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Enzymatic Synthesis of Galactooligosaccharides From Whey Permeate

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Enzymatic synthesis of Galactooligosaccharides from Whey Permeate

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ABSTRACT

Galactooligosaccharides (GOS) are prebiotics that have a beneficial effect on human health by promoting the growth of probiotic bacteria in the gut. GOS are commonly produced from lactose in a reaction catalysed by β-galactosidase, termed transglycosylation.

In the present work the synthesis of GOS from Whey Permeate (WP) using commercially available β-galactosidases was studied. The enzymes used were from *Kluyveromyces lactis* (Maxilact® L2000) and *Escherichia coli*.

Initially, a novel quantitative TLC-based assay to monitor GOS synthesis was developed. This method was employed for kinetic analysis but precision and bias problems in quantification were observed. An HPLC assay was subsequently developed and used to quantitate the kinetics of GOS synthesis.

The influence of substrate concentrations of WP and enzyme concentrations were examined. The reaction kinetics showed an exponential consumption of lactose, while the GOS reached a maximum level and decreased thereafter. The data showed that the enzyme and WP concentrations influenced the maximum level of GOS synthesis. The maximum yield of GOS from WP was found to be 24%.

Modelling of GOS synthesis profiles using a full reaction mechanism (Kim *et al*., 2004) fitted the experimental data. However, high correlation between kinetic parameters and high standard errors in parameter estimates were found. Therefore, a simplified GOS synthesis mechanism based on simplifying assumptions previously identified in literature was devised. This reduced model fitted data appropriately and parameter estimation and associated uncertainty was improved.

The influence of low amounts of organic solvents on GOS synthesis was examined. The progress curve in the presence of solvents was probed using the reduced reaction mechanism model.

To examine the influence of the source of enzyme on GOS synthesis, two β-galactosidases were compared. Data showed that when reaction conditions were identical there was no significant difference in GOS synthesis observed.

These studies show Whey Permeate is a useful material for GOS synthesis. They confirm the literature observations that enzyme and substrate concentrations strongly influence GOS yields. The use of organic solvents was found to modify the reaction kinetics, with promising applications to increase GOS yield. However, the source of enzyme may not influence GOS synthesis to the extent believed in the literature.

Keywords: Galactooligosaccharides, β-galactosidase, Whey Permeate.

DECLARATION

I certify that this thesis which I now submit for examination for the award of MPhil, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for another award in any Institute.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

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Signature _____

Date _____

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List of Abbreviations

ACN	Acetonitrile
Allo	Allolactose
ATP	Adenosine-5-Triphosphate
a_w	Water Activity
BLAST	Basic Logical Alignment Search Tour
BOD	Biochemical Oxygen Demand
β-gal	β-galactosidase
c.f.u.	Colony forming units
CI	Confidence Interval
COD	Chemical Oxygen Demand
dl	Decilitre
DC	Degree of conversion
DLSODA	Double precision Livermore Solver for Ordinary Differential Equations
DM	Dried Matter
DODRC	Double precision Orthogonal Distance Regression
DP	Degrees of Polymerization
E	Enzyme
E:Gal	Enzyme:Galactose complex
E:Lac	Enzyme:Lactose complex
EC	European Community
EU	European Union
EPA	Environmental Protection Agency
EtOH	Ethanol
FAO	Food and Agriculture Organization
FCC	Food Chemical Codex
FE	Free Enzyme
FOS	Fructooligosaccharides
FOSHU	Foods for Specified Health Use
FUFOSE	Functional Food Science in Europe
Gal	Galactose
Gal_{tx}	Galactose concentration at a certain reaction time
Glc	Glucose
Glc_{tx}	Glucose concentration at a certain reaction time
GLL	Galactosyl-Lactose
GOS	Galactooligosaccharides
GOS_{tx}	Galactooligosaccharides concentration at a certain reaction time
GRAS	Generally Recognized As Safe
HPLC	High Performance Liquid Chromatography
I.D.	Internal Diameter
IE	Immobilised Enzyme
ILSI	International Life Science Institute
JECFA	Joint Expert Committee of Food Additives
LAB	Lactic Acid Bacteria
Lac	Lactose

Lac_0	Initial lactose concentration
Lac_{tx}	Lactose concentration at a certain reaction time
Mlt	Maltotriose
MRSA	Methicillin-Resistant *Staphilococcus aureus*
NDOs	Non Digestible Oligosaccharides
ODE	Ordinary Differential Equations
ODEPACK	Ordinary Differential Equation Systems
ODRPACK	Orthogonal Distant Regression System
RI	Refractive Index
R.P.M.	Rotation Per Minute
RSM	Residual sum of squares of the weighted residuals
STR	Stirred Tank Reactor
SCF	Scientific Committee of Food
SCFA	Short-Chain Fatty Acids
T	Temperature
TD	Transgalactosylated Disaccharides
TLC	Thin Layer Chromatography
TOS	Trans-Galactooligosaccharides
UFMR	Ultra Filtrate Membrane Reactor
US	United States of America
vol	Volume
WHO	World Health Organization
WP	Whey Permeate
WPC	Whey Protein Concentrate
WPI	Whey Protein Isolate

Table of Contents

List of Figures

List of Tables

1 Introduction

1.1 General

The dairy industry is one of the oldest and most developed industries in the world. In particular, the European dairy industry is transforming 130 billion litres of raw milk every year into a broad range of products, both for consumption and for application in the production of food, feed and pharmaceutical products (Hilliam, 1990).

The dairy industry produces a large quantity of by-products, which requires that particular attention is paid to their disposal due to the dissolved sugars, proteins, fats, and residues of additives, contained in the effluents. According to the World Bank Group (1996), in the untreated effluents of the dairy industry, the biochemical oxygen demand (BOD) has an average value ranging from 0.8-2.5 kg/t. The effluent chemical oxygen demand (COD) is normally about 1.5 times the BOD level and the total suspended solids are about 100 to 1,000 mg/l, of which phosphorus comprises 10 to 100 mg/l and nitrogen 6% of BOD level.

The major source of BOD in wastewater of dairy industries derives from the production processes for butter, cream, and cheese. The latter gives rise to whey as a by-product. Annual global milk production in 2007 is estimated of over 534 thousands of metric tons, whose transformation to cheese gave up to two thousand of metric tons of whey (Commodity Research of Bureau, 2007). Whey accounts for most of the BOD, between 38,000-40,000 ppm (Bullerman *et al*., 1966), and dissolved salts of dairy industries wastewater. Considering the 3% annual increase in cheese production (Foda *et al*., 2000) whey surplus is a major and increasing concern for the dairy industry.

However, most of the solid waste of the dairy industry can be further utilized or processed. A common use for surplus whey is its addition to animal feed, especially in America, with 90% of American production used (Wastendorf, 2000). Whey has been also been used as a fertilizer, because it improves soil texture and contains nutrients, such as nitrogen, phosphorus, and potassium in the proper proportions (Yang *et al*., 1995). Whey needs to be treated before it can be used as fertilizer. In the past many industries discharged whey into lakes and rivers to remove the economic burden of disposing of whey in waste treatment facilities. In recent years, the Environmental Protection Agency (EPA) has placed restrictions on land-spreading as a method for whey disposal. This serves has served as an important incentive to find other uses for whey (Casper *et al*., 1999). In some European countries, such as Italy, France, Greek and Spain, whey almost entirely used for the production of a typical soft unreaped cheese, called ricotta, with a production of over 450 tons (Fox, 1999).

1.1.2 Whey: definition, components and their use

Whey is defined as the greenish-yellow coloured liquid obtained after the coagulation of casein (Stocking, 2008). It is produced from the process that leads to curds formation during the cheese making process (Smithers *et al*., 1996) (Figure 1.0).

Figure 1.0: Curds and whey. Milk proteins are precipitated leaving a yellowish liquid (whey).

Whey contains nearly half of all the solids found in whole milk (Chandan, 1997). It has about 6.5% solids, of which 4.8% is lactose, 0.6% protein, 0.15% lactic acid, 0.25% non-protein nitrogen compounds and 0.1% fat (Ranken *et al*., 1997).

Lactose is a disaccharide composed of β-D-Galactopyranosyl and β-D-Glucopyranose linked with a β-1→4 bond.

Whey proteins consist predominantly of β-lactoglobulin, α-lactalbumin, immunoglobulins and serum albumin. However, whey also contains minerals (calcium, magnesium, phosphate, citrate, sodium, potassium and chloride), antibacterial peptides (lactoferrin and lactoperoxidase), and vitamins (B_1, B_2, and C) (Wong *et al*., 1978).

Depending on how casein is coagulated (acid or enzymatic coagulation), whey can be classified as sweet (pH 6.4-6.2) with no calcium, or acid (pH 5.0-4.6), which contains a high amount of calcium 92.8 mg/100g (Wong *et al*., 1978; Yang, 2007).

Whey, freshly prepared, has a bland flavour (Laye *et al*., 1993), which allows it to blend well with most products. However, it rapidly oxidizes, giving rise to stale off-flavours (Morr *et al*., 1991). For this reason, together with economic aspects of transport and storage, whey components are generally separated, through filtration techniques such as reverse osmosis or ultrafiltration, and transformed into a dry product, through evaporation techniques, such as spray-drying. According to Tamime, (2009), Whey powders are manufactured as three main products:

- Whey protein concentrate (WPC), which contains 70-85% of the whey proteins of milk and 50% of the lactose of milk.
- Whey protein isolate (WPI), which contains more protein (90-98%) than WPC and very little fat or lactose.

- Whey Permeate (WP), which contains essentially lactose and some minerals and minimal fat and protein.

Whey proteins are commonly used in formulation of infant foods, integrators, bakery products, and meat products.

Currently, whey permeate is mainly used in the manufacturing of dried whey powder for the production of refined lactose. The application of whey described above, however, are often aimed at keeping the surplus whey out of sewers rather producing highly desirable products (Yang, 2007). It is therefore, of interest to investigate novel uses of whey permeate.

1.1.3 Oligosaccharides: definition, production, classification

Oligosaccharides, usually defined as glycosides of different degrees of polymerization (DP), may be synthesised both by enzymatic and chemical means. Examples of oligosaccharides are: lactulose, raffinose, maltooligosaccharides, inulin, fructooligosaccharides (FOS) and galactooligosaccharides (GOS). Lactulose has a mildly purgative action and inhibits the growth of ammonia-producing organisms (Harju, 1993). Lactulose is currently used as a pharmaceutical for the control of constipation and portosystemic encephalophathy (Crittenden *et al*., 1996). Maltooligosaccharides improve colonic conditions by reducing the level of Enterobacteriacea in the gut (Nakakuki, 1993). Inulin and FOS are non-cariogenic, encourage the growth of beneficial bifidobacteria, and decrease the levels of serum cholesterol, phospholipids, and triglycerides (Hidaka *et al*., 1986). Raffinose ingestion increases the number of bifidobacteria (Taizo *et al*., 1999).

Chemical synthesis of oligosaccharides can be carried out through the use of glycosylating agents, such as glycosyl sulfoxides, glycosyl halides and thioglycosides

(Bartolozzi *et al*., 2001). However, the product mixtures obtained are often quite complex and ill defined. Selective synthesis of oligosaccharides requires many reaction steps with the use of protection/deprotection of hydroxyl groups, resulting in low yields of final products, and often, the formation of unwanted enantiomers (Flowers, 1978).

In contrast to chemical synthesis, enzymatic synthesis of oligosaccharides generally produces few by-products, avoids the need for protection/deprotection chemistry, and is environmentally of low impact. For these reasons this procedure is most commonly used. Thus, transglycosylation of lactose by β-galactosidase is widely used to synthesise GOS. Fructanotransferase catalyses the synthesis of FOS from sucrose. Lactosucrose can be synthesised from sucrose through the activity of levansucrase. Transglycosylation of soluble starch by glycosyl hydrolases is used to synthesise glycosylsucrose. Xylo- and chitin- oligosaccharides may be obtained by enzymatic hydrolysis of oligosaccharides using β-xylanase and chitinase respectively. Some oligosaccharides, such as inulin and soybean (raffinose and stachyose), can be extracted from natural sources (Sako *et al*., 1999) (Figure 1.1).

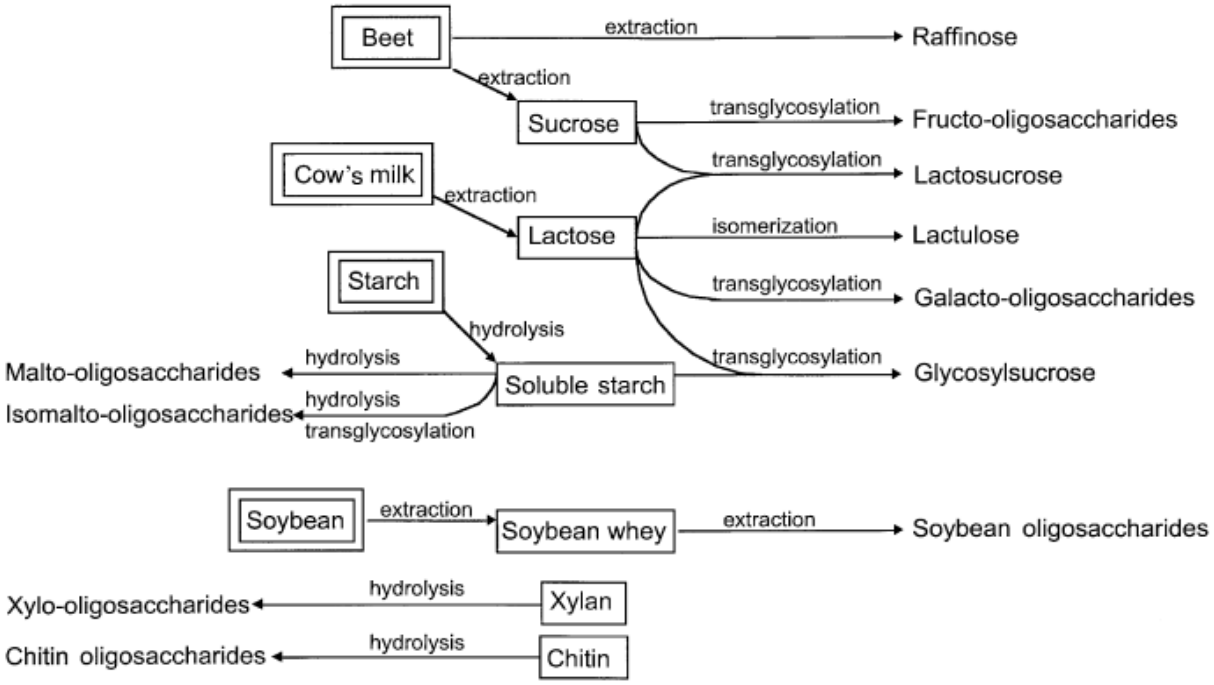


Figure 1.1: Schematic representation of production processes for non-digestible oligosaccharides (NDOs) (from Sako *et al*., 1999).

Non digestible oligosaccharides (NDOs) are useful as prebiotics. In the gastrointestinal tract they serve as substrates for probiotic or "beneficial" bacteria. The most common NDOs used as food ingredients are fructooligosaccharides and galactooligosaccharides. FOS and GOS are generally produced by enzymatic transglycosylation by fructanotransferase and β-galactosidase respectively. The industrial process for enzymatic galactooligosaccharides synthesis is shown in Figure 1.2.

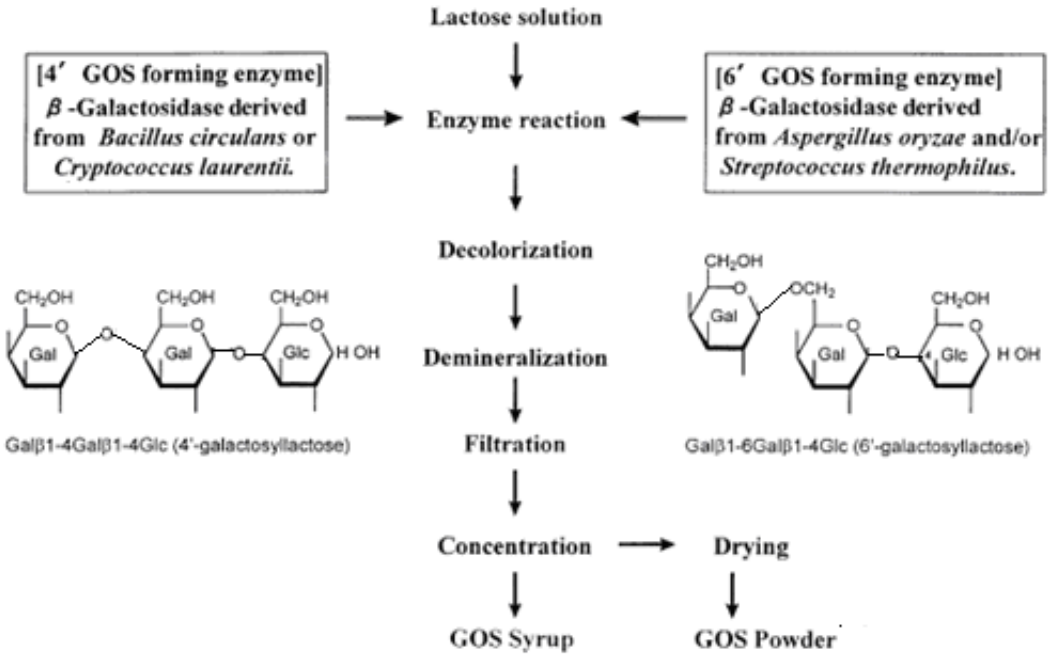


Figure 1.2: Industrial production process for GOS. The figure also shows two possible types of GOS products: Gal-β-1→4-Gal-β-1→4-Glc (4'-galactosyllactose) and Gal-β-1→6-Gal-β-1→4-Glc (6'-galactosyllactose), where Gal: Galactose, Glc: Glucose (from Matsumoto *et al*., 1990).

1.1.3.1 Galactooligosaccharides: definition, functionality and structure

Galactooligosaccharides have a generic formula of D-Glucose-[β-D-Galactose]^{n} where n ranges between three and ten sugar moieties. GOS may be regarded as non digestible oligosaccharides or soluble dietary fibres because they are not digestible by the enzymes of the small intestine, but they are fermentable by bacteria in the large intestine (Champ *et al*., 2003). This is due to the substrate specificity of human gastrointestinal digestive enzymes, which are mostly specific for α-glycosidic bonds whereas GOS glycosidic bonds have a β-configuration. Some β-galactosidases, localized in the small intestine, are able to digest GOS but their activity is usually weak or often deficient (Ito *et al*., 1993).

Oral GOS assumption beneficially affects the human body by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. In this way galactooligosaccharides cause a selective modification of the intestinal microflora, associated with a decrease in faecal pH (Hidaka *et al*., 1988). Such bacteria are able to create an acid medium unfavourable to the growth of many pathogenic microorganisms (Kunz *et al*., 1993). The end products of fermentation of oligosaccharides by colonic bacteria are short chain fatty acids (SCFA) such as acetic, propionic and lactic acid (Hidaka *et al*., 1986; Hidaka *et al*., 1988), which are thought to be efficiently absorbed and utilized by human colonic epithelial cells. In particular, acetic and lactic acids are able to inhibit the growth of undesirable bacteria such as *Esherichia coli* and *Clostridium perfingens* (Tanaka *et al*., 1983).

The carbohydrate composition of food is thought to be an important determinant of the composition of the intestinal flora (Sako *et al*., 1999). The introduction of GOS into foods is considered desirable (Matsumoto *et al*., 1989; Huffman *et al*., 1985; Chen *et al*., 1991).

A large number of GOS species may be synthesized using an enzymatic transglycosylation reaction with lactose as substrate (Tanaka *et al*., 1983; Smart, 1993).

Investigations of GOS synthesis using *Aspergillus oryzae* (Toba *et al*., 1978) and *Streptococcus thermophilus* (Matsumoto, 1990) β-galactosidases have identified disaccharides containing galactose linked to glucose with various types of glycosidic bonds, such as β-1→2, β-1→3, β-1→4, and to galactose through bonds β-1→6 and β-1→3. Glycosidic bonds between two galactose units are mainly β-1→4 bonds when β-galactosidases derived from *Bacillus circulans* (Mozaffar *et al*., 1984) and *Cryptococcus laurentii* (Ozawa *et al*., 1989) are used. The disaccharides synthesized

by β-galactosidase are also called transgalactosylated disaccharides (TD), since the bonds between the monosaccharide moieties are different from those present in nature. TDs may be considered as non digestible oligosaccharides (NDOs), since they have similar physiological characteristics to GOS. Transgalactosylated disaccharides, together with lactose, serve as acceptors for the synthesis of tri- and higher-saccharides. It would seem that galactose can be transferred to any of the hydroxyl groups on acceptor sugars, except for the C^1 hydroxyl (Mahoney, 1998).

The length of the chain of galactooligosaccharides generated by enzymatic reaction depends on the lactose concentration in the media (Huh, 1990; Lopez-Leiva *et al*., 1995; Rustom *et al*., 1998). Quantitatively, the amount of the different GOS products present appears to follow the order: di- > tri- > tetra- > higher- saccharides and the linkages synthesized are predominantly β-(1→6) > β-(1→3) and β-(1→2) (Prenosil *et al*., 1987; Toba *et al*., 1978; Smart, 1993). Trisaccharides, especially galactosyl 1→6 lactose, can be identified at most lactose levels. Tetra- and higher-saccharides have been reported only when using much higher starting lactose levels, although they are considered to be formed at most lactose concentrations but in quantities too small to be detected (Mahoney, 1998). Commercially, short chain oligosaccharides are preferable to long-chain oligosaccharides as human food additives because they are more easily metabolized by the human gut bifidobacteria (Mul, 1997) and also because short-chain oligosaccharides, after metabolism in the human body, are more efficient in the production of short-chain fatty acids (SCFA) (Mul, 1997; Knudsen, 1997).

A list of oligosaccharide structures identified in GOS preparations is given in Table 1.0.

Table 1.0: List of oligosaccharide structures identified in GOS preparations. Where Gal: Galactose, Glc: Glucose. (Adapted from Mahoney *et al*., 1998).

GOS component	Chemical structure
Disaccharides	β-D-Gal (1→6)-D-Glc
	β-D-Gal (1→6)-D-Gal
	β-D-Gal (1→3)-D-Glc
	β-D-Gal (1→2)-D-Glc
	β-D-Gal (1→3)-D-Gal
Trisaccharides	β-D-Gal (1→6)-β-D-Gal (1→6)-D-Glc
	β-D-Gal (1→6)-β-D-Gal (1→4)-D-Glc
	β-D-Gal (1→6)-β-D-Gal (1→6)-D-Gal
	β-D-Gal (1→3)-β-D-Gal (1→4)-D-Glc
	β-D-Gal (1→4)-β-D-Gal (1→4)-D-Glc
Tetrasaccharides	β-D-Gal (1→6)-β-D-Gal (1→6)-β-D-Gal (1→4)-D-Glc
	β-D-Gal (1→6)-β-D-Gal (1→3)-β-D-Gal (1→4)-D-Glc
	β-D-Gal (1→3)-β-D-Gal (1→6)-β-D-Gal (1→4)-D-Glc
Pentasaccharides	β-D-Gal (1→6)-β-D-Gal (1→6)-β-D-Gal (1→6)-β-D-Gal(1→4)-D-Glc

1.1.4 β-galactosidase: reaction mechanism, products, applications

β-galactosidase (β-gal) (EC 3.2.1.23) is a galactosyl hydrolase which cleaves lactose, releasing glucose and galactose. This enzyme was one of the first enzymes isolated and purified from various natural sources, such as plants, animal organs and microorganisms (Richmond *et al*., 1981) (Table 1.1).

