**DP5**	**DP4**	**DP3**	**Lac**	**Glc**	**Gal**
100:0	9.91	9.45	9.82	20.4	-	-	-
90:10	8.92	8.51	8.84	18.36	10.21	5.13	5.30
80:20	7.93	7.56	7.86	16.32	20.42	10.27	10.59
70:30	6.94	6.62	6.87	14.28	30.62	15.40	15.89
60:40	5.95	5.67	5.89	12.24	40.83	20.53	21.18
50:50	4.96	4.73	4.91	10.2	51.04	25.67	26.48
40:60	3.96	3.78	3.93	8.16	61.25	30.80	31.78
30:70	2.97	2.84	2.95	6.12	71.46	35.93	37.07
20:80	1.98	1.89	1.96	4.08	81.67	41.06	42.37
10:90	0.99	0.95	0.98	2.04	91.87	46.20	47.66
0:100	-	-	-	-	102.08	51.33	52.96

The data was analyzed using ANOVA statistical analysis. Lactose is determined using a quadratic fit. Glucose and galactose are determined using a linear fit. Figure 3.6 shows the standard curves and their statistical results are presented in Table 3.11. A P-value of <0.0001 indicates that the models for concentration is significant. The standard error can be used as the standard deviation as indicated by the R Square value. These results are found in Appendix B.

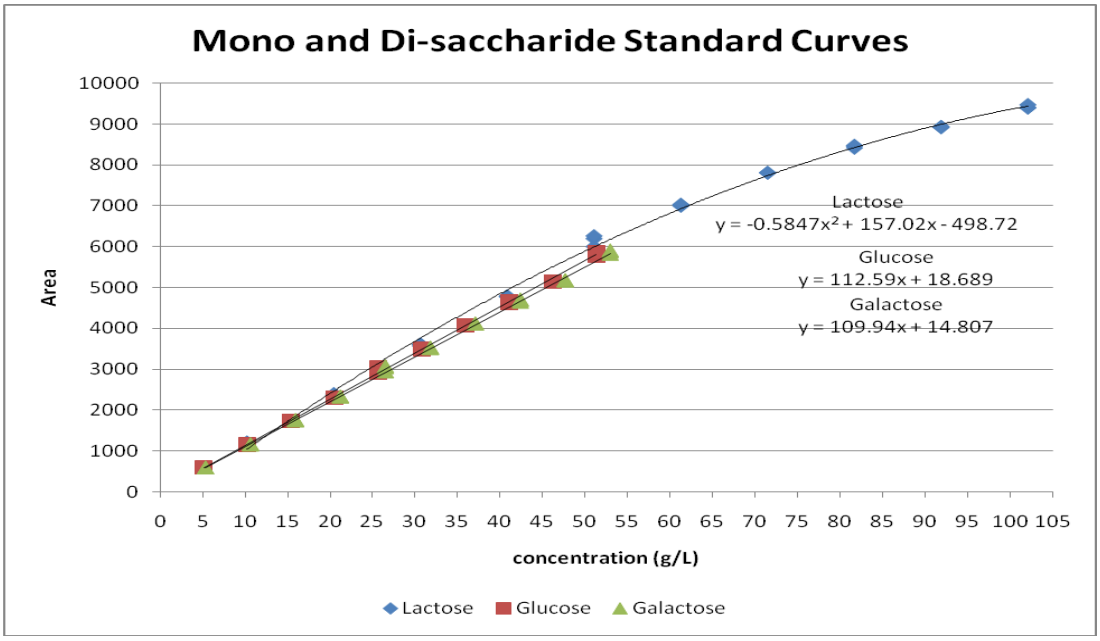


Figure 3.6 Standard Curves with Equations of Fit for Glucose, Galactose and Lactose.

Table 3.11 ANOVA Statistical Analysis Output for Galactose, Glucose and Lactose.

	Degrees of Freedom	*Sum of Squares*	*Mean Squares*	*F-Value*	*P-value*
Lactose					
Model	2	221148151	110574076	6886.007	<.0001
Residual	27	433560	16057.793		
Total	29	221581712			
Glucose					
Model	1	82665096	82665096	36767.46	<.0001
Residual	28	62953	2248.3221		
Total	29	82728049			
Galactose					
Model	1	83899719	83899719	33356.97	<.0001
Residual	28	70426	2515.2082		
Total	29	83970145			

Regression Statistics			
	Lactose	**Glucose**	**Galactose**
R Square	0.998043	0.999239	0.999161
Standard Error	126.719	47.416	50.152

3.5. MEDIA PREPARATION FOR ANAEROBIC CELL CULTURE

Bifidobacteria require an anaerobic growth environment. Media was prepared as follows for culture tubes and vials.

1. Dissolve media components in Milli-Q® water so that the flask contains not more than 75% of the maximum flask volume. Mix by magnetic stirring. For media with high sugar concentration prepare the sugar and nitrogen components separately at appropriate concentrations such that they are the correct concentrations when mixed for the final media.
2. Stop stirring and allow the liquid to come to rest. Mark the liquid edge on the outside of the flask. Add 10-20mL additional water to account for liquid that will be lost during the oxygen purging process.
3. If necessary add methylene blue as an oxygen indicator at 5 milligrams/liter.
4. Turn on the Thermalene® 2110 Tube Furnace and set the temperature to 260°C. Allow the furnace to come to temperature.
5. Strip the furnace of oxygen by flowing "forming" gas (90:10, N_2: H_2) through the heater for 10 minutes. Stop flowing "forming" gas.
6. Purge "forming" gas from the heater by flowing "purge" gas (80:20, N_2: CO_2). This gas is used to purge the oxygen from the media and fill the headspace in the flask.
7. Gently heat the media while stirring slowly using magnetic stirring. If methylene blue is present in the media a lack of blue color indicates oxygen free media.
8. Place a rubber stopper with holes for gas delivery and venting in the flask opening as in Figure 3.7. Ensure the tip of the gas delivery tube is just above the liquid level and the end of the vent tube is as close to the bottom of the rubber stopper as possible to allow for efficient gas exchange.

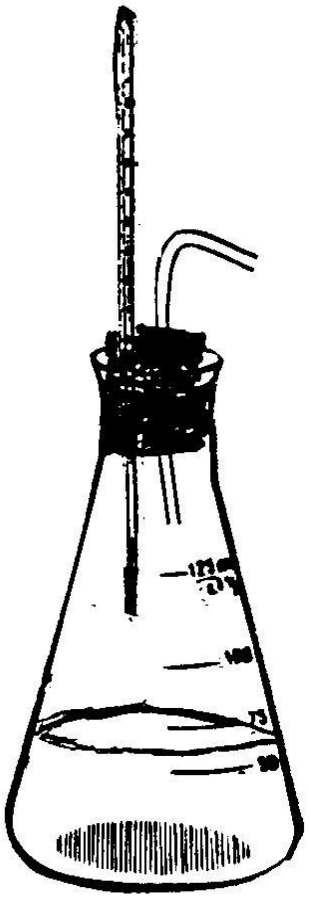


Figure 3.7 Oxygen Purge Setup for Anaerobic Media Preparation.

9. Continue heating and gently stirring the flask allowing the liquid to come to a low boil. Stirring is not necessary however if a stir bar is present it will act as a boiling stone in the bottom of the flask.
10. Direct the purge gas to the gas delivery tube and purge the oxygen from the media and the flask head space for a minimum of 15-20 minutes. Continue to heat the system with gas flow until the volume in the flask reaches the mark made in step 2.
11. Remove the deoxygenated media from the heat source. While the media is still hot exchange the rubber stopper for a solid stopper and seal the container airtight.
12. Transfer the flask to the anaerobic chamber and allow the liquid to cool.

13. Dispense the cooled anaerobic media into clean vials and culture tubes which have been equilibrated in the anaerobic chamber for a minimum of 24 hours. Cap tubes and vials airtight.
    a. For media with high sugar concentration and nitrogen components prepared separately, keep the solutions separate and seal the containers airtight for autoclaving.
    b. Prepare clean empty vials and tubes, sealed airtight for autoclaving, for media preparation after sterilizing the components separately.
14. Sterilize the prepared containers and media in an autoclave for 15-20 minutes at 121°C.
15. Remove the sterile media and containers from the autoclave while still hot. Transfer the containers and media to the anaerobic chamber and allow to cool completely.
    a. Mix media components in the prepared containers to the correct concentrations while still warm to the touch but not hot.
16. Label the tubes and vials with the contents and store at room temperature until ready to use.

Media was prepared as follows for the two-liter bioreactor.

1. Dissolve the media components in Milli-Q® water keeping the carbohydrates separate from the nitrogen components. Divide the volume into halves and make each half 2X concentration. Final volume for most cultures is one liter.
2. If necessary add methylene blue as an oxygen indicator at 5 milligrams/liter.
3. Prepare a 250 milliliter bottle of 1N sodium hydroxide (NaOH).
4. Prepare a 250 milliliter bottle of antifoam solution.
5. Standardize the reactor inline pH probe with pH buffers 4.01 and 7.00.
6. Ensure the reactor is clean then add either the prepared nitrogen based solution or the prepared carbohydrate based solution to the reactor vessel.
7. Put the lid on the reactor and loosely fasten the bolts to hold down the lid.
8. Close or clamp all the ports on the reactor.
9. Put in place the pH probe, the turbidity probe, the antifoam sensors, the oxygen sensor and the septum.

10. Sterilize the reactor, remaining media component, sodium hydroxide solution and antifoam in an autoclave for 30 minutes at 121°C.
11. Remove reactor and other components from the autoclave.
12. Connect the antifoam and sodium hydroxide solution through the Biostat Bplus pump to the reactor feed ports.
13. Tighten down the reactor lid and unclamp the necessary ports for venting and feed solutions.
14. Attach all ancillary components of the reactor.
    a. Attach the water jacket hoses and begin cooling the reactor to 50°C.
    b. Attach the agitator motor and turn on at 100rpm to assist in the cooling of the reactor contents.
    c. Attach gas tubes to gas inlet ports and unclamp ports.
    d. Attach off-gas condenser.
    e. Attach all sensor cables.
15. As aseptically as possible, transfer the remaining media component to the reactor through one of the addition ports.
16. Allow "beer gas" (75:25, N_2:CO_2) flow at low flow to purge oxygen from the media and create a N_2/CO_2 blanket in the headspace of the reactor. Purge for a minimum of one hour.
17. Adjust the control temperature of the reactor to 37°C.
18. Using the pump on the Biostat B Plus gradually add sodium hydroxide solution to reach the desired pH.
19. Allow the reactor to stir at 37°C overnight to verify sterility and ensure the media is anaerobic.
20. Zero the optical density probe.

3.6. ANAEROBIC CELL CULTURE

Cells were cultured anaerobically at 37°C for a minimum of 12 hours. Cells cultured in tubes or vials were gently stirred using an incubating shaker or mixed periodically by hand to minimize the effects of nutritional micro environments. Cells cultured in the Biostat® B plus were mixed using the unit agitator at 150 Rpm and

sparged with beer gas (75:25, N_2:CO_2) to keep the culture oxygen free and the reactor under positive pressure. Cultures were inoculated with 1% total volume using cultures in exponential growth phase. To verify aseptic technique for vial and tube preparations a blank tube was inoculated with sterile media and incubated under the same conditions.

For cultures in tubes and vials pH was not controlled however final pH was recorded. In the bioreactor pH was measured, recorded and in some cases controlled during the culture lifecycle. Cultures were stored at 4°C to serve as future inoculum or for additional testing.

3.7. CELL CULTURE CONDITIONS

In order to understand how changes in culture media influenced cell growth, *B. bifidum* ATCC 700541 was cultured on enhanced reinforced clostridial medium (RCM) as a reference media. A cell bank was started from a single ampule of freeze dried cells and anaerobically cultured using RCM with no additional components through two inoculation cycles before being used to seed batch cultures for evaluation. Table 3.12 lists the formula for the enhanced RCM. For comparison batch cultures in the Biostat® B Plus were cultured with and without pH control. Batches that were controlled were held at pH 6.5 – 6.8 using 1N sodium hydroxide.