Table 1.1: Sources of β-galactosidase (Adapted from Richmond *et al*., 1981).

Plants	Animal organs	Yeast	Bacteria	Fungi
Peach Apricot Almond Kefir grains Tips of wild roses Alfalfa seeds Coffee beans	Intestine Brain Skin tissue Bovine liver	*Kluyveromyces lactis* *Kluyveromyces fragilis* *Candida pseudotropicalis*	*Escherichia coli* *Bacillus megaterium* *Thermus acquaticus* *Streptococcus lactis* *S. thermophilus* *L.. bulgaricus* *L. helareticus*	*Neurospora crassa* *Aspergillus foetidus* *Aspergillus niger* *Aspergillus flavus* *Aspergillus oryzae* *A. phoenicis* *Mucor pucillus* *Mucor meuhei*

The functional form of *E. coli* β-galactosidase is a tetramer of four identical subunits (Appel *et al*., 1965), each consisting of 1,023 amino acid residues (Fowler *et al*., 1970). The tetramer, of 465.412 Da, has a 222-point of symmetry (Jacobson *et al*.,

1994) and consists of five domains, the third of which has the active site (Matthews *et al*., 2005) (Figure 1.3).

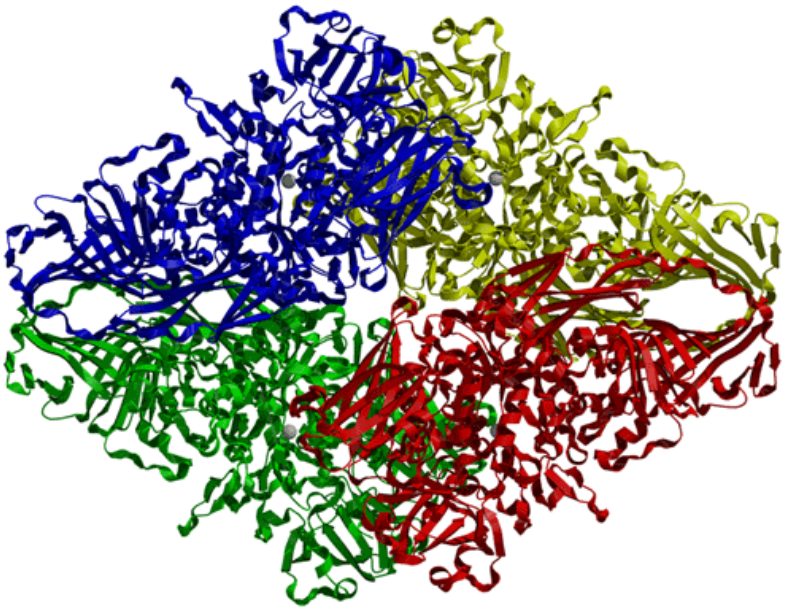


Figure 1.3: 3-D structure of the tetrameric *E coli* β-galactosidase. The colours indicate the four different *E. coli* identical subunits (From Miesfeld, 2001).

β-galactosidase catalyses the transfer of a galactose moiety of a β-galactoside to an acceptor containing a hydroxyl group.

The reaction mechanism for β-galactosidase has been elucidated and proceeds by two steps (Figure 1.4):

- Step (a): enzyme–galactosyl complex formation and simultaneous glucose liberation.
- Step (b): the enzyme–galactosyl complex is transferred to nucleophilic acceptor containing a hydroxyl group. Transfer to water produces galactose (hydrolysis reaction, Figure 1.4 b, where -R is a hydrogen). Transfer to another sugar produces di-, tri- and higher

galactosyl-saccharides, collectively termed galactooligosaccharides (Figure 1.4 b, where -R is a sugar molecule).

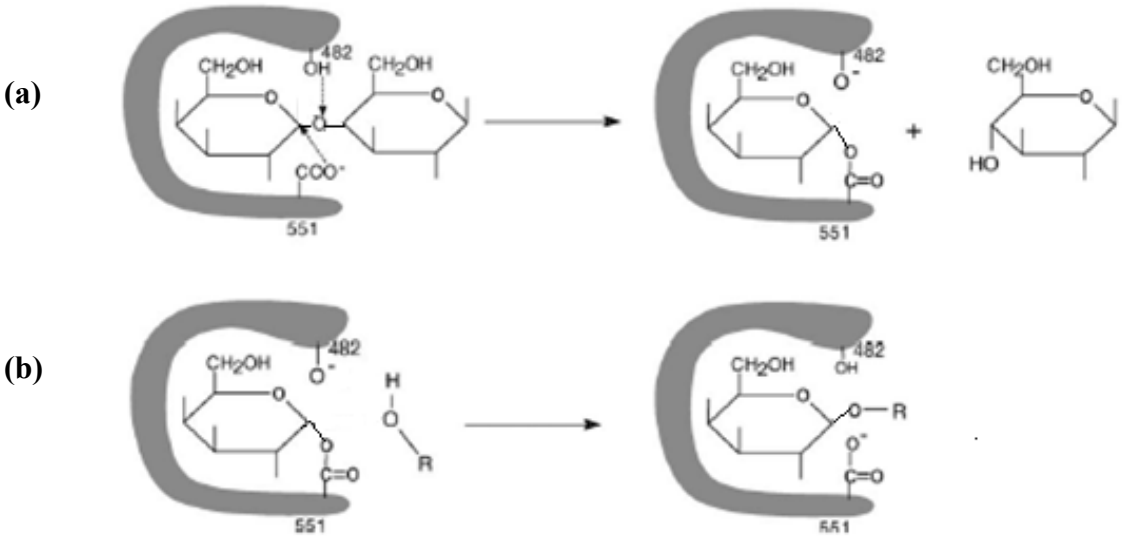


Figure 1.4: Reaction mechanism for the hydrolysis and transglycosylation of lactose by *Kluyveromyces lactis* β-galactosidase. (a): The lactose molecule on the active site of the enzyme forms and acyl-enzyme complex with liberation of glucose. (b): The enzyme-galactose complex, formed by lactose hydrolysis, could react with carbohydrate molecules (where R: mono- or di- saccharides), leading to GOS formation. Glutamate 551 act as the nucleophile and glutamate 482 is the proton donor. (Adapted from Zhou *et al*., 2001).

In 1957, Roberts *et al*. showed that transglycosylation by β-galactosidase from *Saccharomyces fragilis* was useful for the synthesis of GOS from lactose. Thus, a reaction mixture of 15% of lactose with 0.5% *Saccharomyces fragilis* β-galactosidase leads to formation of GOS from lactose as follows (Figure1.5).

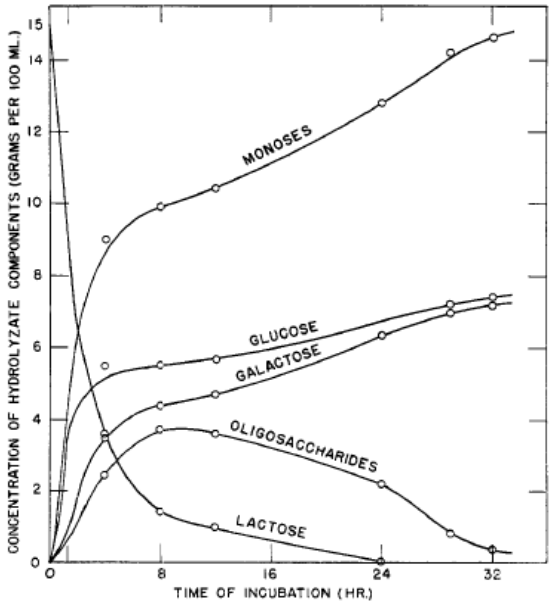


Figure 1.5: GOS synthesis from lactose by *Saccharomyces fragilis* β-galactosidase. Reactions were carried out in phosphate buffer (0.067M, pH 6.2) and 35ºC (Roberts *et al*., 1957).

Therefore, in aqueous solutions with high concentrations of competing hydroxyl groups on sugar moieties, the enzyme catalyses the formation of galactooligosaccharides (GOS) (Huh, 1990; Lopez-Leiva *et al*., 1995; Rustom *et al*., 1998). The ratio of products results from competition between water and the carbohydrate acceptor for enzyme bound substrate (Figure 1.6).

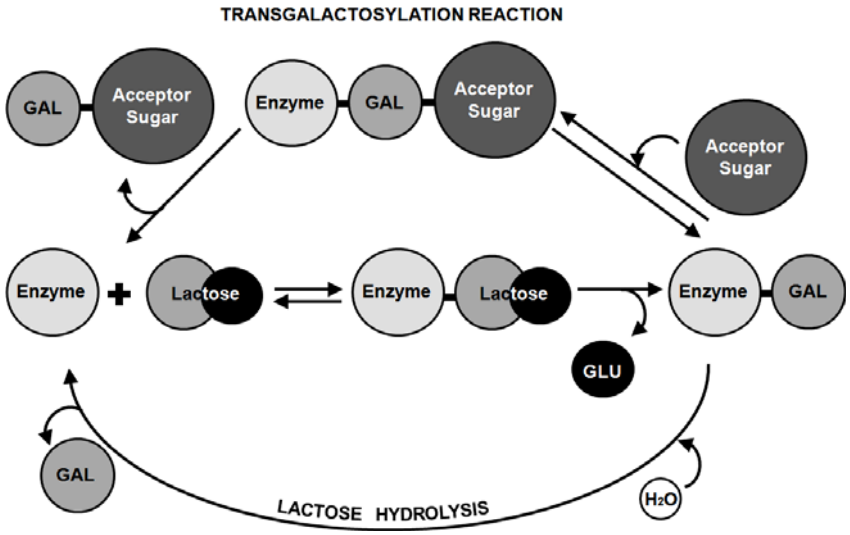


Figure 1.6: Reaction pathway for transglycosylation and hydrolysis β-galactosidase. Lactose hydrolysis (lower path) and transgalactosylation reaction (upper path) are both catalysed by β-galactosidase, depending on the sugar concentration in solution (Neri, 2008).

Therefore, the β-galactosidase reaction mechanism includes both the hydrolysis of lactose and a transglycosylation reaction (Mahoney, 1998). Depending on lactose concentration, the reaction is shifted towards either hydrolysis or transglycosylation. When water concentration in the system, expressed as water activity (a_w), is high, the hydrolysis of lactose occurs predominantly. The transglycosylation reaction increases with a decrease in water activity (Goulas *et al*., 2007). Apart from lactose concentration, other factors influence the reaction, such as: reaction conditions temperature, pH and the presence of inhibitors or activators specific for the enzyme (Zárate *et al*., 1990).

The β-galactosidase reaction mechanism involves two critical amino acid residues on the protein, a proton donor and a nucleophile/base. The mechanism of the reaction, first described by Wallenfels *et al*., (1960), proposed that cysteine and histidine residues acted as proton donor and nucleophile site respectively. This was

subsequently confirmed (Nizizawa *et al*., 1970; Nijipels *et al*., 1981; Prenosil *et al*., 1987). However, recent studies (Huh, 1990; Sheu *et al*., 1998; Mahoney *et al*., 1998; Zhou *et al*., 2001) have show that microbial β-galactosidases have two glutamate residues, one acting as the proton donor and the other as a nucleophile/base (Figure 1.4).

Jobe *et al*. (1972) showed that allolactose, β-D-Galactopyranosyl (1→6)-D-Glucose, is a primary transfer product of the transglycosylation reaction. They demonstrated the capacity of β-galactosidase to modify the 1→4 linkage to a 1→6 linkage (Figure 1.7). The major pathway for production of this compound is direct internal transfer of galactose from the 4 position to the 6 position of the glucose moiety without releasing the glucose from the active site (Huber *et al*., 1976).

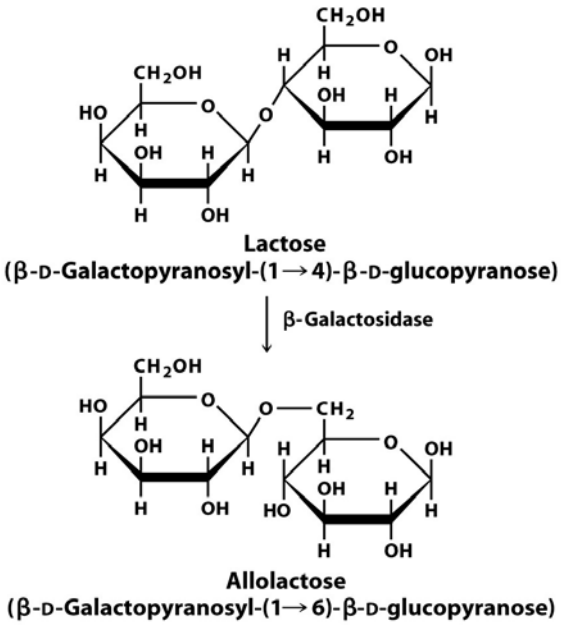
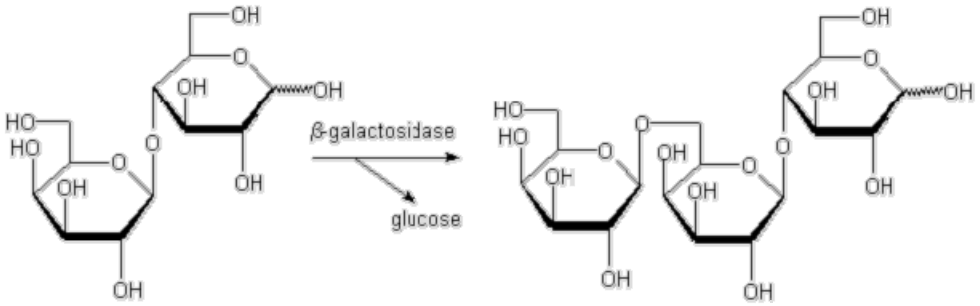


Figure 1.7: Allolactose production from lactose by β-galactosidase. The glycosidic linkage 1→4 is modified to 1→6 linkage (from Horton *et al*., 2006).

Many authors (Bakken *et al*., 1992; Portaccio *et al*., 1998; Shukla *et al*., 1993) have reported galactose to be a competitive inhibitor of lactose hydrolysis and transglycosylation reactions. From a thermodynamic point of view, high galactose concentrations might be expected to favour the transglycosylation reaction, increasing GOS yield. Nevertheless, Neri *et al*., (2009) investigating the effects of galactose and/or glucose addition on transglycosylation, found a simultaneous decrease in lactose hydrolysis and transglycosylation in the presence of galactose, presumably due to galactose inhibition (Prenosil *et al*, 1987; Santos *et al*., 1998).

Glucose also influences the transglycosylation reaction kinetics acting as a non-competitive inhibitor (Shin *et al*., 1998; Cavaille *et al*., 1995).

According to Peinsipp *et al*., (1995), during the transglycosylation reaction, lactose may serve as donor as well an acceptor of a glycosyl group and the isolated product is β-D-Galactose (1→6)-β-D-Galactose-(1→4)-D-Glucose (Figure 1.8).



2 ·Lactose **β-D-Gal-(1→6)-β-D-Gal-(1→4)-D-Glc**

Figure 1.8: β-D-Galactose-(1→6)-β-D-Galactose-(1→4)-D-Glucose synthesis using β-galactosidase. Lactose is hydrolysed to liberate glucose. The resulting enzyme-galactosyl complex reacts to form a 1→6 linkage with the galactose moiety of a second lactose molecule (Peinsipp *et al*., 1995).

The efficiency of transglycosylation, the linkage between the units, the components in the final product and the yield of GOS have been reported to depend upon the source of the enzyme and the reaction conditions used (Sako *et al*., 1999; Boon *et al*., 2000). Indeed, the degree of oligosaccharide formation has been reported to be as high as 40% of the total sugar content of the solution under optimal reaction conditions (Prenosil *et al*., 1987). However, a difficulty with comparison between studies lies in the fact that they have been carried out using different enzymes under widely varying conditions of temperature and pH and substrate concentration without the use of a comparator.

Transglycosylation is an intermediate step of a more complex reaction because, as it progresses, all sugars may be hydrolyzed to their constitutive monosaccharides (Matella *et al*., 2006). Hence, knowledge of the time course of the reaction is required to estimate the point of maximum yield for the desired GOS products.

The process of lactose hydrolysis by β-galactosidase is used industrially whether the interest in removing lactose from milk products is based on nutritional considerations (lactose intolerance) or technological concerns (lactose solubility, sweetness, functionality). Lactose-hydrolyzed milk is used for the preparation of flavoured milk and fermented products (yoghurt, cheese and bakery products) as it accelerates acidification due to the release of glucose. It is also used for ice-creams, as it prevents lactose crystallization; as well as a sugar source in animal feed (Yang *et al*., 1995).

1.2 Functional food

The concept of "functional food" was developed in Japan in the mid 1980s. At that time, the health authorities in Japan decided that greater consumption of certain food types could help to limit or reduce the impact of a number of disease risk factors.

After a relatively slow start, the concept of functional foods has stimulated interest among the major food companies around the world. The functional food market is estimated to be worth $43 billion in the US, with an annual market growth rate of 5-10% (Sloan, 1999). A European survey estimated the functional food market to be worth over about 1€ billion in 1997 (Table 1.2). The global functional food market increased from 10,000 US $ million in 1995 (Byrne *et al*., 1997) to 33,000 $ million in the year 2000 (Hilliam, 2000) and is predicted to be over 50,000 in 2010 (Heller, 2001). Dairy products are one of the most developed sector of the European functional food market.

Table 1.2: Functional dairy products in Europe by country (from Hilliam, 2000).

Country	**Value in 1999 (US $ millions)**	**Share (%)**
Germany	283	21
France	240	18
United Kingdom	222	16
The Netherlands	150	11
Other countries	450	34
Total	1345	100

1.2.1 Definition of functional foods

In 1996, Roberfroid was one of the first to define a functional food as "*a dietary component that may exert physiological effects on the consumer which may eventually lead towards justifiable health claims*". While a globally accepted definition has yet to be agreed, a functional food is broadly regarded as any food or

ingredient that, in addition to providing nutritional benefit, may contribute a health benefit (Marriott, 2000). Those benefits can be of various types. According to Bellisle *et al*., 1998, a functional food affects one or a limited number of functions in the body in a targeted way so as to have positive effects on health. Other workers have defined such foods as having a physiological or psychological benefit (Clydesdale, 1997) and/or reducing the risk of chronic disease beyond their basic nutritional functions (Hasler, 1998).

For the food industry, functional properties are those attributes of food components or additives that, at their proper concentration and under suitable conditions, provide desirable sensory and rheological characteristics (Sikorski, 2001).

Traditionally, fruits and vegetable have been seen as a source of functional food components, but recent investigations have established that animal food derivatives, such as milk and dairy products may also be a valuable source of these components (Bauman *et al*., 2006).

1.2.2 Concepts and legislation for functional foods

The attribution of health claims to foods led to the development of a wide variety of foods claiming such benefits. On the one hand, this indicates the extent of innovation and competitiveness of the food industry, but on the other hand it highlights the need for regulation to ensure legitimacy and that consumers are not misled.

In Europe, the European Commission's concerted action on Functional Food Science in Europe (FUFOSE), involving a large number of the most prominent European experts in nutrition and related sciences, were engaged by the International

Life Science Institute (ILSI) (Diplock *et al*., 1999) to define the claims criteria for functional foods.

European Regulation EC No. 1924/2006 harmonises laws, regulations or administrative actions in Member States which relate to nutrition and health claims of foods in order to ensure the effective functioning of the market whilst providing a high level of consumer protection (Official Journal of the European Union, 2007).

In the European Union (EU) the legal status of functional food is regulated through existing food legislation. However, specific authorisation must be obtained through the process set out in the Novel Food Regulations prior to placing a new food on the EU market (EC No. 258/1997). For these reasons in the EU functional foods are not legally considered as a specific food category, but rather a concept (Coppens *et al.,* 2006; Stanton *et al*., 2005).

In the EU some of the functional foods already available are those with cholesterol lowering plant sterols and stanols, as well as those containing live bacteria (probiotics) that enhance the quality of human gut microflora. During the manufacture of functional fermented milks, oligosaccharides are produced in variable amounts depending on the bacterial strains used (Joung *et al*., 2001; Lamoureux *et al*., 2002; Yadav *et al*., 2007), so the functional properties of fermented milks may be due not only to their probiotic properties but also to the presence of oligosaccharides (Martínez-Villaluenga *et al*., 2008).

Functional food research has moved progressively towards the development of dietary supplementation, introducing the concept of prebiotics, which may affect gut microbial composition (Ziemer *et al*., 1998).

1.3 Oligosaccharides industrial properties or applications and prebiotics properties

In 1991, the Ministry of Health and Welfare in Japan legislated as foods for specified health use (FOSHU): fructo, galacto, xylo-, isomalto- soybean, lactosucrose, raffinose, lactulose and palatinose oligosaccharides (Farnworth, 1997; Sako *et al*., 1999).

Oligosaccharides are water soluble and have a relatively low sweetness (about 0.3-0.6 time that of sucrose), which depends on their chemical structure and molecular mass. For this reason they are used as bulking agents and as carriers for other food flavours, natural or artificial. Because of their high molecular weight, oligosaccharides provide increased viscosity, leading to improved body and mounthfeel (Crittenden *et al*., 1996; Tamine, 2005). Other applications include the alteration of the freezing temperature of frozen foods, and the control of the amount of browning in heat-processed foods. Oligosaccharides have also been shown to be strong inhibitors of starch retrogradation.

As soluble dietary fibre, oligosaccharides are commonly used as low-cariogenic sugar substitutes in confectionery, jams, pastry, chewing gums, yoghurts, drinks, and in low calorie diet and diabetic foods (Matsumoto *et al*., 1995).

Studies regarding the *in vitro* cariogenicity of trans-galactooligosaccharides (TOS) (Hartemink *et al*., 1997) proved that many oral bacteria are able to degrade and ferment TOS and galactosyl-lactose (GLL). Although lactic and acetic acid are produced, the fermentation process is relatively slow. Plaque is not formed, so the risk of caries formation from TOS and GLL is considered rather low.

The caloric value of non digestible oligosaccharides has been estimated to be 1.0-2.0 kcal/g (Roberfroid *et al*., 1993). In particular, Watanuki *et al*. (1996) calculated the caloric value of GOS as 1.73 kcal/g.

GOS are stable compounds, and they remain unchanged even after high temperature treatment and are also quite stable during long-term storage at room temperature. It has been suggested that their stability is better than fructooligosaccharides (Voragen, 1998). This property allows their use in thermally treated foods.

Oligosaccharides are referred as bifidogenic or bifidofactors, referring to their ability to selectively promote the proliferation of: *Bifidobacteria* spp. (such as *B. longum, B. breve, B. pseudolongum, B. infantis* and *B. lactis*) and *Lactobacillus* spp. (such as *L. acidophilus, L. casei, L. reuteri, L. rhamnosus, L. johnsonii*, and *L. plantarum*) which are believed to be beneficial to intestinal health (Shortt, 1999). Such bacteria have been described as friendly bacteria or probiotic (from the Greek, *προ βιοτος*, meaning literally 'for life'). The incorporation of probiotic strains in traditional food products has been well established in the dairy industry, leading to the production of novel types of fermented milks and cheeses (Gomes *et al*, 1999).

Gibson and Roberfroid (1995), defined a prebiotic as a non-digestible food ingredient which beneficially affects the host by selectively stimulating the growth and/or metabolism of one or a limited number of beneficial bacterial species already existent in the colon. Thus, a prebiotic not hydrolyzed and/or absorbed in the upper part of the gastrointestinal tract, serves as a selective substrate for at least one beneficial colon bacterial species in such a way as to alter positively the composition of the microflora.