Since *B. bifidum* readily utilizes glucose as a carbohydrate source, cells were grown on the reference media with increased glucose concentrations in attempt to find a cell density limit for the culture.

One of the challenges to using *Bifidobacteria* cells for the GOS transformation reaction is they are a species that is especially difficult to grow on a purely synthetic medium. The desire to culture the organisms on synthetic media is purely economic. The more components a medium has the more expensive it is to use. Two synthetic media modified from the Norris Medium were chosen in attempts to culture *B. bifidum* ATCC 700541 using minimal medium components. The formulas for each of the selected media can be found in Table 3.13, Table 3.14, and Table 3.15. Since the minimal media did not contain any glucose, complex amino acids or vitamins the cultures needed to be gradually introduced to the medium by stepping down from the RCM by a series of dilutions as listed in Table 3.16.

Table 3.12 Difco™ Reinforced Clostridial Medium (RCM).

Media Component	**Approximate Formula Grams Per Liter**
Pancreatic Digest of Casein	5.0
Proteose Peptone No. 3	5.0
Beef Extract	10.0
Yeast Extract	3.0
Dextrose (glucose)	5.0
Sodium Chloride	5.0
Soluble Starch	1.0
Cysteine Hydrochloride	0.5
Sodium Acetate	3.0
Agar	0.5
Components Added (Enhanced RCM)	
Potassium phosphate, dibasic	4.5
Sodium phosphate, dibasic	6.0

Table 3.13 Minimal Media (Bezkorovainy and Miller-Catchpole 1989).

Media Component	**Approximate Formula Grams Per Liter**
Ammonium Acetate	4
Sodium Acetate	50
Potassium phosphate, dibasic	5
Cysteine	0.4
Lactose	70
Biotin	0.008 mg
Calcium Pantothenate	0.8 mg
Salts “B” Solution	10mL

Table 3.14 Modified Minimal Media (Bezkorovainy and Miller-Catchpole 1989).

Media Component	**Approximate Formula Grams Per Liter**
Ammonium Acetate	4
Potassium phosphate, dibasic	5
Lactose	70
Cysteine	0.4
Tween® 80	2
Calcium Pantothenate	0.8 mg
Biotin	0.008 mg
Ascorbic Acid	0.02
Salts “B” Solution	10mL

Table 3.15 Salts B Solution (Bezkorovainy and Miller-Catchpole 1989).

Media Component	**Approximate Formula Grams Per 250 mL**
Magnesium Sulphate Heptahydrate	10
Iron Sulphate Heptahydrate	0.5
Sodium Chloride	0.5
Manganese Sulphate Dihydrate	0.337

Table 3.16 Dilutions for Media Adaptation to Minimal Media.

Media Ratio	**Volume Complex Media (mL)**	**Volume Minimal Media (mL)**
100:0	15	0
67:33	10	5
33:67	5	10
0:100	0	15

Cell growth was determined by a drop in pH from the starting media after a 24 hour incubation. The results were compared to growth in the RCM. Table 3.17 is a summary of the cell adaptation. The two media conditions chosen were termed “minimal media” and “modified minimal media” and their formula can be found in Appendix C.

Table 3.17 Cell Adaptation Summary Table.

Media	**Starting pH**	**Ending pH**
RCM	6.61	4.46
1/3 Minimal Media, 2/3 RCM	6.80	4.60
2/3 Minimal Media, 1/3 RCM	6.91	4.85
Minimal Media	6.99	6.79
1/3 Modified Minimal Media, 2/3 RCM	6.83	4.21
2/3 Modified Minimal Media, 1/3 RCM	7.05	4.21
Modified Minimal Media	7.21	6.91

4. RESULTS

4.1. CELL GROWTH ON REFERENCE MEDIA

In order to understand how changes in culture media influenced cell growth *B. bifidum* ATCC 700541 was cultured on enhanced reinforced clostridial medium (RCM) as a reference media. For comparison batch cultures in the Biostat® B Plus were cultured with and without pH control. Batches that were controlled were held at pH 6.5 – 6.8 using 1N sodium hydroxide. Figure 4.1 shows the growth profile of the biostat culture without pH control.

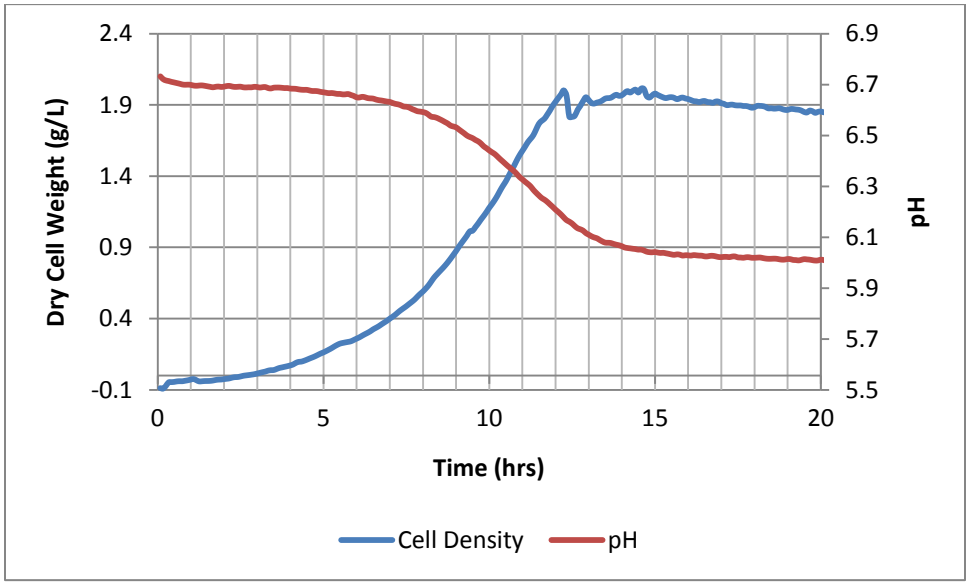


Figure 4.1 Time-Course of Cell Growth in Reference Medium Without pH Control.

B. bifidum readily cells were also grown on the reference media with increased glucose concentrations in attempt to find a cell density limit for the culture. Results are shown in Figure 4.2 and summarized in Table 4.1.

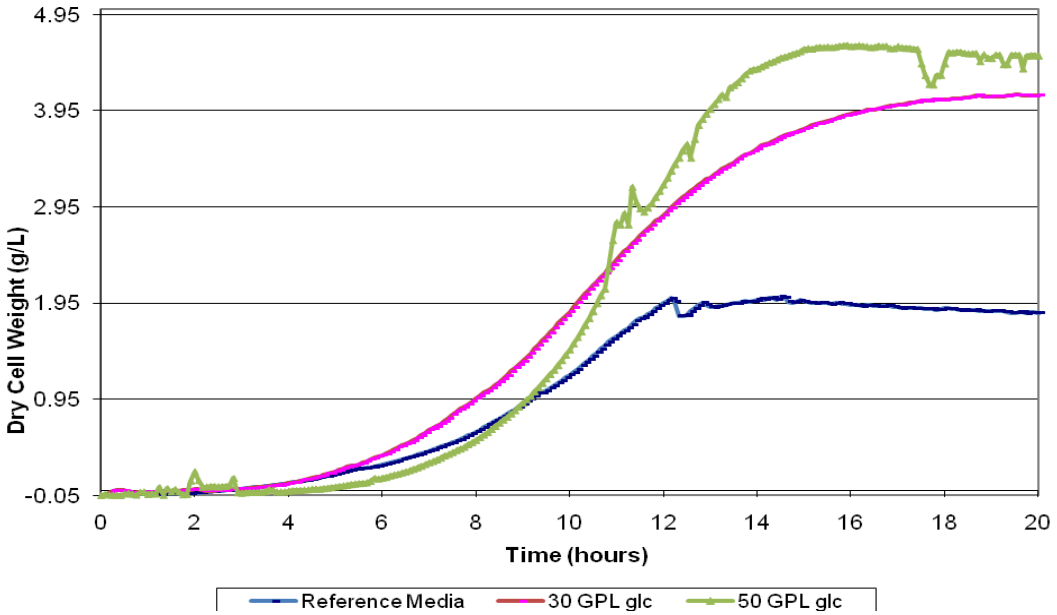


Figure 4.2 Time-Course Plot of Cell Growth in Reference Medium for Increasing Glucose Concentrations. Reference Media is 10 g/L Glucose. Batches are not pH Controlled.

Table 4.1 Reference Media Cell Culture Data Summary.

Glucose Concentration in Media (g/L)	**Maximum Cell Density (g/L)**	**Remaining Glucose Concentration by HPLC (g/L)**
10	2.0	0
30	4.1	20
50	4.6	30

From this plot it can be seen that increasing glucose allows the culture to reach a greater cell density than in the reference culture. As can be expected the cells grown in media containing 50 g/L glucose have a higher growth rate during the exponential phase and they remain in lag phase for a greater amount of time but they reach a greater cell

density overall. Both the 30 g/L and 50 g/L glucose seem to move into stationary phase at approximately the same time indicating that there is either the depletion of a nutritional component limiting further growth or there is an accumulation of a metabolite which inhibits growth. HPLC analysis of broth samples from the end of each fermentation show an excess of glucose remaining in the medium. A comparison plot of a pH controlled fermentation and non controlled fermentation is shown in Figure 4.3. During the pH controlled run there was a disruption of base addition however once the system is brought back under control it recovers and continues to increase in cell density. Figure 4.3 also shows the pH of each batch over time.

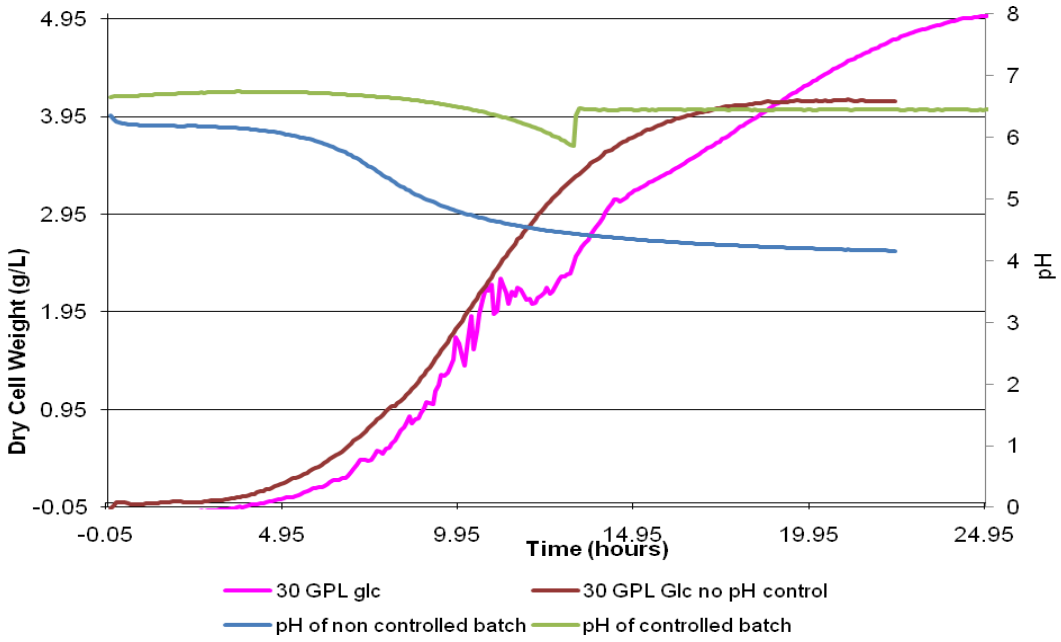


Figure 4.3 Comparison of a pH Controlled Batch Fermentation with a non pH Controlled Batch Fermentation with Overlay of the pH of Each Batch.

4.2. CELL GROWTH ON TEST MEDIA

One of the challenges to using *Bifidobacteria* cells for the GOS transformation reaction is they are a species that is especially difficult to grow on a purely synthetic medium. As expected the cells did not readily grow on the purely synthetic media. For both the minimal media and the modified minimal media the drop in pH could possibly be attributed to the effect of the low pH inoculum however, the modified minimal media which was originally formulated to grow cells in suspension and on solid media did have some turbidity after the incubation period

After adapting the cells to the minimal media an attempt to grow the cells on the two media was made over a 6 day incubation period. The time progression data is shown in Table 4.2.