Many food ingredients such as non-digestible oligosaccharides, some peptides and proteins and certain lipids, could act as prebiotics but only the oligosaccharides are able to fulfil all the criteria of prebiotics as defined above. Oligosaccharides and specifically, galactooligosaccharides achieve this by acting as a selective carbon and energy source that "friendly" bacteria can utilize. Organisms such as *Escherichia coli*, *Clostridium perfringens* or *Streptococcus mutans*, potentially harmful residents of the gut, cannot utilize GOS (Tomomatsu, 1994). This leads to an improvement of the balance of intestinal microflora in the gut.

Another strategy in microflora management is the use of synbiotics, in which prebiotics and probiotics are used in combination (Gibson *et al*., 1995). The live microbial additions may be used in conjunction with specific substrate for growth (*i.e.* Bifidobacteria with GOS or FOS) (Collins *et al*., 1999).

There are studies and advances occurring in the medical applications of specific oligosaccharides. For example, the treatment of gut infectious diseases using oligosaccharides has been proposed by Playne (2002), who discovered the ability of specific oligosaccharides to bind to gut mucosal and epithelial surfaces and thus prevent the attachment of certain microorganisms.

Other investigations established that the presence of *Bifidus* microflora in the intestines of breast-fed infants was attributed to the presence of GOS in human milk (Matsumoto, 1993). Gyorgy (1973) showed that the galactooligosaccharides fraction of human milk (referred as *Bifidus* factor) enhanced the growth of Bifidobacteria in the intestine not only of breast-fed infants but also of infants fed with cow's milk supplemented with GOS.

Galactooligosaccharides have other potential beneficial effects in addition to being bifidofactors. Thus they have been reported to:

- Help synthesis of B-complex vitamins (B_1, B_2, B_3, B_6, B_9, B_{12}), produced by *Bifidobacteria* strains (Perugino *et al*., 2004; Kanbe, 1992).
- Reduce serum cholesterol levels due to assimilation of cholesterol in the diet by some strains of *Lactobacillus acidophillus* (Chonan *et al*., 1995, Gilliland *et al*., 1990).
- Stimulate and enhance mineral absorption of metals such as calcium and magnesium (Sako *et al*., 1999). In particular, calcium solubility increases as a result of SCFA production by *Bifidobacteria* (Chonan *et al*., 1995).
- Affect positively bone mineralization (Chonan *et al*., 1995; Scholz-Arhens *et al*., 2001).
- Improve blood glucose and triglycerides level (Nakakuki, 2002).
- Eliminate toxic compounds (Van den Heuvel *et al*., 1999), such as ammonia (Tamai *et al*., 1992).
- Stimulate intestinal peristalsis as a result of SFCA production thereby, preventing constipation (Deguchi, 1997).
- Have anticariogenic activity (Delzenne, 1999).
- To relieve the symptoms of diabetes mellitus and lactose intolerance (Li *et al*, 2008).
- Prevent colon cancer (Van Dokkum *et al*., 1999).

GOS are generally recognized as safe (GRAS) as they are components of human milk and traditional yoghurt products. Acute and chronic toxicity tests showed no toxicity as well as no mutagenicity for GOS (Sako *et al*., 1999).

1.3.1 Galactooligosaccharides dose-response in prebiotic effect

The prebiotic properties of several oligosaccharides have been demonstrated by the administration of reduced amounts of the products to animals (Kikuchi *et al*., 1993; Korpela *et al*., 1997; Mul, 1997). Caecal enlargement and increases in caecal contents are common in animals after consumption of GOS. Bouhnik *et al*. (1997) compared the *in vitro* activity of a batch human faecal culture in relation to the production of adenosine-5-triphosphate (ATP), acid and gas of healthy humans. The analysis of faeces collected on day 1 (control), 7 days and 14 days after the administration of GOS found that, in response to added GOS, ATP and acid production were stimulated. In addition, the rate of increase of acetic acid in the batch culture in the presence of GOS was higher than that of the control group. This study suggested that the increase in ATP and acid production was due to the change in composition of the faecal flora to a bifidobacteria-predominant one.

A human study with galactooligosaccharides and fructooligosaccharides as prebiotics showed that a daily dose of 4-20 g significantly increases *Lactobacilli* and *Bifidobacteria* levels in the gut (Ryocroft *et al*., 1999). Similarly, earlier studies by Tanaka *et al*. (1983) demonstrated that after a week of intake of β-1→6 GOS at a dose of 3-10 g/day in healthy adults, the faecal count of bifidobacteria increased in a dose-dependent manner. Indeed, a daily intake of 2.5 g of β-1→6 GOS appears to be sufficient to increase the faecal *Bifidobacteria* count when the initial baseline level is low, which is often the case in elderly people (Ito *et al*., 1993). Boehm *et al*., (2000) performed studies in preterm infants where they tested the probiotic capacity of an oligosaccharide mixture consisting of 90% of galactooligosaccharides and 10% fructooligosaccharides. A mixture of 1 g/dl of GOS and FOS, similar to the oligosaccharide content of human milk, cannot stimulate intestinal *Bifidobacteria* in

formula-fed infants. However, the number of *Bifidobacteria* found in the infants fed with the oligosaccharide mixture was in the upper range of the values found in infants fed with human milk. Further studies, Moro *et al*. (2002), demonstrated that a concentration of 0.4 g/dl of the galacto and fructo oligosaccharide mixture is bifidogenic and that doubling the amount of oligosaccharides in the feed increased the effect. Kanamori *et al*., (2003) also showed that oral administration of a synbiotic containing GOS (3 g/day) in combination with vancomycin helped to eradicate methicillin-resistant *Staphilococcus aureus* (MRSA) and re-established an anaerobic-dominant flora in a 3 month old infant suffering from MRSA entercolitis. Deguchi *et al*., (1997) showed that bowel habit is improved after daily ingestion of 5 g GOS for a week. In a study of diabetic subjects with constipation, a correlation was found between the improvement in constipation and the decrease in faecal *Bacteriodaceae* after ingestion of GOS (Narimaya *et al*., 1996).

1.4 Galactooligosaccharides industrial production

Oligosaccharides and their derivatives play a key role in many biochemical reactions and their use in therapeutics, as diagnostic tools, in cosmetics and the food industry is well established (Monsan *et al*., 1995). The estimated production of non digestible oligosaccharides in the world grew since the inclusion of GOS in FOSHU: over 300 products have been approved (Arai *et al*., 2002), more then half of which (except for lactulose) are consumed in Japan, with a market value of approximately 10 billion yen (Sako *et al*., 1999). Furthermore, 60% of FOSHU items so far are products containing non digestible oligosaccharides. Examples of specific products containing galactooligosaccharides recently approved are outlined in Table 1.3 (Tamine, 2005).

Table 1.3: Recent FOSHU introduction containing oligosaccharides (Adapted from Tamine, 2005).

Claim area	Company name	Product detail
Intestinal health	Como	Croissants with lactosucrose
	UCC Ueshima Coffee	Powdered soft drink with lactosucrose
	Nihon Seibutsu Kakagu	Table top GOS
	Nissin Sugar Manufacturing	Table top GOS
	Yakult Honsha	Soft drink with GOS and polydextrose yogurt with prebiotics (*Lactobacillus gasseri* and *Bifidobacterium bifidum*)

Estimated GOS production in 1995 in Europe was about 15,000 tonnes (Playne *et al*., 1996). Examples of companies that are currently involved in GOS production are Friesland Foods Domo in The Netherlands or Snow Brand Milk Products in Japan.

Vivinal® GOS (from Friesland Foods) or P7L® GOS (Snow Brand Milk), whose production process are patented, are used to formulate products targeting specific groups such as infants, children, women and the elderly. Commercially available GOS is a mixture of several species of GOS. The typical composition of Vivinal is GOS (more than ~55%), lactose (~20%), glucose (~20%) and a small amount of galactose (less than 1%).

Worldwide, there are 12 classes of food grade oligosaccharides in commercial production and the production in 1995 (latest data available with details from manufacturers) can be seen in Table 1.4.

Table 1.4: Yields of produced food-grade oligosaccharides in 1995 by manufacturer (Adapted from Crittenden *et al*., 1996)

Class of oligosaccharides	**Estimated production in 1995 (t)**	**Major manufacturers**	**Trade names**
GOS	15000	Yakult Honsha (Japan)	Oligomate
		Nissin Sugar Manufacturing Company (Japan)	Cup-Oligo
		Snow Brand Milk Products (Japan)	P7L and others
		Borculo Whey Products (The Netherlands)	TOS-Syrup
Lactulose	20000	Morinaga Milk Industry Co. (Japan)	MLS/P/C
		Solvay (Germany)	
		Milei GmbH (Germany)	
		Canlac Corporation (Canada)	
		Laevosun (Austria)	
		lnalco SPA (Italy)	
Lactosucrose	1600	Ensuiko Sugar Refining Co. (Japan)	Newka-Oligo
		Hayashibara Shoji Inc. (Japan)	Newka-Oligo
FOS	12000	Meiji Seika Kaisha (Japan)	Meioligo
		Beghin-Meiji Industries (France)	Actilight
		Golden Technologies (USA)	NutraFlora
		Cheil Foods and Chemicals (Korea)	Oligo-Sugar
		ORAFTI (Belgium)	Rafilose and Raftiline
		Cosucra (Belgium)	Fibruline
Isomaltulose oligosaccharides	5000	Mitsui Sugar Co. (Japan)	ICP/O
Glucosyl sucrose	4000	Hayashibara Shoji Inc. (Japan)	Coupling Sugar
Maltooligosaccharides	10000	Nihon Shokuhin Kako (Japan)	Fuji-Oligo
		Hayashibara Shoji Inc. (Japan)	Tetrup
Isomaltooligosaccharides	11000	Showa Sangyo (Japan)	lsomalto-900
		Hayashibara Shoji Inc. (Japan)	Panorup
		Nihon Shokuhin Kako (Japan)	Biotose and Panorich
Cyclodextrine	4000	Nihon Shokuhin Kako (Japan)	Celdex
		Ensuiko Sugar Refining Co. (Japan)	Dexy Pearl
		Asahi Kasei Kagyo Co. (Japan)	
Gentiooligosaccharides	400	Nihon Shokuhin Kako (Japan)	Gentiose
Soybean oligosaccharides Xylo-oligosaccharides	2000	The Calpis Food Industry Co. (Japan) Suntory Ltd (Japan)	Soya-oligo Xylo-oligo

Both the volume and the diversity of oligosaccharide products are increasing rapidly as their functional proprieties become better understood (Crittenden *et al*., 1996).

There are many foods in which GOS can be included, such as bread and fermented dairy products. During yeast fermentation and the baking of bread, GOS are not decomposed and may influence positively the taste and the texture of the product. Fermented dairy products with *Bifidobacteria* spp. or other Lactic Acid

Bacteria (LAB) with added GOS are commercially available in Japan as well as in Europe.

1.5 Galactooligosaccharides production in a research context

Galactooligosaccharides produced by the action of β-galactosidase on lactose were identified for the first time in the early 1950s. Four species of GOS were formed using *Kluyveromyces lactis* β-galactosidase (Aronson, 1952; Pazur, 1954), and three using *E. coli* β-galactosidase (Aronson, 1952). Experiments conducted with high lactose concentrations detected eleven species of GOS (Roberts *et al*., 1957). In the same study, the total concentrations of synthesised GOS was also quite high, as the hydrolysis of a 35% lactose solution contained up to 44% of the total sugar in the form of di- and higher- saccharides.

Since then, there have been several studies of the enzymatic synthesis of GOS by β-galactosidase. The mains findings were that GOS production increased with initial lactose concentration (Wienbicki *et al*., 1973; Burvall *et al*., 1979) and that GOS production declined as the reaction progresses (Burvall *et al*., 1980). Also, different species of GOS were synthesized with different sources of enzyme, 13 *Lactobacillus* strains showed that each enzyme produced a different spectrum of GOS (Toba *et al*., 1981), and more then 20 GOS species were found to be synthesized using *Aspergillus oryzae* β-galactosidase (Toba *et al*., 1985). Yang *et al*. (1988) indicated that trisaccharide GOS was formed for all reaction condition studied (lactose concentrations: 2.5, 5, 10, 15, 20 and 25%), whereas tetrasaccharide GOS was formed only when the starting lactose concentration was greater than 20%.

A large number of studies to date have shown the formation of GOS using β-galactosidase from different bacteria, such as *Candida pseudotropicalis* and

Kluyveromyces lactis (Jeon *et al*., 1984), *S. fragilis* (Toba *et al*., 1978), *Escherichia coli* (Huber *et al*., 1976), *Aspergillus oryzae* (Betschart *et al*., 1984) *Penicillium chrysogenum* (Ballio *et al*., 1960) and *Bacillus circulans* (Mozaffar *et al*., 1984) (Table 1.5).

In the tables that follow, the percentage of maximum GOS on initial lactose used was calculated where possible. Otherwise, the maximum GOS was presented following the author reference.

Table 1.5: Studies on transglycosylation reaction using lactose as substrate. Where Lac: lactose, E: enzyme, and Lac_0: initial lactose concentration.

Enzyme source	**[Substrate] and/or [Enzyme] studied**	**Assay conditions**	**Maximum synthesized GOS (% of $[Lac]_0$)**	**Reference**
Saccharomyces fragilis	[Lac]=50, 100, 150, 200, 250, 300, 350, 400 and 500 g/l [E]= 0.5 and 0.58%	T=35°C in phosphate buffer (0.067M, pH 6.2) for 32 hours	1.1-21.2% depending on [Lac]	Roberts *et al*., 1957
Escherichia coli	[Lac]=171g/l [E]=130µg/ml	T=30°C in imidazole hydrochloride buffer (pH 7.2) with 0.01M NaCl and 0.0067M M_gSO_4 for 14 hours	~20%	Huber *et al*., 1976
Bacillus circulans	[Lac]=45.6 g/l [E]= 3U/ml	T=40°C in buffer (pH 6.0) for 5 hours	6%	Mozaffar *et al*., 1984
Candida pseudotropicalis and *Kluyveromyces lactis*	[Lac]=50 and 200 g/l [E]=1.0-2.0 U/ml	T=37°C in phosphate buffer (0.25M, pH 6.6) for 4 hours with gentle agitation or at T=4°C for 24 hours	11.3-16.3% depending on [Lac] and [E]	Jeon *et al*., 1984
Aspergillus niger	[Lac]=25, 50, 100, 150, 200 and 250 g/l [E]=1.25 mg/ml	T=50°C in buffer	~10%	Yang *et al*., 1988

Most recent studies of GOS production by β-galactosidase are focused on improving or maximizing GOS yields from lactose.

1.5.1 Research on GOS production using thermophilic enzymes.

At high temperature lactose solubility increases and the viscosity of the media decreases which eliminates the possibility of microbial contamination of the reaction system. Thus transglycosylation reaction with high lactose content can be performed. Hence, enzymes that act at high temperatures can be used under conditions that favour GOS production (Table 1.6).

Table 1.6: Transglycosylation reaction of lactose carried out with thermophilic enzymes. Where Lac: lactose, E: enzyme, and Lac_0: initial lactose concentration.

Enzyme source	[Substrate] and/or [Enzyme] studied	Assay conditions	Maximum synthesized GOS (% of $[Lac]_0$)	Reference
Saccharopolyspora rectivirgula strain V2-2	[Lac]= 599 g/l [E]=not well specified	T=70°C in buffer (pH 7.0) for 22 hours	41%	Nakao *et al*., 1994
Sulfolobus solfataricus (SsβGly) and *Pyrococcus furiosus* (CelB)	[Lac]= 70, 170 and 270 g/l [E]= 20 U/ml	T=70°C in sodium citrate buffer (20 mM, pH 5.5), with agitation (400 R.P.M.)	Depending on [Lac]: ~14, 23, 33% for *Pyroc. furiosus* ~7, 17, 26 g/l for *Sulfol. solfataricus*	Petzelbauer *et al*., 2000
Sulfolobus solfataricus	[Lac]= 300, 400, 500 and 600 g/l [E]= 1.2, 2.4, 3.6 and 4.8 U/ml	T=70, 75, 80, 85 and 90°C in phosphate buffer (50 mM, pH 6.0) for 25, 60 and 80 hours	~52% after optimization of [Lac], [E], T°C, pH and reaction time.	Ha-Young *et al*., 2008
Thermotoga maritima	[Lac]= 200, 300, 400 and 500 g/l [E]= 1.0, 1.5, 2 U/ml	T=50-100°C in phosphate buffer (50 mM, pH 6.0) for 360 minutes	~ 10-18% depending on [Lac] and [E]	Eun-Su *et al*., 2005
Sirobasidium magnum CBS6803	[Lac]= 20 g/l [E]= 0.25 U/dl	T= 60°C in sodium acetate buffer (100 mM, pH 5.0)	27%	Onishi *et al*., 1997

For some β-galactosidase, such as *Sulfolobus solfataricus*, it was found that GOS production increased linearly with temperature (30-95ºC) (Pisani *et al*., 1990). However, temperature did not influence some other enzymes, such as *Aspergillus niger* β-galactosidase, as oligosaccharides production was found to be constant between 8-50ºC (Yang *et al*., 1988).

1.5.2 Research on GOS production using immobilized enzymes.

Immobilization is important in commercial enzymology allowing the repetitive and economic utilization of enzymes (Oliveira *et al*., 2008). Compared with free enzyme in solution, enzyme immobilized on a solid support provides many advantages, including β-galactosidase reusability, continuous operation, controlled product formation, and simplified and efficient processing (Albayrak *et al*., 2002). For these reasons, there are studies focused on the immobilization of enzymes on a stationary phase while substrate is continually fed through the reaction medium (Table 1.7). Consequently an appropriate immobilized system for transglycosylation is desirable (Petzelbauer *et al*., 2000).

Table 1.7: Transglycosylation reactions of lactose carried out with immobilized enzymes. Where Lac: lactose; E: enzyme; FE: free enzyme; IE: immobilised enzyme, and Lac_0: initial lactose concentration.

Enzyme source	[Substrate] and/or [Enzyme] studied	Assay conditions	Maximum synthesized GOS (% of $[Lac]_0$)	Reference
Thermus aquaticus YT-I free (FE) and immobilized (IE)	[Lac]= 160 g/l [E]= 100 ml (IE)	T=70°C in buffer (pH 4.6, 6.0) with agitation (60 R.P.M.)	with FE: -32.8% at pH 6.0; - 32.4% E at pH 4.6; with IE: -32.7% at pH 6.0; -34.8% at pH 4.6	Berger *et al*., 1995
Aspergillus oryzae free (FE) and immobilized (IE) on cotton cloth in a recycle batch reactor	[Lac]=43, 133 270 g/l (FE), 2.7 g/l for (IE) [E]= 4.5, 11.8, 23.6 g/l (FE), 5 mg/ml (IE)	T=40°C in acetate buffer (0.1 M, pH 4.5) for 15, 30 and 50 minutes with shaking (150 R.P.M.)	~ 22% FE, ~ 20% IE	Matella *et al*., 2006
Aspergillus oryzae free (FE) and immobilized (IE) on mPOS-PVA	[Lac]= 50, 100, 200, 300, 400, 500 g/l [E]= 0.149 mg/ml (FE), 0.383 mg/ml (IE)	T= 30, 40, 60 °C in citrate-phosphate buffer solution (20 mM, pH 3.5, 4.0, 4.5, 5.0, 5.5)	26.1% FE 26.0% IE	Neri *et al*., 2009
Escherichia coli in reverse micelles	[Lac]= 22.11 g/l in AOT/isooctane, 22.11 g/l and 238 g/l in aqueous system [E]= 16.7 µg/ml in AOT/isooctane, 16.7 and 18 µg/ml in aqueous system	T= 37°C in buffer with 338mM AOT/isooctane, 36mM 2-mercaptoethanol and 0.6mM $MgCl_2$, (pH 4.3–11.2); T=37°C in aqueous system with Tris–HCl buffer (0.1M, pH 7.3)	~ 5-10% depending on the conditions	Chen *et al*., 2003

Kluyveromyces lactis (Maxilact LX 5000) immobilized on cotton cloth	[Lac]= 30, 50, 75, 100 and 125 g/l, pumped at flow rate 2.8 ml/min [E]= 280U	T= 37°C in potassium phosphate buffer (0.1 M, pH 6.6) with M_gCl_2 (1.5 mM) for 60-120 minutes	2-18 mM depending on [Lac]	Zhou *et al*., 2003
Saccharomyces cerevisiae L_1, *Penicillium expansum* F3 and *Kluyveromyces lactis* L_3 immobilized in calcium alginate	[Lac]= 50, 100, 180, 270, 320, 380, 450 and 480 g/l [E]= 10U of immobilized E	T= 37, 45, 50 and 55°C in acetate buffer (pH 3.6, 4.5, 5.4) and phosphate buffer (pH 6.4, 7.2, 8.2) for 24 hours	28.7% for *P. expansum* F3; 28.3% for *S. cerevisiae* L_1; 23.0% for *Kl. lactis* L_3;	Li *et al*., 2008
Aspergillus oryzae immobilized on cotton cloth	[Lac]= 50, 100, 200, 300, 400 and 500 g/l at flow rate 1 ml/min [E]= 50 mg/g of cotton cloth	T= 30, 40, 50°C in acetic acid buffer (0.1M, pH 4.5, 5.2, 6.0) for 11-25 hours depending on T°C incubation	~ 26%	Albayrak *et al*., 2002

The state of the enzyme (free vs. immobilized) appears to affect GOS formation. Some authors reported enzyme inactivation during the immobilization procedure and/or after use versus the free form: ~50-90% on polyethyleneimine and glutaraldehyde for a β-galactosidase from *Aspergillus oryzae* (Matella *et al*., 2006); ~50% on silica-alumina for a β-galactosidase from *Kluyveromyces fragilis* (Ladero *et al*., 2000). This change may be due to mass transfer limitations for the larger sugar molecules (Yang *et al*., 1988). On the other hand, some authors (Berger *et al*., 1995; Neri *et al*., 2009; Gaur *et al*., 2006) did not find significant inactivation of the enzyme when immobilized or found that the immobilized enzyme gave a higher yield of GOS.

1.5.3 GOS production using different substrate concentrations or assay conditions

Other researches have focused their studies on the effect of substrate concentration on GOS production without changing enzyme concentration or assay conditions, such as temperature, pH, agitation, reaction time (Table 1.8).

Table 1.8: Transglycosylation reaction carried out with different lactose concentrations and fixed assay conditions. Where Lac: lactose, E: enzyme Lac_0: initial lactose concentration.