Table 4.2 Synthetic Media Growth Study Results.

Media	Day	pH	Cell Density (g/L)
Minimal Media RCM	0	7.04	-
	1	6.94	-
	2	6.93	-
	3	6.94	-
	4	6.85	-
	5	6.85	-
	6	6.85	0.005
Modified Minimal Media	0	7.31	-
	1	6.95	-
	2	6.91	-
	3	6.52	0.005
	4	6.16	0.017
	5	5.57	0.124
	6	5.48	0.389

The results of this trial show that this strain of *Bifidobacteria* requires media enhancements such as the bifid factors of yeast extract in order to be able to grow for GOS production.

Bifidobacteria bifidum NCIMB41171 has been successfully cultured using a medium containing yeast extract, meat extract and tryptone. The media used by Goulas et al. were selected as media for enumeration and enzyme production. The media are termed "propagation medium" and "enzyme medium" and their formula can be found in Table 4.3. Cells were adapted to the media by culturing them in enhanced RCM with 10 grams per liter lactose added to the media. A cell bank was started by culturing the cells through two inoculation cycles on propagation medium before being used to seed batch cultures for evaluation. The cells were cultured in a similar fashion as with the reference media. The growth curves for each media are presented in Figure 4.4.

Table 4.3 *B. bifidum* media. Approximate Formula Grams Per Liter (Tzortis et al. 2005).

Media Component	**Propagation**	**Enzyme**
Tryptone	15	7.5
Meat Extract	2.5	7.5
Yeast Extract	7.5	7.5
Potassium phosphate, dibasic	4.5	2
Cysteine Hydrochloride	0.05	0.5
Lactose	2.5	4
Glucose	7.5	6
Tween™ 80	1 mL	0.5 mL

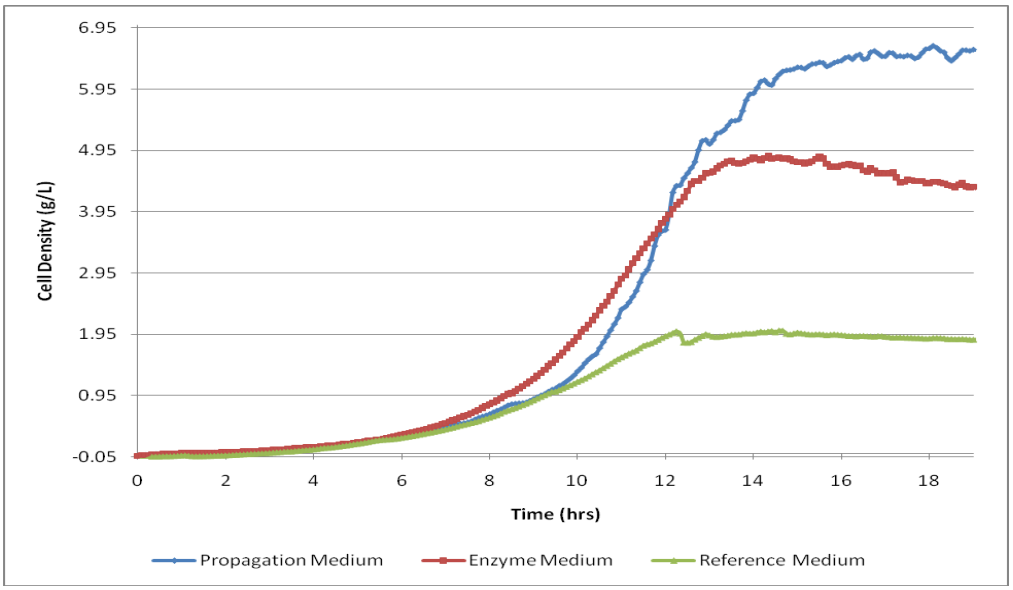


Figure 4.4 Time-Course of Cell Growth in Propagation and Enzyme Media. Reference Media is 10 g/L Glucose. Batches are pH Controlled.

From this plot it can be seen that this strain of Bifidobacteria responds very well to the bifid factors in the media with the same sugar concentration as in the reference medium. The cells grown in propagation medium reach a maximum cell density of 6.5 grams per liter and the cells grown in enzyme cell medium reach a level of 4.8 grams per liter. Both media outperform the RCM at all glucose concentrations.

Using the propagation medium, a study was performed to look at varying the concentration of lactose for growth and how it affected cell growth and enzyme activity. The concentrations selected for study were 10g/L, 30 g/L, 50 g/L, 100 g/L and 250 g/L (1%, 3%, 5%, 10%, and 25%) lactose with glucose concentration constant at 7.5 g/L. For each lactose concentration a set of eight culture tubes were prepared. The initial pH of the medium was not adjusted prior to inoculation and the pH of the cultures was not adjusted during the study. All the tubes were inoculated with 1% inoculum at the same time from the same base culture and incubated at 37°C for the duration of the trial. Samples were taken by removing a tube from the incubator and measuring the turbidity and pH immediately. Figure 4.5 shows the growth curves of each of the lactose concentrations. The results for the study are presented in Table 4.4.

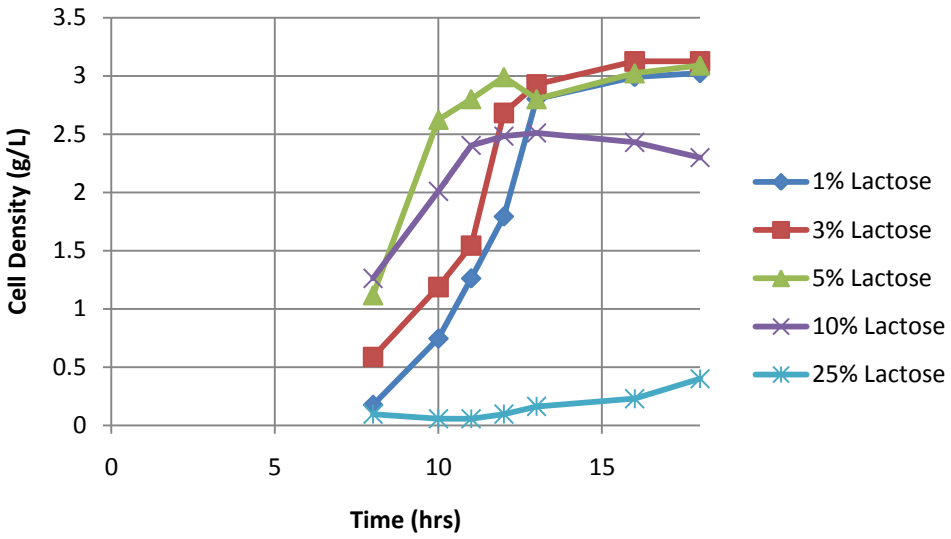


Figure 4.5 Cell growth of *B. bifidum* ATCC 700541 on Varying Concentrations of Lactose in Propagation Medium.

Table 4.4 Lactose in Propagation Media Results.

	1%		3%		5%		10%		25%	
Time (hrs)	pH	Cell Mass (g/L)	pH	Cell Mass (g/L)	pH	Cell Mass (g/L)	pH	Cell Mass (g/L)	pH	Cell Mass (g/L)
0	7.08		6.94		6.88		6.59		6.5	
8	6.85	0.175	6.39	0.586	5.59	1.116	4.99	1.261	6.04	0.096
10	6.38	0.745	5.62	1.187	4.75	2.624	4.75	2.009	6.06	0.056
11	5.54	1.261	5.31	1.543	4.67	2.802	4.62	2.402	6.05	0.056
12	5.13	1.793	4.77	2.682	4.59	2.991	4.5	2.483	5.81	0.096
13	4.74	2.802	4.67	2.927	4.51	2.802	4.54	2.511	5.59	0.162
16	4.56	2.991	4.57	3.124	4.49	3.024	4.47	2.429	5.42	0.228
18	4.54	3.023	4.48	3.124	4.42	3.090	4.41	2.298	5.11	0.401
37									4.38	1.143

Looking at the data it appears that cells grown on 1%, 3% and 5% reach a maximum cell density of approximately 3 g/L and a minimum pH of 4.4 – 4.5. The initial growth rate increases as the lactose concentration increases until the concentration exceeds 10% then it appears that the cell growth is inhibited by the concentration of sugar in the medium.

4.3. CELLULAR BETA GALACTOSIDASE ACTIVITY

In order to determine if *Bifidobacteria bifidum* ATCC 700541 is an economically viable option for producing GOS, the amount of β-galactosidase activity by the cells is determined by an endpoint colorimetric assay. The cells were separated from the fermentation broth by centrifugation and resuspended in phosphate buffer at approximately 1mg/mL cell density. The activity of the cells cultured on propagation medium was found to be 10.94 EU/gram/min. The activity of the cells cultured on enzyme medium was found to be 23.61 EU/gram/min. For comparison *Bifidobacteria bifidum* NCIMB 41171 cells were tested and found to have activity levels of ~400 EU/gram/min. The time point data from the 1% and 3% samples in the lactose growth study were tested for β-galactosidase activity. As can be seen in Figure 4.6 in the 1% samples β-galactosidase activity increases with time whereas in the 3% samples the activity remains fairly constant.

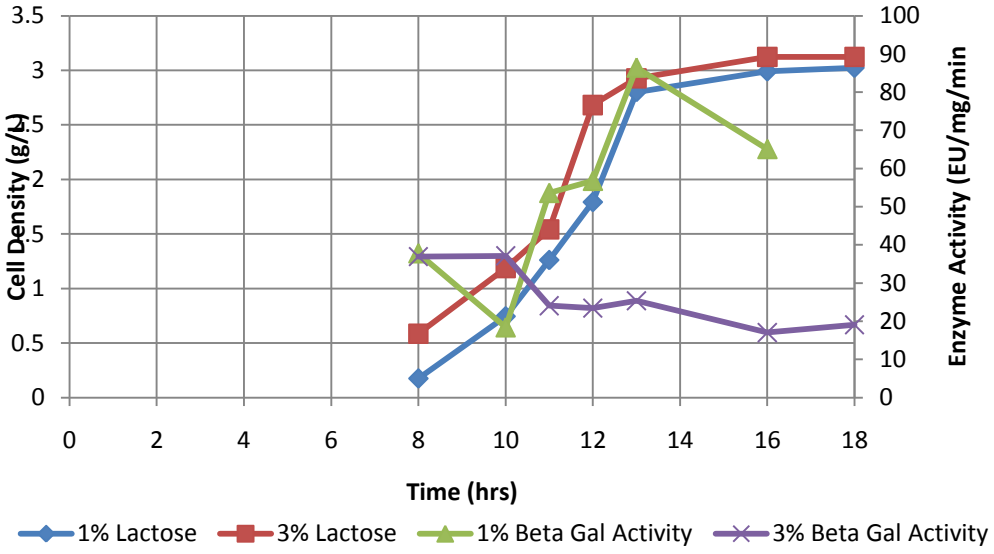


Figure 4.6 β-Galactosidase Activity and Cell Density of *B. bifidum* ATCC 700541 Cultured With 1% and 3% Lactose in Propagation Medium.

4.4. CELLULAR ALPHA-GALACTOSIDASE ACTIVITY

Bifidobacteria which have α-galactosidase activity make it possible for the cells to utilize alpha-linked sugars and if the α-galactosidase has transgalactosylation capabilities the organism can be used to make alpha-linked GOS molecules from lactose. To determine if *B. bifidum* ATCC 700541 had α-galactosidase activity the 1% culture from the lactose growth study was tested using the α-galactosidase endpoint assay. Figure 4.7. shows the results of the study.

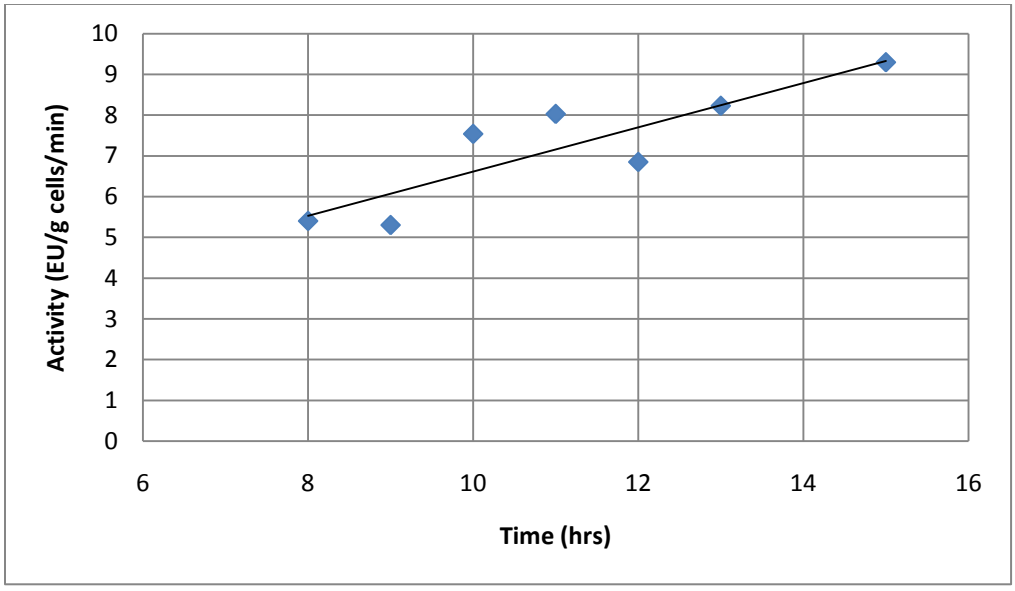


Figure 4.7 α-Galactosidase Activity of *B. bifidum* ATCC 700541 Cultured With 1% Lactose in Propagation Medium.

As was seen with β-galactosidase there is an increase in activity with time. Cells cultured on enzyme medium expressed similar levels of activity as those cultured on propagation medium. The α–galactosidase activity of *B. bifidum* ATCC 700541 was found as 6.37 EU/gram/min.

4.5. UNBOUND ENZYMEACTIVITY

In order to compare the economics of making GOS using *Bifidobacteria bifidum* a baseline for comparison is established by determining the rates for the unbound enzymes. The enzyme activities for both galactosidases were determined experimentally by reacting a constant enzyme concentration with varying substrate concentrations. Michaelis–Menten kinetics (9) were used as the model and plotted using an Eadie–Hofstee diagram to determine Vmax (10) and Km (11). Figure 4.8 and Figure 4.9 show the saturation curves for α-galactosidase and β –galactosidase respectively and Figure 4.10 and Figure 4.11 are the Eadie-Hofstee diagrams for α-galactosidase and β –galactosidase respectively.

(9)

(10)

(11)

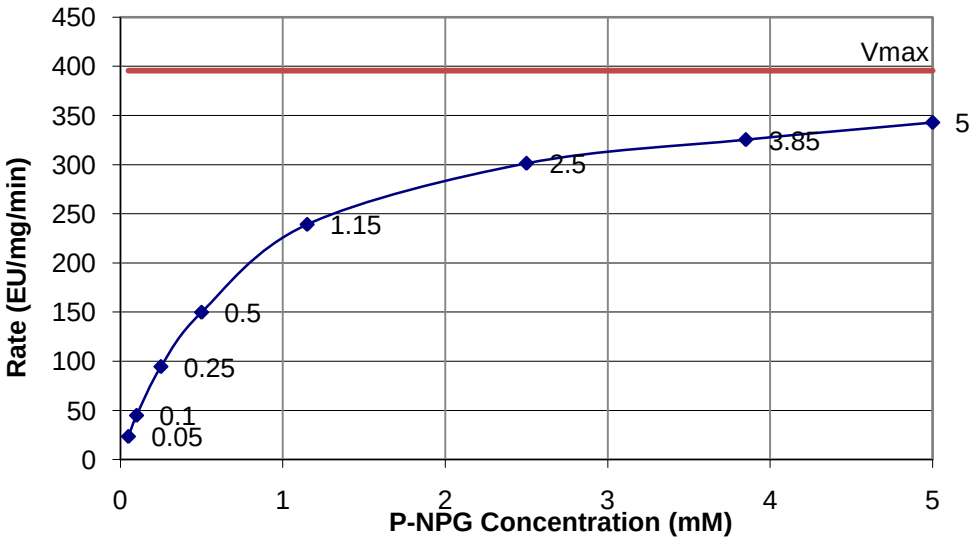


Figure 4.8 α-Galactosidase Saturation Curve.

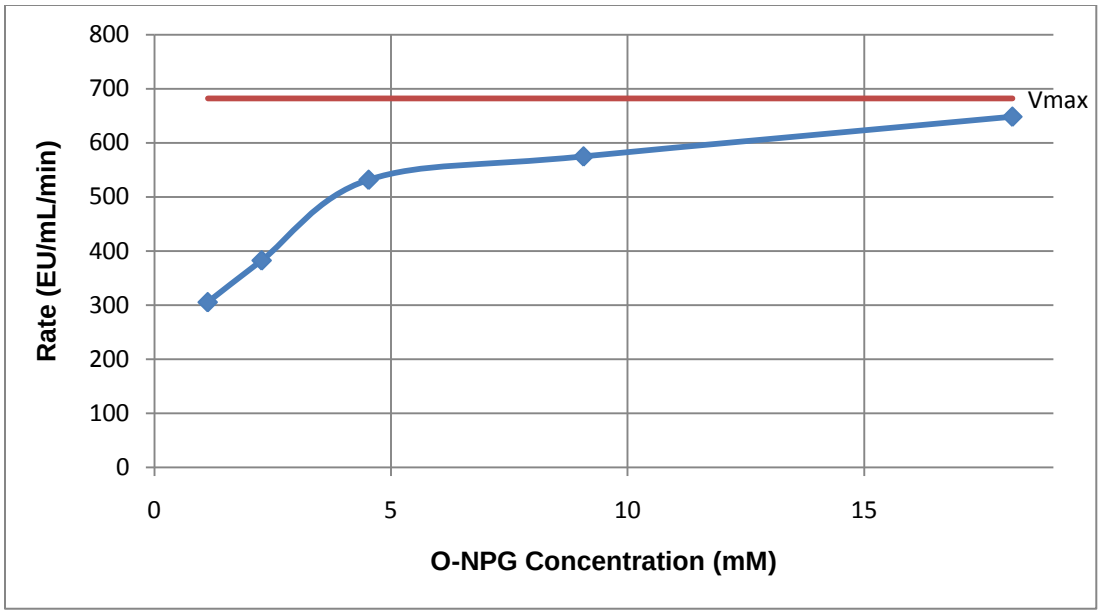


Figure 4.9 β-Galactosidase Saturation Curve.

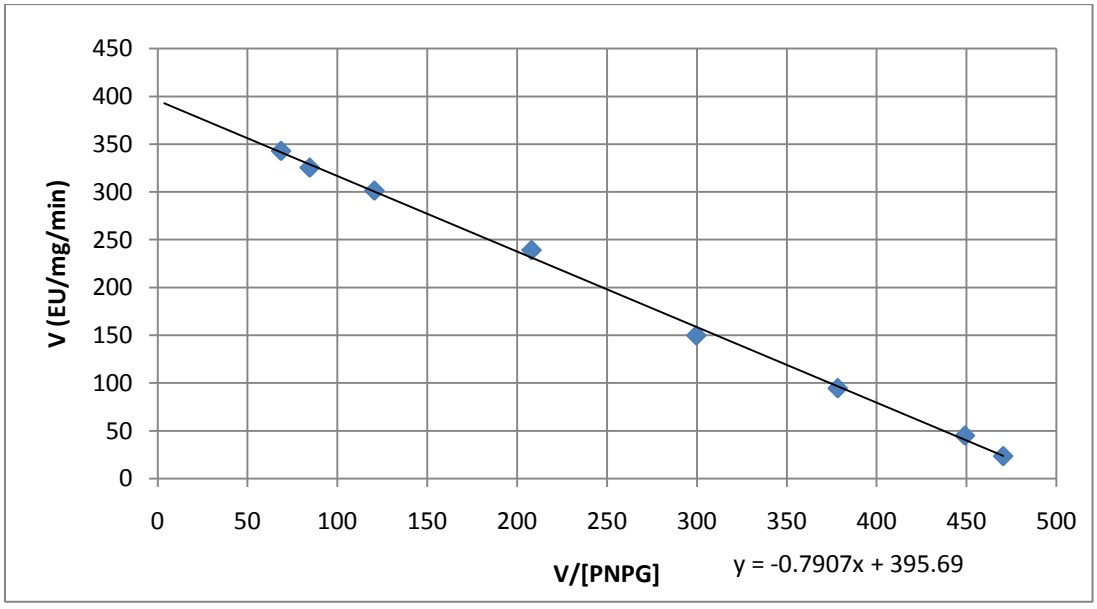


Figure 4.10 Eadie–Hofstee Diagram for α-Galactosidase at 27°C.

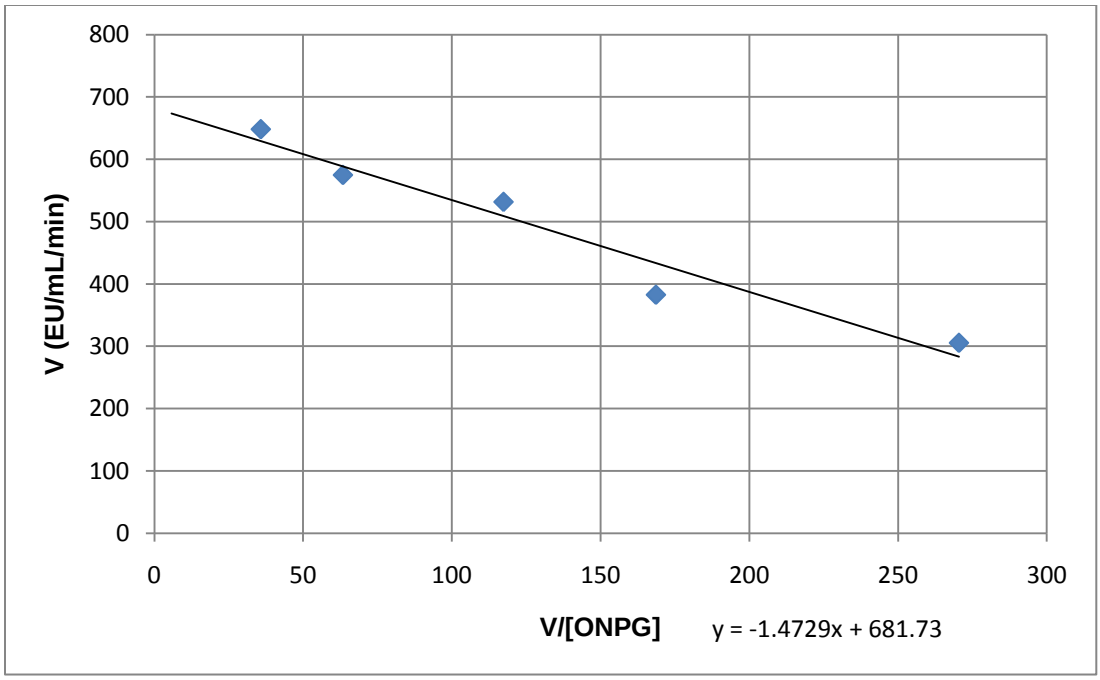


Figure 4.11 Eadie–Hofstee Diagram for β -Galactosidase at 37°C.