Enzyme source	[Substrate] and/or [Enzyme] studied	Assay conditions	Maximum synthesized GOS (% of $[Lac]_0$)	Reference
Kluyveromyces lactis expressed in *Escherichia coli*	[Lac]= 51.63, 95.84, 301.22 g/l [E]= 0.029 mM	T= 37 °C for 5 hours, in potassium phosphate buffer (50mM, pH 7.0), containing 10mM N_aC_l and 1.5mM $MgCl_2$	~ 6-16% depending on [Lac]	Kim *et al*., 2004
Bifidobacterium bifidum NCIMB 41171	[Lac]= 45, 55 g/l [E]= 2.5% (344U/g)	T= 40°C with shaking (100 R.P.M) for 25 hours, in phosphate buffer (0.1M, pH 6.8) or citric acid/trisodium citrate (0.1 M, pH 6.2)	~10-17% depending on [Lac]	Goulas *et al*., 2007
Bifidobacterium bifidum NCIMB 41171	[Lac]= 100-500 g/l [E]= $2x10^8$ c.f.u.	T=39°C in potassium phosphate buffer (0.2 M, pH 6.8) for 7 hours	5%	Tzortzis *et al*., 2005
Bacillus circulans	[Lac]=~0.l9-0.59 g/l [E]= 0.4g in 2 ml H_20	T= 40°C in sodium phosphate buffer (0.02M, pH 5) for 90-340 min	~20-26% depending on [Lac]	Boon *et al*., 1999
Aspergillus oryzae	[Lac]= 47.57, 90.15, 191.68, 359.97, 571.64 g/l [E]= 0.1% (4.2 U/ml)	T= 40°C in pH 4.5 for 15 min	~11-35% depending on [Lac]	Iwasaki *et al*., 1996
Bifidobacterium infantis HL96, expressed in *Escherichia coli*	[Lac]= 20-30 g/l [E]= 2.5 U/ml	T= 30-60°C in Na-phosphate buffer (50 mM, pH 7.5) for 30 hours with agitation (100 R.P.M.)	6%	Hung *et al*., 2002
Bifidobacterium bifidum	[Lac]= 34.23, 85.57, 117.14 and 136.92 g/l [E]= 25 µl (50munits)	T= 45°C in sodium citrate buffer (0.1 M, pH 4.25) for 50 hours	29%	Dumortier *et al*., 1994

Others attempts to improve GOS synthesis examined the effect of lactose and enzyme concentrations or sources, or assays conditions, such as temperature, solvent or added metal cations (Table 1.9).

Table 1.9: Transglycosylation reaction carried out with different lactose, and/or enzyme concentrations, and/or enzyme source, and/or assay conditions. Where Lac: lactose; E: enzyme; STR: stirred tank reactor; UFMR: ultra filtrate membrane reactor, and Lac_0: initial lactose concentration.

Enzyme source	**[Substrate] and/or [Enzyme] studied**	**Assay conditions**	**Maximum synthesized GOS (% of $[Lac]_0$)**	**Reference**
Kluyveromyces lactis (Lactozym 3000 L HP G)	[Lac]= 150, 250, 350 g/l [E]= 3, 6, 9 U/ml	T= 40, 50, 60°C in phosphate buffer (50 mM, pH 5.5, 6.5, 7.5) with shaking at 300 rpm	~ 5-17.1% depending on [Lac] and pH	Martinez-Villaluenga *et al*., 2007
Aspergillus oryzae	[Lac]= 51.34 g/l [E]= 0.025 mg (>8U/mg)	T= 40°C in citrate buffer (50 mM, pH 4.5) or mixture organic solvent (1,4-butanediol, 1.5-pentanediol, methoxyethyl acetate, triethyl phosphate, acetonitrile) for 48 hours	~ 0.25-25% on lactose substrate at T_0, depending on the solvent used	Srisimarat *et al*., 2008
Kluyveromyces lactis (Maxilact LX 5000)	Reconstituted dried buttermilk, whose [Lac]= 219 g/l [E]= 0.1, 0.4, 0.8, 1.2, 1.6, 2%	T= 38°C for 80 min	~ 13%	Čurda *et al*., 2006
Bifidobacterium bifidum truncated produced in *E. coli*	[Lac]= 100, 200, 400 g/l [E]= not specified, different [E] used	T= 38°C for 20 hours	~ 38-42% depending on the [Lac]	Jørgensen *et al*., 2001
Kluyveromyces maxianus var. *lactis* OE-20	[Lac]= 10, 39, 50, 100, 200 g/l [E]= 1.0 U/ml	T= 25-40°C in phosphate buffer (10 mM, pH 7.0) for 3 hours	1.5-13% depending on [Lac] and T °C	Kim *et al*, 2001
Sterigmatomyces elviae CBS8119	[Lac]= 20 g/l [E]= 5 ml of toluene-treated suspension cells	T= 60°C in potassium phosphate buffer (100 mM, pH 6.0) for 2 hours	24% adding Fe^{2+}, Zn^{2+} and Cu^{2+} in the media	Onishi *et al*., 1998
Kluyveromyces lactis (Maxilact L2000) in stirred tank reactor (STR) and cross-flow ultrafiltration membrane reactor (UFMR)	[Lac]= 220, 280, 340 and 400 g/l in STR, 0.25 g/l in UFMR [E]= 2.9, 5.8 and 8.7 U/ml in STR, 8 U/ml in UFMR	T= 40°C in potassium phosphate buffer (0.2M, pH 7.0) with $MgCl_2$ (2mM) for 4 hours with agitation (200 R.P.M.)	in STR: ~22-25% depending on [Lac] for [E]= 5.8U/ml in STR; ~ 70-100 mg/ml depending on [E] for [Lac]= 340 mg/ml; in UFMR: 26.05 mg/ml with [Lac]=250 mg/ml	Chockchaisawasdee *et al*., 2005

Penicillium semplicissimus	[Lac]= 200, 300, 400, 500 and 600 g/l [E]= 18 and 26.6 U	T= 40, 45, 50 and 55°C in McIlvaine buffer (75 and 150 mM, pH 2.6-7.0) for 8 hours	25.63-30-48% depending on T °C and [Lac]	Cruz *et al*., 1999
Bacillus circulans, Aspergillus oryzae, Kluyveromyces lactis, K. fragilis	[Lac]=~ 0.20-0.60 g/l [E]= 62.5 mg/ml *for B. circulans* and *A. oryzae*, 100 µl for *K. lactis*, 75 µl for *K. fragilis*	T= 40°C for 360 min - in McIlvaine standard citrate buffer (0.02 M) pH 4.5 for *A. oryzae* and pH 5.0 for *B. circulans*; - in potassium phosphate buffer (0.025 M, pH 7.3) for *K. lactis* and pH 6.5 for *K. fragilis*;	~ 8-15% depending on the source of the enzyme	Boon *et al*., 2000
Kluyveromyces marxianus ATCC 56497, *Kluyveromyces lactis, Aspergillus oryzae*, *Bacillus* spp.	[Lac]= 330 g/l [E]=1% (w/v) *K. marxianus*, 10 and 13 U/g Lac for *K. lactis*, 6.2 U/g Lac for *A. oryzae*, 4.5 and 5.6 U/g Lac for *Bacillus* spp.	T= 30, 40 and 50°C with agitation (200-300 R.P.M.) for 24 hours; for *Bacillus* spp. 15U gluzyme/g Lac were added to the β-galactosidase at 0, 6, 12 and 18 hours and T= 50°C pH 5.0 by adding 40% (w/w) $CaCO_3$; for *K. marxianus* 1% (w/v) of malt extract was added, T= 30 °C pH 5.0-5.5 by 5mM NaOH	*A. oryzae*: 17-21%; *K. lactis*: 21-35%; *Bacillus* spp.: 27-33%; *K. marxianus* ATCC 56497: ~6% trisaccharides and ~ 15% tetrasaccharides	Cheng *et al*., 2006

Studies on laboratory selected bacterial enzymes have been also carried out (Table 1.10), without changing the assay conditions (temperature and pH).

Table 1.10: Transglycosylation reactions carried out with selected bacteria or uncommon strains enzymes. Where Lac: lactose, E: enzyme, and Lac_0: initial lactose concentration.

Enzyme source	**[Substrate] and/or [Enzyme] studied**	**Assay conditions**	**Max synthesized GOS (% of $[Lac]_0$)**	**Reference**
Sporobolomyces singularis	[Lac]= 200 g/l [E]= 0.16 U/ml	T= 40°C in phosphate citrate buffer (50 mM, pH 6.0)	~ 35%	Ishikawa *et al*., 2005
Sterigmatomyces elviae CBS8119	[Lac]= 400 g/l	T= 30°C in buffer (pH 6.0, with $(NH_4)_2SO_4$, K_2SO_4, KH_2SO_4, M_gSO_4, C_aCO_3) with shaking for 60 hours.	~ 37.5%	Onishi *et al*., 1995
Penicillium semplicissimum	[Lac]= 600 g/l [E]= 26.6 U/50 ml	T= 50°C in McLlvaine buffer (150 mM, pH 6.5) for 8 hours	30.5%	Cruz *et al*., 1999
Bullera singularis	Pure lactose and whey, whose [Lac]= 200 g/l [E]= 5.4 U/g Lac	T= 37°C in sodium phosphate buffer (50 mM, pH 6.0) for 30 hours	34-41%	Cho *et al*., 2003

The proportion of transgalactosylation to hydrolysis reactions varies, depending on different sources of the enzymes. Some β-galactosidases, from *E. coli* or *Aspergillus niger*, appear to promote strong hydrolytic activity, whereas the β-galactosidase from *Aspergillus oryzae* or *Bacillus circulans* exhibit strong transglycosylation (Mahoney, 1998).

From the analysis of most of the present literature until now, it is possible to conclude that only a few researchers claim to have reached GOS synthesised level higher than 40%, while most of the research reached a GOS synthesis of around 20-25%. The production of higher levels of GOS is a challenge and may require new approaches.

Although the enzymes derived from various microbial origins have different properties, many use glutamic acid, as a key catalytic residue of their active site, as shown in Table 1.11.

Table 1.11: Physical properties and catalytic residues of β-galactosidases from various microbial origins (Adapted from Zhou *et al*., 2001).

Enzyme Origin	*Kluyveromyces lactis*	*Escherichia coli*	*E. coli* (subunits)	*Aspergillus niger*
Molecular weight (Da)	117618	116351	118016	119160
Length (AA)	1025	1023	1031	1006
Proton donor	Glutamate^{482}	Glutamate^{461}	Glutamate^{449}	Glutamate^{200}
Nucleophile/base	Glutamate^{551}	Glutamate^{537}	Glutamate^{512}	Glutamate^{298}

Several β-galactosidases have been purified, sequenced and extensively characterized. Some β-galactosidases are commercially available. Although there may be sequence differences in enzymes across species, the active site and the two catalytic glutamate residues are highly conserved. Thus, fundamentally the problem of enhancing GOS synthesis is to influence attack of sugar rather then water on the enzyme-galactose complex.

1.6 Modelling of GOS production kinetics

Modelling the synthesis of galactooligosaccharides has been investigated in the past by different authors. Some authors (Iwasaki *et al*., 1996) considered the chain length of the synthetised galactooligosaccharides, involving a complex model based on 19 ordinary differential equations. Although a separation of GOS considering the chain length is the most complete approach to describe transglycosylation reaction, from a theoretical point of view, the application of the model could be difficult and lead to ill conditioned systems (Boon *et al*., 1999).

Other authors (Boon *et al*., 1999 and 2000; Neri *et al*., 2009) proposed simplified mechanisms, with the consideration of the GOS family of compounds as a single moiety and ignoring the formation of allolactose or other intermediate compounds. The solution of the presented models involved the application of the King-Altman method (King *et al*., 1956) to simplify the system and reduce the number of equations. However, the King-Altman simplification lead to a model where the enzyme concentration is not considered as interactive part of the transglycosylation reaction.

Kim *et al*. (2004) proposed a GOS reaction mechanism that included the enzyme concentration as well as the synthesis of allolactose in the system.

To date, and to the best of our knowledge, no error associated to the estimated kinetic reaction rates has been reported in literature, other than the values reported by Neri *et a*l. (2009) and Boon *et al*. (1999 and 2000). The procedure employed to report the parameters was to estimate the parameter and its standard error as the mean and the standard deviation of a series of individual fittings. With present statistical methods available it would be possible to report statistical errors from individual

experiments by applying a multiresponse nonlinear regression method (Bates *et al*., 1984).

1.7 Overview of the literature on GOS production by β-galactosidase

Although there has been extensive research on better utilization of whey derived lactose, the dairy industry is still in need of new technologies for converting lactose into marketable products (Yang *et al*., 1995). Thus, converting lactose into a product that contains a prebiotic food ingredient and is free of problems associated with lactose intolerance is highly desirable (Playne *et al*., 1996). A recent study (Cho *et al*., 2003) comparing the transglycosylation reaction using pure lactose and cheese whey as substrate, found out that GOS conversion (%) and reaction rate of the whey reaction were slightly higher with cheese whey than when pure lactose was used as substrate.

The β-galactosidase most studied for GOS production is from *Escherichia coli* and is encoded by the lacZ gene. It is not considered suitable for use in foods owing to toxicity problems associated with the host coliform (Mahoney, 1997). Hence, the β-galactosidase from *E. coli* is generally not preferred for use in food industry (Joshi *et al*., 1987; Stred'ansky *et al*., 1993; Mahoney, 2003). Furthermore, many of the enzymes used in previous studies (Table 1.10) are not from sources commercially available or are not available in sufficient quantities for industrial applications.

In contrast, relatively little is known about the enzymes from eukaryotes, such as *Kluyveromyces lactis*. Previous studies showed that β-galactosidase from *B. circulans* produces the largest sized oligosaccharides (Neri, 2008). However, the enzyme from *Kluyveromyces* spp. produces comparably large amounts of glucose and galactose as indicated by its strong hydrolytic activity and production of high

proportion of trisaccharides in the synthesis mixture (Boon *et al*., 2000; Matsumoto *et al*., 1993; Nakanishi *et al*., 1983; Prenosil *et al*., 1987). A recent study (Cheng *et al*., 2006) compared GOS production by β-galactosidases from *Aspergillus oryzae*, *Kluyveromyces lactis* and *Bacillus* spp., in a combined system with *K. marxianus* β-galactosidases, found that *K. marxianus*, increasing the final mass production of GOS by 3%. This is because more GOS-4 and less GOS-2 are obtained by the mixed enzyme system, as GOS-2 is consumed by *K. marxianus*, while GOS-4 is not.

1.8 Objectives and aims of the work

The aim of the research in this thesis is to investigate GOS production with commercially available β-galactosidases with a view to understanding the factors that influence GOS yield. In order to achieve this the following objectives of the work were:

- To propose a model of the reaction system of GOS production that can be identified under normal conditions.
- To investigate the effect factors that may influence and improve the GOS yield. As such the enzyme concentration, substrate concentration and solvent usage in the kinetics of GOS production were investigated.
- To investigate the standardisation of GOS production research assays and the influence of the enzyme source in the GOS kinetics and yield.

The enzymes were obtained from *Kluyveromyces* spp. and *Escherichia coli*. The substrate of the reaction was a waste by-product of the dairy industry, Whey Permeate. This material has recently been shown to be a good substrate for GOS synthesis (Cho *et al*., 2003).

We sought in the first place to devise an assay method that would allow rapid, convenient monitoring of the GOS synthesis reaction progress and the quantitation of key components using Thin Layer Chromatography, TLC.

In order to quantify the GOS synthesis reaction products with greater accuracy and precision we devised a High Performance Liquid Chromatography (HPLC) assay method. This allowed accurate quantitation of reaction component profiles that were used to compare assays under different enzyme and substrate concentrations. Furthermore, it was possible to examine the effect of enzymes from different species, such as *Kluyveromyces* spp. and *Escherichia coli*, on the GOS synthesis with the aim of identifying those that gave higher yields.

In order to reduce the water activity of the system, we have examined the influence of water miscible solvents on GOS synthesis.

Finally, a reaction scheme for lactose hydrolysis and GOS production based on transglycosylation mechanisms previously described in the literature was proposed in order to construct a mathematical model of the experimental data. The reaction mechanism modelling will allow for:

1. The analysis of yield and mass balance and thereby assess the efficacy of analytical methods to monitor the reaction progress.
2. The estimation of the reaction rate constants for the proposed mechanism that would facilitate prediction of GOS yield at any point in time during the reaction.
3. The optimisations of the GOS yield using the model.
4. The assessment of the effect of the manipulation of reaction conditions (*i.e* different enzyme/substrate concentration, solvent addition, and different enzyme source) on GOS production.

2 Materials and Methods

2.1 Substrates

2.1.1 Whey permeate

The Whey Permeate (WP), used as substrate for GOS synthesis in these, was provided by Kerry Group plc (Prince's Street Tralee, Co. Kerry, Ireland). This product, a fine powder of pale yellow colour, is characterised by a lactose content of over 90%. The spray dried whey permeate provided was a demineralised WP (product code W469), whose mineral concentration has been reduced by Ion Exchange. This product has a lactose content of up to 92%, a protein content of 2.5%, and a fat and mineral content of 1% each.

The whey was stored in a multi-walled paper sack with inner polyethylene liner to avoid moisture absorption, and kept in a cool dry store, odour free, with maximum humidity of 65%. The product is suitable for food industry use.

2.1.2 β-galactosidases

Two β-galactosidases were used to carry out transglycosylation reactions. The first is commercially available under the name Maxilact® and was provided by Carbon Group (Ringaskiddy, Co. Cork, Ireland). Maxilact L2000 (G003-MLT-991) is a purified liquid lactase preparation derived from the dairy yeast *Kluyveromyces lactis*. Its activity is ≥ 2,000 Neutral Lactase Units/g. A Neutral Lactase Unit is defined as the quantity of enzyme that will liberate 1.0 μmol of o-nitrophenol from o-nitrophenyl β-D-galactoside at pH 7.0 and 37ºC. The enzyme is supplied as a glycerol solution. The other chemical components present in Maxilact L2000 and its microbiological properties are listed in the Table 2.0. Maxilact L2000 complies with the purity specifications of the FAO/WHO's Joint Expert Committee of Food

Additives (JECFA), with the Food Chemical Codex (FCC) and with the guidelines for Food Enzymes of the Scientific Committee of Food (SCF) in the EU.

Table 2.0: Chemical and microbiological properties of Maxilact L2000.

Specification	**Value**
Heavy Metals	≤ 30 ppm (as Pb)
Lead	≤ 5 ppm
Arsenic	≤ 3 ppm
Mercury	≤ 0.5 ppm
Cadmium	≤ 0.5 ppm
pH	7.0-7.5
Glycerol	≥ 50% (vol/vol)
Total bacterial count	≤ 10 in 1 ml
Coliforms	≤ 30 in 1 ml
Salmonella	Absent in 25 ml
Staphyloccoccus aureus	Absent in 1ml
Escherichia coli	Absent in 25 ml
Lysteria monocytogenes	Absent in 25 ml
Yeasts	≤ 10 in 1 ml
Moulds	≤ 10 in 1 ml
Antibioticy activity	Absent by test
Mycotoxins	Absent by test

The enzyme preparation was stored in its original sealed container at 4ºC, as specified in the data sheet for the product. Under these conditions the loss of activity was less then 1% per month.

The second enzyme used in these studies was an *Escherichia coli* β-galactosidase, purchased from Sigma-Aldrich. The bacterial β-galactosidase, grade VIII, was a lyophilized powder with an activity of 600-1200 units/mg, where one unit was defined as the quantity of enzyme that hydrolyzes 1.0 μmole of o-nitrophenyl β-D-galactoside to o-nitrophenol and D-galactose per minute at pH 7.3 and 37ºC.

2.2 Chemicals

All the chemicals, including the chromatography standards: maltotriose, lactose, galactose and glucose; all solvents, such as ethanol, methanol, butanol, acetonitrile acetone, diethyl ether, dioxane and sulphuric acid; Thin Layer Chromatography plates (Fluka, ref. no. 02599); micropipettes (Blaubrand®, intramark, 1-5 μL, catalogue number 708707); were supplied by Sigma-Aldrich (Dublin, Ireland).

2.3 Commercially available GOS syrup used for comparison studies

The synthesised products of the reaction were compared with the pre-biotic galactooligosaccharide syrup commercially available under the name Vivinal GOS® (Friesland Foods Domo®, P.O. Box 449, Zwolle, 8000 AK, The Netherlands). The typical composition of this product is 75% dry matter, of which 59% is GOS, 21% lactose, 19% glucose and 1% galactose. The chemical, physical and microbiological specifications of Vivinal GOS® are listed in Table 2.1.

.

Table 2.1: Chemical, physical and microbiological characteristics of Vivinal GOS®. Where D.M.: dried matter; c.f.u.: colony forming units.

Specifications	Value
Dried matter (D.M.)	74-76%
Galactooligosaccharides	Min 57% on D.M.
Nitrogen	Max 0.016% on D.M.
Sulphated ash	Max 0.3% on D.M.
Lactose anhydrous	Max 23% on D.M.
Glucose anhydrous	Max 22% on D.M.
Galactose	Min 0.8% on D.M.
Viscosity	1000-5000 cPs
Nitrite	Max 2 ppm on D.M.
pH	3.2-3.8
Total plate count T=30ºC	Max 3000 c.f.u./g
Enterobacteriaceae	Absent in 1 g
E. coli	Absent in 5 g
Yeasts	Max 50 c.f.u./g
Moulds	Max 50 c.f.u./g
Staphylococci Coagulase +	Absent in 1 g
Salmonellae	Absent in 25 g

2.4 GOS synthesis reaction

Laboratory scale reactions for GOS synthesis were carried out by dissolving demineralised Whey Permeate in phosphate buffer (0.1 M, pH: 6.8) to which β-galactosidase at varying levels was added. As indicated by studies in the literature (Huh, 1990; Lopez-Leiva *et al*., 1995; Rustom *et al*., 1998), high lactose concentrations facilitate transglycosylation reactions. Therefore, the lactose concentration used was 200 g/l, corresponding to the maximum aqueous solubility of lactose. In addition to the concentration of 200 g/l, a higher concentration was tested. A concentration of 350 g/l was reached by adding the Whey Permeate to the buffer system at its boiling point in order to create a supersaturated solution.

For GOS synthesis reactions were carried out in an Erlenmeyer volumetric flask immersed in a thermostatic bath (GRANT OLS2000) at 40°C, with agitation at 80 r.p.m., in order to allow for continuous mixing of the media without the formation of air bubbles.

Typically, the reactions were carried out in a volume of 100 ml for five hours to ensure completion of the synthesis/degradation reaction. Triplicate samples (1 ml) were withdrawn every 30 minutes. The enzymatic reaction was quenched by boiling for 10 minutes, followed by frozen storage at -18°C.

2.4.1 Use of solvents

Reduced water activity (a_w) may enhance the synthesis of GOS (Goulas *et al*., 2007). Moreover, many enzymes have altered specificity in the presence of organic solvents. To enhance GOS synthesis the effect of adding solvents to the GOS synthesis reaction mixture assay was investigated. The solvents were used in relatively low concentrations, to avoid inhibiting enzyme activity. The solvents used were ethanol, acetonitrile, acetone, diethyl ether and dioxane.

2.4.2 Enzyme comparison studies

GOS synthesis reaction was carried out to compare two β-galactosidases from different sources were carried out. The enzymes compared were *E. coli* β-galactosidase and *Kluyveromyces lactis* β-galactosidase (Maxilact L2000). Thus, these enzymes were sourced from a prokaryote and a eukaryote microorganism. The reactions were carried out by dissolving demineralised Whey Permeate (200 g/l) in phosphate buffer (0.1 M, pH 7.0) with agitation (80 R.P.M.). The concentrations used were 0.1 mg/ml and 0.2% for *E. coli* and Maxilact L2000 respectively. The initial rates of lactose degradation were used to normalise the enzymes to the same activity.

2.5 Methods

2.5.1 pH measurement

pH was measured using an Orion pH meter model 420A (Orion research Inc, Beverly, MA. US). The pH meter was calibrated with pH 4.0, 7.0 and 10.0 standard buffers before use.

2.5.2 Water activity measurement

Water activity was measured using an AQUALAB model 3 TE (Decagon Devices, Inc.), with the temperature of the internal chamber set as the assay temperature. Before measurement, the water activity of pure distilled water and activated charcoal were checked.

2.5.3 Thin Layer Chromatography (TLC)

The products obtained through enzymatic hydrolysis of Whey Permeate were analysed by Thin Layer Chromatography (TLC), using a silica gel matrix on Alu-foil TLC plate (20x10cm, 60 Å medium pore diameter).

A small portion of the samples (1 µl), was diluted 1:10 and applied to a TLC plate by capillary injection with a disposable micropipette. The samples were applied over 1 cm from the bottom of the plate and, at least 1 cm from each other.