From these plots, it is determined that Vmax is 395.69 EU/mg/min and Km is 0.7907 mM for α-galactosidase and Vmax is 681.73 EU/mL/min and Km is 1.4729mM for β –galactosidase. As has been determined, temperature and pH influence the rate of reaction. The GOS production reaction is carried out at 40°C, pH 6.5 – 7.2. The cellular bound enzymes and the unbound β –galactosidase enzyme kinetics were determined using these conditions however the α-galactosidase kinetics were determined at a much lower temperature. The α-galactosidase is not the primary enzyme of interest however it is important to see how temperature and pH will affect the reaction rate. Figure 4.12. shows the relationship of temperature and pH on enzyme reaction rate for α-galactosidase. It is clear from this graph that enzyme activity increases with temperature and pH has an optimum at pH 6.8 – 7.0. At 40°C the enzyme activity is 2.5 times the activity at 25°C.

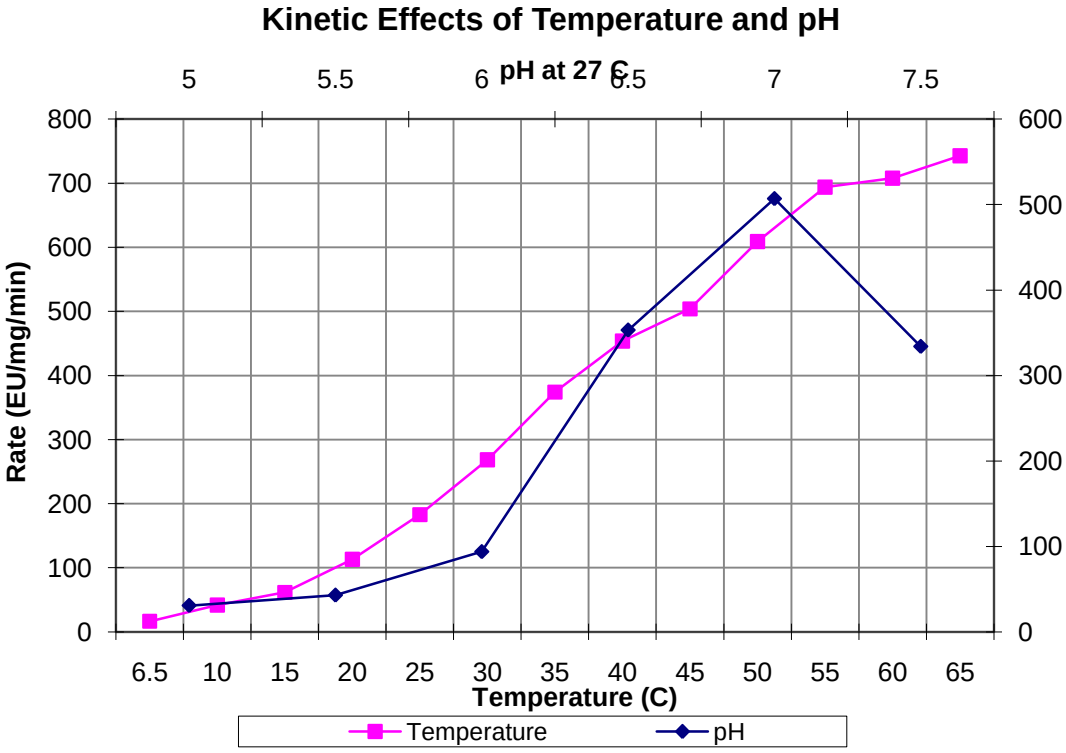


Figure 4.12 Effects of pH and Temperature on α-Galactosidase at 0.01mg protein/mL and 1mM PNPG.

4.6. ECONOMIC ANALYSIS

The costs for batch production of GOS by *B. bifidum* ATCC 700541 was calculated using the β-galactosidase activity found in this study. Tzortzis et al. describe a GOS process using 40% lactose in 100mM phosphate buffer with enough cell mass to provide 850 EU in 100 mL of solution. The cells are recycled and used eight times with 10% loss of activity after each synthesis batch. This process yields 80-85% lactose conversion with a GOS yield of 28 - 30%. These conditions provide the basis for calculation of GOS production. Using the yield numbers from whey permeate reported by Tzortzis a reactor needs to be batched with 8,600 U β-galactosidase /L to produce 130 - 150 g/L GOS. Dosing the reaction at this level the α-galactosidase levels in the mixture are 7000 EU / 100L. The calculations include the potential for recycle of the cells with a

loss ratio of 15% per cycle. Table 4.5 details the costs for GOS production using *B. bifidum* ATCC 700541 and Table 4.6 details the costs for GOS production using unbound enzymes. Prices for raw materials were obtained from common distributers of chemicals and media components.

Table 4.5 Cost Details for Cell Bound Enzymes.

Media	Cell Density (g/L)	Liters / kg Cells	Media Cost ($/100L)	Cost($/kg cells)
Propagation	6.5	154	$ 402.30	$ 618.92
Enzyme	4.5	222	$ 398.32	$ 885.16

Cost Without Cell Reuse	β –gal U/kg	β –gal U/100L GOS Batch	kg cells/100L GOS Batch	Cost ($/100L GOS Batch)
Propagation	1.1×10^4	8.6×10^5	78.2	$48,400
Enzyme	2.36×10^4	8.6×10^5	36.4	$32,220
Cost With Cell Reuse	kg cells/100L GOS Batch	Cost ($/100L GOS Batch)	α –gal U/kg	α –gal U/100L GOS Batch
Propagation	78.2	$12,100	-	-
Enzyme	36.4	$8,055	6.37×10^3	2.32×10^5

Table 4.6 Cost Details for Unbound Enzymes.

Enzyme	Activity per Unit	Units per 100L GOS Batch	Cost ($/Unit)	Cost($/100L GOS Batch)
β –galactosidase	681	1263	$28	$35,364
α –galactosidase	395	19	$17	$325

5. DISCUSSION

The ability to regulate human health through manipulation of the intestinal microbiota has become an area of intense study. A particular area of study is the use of prebiotics to selectively promote the growth of beneficial bacteria which are indigenous to the lower intestines. Specifically, the use of galactooligosaccharides (GOS) has been used to target species of *Bifidobacteria.*

Synthesis of GOS using β –galactosidase is presently the most feasible industrial synthesis method due to the availability and cost of the starting material, lactose rich cheese whey. β –galactosidase hydrolyzes lactose and transfers galactose to another carbohydrate unit or water. GOS are formed when galactose are transferred to another carbohydrate. Transfer to a water molecule results in complete hydrolysis and monomer sugar units (Pazur 1954; Juers et al. 2001). It has been shown that in order to increase the transgalactosylation reaction a high concentration of lactose must be present in solution so that lactose is the preferred receptor for the galactose (Hsu et al. 2007).

GOS synthesis can be carried out using cellular bound enzymes or purified enzymes. Research indicates that *Bifidobacteria* in the lower intestine show a preference for GOS synthesized using enzymes from a homologous source (Rabiu et al. 2001). Work done by Tzortis et al. show that *Bifidobacterium bifidum* NCIMB 41171 produces a GOS mixture that promotes increased levels of indigenous *Bifidobacteria* growth as well as increases in short chain fatty acid production. The strain exhibited both β –galactosidase and α –galactosidase which produced a unique mixture of GOS (Tzortis et al. 2005). We investigated *B. bifidum* ATCC 700541 using substrate analogs to determine if it had similar enzyme activity characteristics. The findings were then compared to the activity levels of purified enzymes from *E. coli* and *A. niger* to determine the economic feasibility of further investigating *B. bifidum* ATCC 700541 as a GOS producing organism.

The most cost effective media for cell culture would be a simplified media which contained no complex vitamins, amino acids or protein fractions to enhance growth. Previous work shows that *Bifidobacteria* are difficult to culture on minimal media however, each strain of *Bifidobacterium* behaves differently under the same culture

conditions. Results from our study show that *B. bifidum* ATCC 700541 does not grow or has limited growth using minimal media. In an experiment performed over 8 days cultures showed indications of growth on the 7^{th} and 8^{th} days of the experiment. Growth was significantly less than 1 g/L which would not be cost effective considering the volume that would be necessary to attain the number of cells that would be necessary for GOS production.

Most *Bifidobacteria* require growth promoting factors termed "bifid" factors to enhance growth. These factors include milk casein fractions, yeast extract, and complex amino acid mixtures. Two media used by Tzortis et al. were selected to culture the cells. *B. bifidum* ATCC 700541 responded very well to both the culture media. Each medium contained different levels of yeast extract, meat extract and tryptone. The media were termed "propagation medium" which yielded 6.5 g/L cell mass and 10.94 EU/g cells β – galactosidase activity and "enzyme medium" which yielded 4.5 g/L cell mass and 23.6 EU/g cells β –galactosidase activity. The level of cell mass when compared to *B. bifidum* NCIMB 41171 are in the same range or possibly higher; however, the level of β – galactosidase activity is much lower.

Work by Astapovich and Ryabaya show a relationship between the types of sugar in the culture medium and growth rate. There has also been work that has shown β – galactosidase activity over time and its association with growth. *B. bifidum* ATCC 700541 was cultured using different levels of both glucose and lactose on a commercially prepared medium as well as the enzyme medium to compare growth on the sugars as well as the enzyme activity during growth. Cells were cultured on the prepared medium with only glucose at 3% and 5% the end samples were analyzed using HPLC. Each culture had differing growth rates but in both cases there was an excess amount of glucose present indicating that the sugar concentration was not the only factor in the increased growth. In the "enzyme" medium, the culture had a glucose level held constant with differing levels of lactose. The levels of lactose in the medium also showed an increase in growth rate with an increase in concentration. Cells grown at 1% and 3% lactose were then tested for enzyme activity and it is clear that at 1% the level of activity increases with growth however at 3% the level remains fairly constant. The cells in the 3% culture

reaches stationary phase sooner than the cells in the 1% medium and this could be a reason for this observation.

The current method for GOS production is to use a purified enzyme extracted from a fungal or bacterial source. The enzymes can only be used a single time since the termination step in the GOS reaction is to heat denature the enzyme. To determine if *B. bifidum* ATCC 700541 is an economically feasible strain to use for GOS conversion the enzyme levels determined are compared to purified enzymes. The activity levels of an α –galactosidase and a β –galactosidase were determined using kinetic assay measurements. Looking at the primary functioning enzyme, β –galactosidase , and comparing the two sources of enzymes, bound and unbound, enzymes bound in *B. bifidum* ATCC 700541 are as cost effective as the unbound enzymes. In order to be an economically viable solution growing the cells needs to be more cost effective than the enzymes. In order for this to be a possibility the costs were compared using data from Tzortis et al. with 15% loss per cycle. Using this assumption in calculation the *B. bifidum* ATCC 700541 becomes a viable option, with further research, as a candidate for GOS conversion. Looking also at the α –galactosidase activity levels between *B. bifidum* ATCC 700541 and *B. bifidum* NCIMB 41171, there is significantly more α –galactosidase present in *B. bifidum* ATCC 700541. The overall effect this would have in the GOS conversion reaction needs to be investigated.

To fully understand the potential of *B. bifidum* ATCC 700541 as an organism for GOS production investigation into several factors is still necessary. Some of the factors to determine viability for production include: determining what media components cause increases in enzyme activity during cell culture and an evaluation of tradeoff between enzyme expression and cell mass, metabolites in the culture media that cause growth inhibition, gaining an understanding of how α –galactosidase activity present in *B. bifidum* ATCC 700541 differs from α –galactosidase activity present in *B. bifidum* NCIMB 41171 and what are the overall effects on the GOS mixture, determining if there is any method of treatment for the cells which would open the cell wall to gain access to β –galactosidase activity inside the cell, and verification of actual loss of activity levels with each GOS production cycle.

6. CONCLUSION

The investigation into the growth characteristics of this organism shows that *B. bifidum* ATCC 700541 cannot be cultured on a medium that does not contain bifid factors. The complex media presented in this work will culture *B. bifidum* ATCC 700541 with β –galactosidase activity. The studies indicate the following:

- Sugar type and concentrations are important for growth and enzyme production in the culture.
- Nitrogen growth components type and concentrations are important for producing a cell culture suitable for GOS production.
- Under non-optimized growth conditions and measured enzyme activity levels using the substrate analog ONPG, *B. bifidum* ATCC 700541 can be considered as an organism for GOS production.
- Optimization work should be done to find the right culture conditions which achieve a balance between cell mass and enzyme activity level to produce a culture economically capable of commercially producing GOS.