Development of the TLC plate was carried out in the Twin Trough Chamber light-weight CAMAG® (20x10 cm, product number 022.5254, Mason Technology, Dublin, Ireland), at room temperature, under a fume hood. Before application of samples the TLC plate was placed in the chamber, which contained the solvent, for pre-equilibration (Figure 2.0). This step, called conditioning, took 1 hour and helped to increase the reproducibility of the analysis (CAMAG protocol A 07.3).

Figure 2.0: TLC development steps. Conditioning is shown on the left and development on the right (From CAMAG protocol A 07.3).

The solvent system used to separate the carbohydrate mixture, of glucose, galactose, lactose and galactooligosaccharides, was a butanol/methanol/H_2O (70:20:10 [vol/vol/vol]) mixture.

When development was completed, plates were dried using a Qualivac vacuum oven at 100 °C and -760 mmHg for 2 minutes approximately. To visualise the separated carbohydrates the plates they were sprayed with a fine spray of 35% H_2SO_4 in Ethanol. Finally, the plate was dried in a vacuum oven (100 °C and -760 mmHg) for 5 minutes.

Since the retention factor depends on many variables, such as temperature and solvent composition, an internal standard of lactose, glucose and galactose at a known concentration was spotted on every TLC plate.

The analysis of the plate was achieved by the scanning the developed TLC plate using a optical scanner (HP series 5300) and subsequent analysis with image analysis software (Image J, version 1.38X).

2.5.4 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) analysis was used to accurately quantify GOS synthesis products.

HPLC was carried out using a SUPELCOGEL Ca^{2+} column (product no. 5930-U), 30 cm x 7.8 mm I.D., and a flow rate of 0.5 ml/min. A column heater (Waters Temperature Control Module I and II) was used to maintain the column temperature at 80 º C. The column is a cation exchange resin consisting of sulfonated cross-linked styrene-divinyl benzene copolymer in the calcium form, of 9 μm particles diameter.

The mobile phase used was distilled, ultrapure water (Waters Purification System Simplicity 185). The mobile phase was filtered through Nylon filter (MAGNA 0.22 micron, 47 mm) and degassed for 20 minutes in an ULTRAsonick bath 57X (NEY) before use.

The detector used was a Refractive Index (RI) Detector (Waters 410), with an internal temperature of 34 ºC.

For automatic injection, the injection volume used was 10 μl. When the manual injector was used, 60 μl was injected.

Instrument control, data acquisition and analysis were performed using the Empower 2 Enterprise Build 2154 (2005, 2006) software.

The samples for HPLC analysis were diluted 1:100 or 1:500 and filtered through a Nylon membrane (SUPELCO, 25 mm x 0.45μm) before injection.

2.6 Numerical methods

Model building and individual fitting of data from each of the experiments were performed using JSim version 1.6.82 (Physiom Project, Washington) (Bassingthwaighte, 2000).

JSim estimated the values of reaction rate constants by fitting the proposed kinetic models to the experimental data using a mixture of a non-linear steepest-descent, and an adaptive nonlinear least-squares and SENSOP, a variant of the Levenberg-Marquardt algorithm (Chan *et al*., 1993).

The global fitting of the model to the experimental data was performed using software built in Fortran 77, employing the subroutine DLSODA May 2005 version of the ODEPACK library (Hindmarsh, 1983) for simulation of the ordinary differential equations (ODE) system and the multiresponse nonlinear regression subroutine DODRC from the ODRPACK library (Boggs *et al*., 1992).

The integration of initial value ODE systems resulting from mathematical modelling was performed using a multistep backward differentiation formula for stiff systems in ODEPACK and a implicit Runge-Kutta method of order 5 for stiff systems (RADAU5) in JSim.

The ODRPACK package used a derivative of the Levenberg-Marquardt algorithm to perform the fitting of experimental data to a biochemical reaction model.

JSim simulation settings are shown in Table 2.2.

Table 2.2: JSim simulation constants setting: JSim simulation ODE (a) and JSim fitting (b).

(a)

JSim simulation ODE	Value
Relative tolerance	10^{-4}
Absolute tolerance	10^{-7}
Number of steps	100000

(b)

JSim fitting	Value
Maximum number of iterations	2000
Min RMS error	0.001
Minimum gradient	10^{-6}
Parameter tolerance	10^{-8}

The ODEPACK package was set with the following options shown in Table 2.3.

Table 2.3: ODEPACK package constants setting.

ODEPACK package	Value
Atoll	10^{-4}
Rtoll	10^{-7}
Number of steps	500000

In the ODRPACK package Jacobian calculation was carried out by a central differences scheme. The precision of regression estimated automatically by ODRPACK is shown in Table 2.4.

Table 2.4: ODRPACK precision of regression setting.

ODRPACK package	Value
Significant digits	up to 8
Tolerance of sum of squares	1.5×10^{-8}
Parameter tolerance	3.6×10^{-11}
Maximum number of iterations	10000

3 Results

3.1 Development of a TLC method for analysis of GOS synthesis

Thin Layer Chromatography (TLC) was initially explored as a screening technique to monitor the synthesis of GOS. This technique is simple, quick, and inexpensive. TLC offers the possibility of observing all the components in the synthesis of GOS at the same time. Thus, under suitable conditions, lactose, glucose, galactose and GOS may be monitored simultaneously.

Different solvent systems were explored to achieve optimum spot resolution. A literature review identified several examples of previous studies of TLC analysis of sugars, carried out using different solvent systems.

For example, Rabiu *et al*., (2001) separated carbohydrates mixtures, such as glucose, galactose, lactose and galactooligosaccharides, by TLC using butanol-ethanol-water (5:3:2 [vol/vol/vol]) as the mobile phase. Petzelbauer *el al*., (2000), used 2-methyl-1-propanol/pyridine/H_2O (6:4:3) as eluent. Jørgensen *et al*. (2001) used a solvent system containing butanol/2-propanol/H_2O (3:12:4 [vol/vol/vol]); while Fischer *et al*. (2006) achieved separation of oligosaccharides in an acetone/n-butanol/water (70:15:15) mixture. Ohmiya *et al*., (1977), analysed the products of lactose hydrolysis with an *n*-butanol/methanol/boric-acid TLC system (5:3:1 [vol/vol/vol]) at room temperature. The CAMAG protocol (Materials and Methods, section 2.5.2) advised the use of a mixture of acetonitrile/H_2O (85:15) for the analysis of mono-, di- and trisaccharides and a butanol/methanol/H_2O system (50:25:20) for the separation of polysaccharides.

In our hands, most of those solvent systems were found to yield unsatisfactory results in terms of resolution, mobility and streaking with the sugars used in this study. Our optimisation studies showed a solvent system composed of

butanol/methanol/H_2O (70:20:10), achieved good resolution and minimised streaking. Equilibration of the TLC plate with the solvent system gas phase, prior to chromatographic development, was essential to achieve good resolution, using the CAMAG protocol (A 07.3, www.camag.com).

To visualise the separated sugars on the TLC plate, different solutions may be used, such as: 5% ceric sulphate in 15% concentrated H_2SO_4 (Rabiu *et al*., 2001), orcinol reagent (Jørgensen *et al*., 2001), anthrone-H_2SO_4 reagent (Ohmiya *el al*., 1977), 5% H_2SO_4 in ethanol containing α-naphtol (Tanriseven *et al*., 2002), 3% *para*-anisaldehyde in ethanol containing 5% H_2SO_4 (Naudorf *et at*., 1998), The visualization could be achieved either by dipping the plate into the staining mixture or by spraying it. After several investigations, a solution of H_2SO_4 in ethanol was found to be the most appropriate detection mixture for these studies. However the concentration of H_2SO_4 was increased to 35% compared to literature protocols (Tanriseven *et al*., 2002; Naudorf *et al*., 1998; Rabiu *et al*., 2001). The visualization solution was sprayed on the TLC plates since this method gave a better visualization than the dipping method.

TLC has been used as a qualitative method to detect galactooligosaccharides by many authors (Rabiu *et al*., 2001; Jørgensen *et al*., 2001; Ohmiya *et al*., 1977; Tanriseven *et al*., 2002; Petzelbauer *el al*., 2000; Naudorf *et al*., 1998), but not as a quantitative method. In this study we investigated the possibility of using this method to quantify components as they changed during GOS synthesis.

Therefore, qualitative analysis of GOS synthesis was carried out by scanning TLC plates, followed by image analysis with Image J software. This program allows quantitative analysis of the spots on the TLC plates which are related to their darkness and area.

Standards solutions of glucose, galactose, lactose and maltotriose were spotted on TLC plates under the previously described conditions (Materials and Methods, section 2.5.3). The TLC plates showed a linear correlation between the concentration of the sugars and the darkness and area of the spot (Figure 3.0).

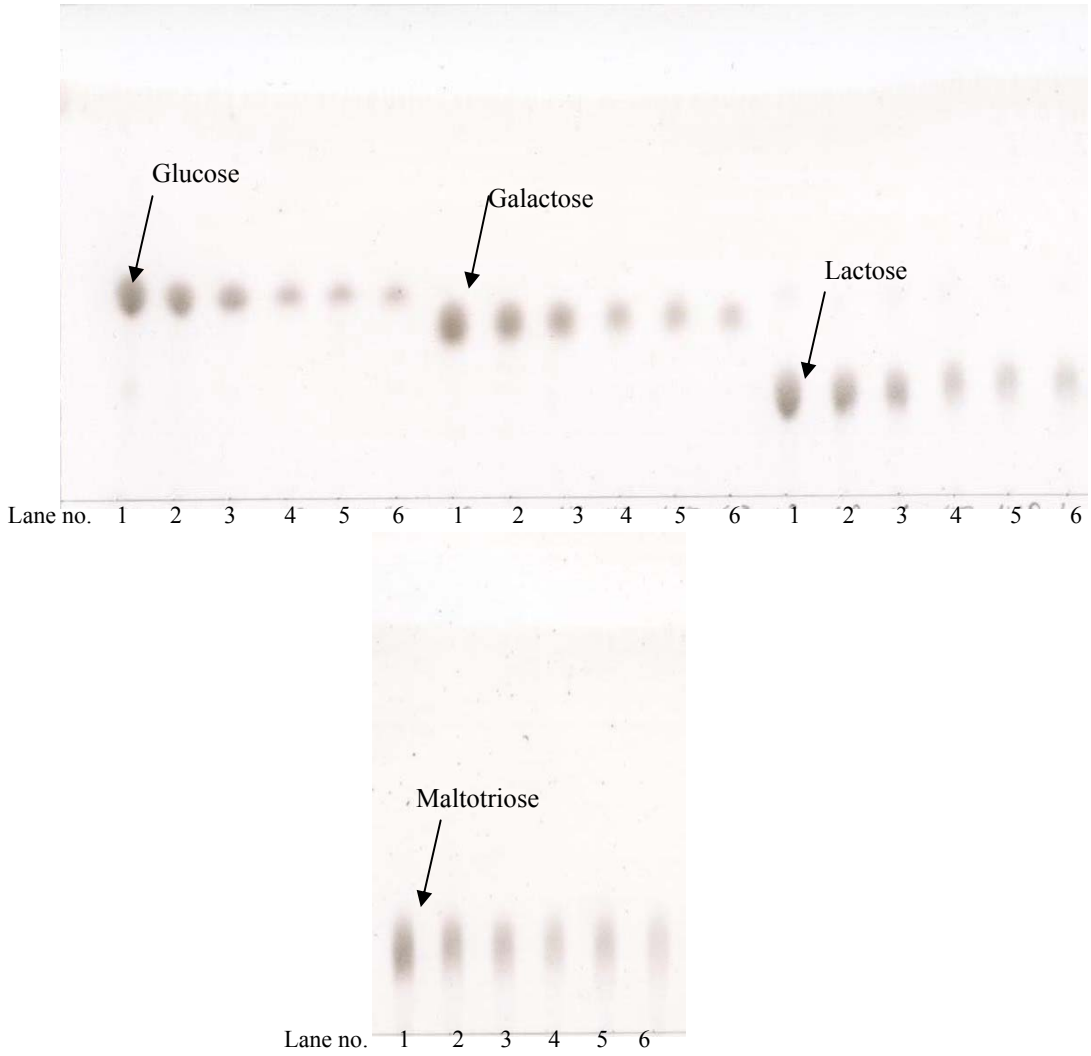


Figure 3.0: TLC analysis of standard solutions. The densities of the spots of glucose, galactose, lactose and maltotriose standard solutions are proportional to their concentrations. Each starting point is a different concentration of a pure standard solution (line 1: 25 g/l; line 2: 16.6 g/l; lane 3: 12.5 g/l; lane 4: 6.25 g/l; lane 5: 5 g/l; line 6: 4.16 g/l). The TLC plates were developed with a butanol/methanol/H_2O (70:20:10 [vol/vol/vol]) mixture, stained with a solution of 35% H_2SO_4 in ethanol and dried in a vacuum oven (100 °C and -760 mmHg) for 5 minutes.

Data from the TLC plates shown in Figure 3.0, were used to construct calibration curves for lactose, galactose, glucose and maltotriose (4.16, 5, 6.25, 12.5, 16.6 and 25 g/l) (Figure 3.1). Each calibration curve was constructed for six concentration levels, with each concentration spotted in triplicate.

The response factor of maltotriose was used to calibrate galactooligosaccharides. There is not a commercial GOS available for calibration and because GOS is a heterogeneous mixture, maltotriose was the closest oligosaccharide to GOS available.

Each TLC plate was run using an internal standard lane to correct for day to day variations in response to visualization staining. This mechanism allowed the reduction of the variability of each analysis due to factors such as: quantity of stain sprayed on the TLC plates, temperature and time of drying. The internal standards used were: (i) glucose which was used for the glucose calibration; (ii) galactose, which was used for the galactose calibration; and (iii) lactose which was used as internal standard for lactose and GOS. The reasons for using lactose as an internal standard instead of maltotriose is that it represented the variation in the assay in the same way as maltotriose, and was a cheaper alternative.

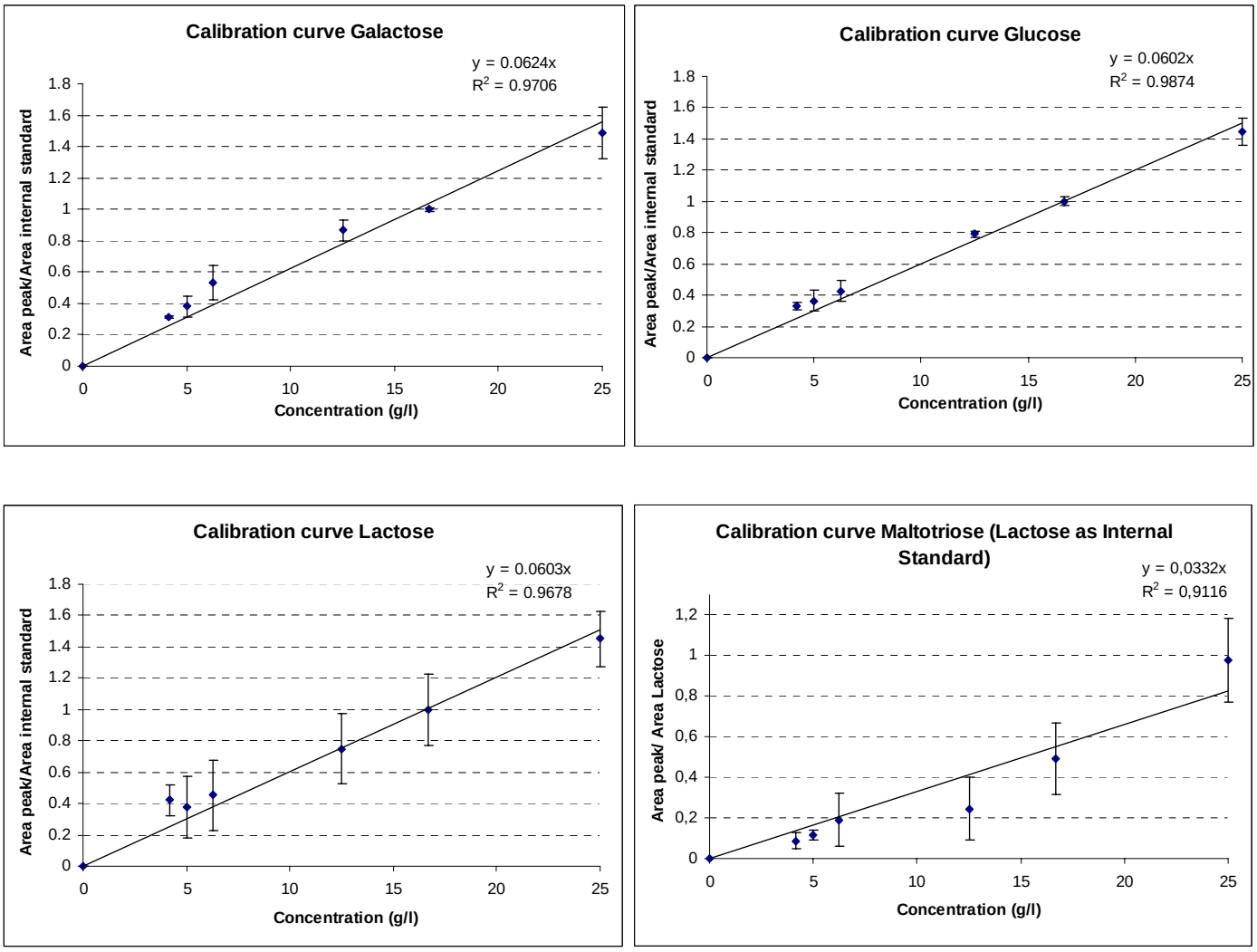


Figure 3.1: TLC standards calibration curves. Galactose, glucose, lactose and maltotriose curves are the average of the three replicates (± standard deviations). The linear trend line and relative R^2 is shown**.**

In all cases, calibration curves were linear over the range of concentrations used. However, it was apparent that the calibrations for monosaccharides were more reproducible than for lactose and maltotriose. Significant errors were associated with estimation of maltotriose and lactose concentration in particular.

3.1.1 Monitoring Maxilact-catalysed GOS synthesis from Whey Permeate by TLC

The TLC assay method developed was used to monitor Maxilact-catalysed GOS synthesis form Whey Permeate. Maxilact is a commercial preparation of β-galactosidase from *Kluyveromyces lactis*, which has been used previously by other authors for GOS synthesis (Zhou *et al*., 2003; Čurda *et al*., 2006; Chockchaisawasdee *et al*., 2004). Whey Permeate is not a very common substrate for GOS synthesis, pure lactose is more commonly used (Tables 1.5-1.10).

Figures 3.2 and 3.3 show a typical TLC plate obtained for the synthesis of GOS using 0.1, 0.4, 0.8, 1.2, 1.6 and 2% Maxilact and a Whey Permeate solution (200 g/l) as substrate at 37 ºC over a 5 hour reaction time at pH 6.8. On each TLC plate an internal standard, composed of lactose, galactose and glucose at a fixed concentration (Control), has been spotted in the first lane.

The assays with 0.1, 0.4, 0.8, 1.2, 1.6 and 2% Maxilact showed the typical profile described for GOS synthesis by other researchers (Čurda *et al*., 2006; Chockchaisawasdee *et al*., 2004). It was possible to separate and distinguish all components during GOS synthesis. The TLC analysis clearly shows lactose decreasing while GOS, galactose and glucose all increase.

The TLC plates in Figures 3.2 and 3.3, show the influence of enzyme concentration on GOS synthesis. The reaction was monitored every 30 minutes for 5 hours. For all concentrations used GOS formation was observed within 30 minutes. The spot corresponding to glucose is clearly visible from the beginning of the enzymatic reaction and the density of glucose spot is always denser than the galactose spot. This is consistent with the mechanism of the transglycosylation reaction (Figures 1.4 and 1.6).

The heterogeneous nature of GOS species gives streaking of its spot in the TLC plates, making GOS estimation difficult. Resolution of GOS from lactose becomes more difficult as synthesis progresses (see Figure 3.2 and Figure 3.3). It is clear that GOS synthesis reaches a maximum whereafter it declines. This is clear that the enzyme hydrolyses GOS species and that degradation to monosaccharides is favoured as lactose concentration decline (Figure 3.3). By comparison of Figures 3.2 and 3.3, it is clear that the hydrolysis process is faster in Figure 3.3 due to the higher enzyme concentration used.

In conclusion, comparing the assays of GOS synthesis at different enzyme concentrations revealed similar profiles and showed that the enzyme concentration influences the rate of the degradation of lactose and as a consequence, the production of GOS.

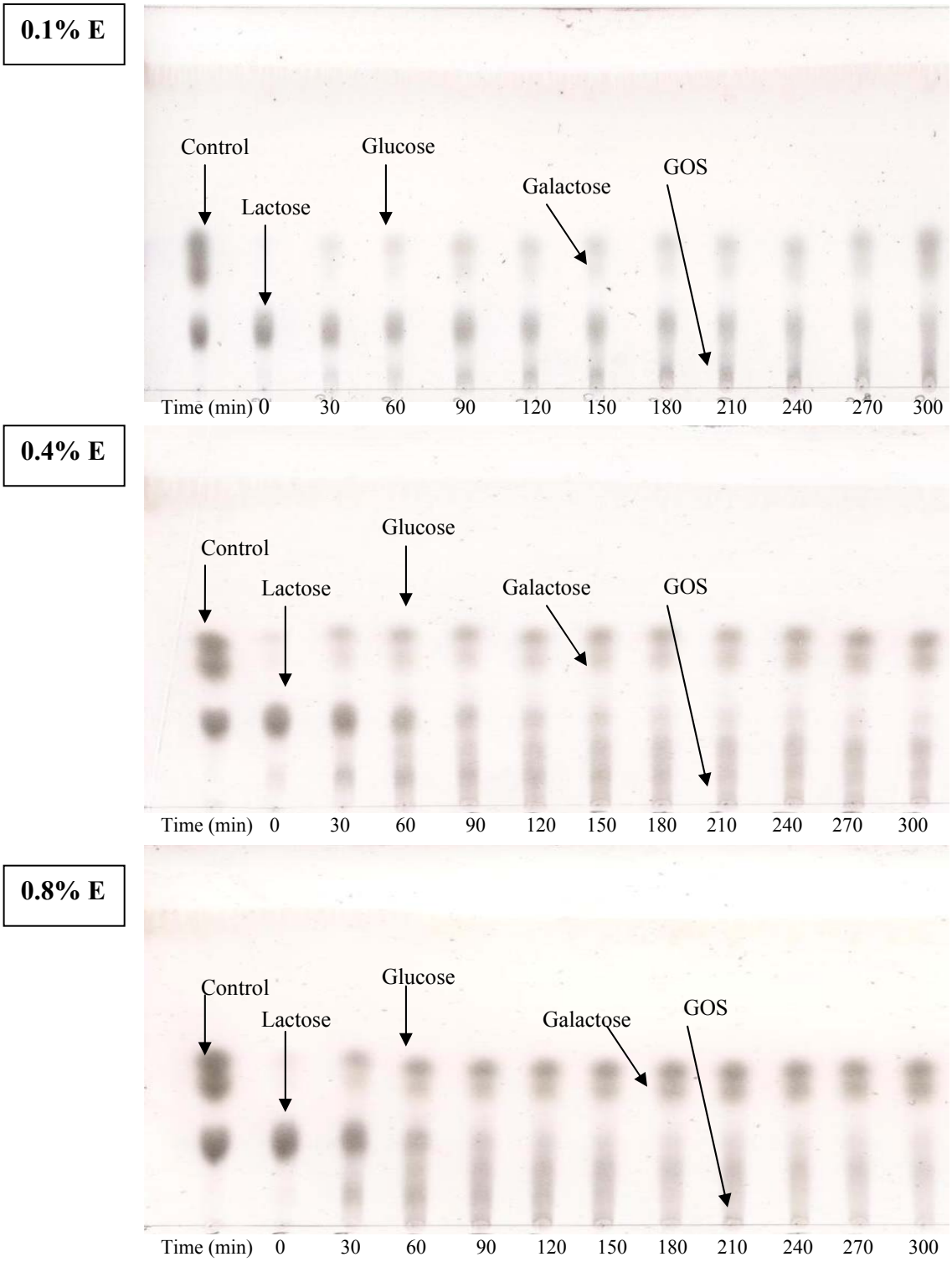


Figure 3.2: TLC analysis of GOS synthesis. Enzyme (E) concentrations: 0.1, 0.4 and 0.8% respectively, using Whey Permeate as substrate. Samples were withdrawn every 30 min of the 5 hour reaction. Control was an internal standard composed of a mixture of lactose, glucose and galactose at 16.6 g/l, spotted in the first lane of every TLC plate. Each starting point is a consecutive sample of the reaction.

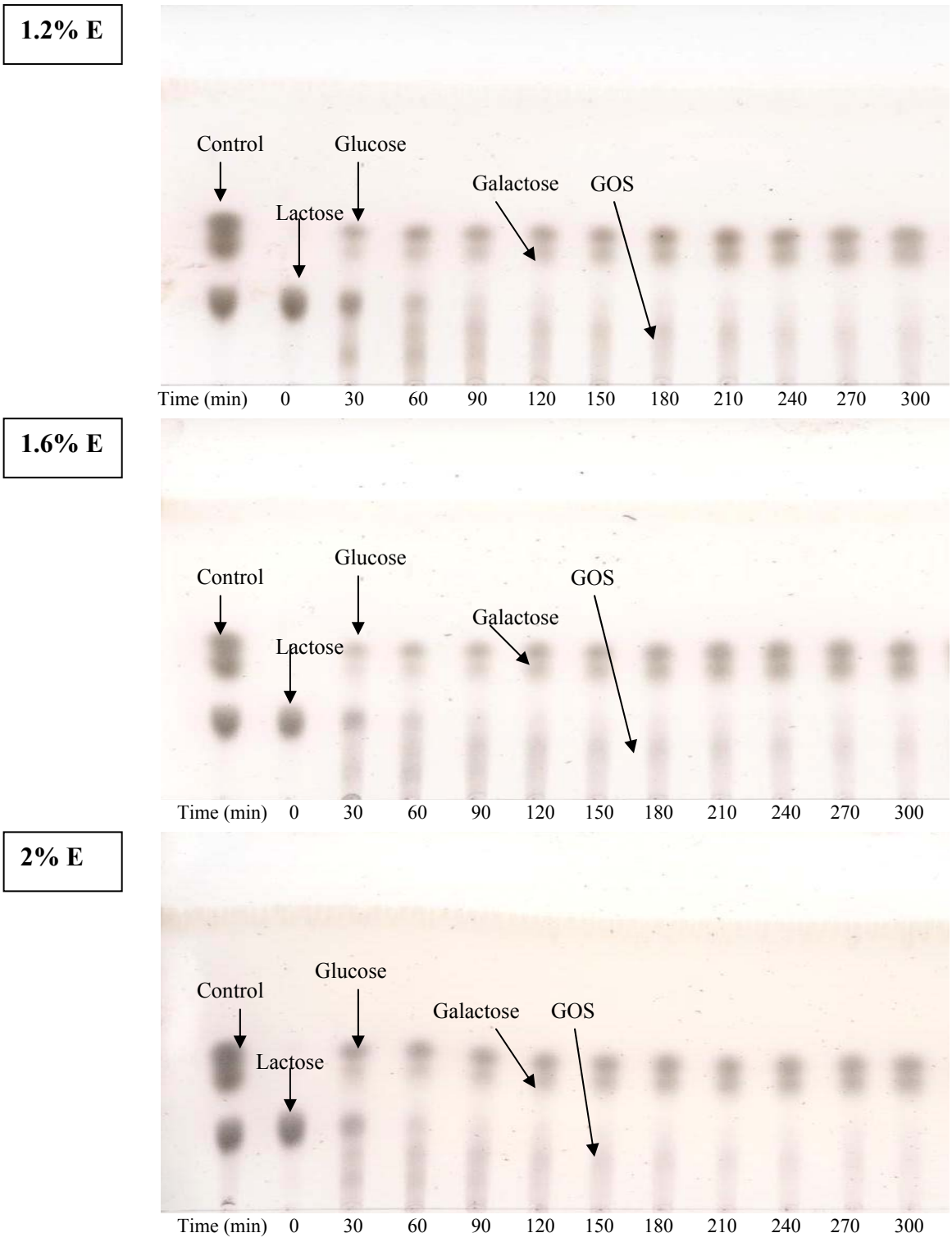


Figure 3.3: TLC analysis of GOS synthesis. Enzyme (E) concentrations: 1.2, 1.6 and 2% respectively, using Whey Permeate as substrate. Samples were withdrawn every 30 min of the 5 hour reaction. Control was an internal standard composed of a mixture of lactose, glucose and galactose at 16.6 g/l, spotted in the first lane of every TLC plate. Each starting point is a consecutive sample of the reaction.

A comparison of the synthesised GOS in a sample assay (0.4% Maxilact) and the commercially available GOS solution (Vivinal GOS®) was carried out (Figure 3.4).

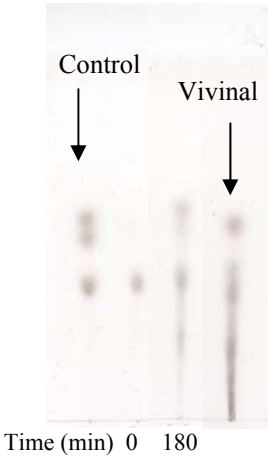


Figure 3.4: TLC comparison of synthesised GOS in a sample assay with a commercially available GOS source. It can be seen that the retention time of the GOS in the sample assay is the same of the GOS in Vivinal GOS® solution.

The data from Figures 3.2 and 3.3 were analysed as indicated in Materials and Method's chapter (section 2.5.3) and an attempt was made to quantify the changes in reaction species. The data are shown in Figure 3.5.

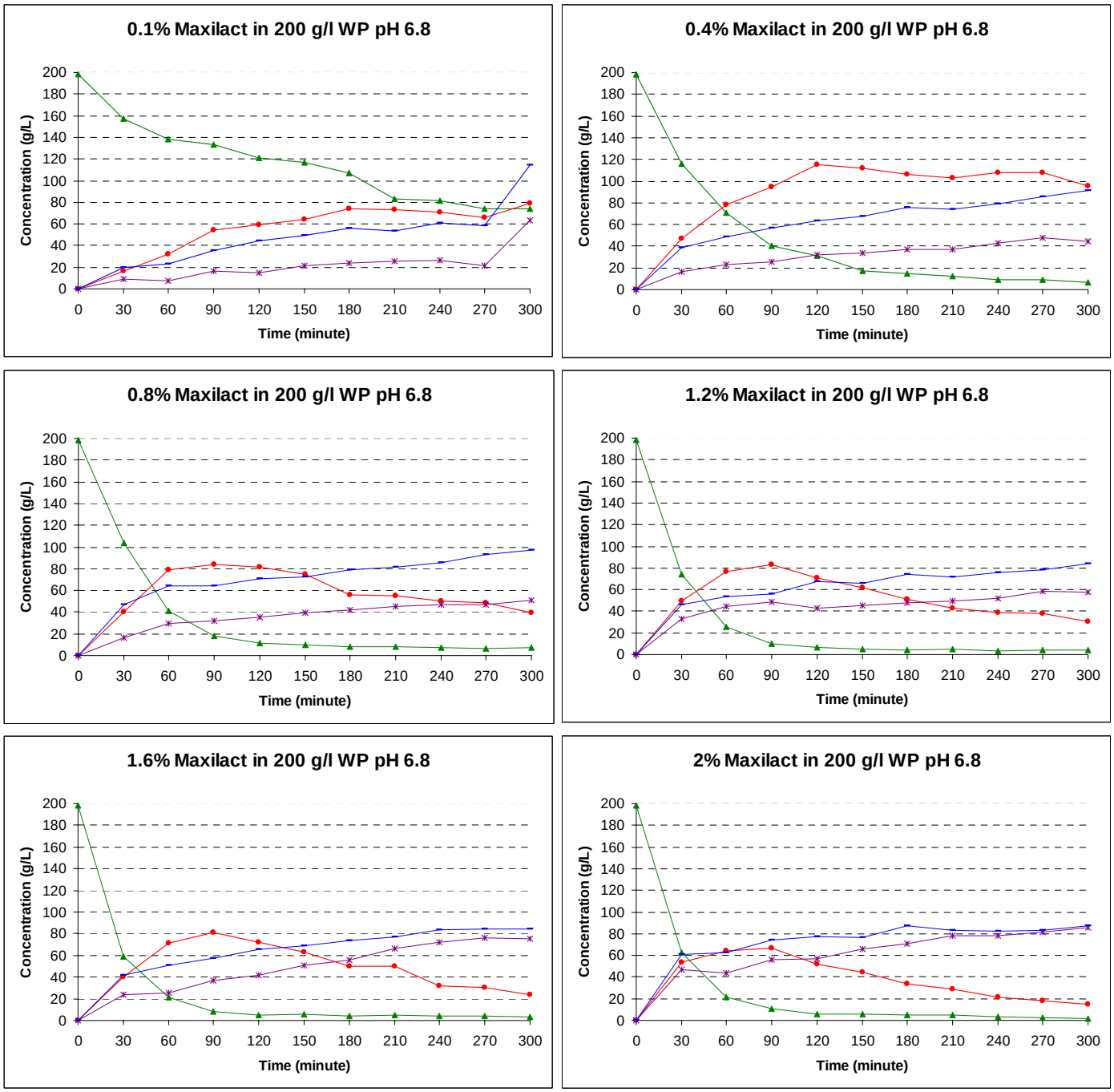


Figure 3.5: Quantitative analysis of GOS synthesis reaction progress. Assays were carried out in phosphate buffer (0.1M, pH 6.8) with Whey Permeate (200 g/l) at 40ºC, for 300 minutes reaction time; enzyme concentrations used were: 0.1, 0.4, 0.8, 1.2, 1.6 and 2% Maxilact (Where ▲: Lac, —: Glc, *: Gal, and ●: GOS).

From Figure 3.5, at 0.1 and 0.4% Maxilact concentrations, it appears that GOS synthesis reaches a maximum level. At higher enzyme concentrations, it is observed a maximum in GOS production is observed, followed by a slow decline due to the hydrolysis of GOS.

Figure 3.6 shows how the enzyme concentrations influence the initial rate of lactose consumption. By increasing Maxilact concentration, the difference between the initial lactose and the lactose left after 30 minute of reaction increases.

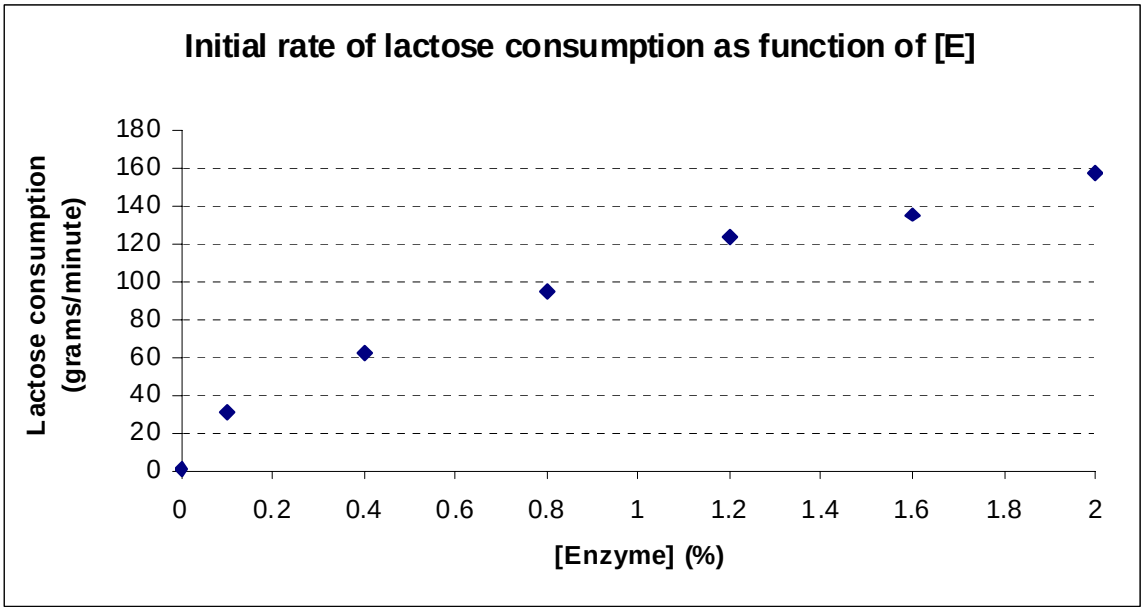


Figure 3.6: Influence of enzyme concentration on lactose depletion rate. It can be seen how increasing doses of enzyme will increase the rate of lactose hydrolysis, therefore making this an enzyme catalysed reaction.

From these data, it is clear that optimization of GOS production will require a thorough knowledge of the kinetics of the GOS synthesis reaction in order to identify the time at which GOS production is optimal for a given level of enzyme.

3.2 Mathematical modelling of the enzymatic synthesis assays

3.2.1 Full reaction mechanism model

The transglycosylation reaction mechanism could be described by the following equations (see equations 3.1-3.4 below) (Kim *et al*. 2004) (this model will be referred in all further discussion as the full model).

This model was based on the following assumptions:

- Any effect of diffusive transport has not been considered.
- Only one rate-limiting step is involved in the reaction mechanism and all the other steps are reversible.
- Lactose acts as both a substrate and a glycosyl acceptor, depending on its concentration.
- Lactose binds the free enzyme to form the E:Gal complex, which interacts with lactose and glucose for the transglycosylation reaction, but not with galactose.
- Glucose reacts with the E:Gal complex to form glucose-galactose disaccharides.
- In order to estimate the molar concentration of GOS a molecular weight of 504.32 g/Mol was assumed estimating a chain of 2 galactose with one glucose unit.
- The β-galactosidase molecular weight was obtained from BRENDA (Tello-Solís *et al*., 2005) and assumed to be 117619 Da.

The model reaction mechanism is described below in equations (3.1) to (3.4)

$$E + Lac \underset{k_{r1}}{\overset{k_1}{\rightleftharpoons}} E{:}Lac \xrightarrow{k_2} E{:}Gal + Glc \quad (3.1)$$

$$E{:}Gal \underset{k_{r3}}{\overset{k_3}{\rightleftharpoons}} E + Gal \quad (3.2)$$

$$E{:}Gal + Glc \underset{k_{r4}}{\overset{k_4}{\rightleftharpoons}} E + Allo \quad (3.3)$$

$$E{:}Gal + Lac \underset{k_{r5}}{\overset{k_5}{\rightleftharpoons}} E + GOS \quad (3.4)$$

Where: E: enzyme; Lac: lactose; E:Lac: enzyme-lactose complex; E:Gal: enzyme-galactose complex; Gal: galactose; Glc: glucose; Allo: allolactose.

The material mass balances are described by the following equations (3.5) to (3.12):

$$\frac{dE}{dt} = -k_1 \cdot E \cdot Lac + k_{r1} \cdot E:Lac + k_3 \cdot E \cdot Gal - k_{r3} \cdot E:Gal + k_4 \cdot E:Gal \cdot Glc - \\ - k_{r4} \cdot E:Gal \cdot Allo + k_5 \cdot E:Gal \cdot Lac - k_{r5} \cdot E \cdot GOS \quad (3.5)$$

$$\frac{dE:Gal}{dt} = k_2 \cdot E:Lac + k_{r3} \cdot E \cdot Gal - k_3 \cdot E \cdot Gal + k_{r4} \cdot E \cdot Allo - k_4 \cdot E:Gal \cdot Glc + \\ + k_{r5} \cdot E \cdot GOS - k_5 \cdot E \cdot Gal \cdot Lac \quad (3.6)$$

$$\frac{dE:Lac}{dt} = -k_2 \cdot E:Lac - k_{r1} \cdot E:Lac + k_1 \cdot E \cdot Lac \quad (3.7)$$

$$\frac{dGal}{dt} = k_3 \cdot E:Gal - k_{r3} \cdot E \cdot Gal \quad (3.8)$$

$$\frac{dGlc}{dt} = k_2 \cdot E:Lac - k_{r4} \cdot E \cdot Allo - k_4 \cdot E:Gal \cdot Glc \quad (3.9)$$

$$\frac{dLac}{dt} = k_{r1} \cdot E:Lac - k_1 \cdot E \cdot Lac + k_{r5} \cdot E \cdot GOS - k_5 \cdot E:Gal \cdot Lac \quad (3.10)$$

$$\frac{dAllo}{dt} = -k_{r4} \cdot E \cdot Allo + k_4 \cdot E : Gal \cdot Glc \tag{3.11}$$

$$\frac{dGOS}{dt} = -k_{r5} \cdot E \cdot GOS + k_5 \cdot E : Gal \cdot Lac \tag{3.12}$$

The kinetic parameters k_1, k_{r3}, k_4, k_{r4}, k_5 and k_{r5} are expressed in $M^{-1}min^{-1}$ and k_{r1}, k_2 and k_3 are expressed in min^{-1}.

3.2.2 Fitting of GOS synthesis reaction using the full model

The data obtained from the TLC analysis assays have been modelled using the full model: equations (3.1) to (3.4).

Figure 3.7 shows a typical fitting of the TLC data using the full model (equations 3.1-3.5). The full model described the experimental data well.

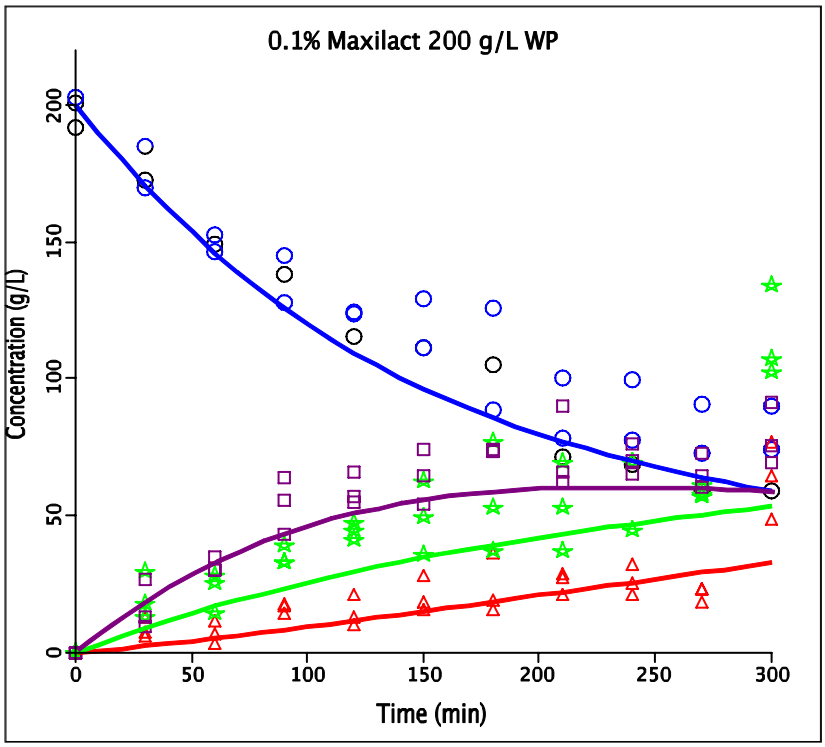


Figure 3.7: Measurement and model prediction with full model. HPLC assay with 0.1% Maxilact in WP (200 g/l) is shown. Symbols are the points of experimental data and lines are the curve fits of the species for the proposed model. Where ○: Lactose; □: Glucose; ☆: Galactose and Δ: GOS. Assay conditions are described in Materials and Methods (Section 2.4).

Table 3.0 shows the fitted kinetic parameters obtained. However, a high correlation between the kinetic parameters was found and sometimes also a high standard error (Table 3.0).

Table 3.0: Fitted parameter values of the enzymatic assays with Maxilact (0.1, 0.4, 0.8, 1.2, 1.6 and 2%) and Whey Permeate (200 g/l). All figures are mean ± standard deviation**.**

Parameters	**0.1%E 200 g/l WP**	**0.4%E 200 g/l WP**	**0.8%E 200 g/l WP**	**1.2%E 200 g/l WP**	**1.6%E 200 g/l WP**	**2%E 200 g/l WP**
k_1 ($M^{-1}min^{-1}$)	2052±3	1285±3	1721±8	1641±20	1478±6	1666±3
kr_1 (min^{-1})	23872±4	25459.1±0.5	25443±11	25447±10	25458±13	25707±3
k_2 (min^{-1})	3550±4	4178±2	1388±16	1268±14	2440±20	1580±2
k_3 (min^{-1})	$1.3x10^3$±6 $x10^3$	2859.2±1.7	3763±5	2786±2	2468±2	2861±6
k_{r3} ($M^{-1}min^{-1}$)	139±2	703.6±1.0	419±25	375±3	877±3	406±7
lk_4 ($M^{-1}min^{-1}$)	-15±2	-25±4	-17±10	-18±15	-26±30	-21±4
k_{r4} ($M^{-1}min^{-1}$)	180±3	0.480±150	86±20	100±30	6.440±7	92±2
k_5 ($M^{-1}min^{-1}$)	7.2±1.6	3.1±0.9	4±1.5	3.6±0.7	1.7±0.6	2.1±0.7
k_{r5} ($M^{-1}min^{-1}$)	687±3	55 ±7	43±7	48±3	77 ±10	50±3

A significant difficulty when modelling of experimental data was encountered. This was largely due to the large errors associated with TLC measurement which made reliable estimates of GOS synthesis difficult. However, the TLC method was useful as a way to monitor trends of the reaction mechanism.

Table 3.1 shows data for TLC analysis of GOS production at 0.4% Maxilact.

Table 3.1: TLC results of the assays 200 of Whey Permeate (WP) with 0.4% Maxilact concentration. All figures are mean ± standard deviation.

Time (min)	**GOS (g/l)**	**Lactose (g/l)**	**Glucose (g/l)**	**Galactose (g/l)**	**Total (%)**
0	0	198±5	0	0	100±5
30	48±14	116±22	38±12	16±5	109±30
60	77±28	71±18	48±11	23±5	111±36
90	94±36	40±13	56±12	25±1.9	109±41
120	115±29	31±9	63±13	31±2	121±33
150	11±7	17±4	67±10	33±4	161±32
180	106±33	14±4	75 ±19	37±1.0	118±38
210	102±27	12 ±4	73±19	37±3	114±34
240	107±23	9±5	79±20	42±5	120±32
270	107±20	8 ±4	85±20	47 ±11	126±32
300	95±29	6±3	91±24	44±12	119±39

The large errors associated with the measurement of GOS species it made difficult to use the TLC assay for studies of reaction mechanism. In particular, there was an overestimation of GOS in comparison with literature (Zhou *et al*., 2003; Kim

et al., 2004). Based on this, it was decided to develop a HPLC method as a more precise and accurate way of monitoring the reaction.