APPENDIX A

DATA TABLES FOR STANDARD CURVES

This appendix contains data used to generate standard curves for enzyme activity, dry cell weight determination and sugar analysis by HPLC.

Table A.1 Absorption Data, 415nm for ONP Standard Curve.

[ONP] (mM)	A1	A2	A3	Mean		Standard Dev
1	1.286	1.2622	1.2829	1.27703		0.0129
0.9	1.1733	1.1759	1.1666	1.17193		0.0048
0.8	1.0414	1.0037	1.0389	1.02800		0.0211
0.7	0.88272	0.88213	0.89317	0.88601		0.0062
0.6	0.76014	0.76464	0.76916	0.76465		0.0045
0.5	0.63333	0.6407	0.64392	0.63932		0.0054
0.4	0.51119	0.50887	0.50764	0.50923		0.0018
0.3	0.37982	0.38021	0.37623	0.37875		0.0022
0.2	0.24653	0.24192	0.25914	0.24920		0.0089
0.1	0.12194	0.13044	0.12782	0.12673		0.0044

Table A.2 Absorption Data, 400nm for PNP Standard Curve

[PNP] (mM)	A1	A2	A3	Mean		Standard Dev
0.5	NA	NA	NA	NA		NA
0.4	2.0137	2.0351	2.0104	2.01973		0.01341
0.3	1.5049	1.5431	1.4598	1.50260		0.04170
0.2	0.99768	0.99464	1.0012	0.99784		0.00328
0.1	0.48963	0.4803	0.49336	0.48776		0.00673

Table A.3 Turbidity Data, 910nm for Dry Cell Weight Standard Curve.

Cell Density (g/L)	OD1	OD2	OD3	Mean		Standard Dev
7.5	2.63	2.62	2.62	2.62		0.01
5	2.25	2.25	2.24	2.25		0.01
4	2.05	2.06	2.05	2.05		0.01
3	1.77	1.78	1.78	1.78		0.01
2.5	1.56	1.57	1.58	1.57		0.01
2	1.38	1.37	1.38	1.38		0.01
1.5	1.14	1.15	1.14	1.14		0.01
1	0.78	0.80	0.77	0.78		0.02
0.5	0.44	0.43	0.45	0.44		0.01
0.25	0.21	0.23	0.20	0.21		0.02
0.1	0.11	0.10	0.12	0.11		0.01

Table A.4 Area Data for Lactose HPLC Standard Curve.

Concentration (g/L)	Area 1	Area 2	Area 3	Mean		Standard Dev
10.21	1205.72	1208.413	1209.311	1207.815		1.87
20.42	2353.257	2386.781	2397.956	2379.331		23.26
30.62	3570.181	3600.313	3610.357	3593.617		20.91
40.83	4770.027	4783.453	4787.928	4780.47		9.32
51.04	5997.861	6193.249	6258.378	6149.83		135.58
61.25	6990.694	7018.685	7028.016	7012.465		19.42
71.46	7803.779	7803.939	7803.993	7803.904		0.11
81.67	8409.885	8454.983	8470.016	8444.961		31.29
91.87	8910.779	8928.339	8928.339	8922.486		10.14
102.08	9393.803	9393.803	9465.711	9417.772		41.52

Table A.5 Area Data for Glucose HPLC Standard Curve.

Concentration (g/L)	Area 1	Area 2	Area 3	Mean		Standard Dev
5.13	591.181	592.1505	592.4736	591.935		0.67
10.27	1144.065	1157.854	1162.45	1154.789		9.57
15.4	1728.168	1740.501	1744.611	1737.76		8.56
20.53	2308.597	2312.179	4787.928	3136.235		1430.41
25.67	2917.307	3015.954	2313.373	2748.878		380.37
30.8	3479.81	3493.242	3497.719	3490.257		9.32
35.93	4078.241	4078.707	4080.105	4079.018		0.97
41.06	4607.423	4658.209	4675.137	4646.923		35.24
46.2	5135.346	5139.369	5151.436	5142.05		8.37
51.33	5774.066	5840.219	5862.27	5825.518		45.90

Table A.6 Area Data for Galactose HPLC Standard Curve.

Concentration (g/L)	Area 1	Area 2	Area 3	Mean		Standard Dev
5.3	587.5096	588.1352	588.3437	587.9962		0.43
10.59	1146.574	1160.676	1165.377	1157.542		9.79
15.89	1736.779	1749.775	1754.107	1746.887		9.02
21.18	2324.521	2327.419	2328.385	2326.775		2.01
26.48	2939.6	3038.589	3071.585	3016.591		68.69
31.78	3505.491	3519.35	3523.969	3516.27		9.62
37.07	4108.457	4109.237	4111.578	4109.757		1.62
42.37	4641.737	4687.291	4702.476	4677.168		31.61
47.66	5158.222	5170.575	5174.237	5167.678		8.39
52.96	5814.234	5879.985	5901.901	5865.373		45.62

APPENDIX B

STATISTICAL ANALYSIS FOR STANDARD CURVES

This appendix contains the statistical analysis of the data used to generate standard curves for enzyme activity, dry cell weight determination and sugar analysis by HPLC.

B.1. Statistical Analysis of ONP Standard Curve

The assumptions of constant variance and normality made for ANOVA analysis are verified in Figure B.1 and Figure B.2. The plot of the residuals in Figure B.1shows random and independent distribution of errors. Linearity in Figure B.2 indicates the residuals are normally distributed.

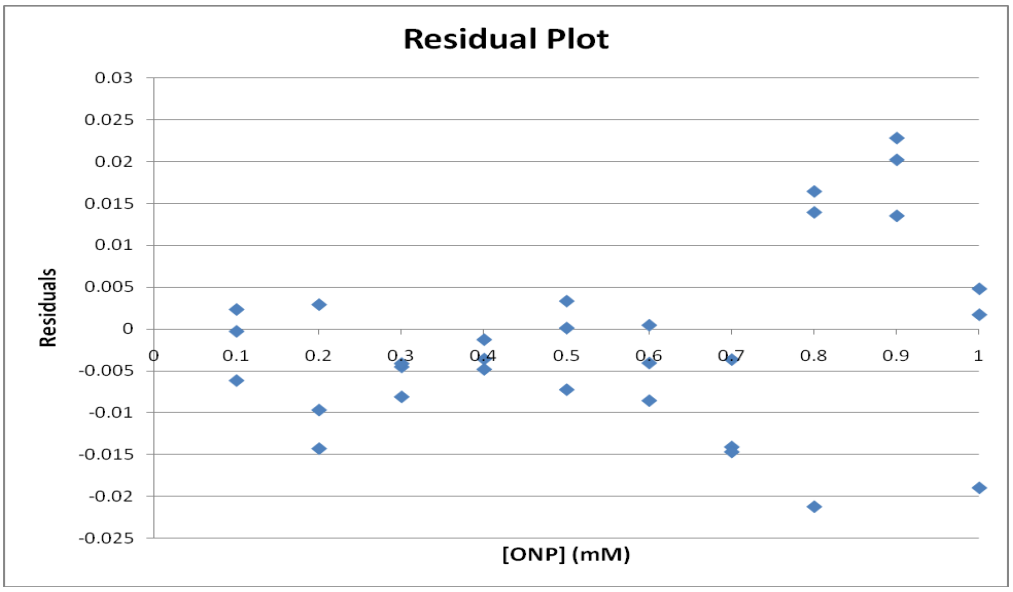


Figure B.1 Plot of Variation of Residuals for Each Concentration of ONP.

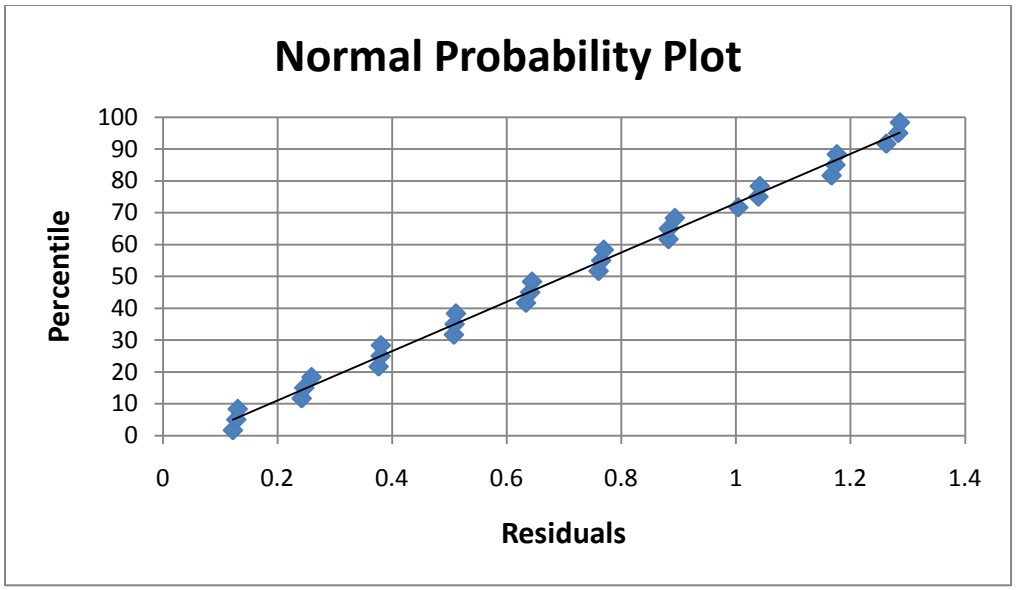


Figure B.2 Normal Probability Plot for ONP Standard Curve.

Table B.1 ANOVA Statistical Analysis Output for ONP Standard Curve.

	Degrees of Freedom	*Sum of Squares*	*Mean Squares*	*F-Value*	*P-value*
Regression	1	18.95929753	18.95929753	157408.5	
Residual	29	0.003492948	0.000120446		
Total	30	18.96279048			

	Coefficients	*Standard Error*	*Lower 95%*	*Upper 95%*
Absorbance 415	1.281209437	0.003229283	1.274604812	1.287814062

Regression Statistics	
Multiple R	0.999907896
R Square	0.9998158
Standard Error	0.010974812

B.2. Statistical Analysis of PNP Standard Curve

The assumptions of constant variance and normality made for ANOVA analysis are verified in Figure B.3 and Figure B.4. The plot of the residuals in Figure B.3 shows random and independent distribution of errors. Linearity in Figure B.4 indicates the residuals are normally distributed.

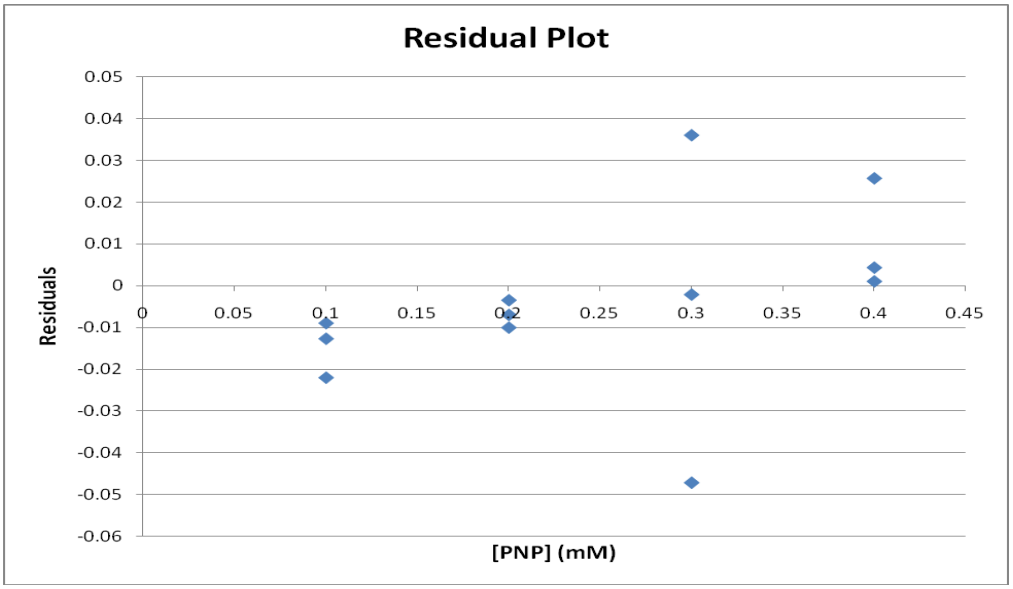


Figure B.3 Plot of Variation of Residuals for Each Concentration of PNP.