3.3 Development of HPLC assay to monitor GOS synthesis

To solve the mass balance problem with TLC assay method, a HPLC method was devised. HPLC was carried out on a SUPELCOGEL Ca^{2+} column at 80 ºC, using distilled, ultrapure degassed water as the mobile phase at a flow rate of 0.5 ml/min. Components were monitored using a Refractive Index Detector. HPLC analysis gave more accurate and reproducible quantitation of the species in the GOS synthesis reaction mixture and a more reliable mass balance. However, the general trend of the reactions was the same as observed previously by TLC.

The mass balances obtained with the HPLC method have a smaller standard deviation in comparison with the TLC method. The data obtained in a typical assay (0.4% enzyme), including the mass balance, are presented in Table 3.2. The total of the species present in the reaction is within 10% error of the starting value. Thus, the HPLC assay may be considered a more reliable method than TLC assay for the quantitation of the changing components of the GOS synthesis reaction. Monitoring using this assay is expected to yield data that are more suitable for modelling studies.

Table 3.2: HPLC results of the assays with different concentration of Whey Permeate 200 g/l (a) and 350 g/l (b) with 0.4% Maxilact. All figures are mean ± standard deviation.

(a)

Time (min)	GOS (g/l)	Lactose (g/l)	Glucose (g/l)	Galactose (g/l)	Total (%)
0	3.4±0.6	197.9±0.9	0	0	100.0±1.0
30	22.4±0.5	108.9±0.3	52.3±0.3	30.09±0.7	106.24±1.0
60	37.5±0.8	75.9±0.7	59.6±1.2	42.5±0.4	107.12±1.6
90	29.3±0.3	56±3	68.3±1.5	49±2	100±3
120	27.4±1	47.7±1.4	66.6±0.8	59.1±1.7	99±2
150	31.4±1.7	50±2	76.9±1.9	50±2	103±4
180	30.7±1.8	50±2	75.2±1.6	61±2	108±4
210	29.3±0.9	46.6±0.6	76.5±1.9	64.1±0.6	107±2
240	25.4±0.7	43.8±0.3	77±2	70.5±0.7	108±2
270	23.8±0.9	37.3±0.3	76.2±0.8	74±2	105±2
300	21.8±0.4	38.3±1.1	70.7±0.9	70.6±1.4	100±2

(b)

Time (min)	GOS (g/l)	Lactose (g/l)	Glucose (g/l)	Galactose (g/l)	Total (%)
0	5.5±0.1	343±4	0	0	100.0±0.9
30	26.8±1.4	292.9±1.4	38.±2	28.4±1.6	110.7±0.8
60	60.6±0.9	230.4±0.9	63±2	39.9±0.8	113.0±1.5
90	57.9±0.4	187.1±1.9	84.8±1.1	43.1±0.6	106±4
120	66.4±0.7	166.7±0.9	102.4±1.2	60.2±1.4	113±2
150	66.5±1.4	141.9±1.2	109.4±1.1	57.1±0.9	107±4
180	75.3±1.6	136.2±0.6	127.2±1.7	67.7±1.3	116±3
210	79.5±0.5	128.1±1.8	130.5±0.2	68.0±1.3	116 ±2
240	77.7±1.4	123.8±1.5	131.6±1.7	70.2±2	115±2
270	77.2±0.5	118.5±0.3	136.6±1.5	76.3±1.3	117±2
300	68.5±1.1	109.8±0.5	134.2±0.2	80.7±1.4	112±2

A typical chromatogram of the standard elution times for a solution of standards is presented in Figure 3.8. Maltotriose, used as reference for GOS because of its chemical structure, eluted at 10 minutes, followed by lactose (11 minutes), glucose (13 minutes) and galactose (14 minutes).

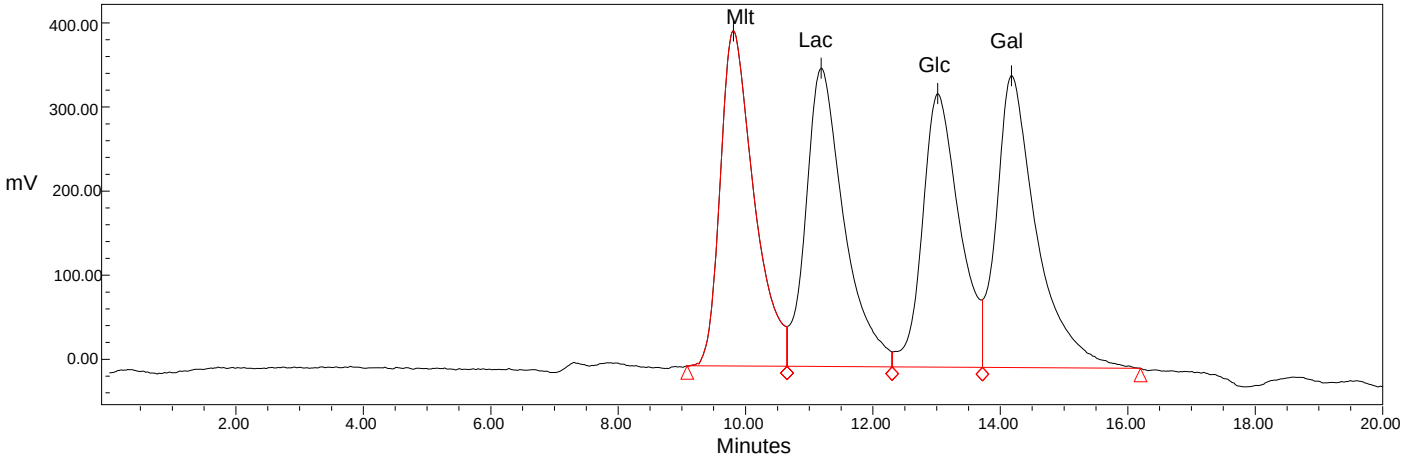


Figure 3.8: Typical HPLC profile for standards solution. The standard mixture retention times in minutes were: Maltotriose (Mlt) 9.9808; lactose (Lac) 11.188; glucose (Glc) 13.017; galactose (Gal) 14.176. Each component of the standard mixture was present at a concentration of 0.1 g/l. The standard mixture was eluted at 0.5 mil/min using ultrapure, distilled and degassed water as mobile phase on a SUPELCOGEL Ca^{2+} column at 80 ° C, and a RI detector.

Although baseline resolution of peaks was not obtained, it was possible to obtain linear standard curves with a smaller standard deviation than those obtained using the TLC method. That was due to lower variability of HPLC methodology (Table 3.2, Figure 3.9) and to the favourable HPLC peak resolution (Figures 3.8 and 3.9).

Standard curves for maltotriose, lactose, glucose and galactose were carried out over a concentration range of 0.01-1.0 g/l (Figure 3.9) using the HPLC analysis method.

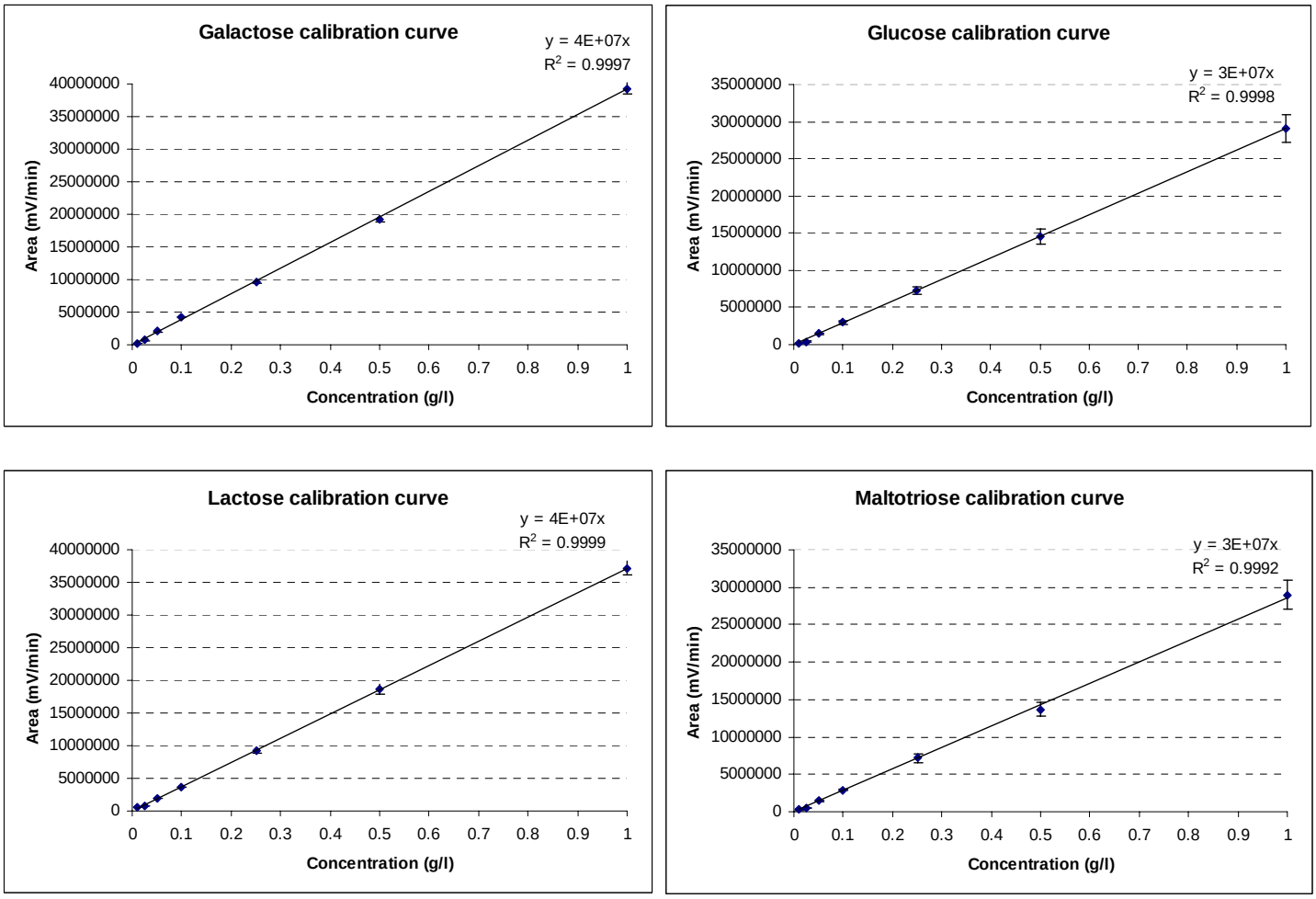


Figure 3.9: HPLC Standards calibration curves. Galactose, glucose, lactose and maltotriose are represented as average of the three replicates with their standard deviations. The linear trend lines, the equations and their relative R^2 are also represented**.**

A typical chromatogram obtained from a GOS synthesis reaction mixture is shown below at time 0 and after 210 minutes of reaction (Figure 3.10). Under these conditions, the first components to elute are oligosaccharides (GOS eluted at 10.69 minutes), followed by disaccharides (lactose eluted at 12.06 minutes), then monosaccharides (glucose eluted at 14.02 minutes and galactose at 15.28 minutes).

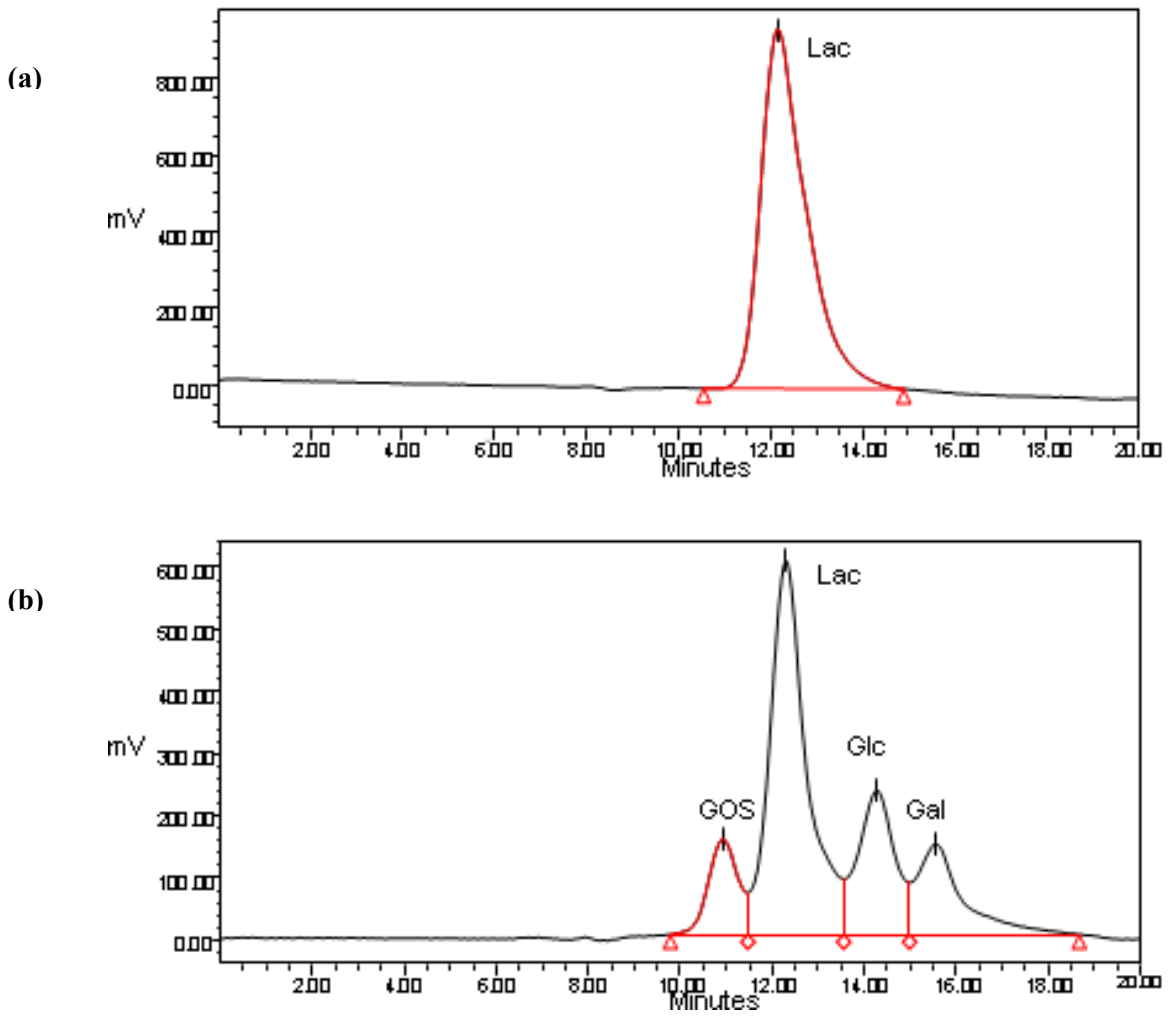


Figure 3.10: Chromatograms of GOS synthesis at different reaction times. Assay with 0.1% Maxilact in Whey Permeate (200 g/l) at the beginning of the reaction (a) and after 210 minutes (b). Elution times at the beginning of the reaction: Lactose 12.158 minutes. Elution times, in minutes, after 150 minutes of reaction: GOS 10.69 minutes; lactose 11.335; glucose 13.217; galactose 14.404. Where: Lac: lactose, Glc: glucose and Gal: galactose. Samples eluted with 0.5 mL/min ultrapure, distilled and degassed water in a SUPELCOGEL Ca^{2+} column at 80 º C, RI detector.

3.3.1 GOS synthesis reaction mixture at different Maxilact and Whey Permeate concentrations

Enzymatic assays with different Maxilact concentration (0.1, 0.4, 0.8, 1.2, 1.6 and 2%) and Whey Permeate (200 and 350 g/l) were carried out (Figures 3.9, 3.10, 3.11, 3.12).

Comparing Figure 3.5 with Figures 3.11 and 3.12 it is possible to notice that TLC analysis caused an overestimation of the quantitation of some of the species in the reaction, especially GOS.

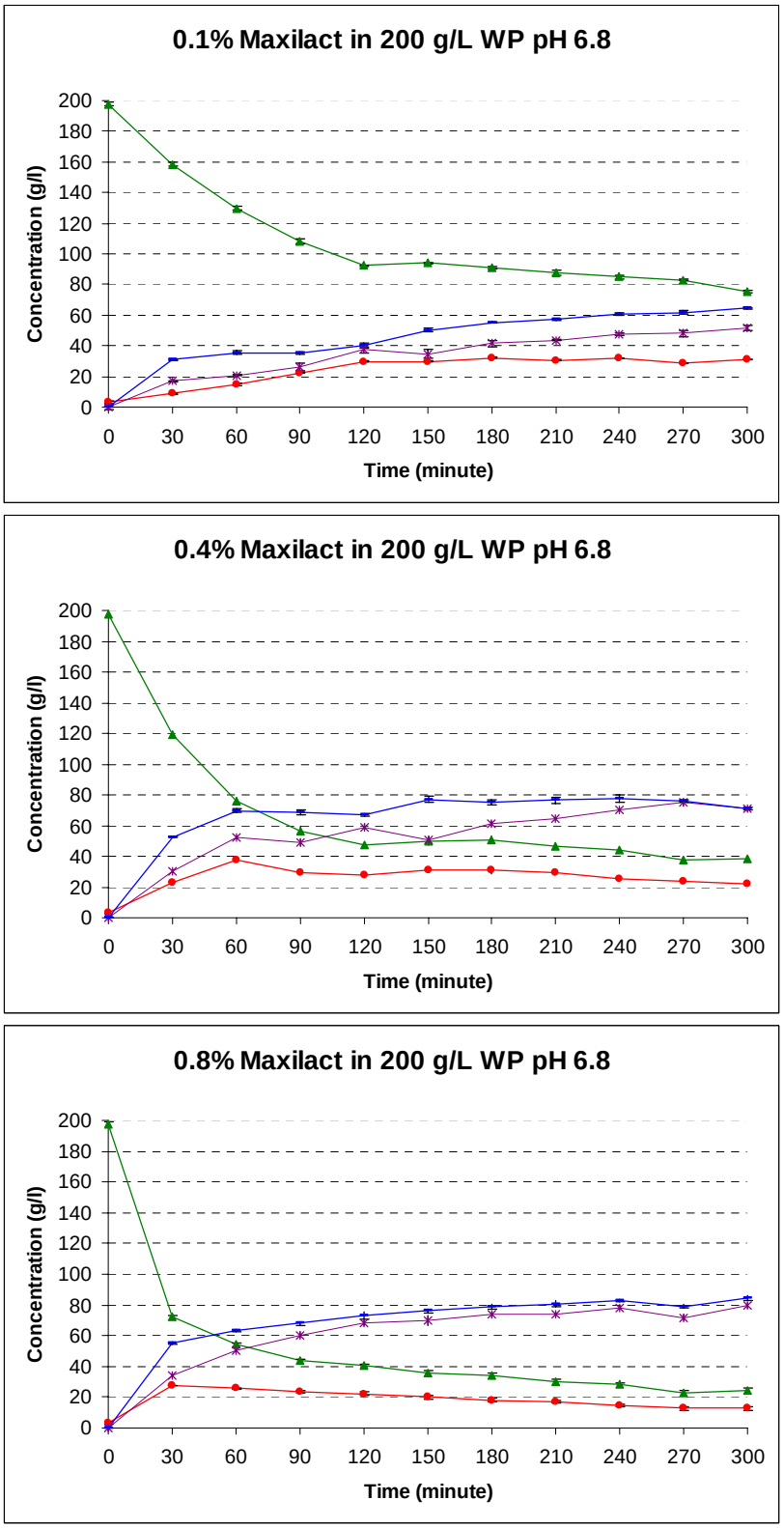


Figure 3.11: Quantitative analysis of GOS synthesis at different Maxilact concentrations. Assays were carried out in phosphate buffer (0.1 M, pH 6.8) with Whey Permeate (200 g/l), at 40ºC, for 300 minutes reaction; enzyme concentrations used were 0.1, 0.4 and 0.8 % Maxilact. (Where ▲: Lac, ▬: Glc, *: Gal, and ●: GOS)

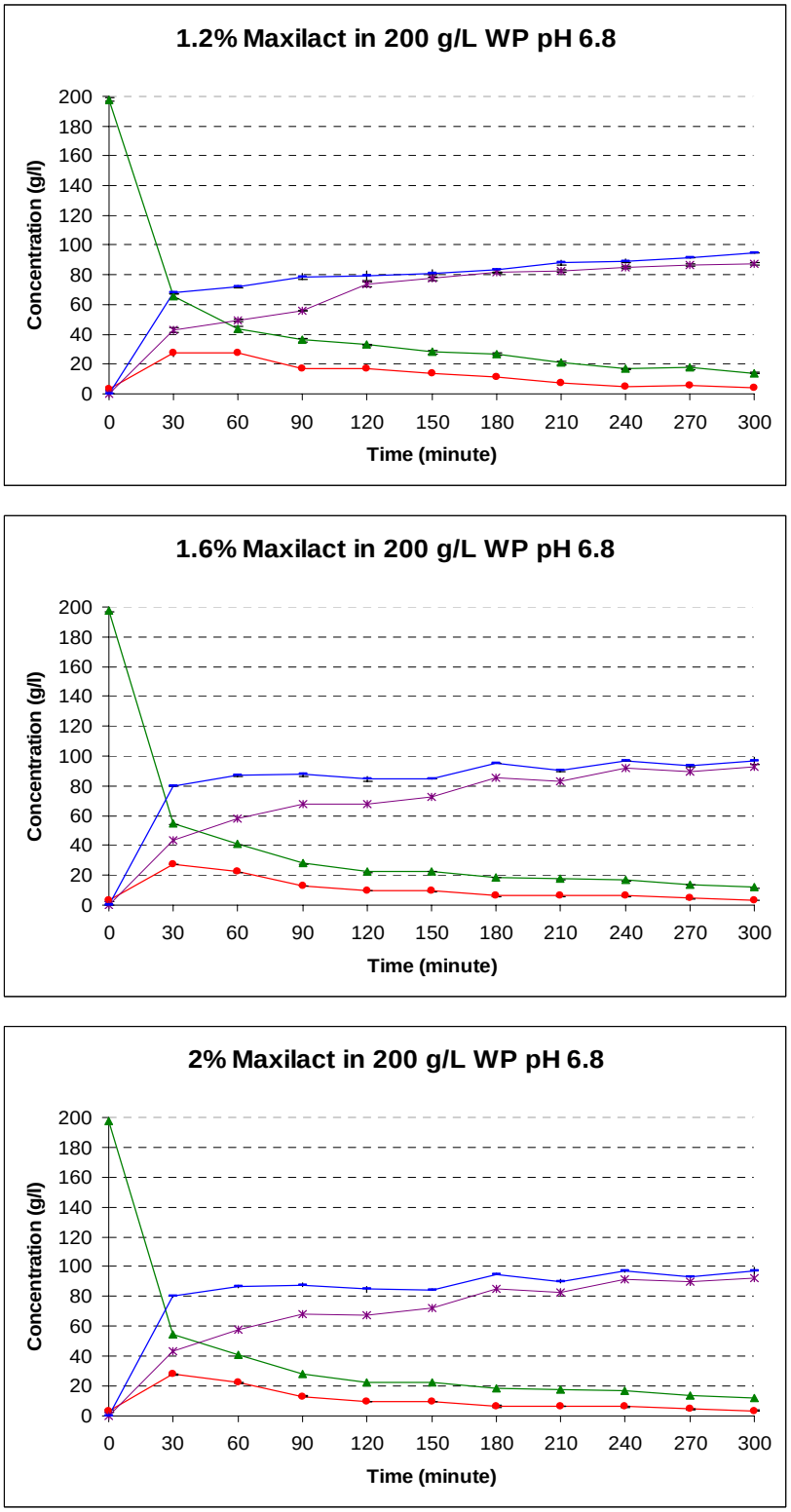


Figure 3.12: Quantitative analysis of GOS synthesis at different Maxilact concentrations. Assays were carried out in phosphate buffer (0.1 M, pH 6.8) with Whey Permeate (200 g/l), at 40ºC, for 300 minutes reaction; enzyme concentrations used were 1.2, 1.6 and 2 % Maxilact. (Where ▲: Lac, ▬: Glc, *: Gal, and ●: GOS)