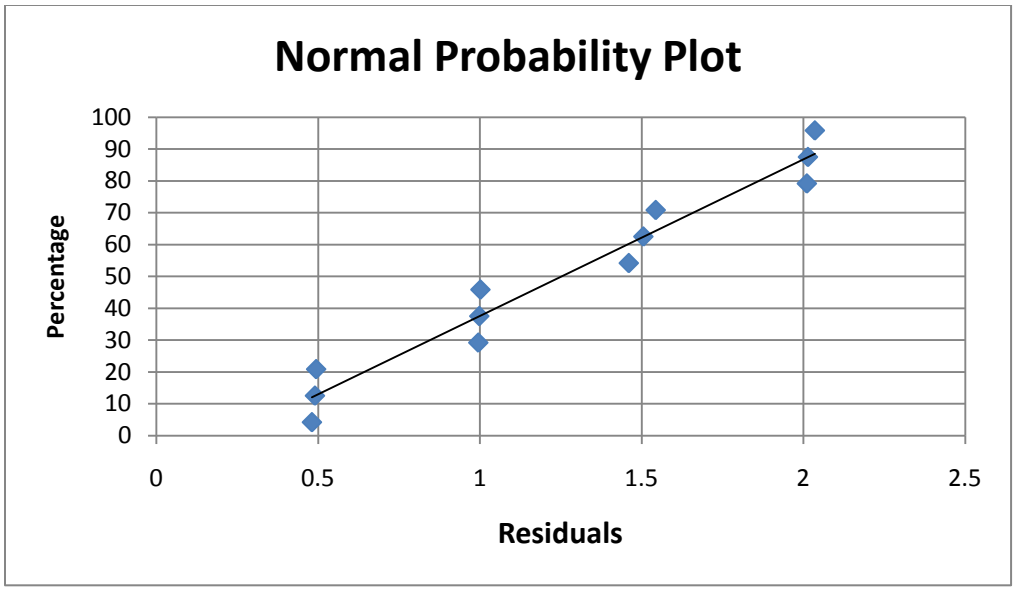


Figure B.4 Normal Probability Plot for PNP Standard Curve.

Table B.2 ANOVA Statistical Analysis Output for PNP Standard Curve.

	Degrees of Freedom	*Sum of Squares*	*Mean Squares*	*F-Value*	*P-value*
Regression	1	22.71102	22.71102	48904.85	<<0.00001
Residual	11	0.005108	0.000464		
Total	12	22.71613			

	Coefficients	*Standard Error*	*Lower 95%*	*Upper 95%*
Absorbance 400	5.023392222	0.022715438	4.973395881	5.073388564

Regression Statistics	
Multiple R	0.999887556
R Square	0.999775124
Standard Error	0.021549756

B.3. Statistical Analysis of Dry Cell Weight Standard Curve

The assumptions of constant variance and normality made for ANOVA analysis are verified in Figure B.3 and Figure B.4. The plot of the residuals in Figure B.3 shows random and independent distribution of errors. Linearity in Figure B.4 indicates the residuals are normally distributed.

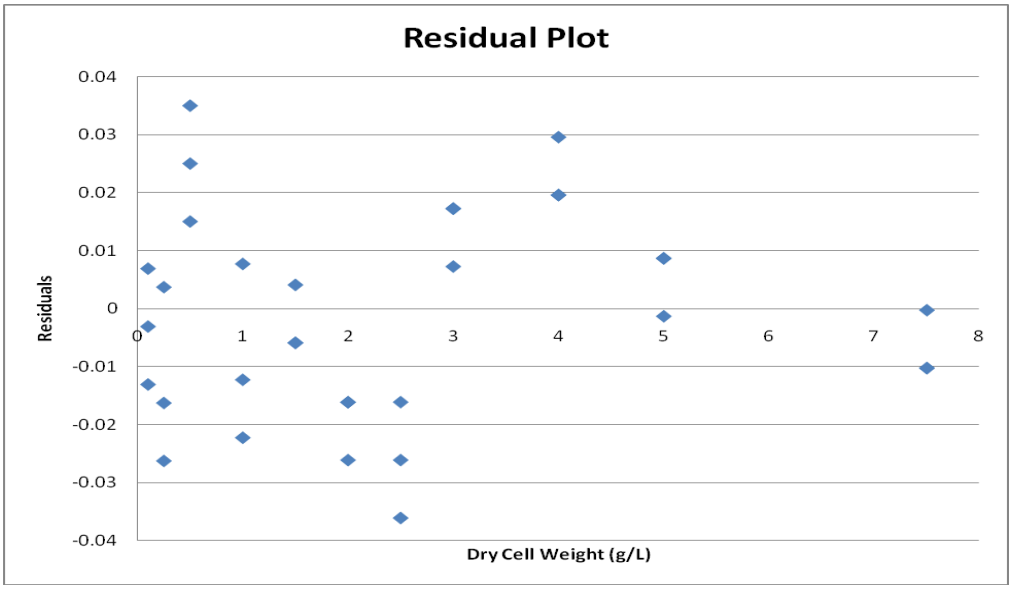


Figure B.5 Plot of Variation of Residuals for each Cell Density.

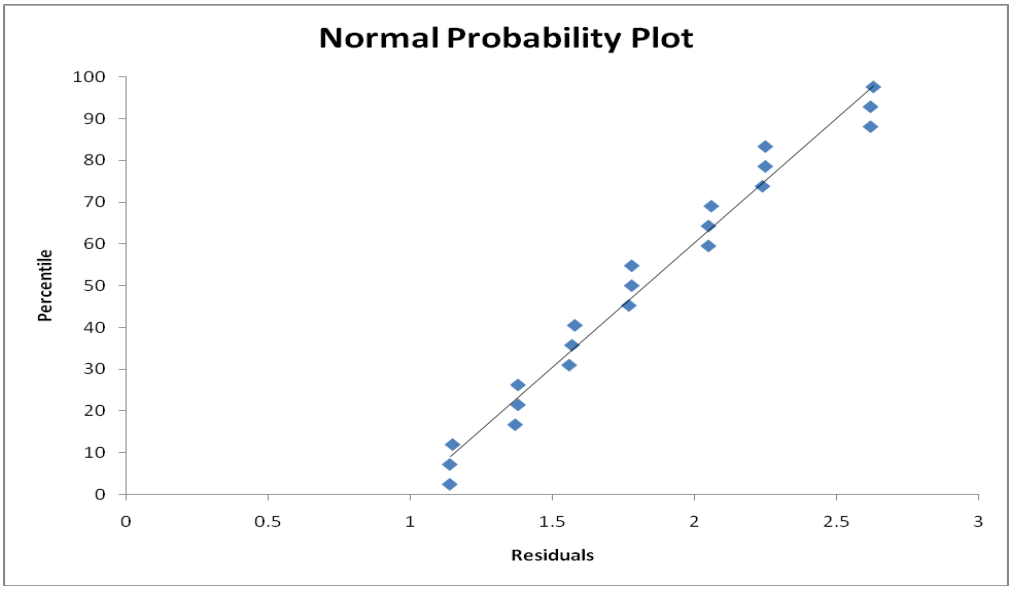


Figure B.6 Normal Residual Plot for Exponential Fit Dry Cell Weight Standard Curve.

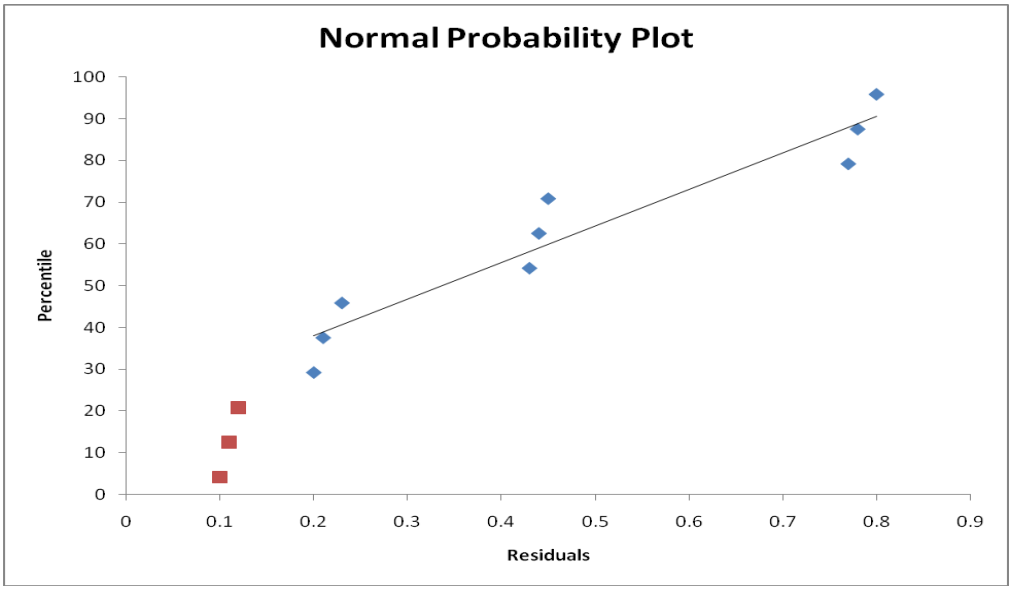


Figure B.7 Normal Residual Plot for Linear Fit Dry Cell Weight Standard Curve.

Table B.3 ANOVA Statistical Analysis Output for Dry Cell Weight Standard Curve (Linear).

	Degrees of Freedom	*Sum of Squares*	*Mean Squares*	*F-Value*	*P-value*
Model	1	2.5858120	2.58581	2847.803	<.0001
Residual	11	0.0099880	0.00091		
Total	12	2.5958000			

Regression Statistics	
R Square	0.999004
Standard Error	0.114113

Table B.4 ANOVA Statistical Analysis Output for Dry Cell Weight Standard Curve (Exponential).

	Degrees of Freedom	*Sum of Squares*	*Mean Squares*	*F-Value*	*P-value*
Model	1	248.19015	248.190	19059.70	<.0001
Residual	19	0.24741	0.013		
Total	20	248.43756			

Regression Statistics	
R Square	0.995026
Standard Error	0.019969

B.4. Statistical Analysis of HPLC Standard Curves

The assumptions of constant variance and normality made for ANOVA analysis are verified in Figure B.8, Figure B.9, and Figure B.10. The plots of the residuals in. Figure B.8, Figure B.9 and Figure B.10 show random and independent distribution of errors. Linearity in Figure B.11, Figure B.12, and Figure B.13 indicates the residuals are normally distributed.

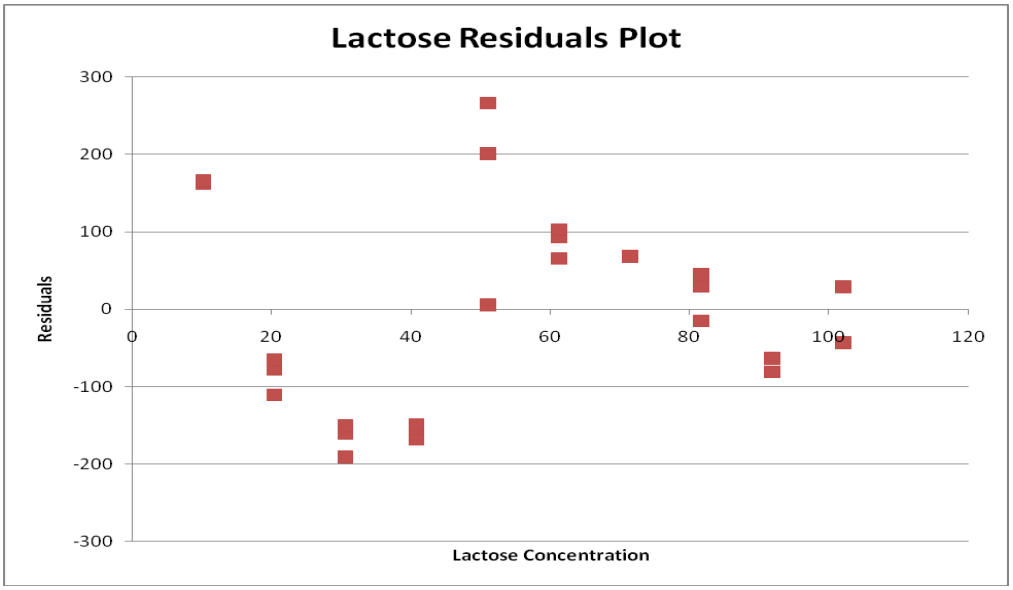


Figure B.8 Plot of Variation of Residuals for Each Concentration of Lactose.

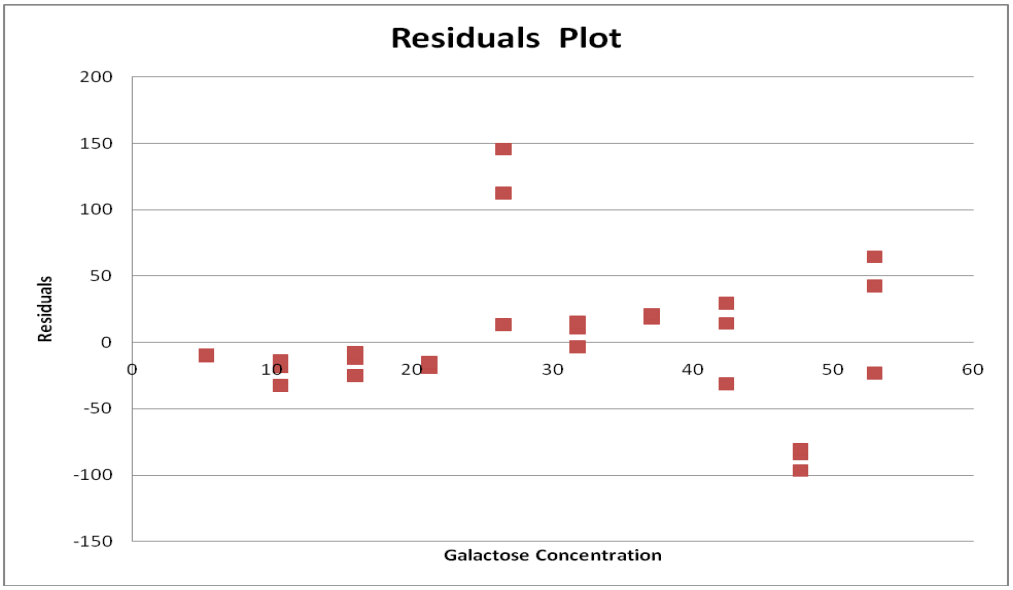


Figure B.9 Plot of Variation of Residuals for Each Concentration of Galactose.

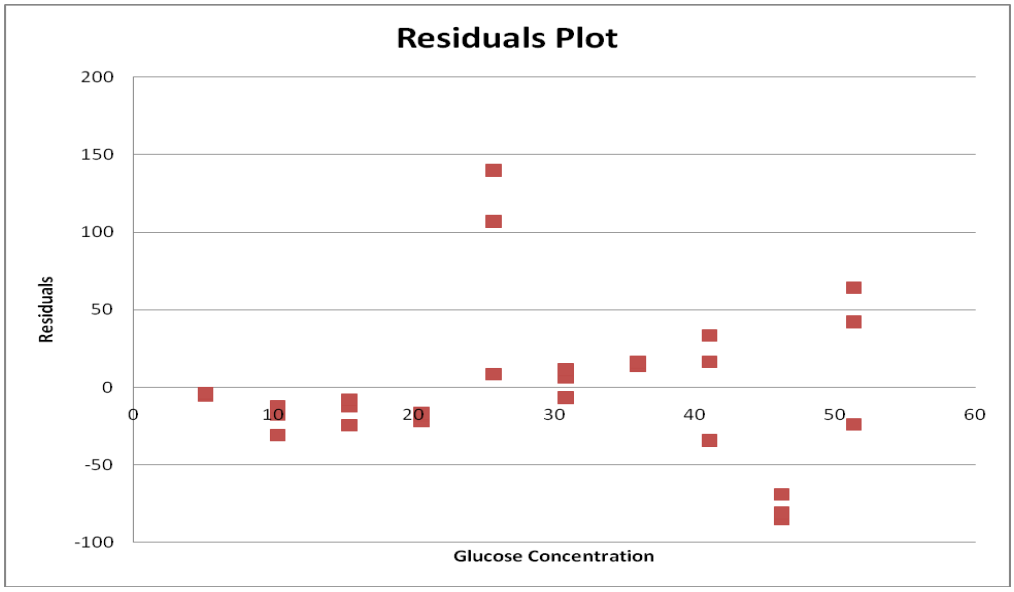


Figure B.10 Plot of Variation of Residuals for Each Concentration of Glucose.

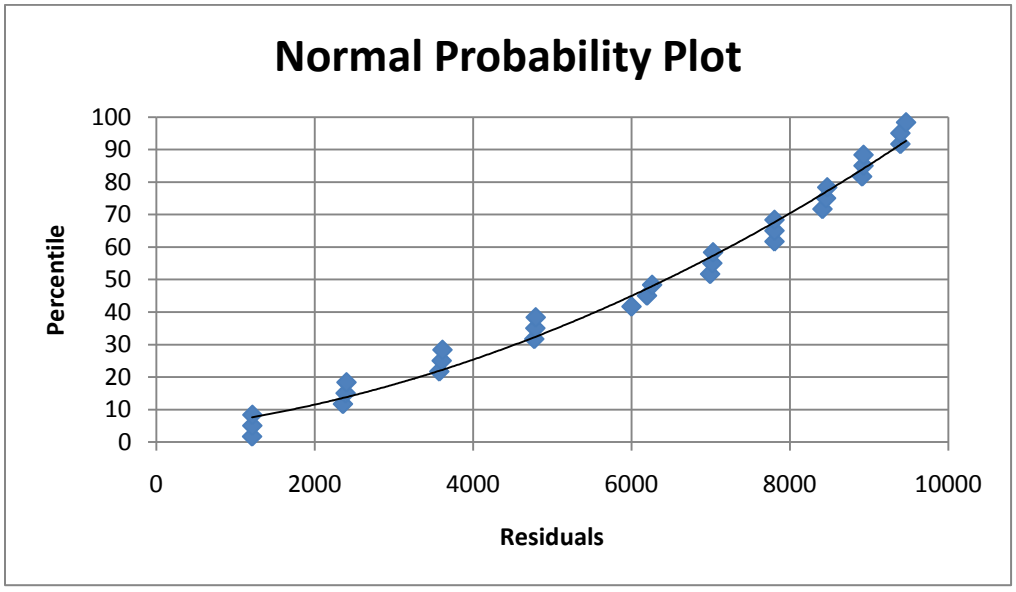


Figure B.11 Normal Residual Plot for Lactose.

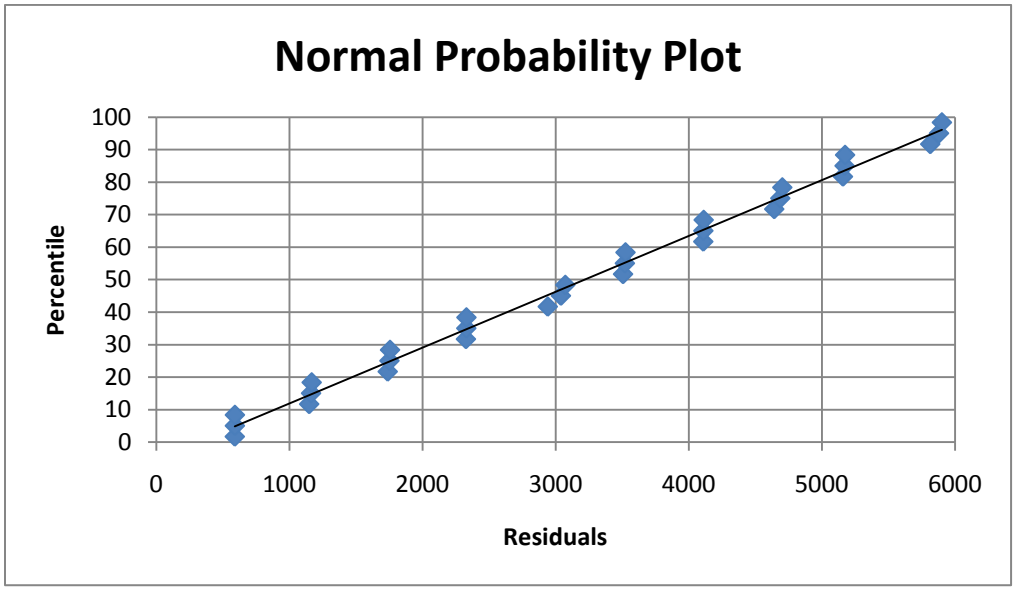


Figure B.12 Normal Probability Plot for Galactose.

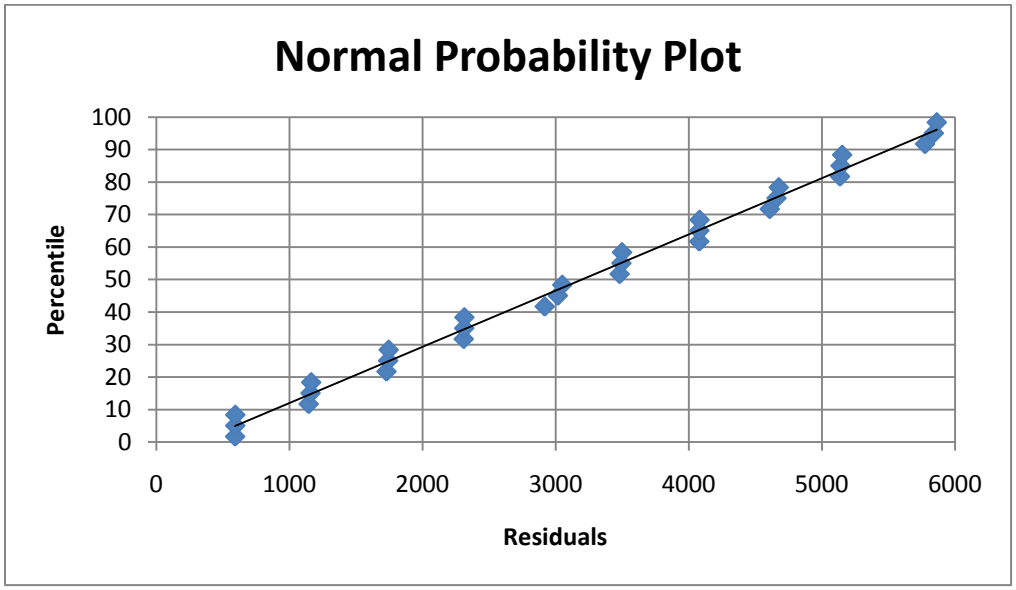


Figure B.13 Normal Probability Plot for Glucose.

Table B.5 ANOVA Statistical Analysis Output for Galactose, Glucose and Lactose.

	Degrees of Freedom	*Sum of Squares*	*Mean Squares*	*F-Value*	*P-value*
Lactose					
Model	2	221148151	110574076	6886.007	<.0001
Residual	27	433560	16057.793		
Total	29	221581712			
Glucose					
Model	1	82665096	82665096	36767.46	<.0001
Residual	28	62953	2248.3221		
Total	29	82728049			
Galactose					
Model	1	83899719	83899719	33356.97	<.0001
Residual	28	70426	2515.2082		
Total	29	83970145			

Regression Statistics			
	Lactose	**Glucose**	**Galactose**
R Square	0.998043	0.999239	0.999161
Standard Error	126.719	47.416	50.152

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