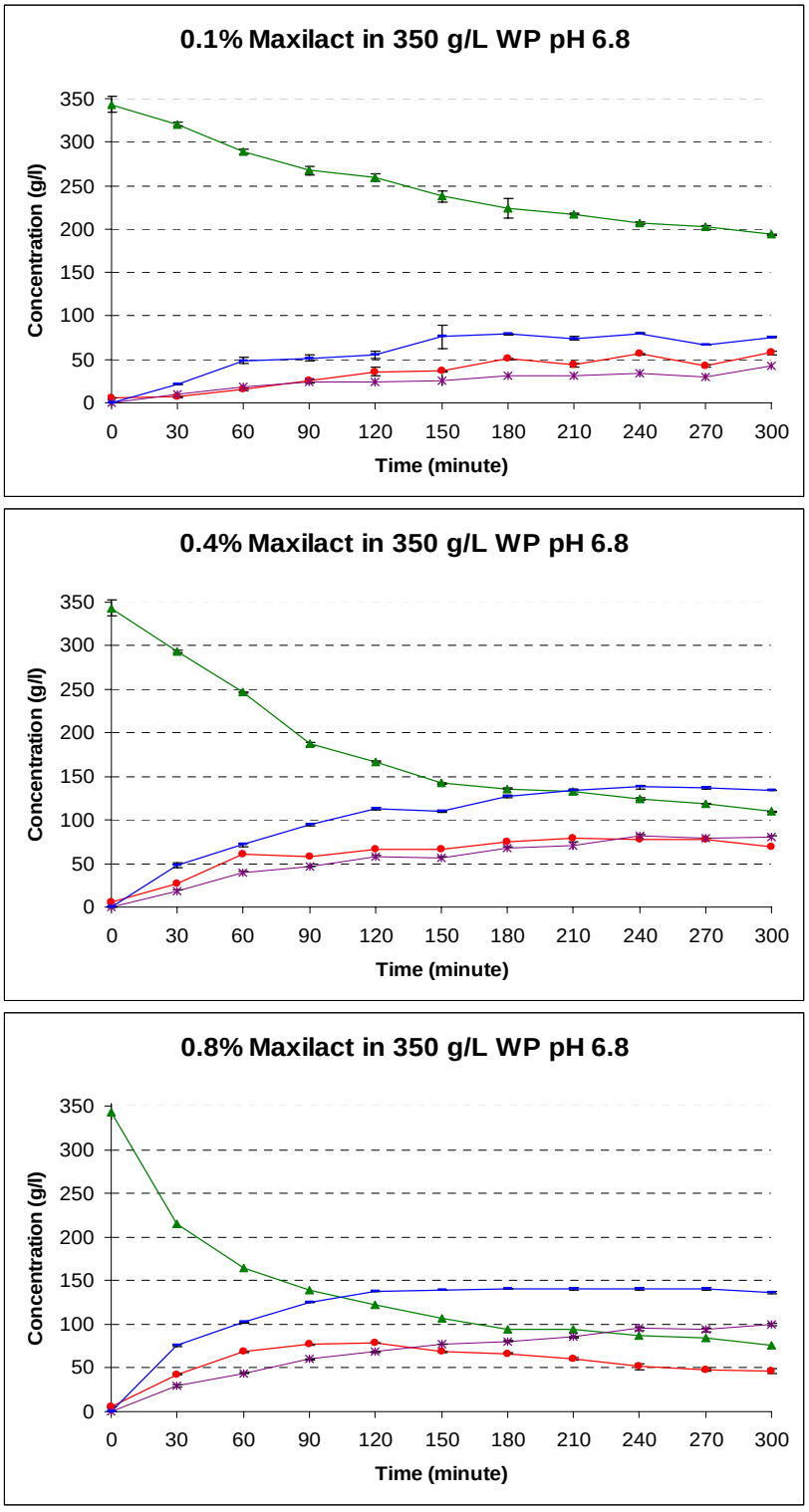


Figure 3.13: Influence of increased lactose concentration on GOS synthesis at different Maxilact concentrations. Assays were carried out in phosphate buffer (0.1 M, pH 6.8) with Whey Permeate (350 g/l), at 40ºC, for 300 minutes reaction; enzyme concentrations used were 0.1, 0.4 and 0.8 % Maxilact. (Where ▲: Lac, ▬: Glc, *: Gal, and ●: GOS)

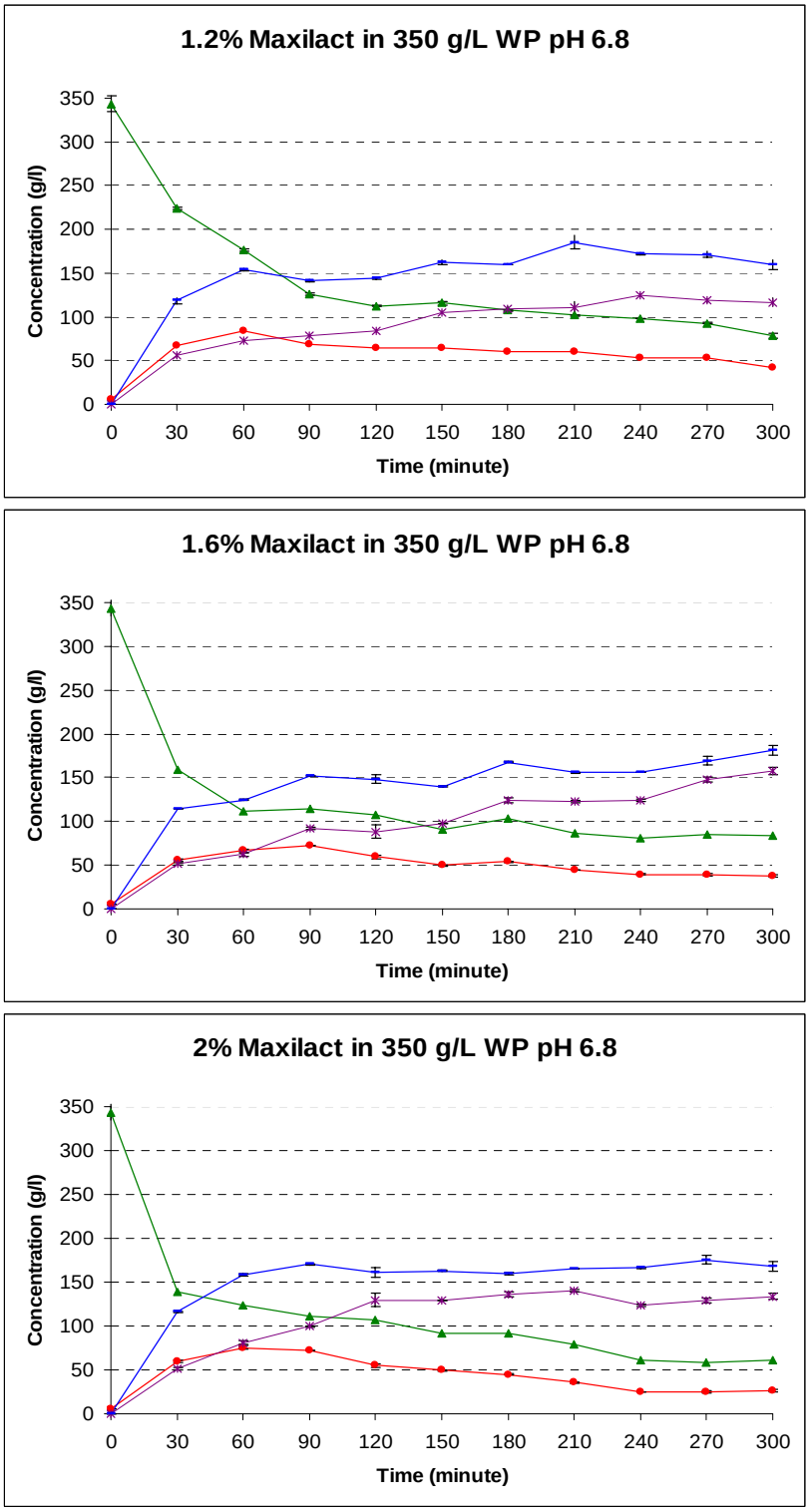


Figure 3.14: Influence of increased lactose concentration on GOS synthesis at different Maxilact concentrations. Assays were carried out in phosphate buffer (0.1 M, pH 6.8) with Whey Permeate (350 g/l), at 40ºC, for 300 minutes reaction; enzyme concentrations used were 1.2, 1.6 and 2 % Maxilact. (Where ▲: Lac, ▬: Glc, *: Gal, and ●: GOS)

Figures 3.11, 3.12, 3.13 and 3.14 showed how the initial substrate concentration influences both the transglycosylation and hydrolysis reactions. The synthesis of GOS is increased probably due to the decrease of the water activity in the media. The water activity of the assays with 350 g/l is 0.968, while the one of the assays with 200 g/l is 0.984. The influence of initial lactose concentration on β-galactosidase activity is consistent with similar works in literature (Goulas *et al*., 2007; Maugard *et al*., 2003).

By comparing assays with different Whey Permeate concentration, at low enzyme concentration, *i.e.* 0.1% (Figure 3.11), it is possible to observe that GOS synthesis has a increasing trend throughout the reaction time. Furthermore, the maximum GOS achieved at the end of the reaction is almost doubled by increasing the starting lactose concentration (Figure 3.13). By increasing enzyme concentration, *i.e.* 0.4%, in the assay with 350 g/l, as an increase in GOS synthesis is observed (Figure 3.13). In the assay with 200 g/l GOS starting with 0.4% of enzyme (Figure 3.11), GOS reached a maximum and then declined. At 0.8 and 1.2% enzyme concentrations and 350 g/l WP (Figures 3.13-3.14), GOS synthesis starts to decrease slowly after 4 hours of reaction, while at 1.6 and 2% (Figure 3.14) it decrease after 2 hours. The different trends in GOS synthesis/degradation between the assays with 200 and 350 g/l at the same enzyme concentration may be due to the higher lactose available as acceptor for the transglycosylation reaction with 350 g/l Whey Permeate.

The trend of GOS synthesis in the assays with 200 g/l Whey Permeate (Figures 3.11-3.12) is consistent with the results obtained using the TLC assay (Figure 3.5), although the HPLC method shows a lower quantity of GOS production.

The GOS yield, as a percentage of all the sugars present in the media ($GOS\%_{TOT}$) (Dumortier *et al*., 1994) (Formula 3.13), has been calculated as the value

(%) of the GOS present at a certain time of the reaction (GOS_{tx}) divided by the sum of all the other species presents in the media at the same time (Lac_{tx}, Glc_{tx} and Gal_{tx}).

$$GOS\%_{TOT} = \frac{GOS_{tx}}{(Lac_{tx} + Glc_{tx} + Gal_{tx})}\% \quad (3.13)$$

The degree of lactose conversion (*DC*) has been defined as a conversion of lactose to GOS and monosaccharides in percentage (Chockchausawasdee *et al*., 2004). It has been calculated as the initial quantity of lactose (Lac_0) minus the lactose present in the solution at a certain time (Lac_{tx}) divided by the initial lactose (Formula 3.14).

$$DC = \frac{Lac_0 - Lac_{tx}}{Lac_0}\% \quad (3.14)$$

In Figure 3.15 the influence of enzyme concentration and Whey Permeate concentration is related to the GOS % of total sugars and lactose conversion (%). In the assay with 200 g/l of Whey Permeate, the maximum GOS synthesis (18%) is achieved when 62% of lactose is converted with 0.4% of Maxilact used. By increasing substrate concentration to 350 g/l of Whey Permeate, the maximum synthesised GOS increases to 24%, when 65% of lactose is converted with 0.8% of Maxilact. In both of the assays, by increasing of the enzyme concentration a higher percentage of lactose is converted but a smaller GOS percentage obtained. This may be due to a shift of the equilibrium of β-galactosidase reaction towards hydrolysis rather then transglycosylation (Figure 1.6).

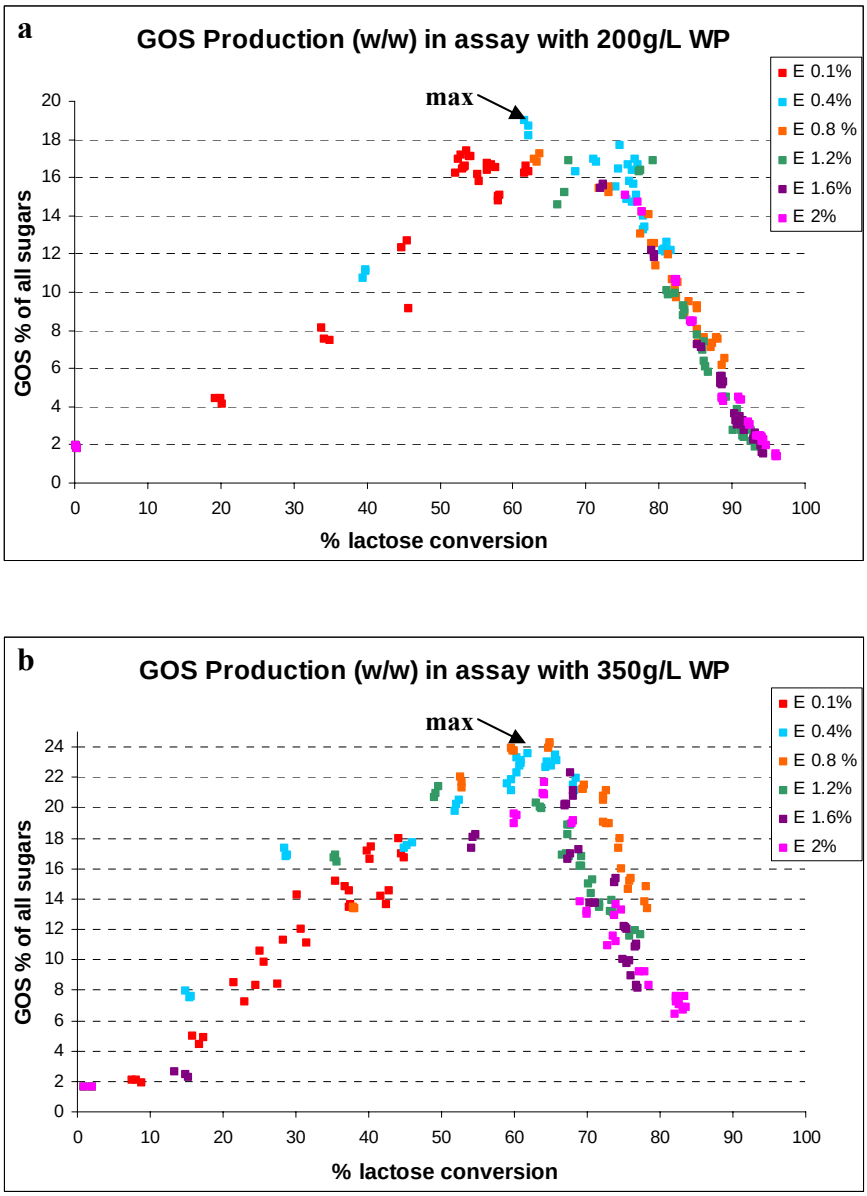


Figure 3.15: Comparison of the effect of enzyme concentration on GOS % of total sugars at different Whey Permeate concentrations (200 -a- and 350 -b- g/l). Where: E: enzyme; ■: E 0.1; ■: E 0.4; ■: E 0.8; ■: E 1.2; ■: E 1.6; and ■: E 2%.

3.4 Model reduction of the GOS synthesis mechanism

3.4.1 Reduced reaction mechanism model

Previous studies (Iwasaki *et al*., 1996 and Kim *et al*., 2004) have investigated the modelling of GOS formation using the full feature mechanism model of reaction. Generally, this has resulted in an ill-conditioned system, where strong correlation between parameters and variables has resulted in no statistically meaningful results. The main approach to avoid this obstacle in this study focused on simplifying the reaction mechanism and tried to explain GOS synthesis with a reduced set of reaction steps (Boon *et al*., 1999 and 2000; Zhou *et al*., 2003; Neri *et al*., 2009).

In this work, the GOS synthesis mechanism has been simplified on the basis of the following considerations based on previous studies by Boon *et al*., (1999 and 2000) and Zhou *et al*., (2003):

- Enzymatic hydrolysis is assumed to be rapidly equilibrated, lumping therefore the whole enzymatic hydrolysis mechanism into a single first order step.
- There is no GOS synthesis inhibition process due to re-arrangement of the E:Gal complex with glucose by reaction of the E:Gal complex with glucose, to form allolactose. Therefore the step of allolactose formation is considered of negligible influence.

From these hypotheses the following system of ordinary differential equations (ODE) was constructed (equations 3.15-3.17) (this model will be further referred in the following discussion as the reduced model):

$$E + Lac \xrightarrow{k_1} E:Gal + Glc \quad (3.15)$$

$$E:Gal \underset{k_{r3}}{\overset{k_3}{\rightleftharpoons}} E + Gal \quad (3.16)$$

$$E:Gal + Lac \underset{k_{r5}}{\overset{k_5}{\rightleftharpoons}} E + GOS \quad (3.17)$$

The material mass balances are described by the following equations (3.18-3.23):

$$\frac{dE}{dt} = -k_1 \cdot E \cdot Lac + k_3 \cdot E:Gal - k_{r3} \cdot E \cdot Gal + k_5 \cdot E:Gal \cdot Lac - k_{r5} \cdot E \cdot GOS \quad (3.18)$$

$$\frac{dE:Gal}{dt} = k_1 \cdot E \cdot Lac + k_{r3} \cdot E \cdot Gal - k_3 \cdot E:Gal + k_{r5} \cdot E \cdot GOS - k_5 \cdot E \cdot GOS - k_5 \cdot E:Gal \cdot Lac \quad (3.19)$$

$$\frac{dGal}{dt} = k_3 \cdot E:Gal - k_{r3} \cdot E \cdot Gal \quad (3.20)$$

$$\frac{dGlc}{dt} = k_1 \cdot E \cdot Lac \quad (3.21)$$

$$\frac{dLac}{dt} = -k_1 \cdot E \cdot Lac + k_{r5} \cdot E \cdot GOS - k_5 \cdot E:Gal \cdot Lac \quad (3.22)$$

$$\frac{dGOS}{dt} = -k_{r5} \cdot E \cdot GOS + k_5 \cdot E:Gal \cdot Lac \quad (3.23)$$

The kinetic parameters k_1, k_{r3}, k_5 and k_{r5} are expressed in $M^{-1}min^{-1}$ and k_3 is expressed in min^{-1}.

In this reduced model, lactose can react with the galactosyl-enzyme complex and the synthesis of galactooligosaccharides is assumed to be reversible. Lactose inhibition, allolactose production (Huber *et al.*, 1976), mutarotation of galactose

(Bakken *et al*., 1992), separate production of tri- and tetrasaccharides (Iwasaki *et al*., 1996), diffusional limitation and enzyme inactivation with time were not considered in the model. Also, temperature and pH effects on lactose hydrolysis and oligosaccharides synthesis were not included (Neri *et al*., 2009).

3.4.2 Comparison of full model and reduced model set

The kinetics of a general experiment for GOS synthesis (0.1% Maxilact concentration 200g/l of WP) together with the full model prediction are shown in Figure 3.16.

Although the full model fitted well the HPLC data (Figure 3.16), the parameters were correlated highly with large standard errors (data not shown). This precluded from obtaining sound estimates of reaction rate constants that might be used to predict GOS synthesis under other conditions.

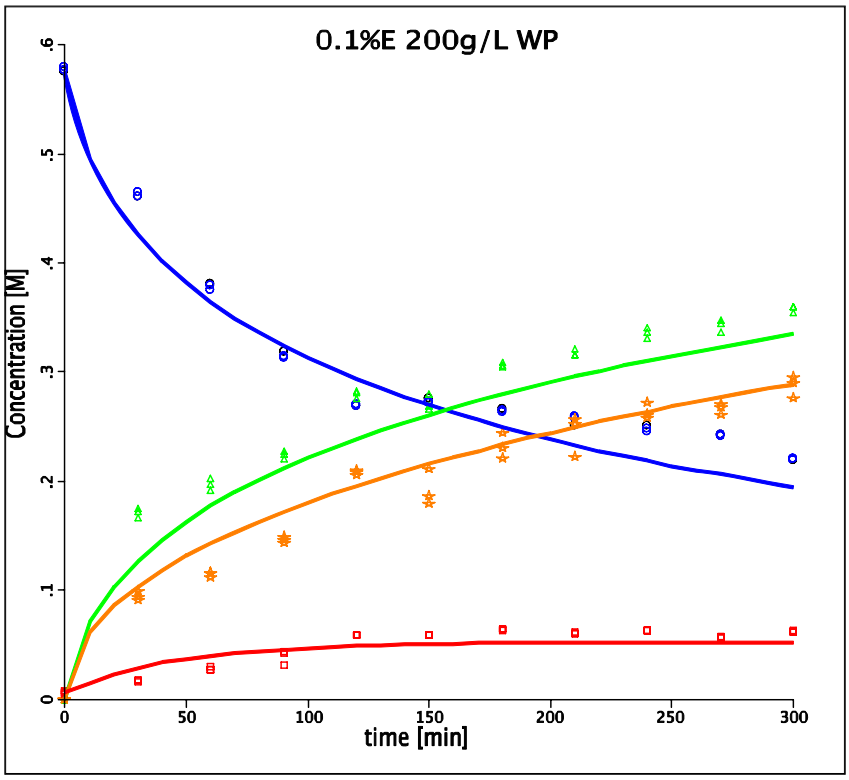


Figure 3.16: Measurement and model prediction with full model. HPLC assays with 0.1% Maxilact in WP (200 g/l) are shown. Symbols are the points of experimental data and lines are the curve fits of the species for the proposed model. Where ○: Lactose; Δ: Glucose; ★: Galactose and □: GOS. Assay conditions described in Materials and Methods (Section 2.4).

In Table 3.3 the comparison of the residual sum of the squares between the full model and the reduced model is shown. It is possible to notice that the reduced model explained the data at least as well as the full model, with a minor increase in deviance and a considerable decrease in the correlation between parameters (no parameters with a correlation higher than 0.95 were found). This indicates that the reduced model could properly explain the GOS synthesis reaction, even with the elimination of four kinetic parameters from the nine parameters of the full model.

Table 3.3: Residual sum of squares of the weighted residuals (RSM) comparison between the full model -a- and the reduced model -b- of the enzymatic assays with Maxilact (0.1, 0.4, 0.8, 1.2, 1.6 and 2%) and Whey Permeate (200 and 350 g/l).

(a)

Substrate concentration	***200 g/l Whey Permeate***					
Enzyme concentration	***0.1%E***	***0.4%E***	***0.8%E***	***1.2%E***	***1.6%E***	***2%E***
Full Model RSM	0.197	0.309	0.189	0.205	0.287	0.344
Substrate concentration	**350 g/l Whey Permeate**					
Enzyme concentration	**0.1%E**	**0.4%E**	**0.8%E**	**1.2%E**	**1.6%E**	**2%E**
Full Model RSM	0.569	0.808	0.713	0.1135	0.941	0.958

(b)

Substrate concentration	***200 g/l Whey Permeate***					
Enzyme concentration	***0.1%E***	***0.4%E***	***0.8%E***	***1.2%E***	***1.6%E***	***2%E***
Reduced Model RSM	0.196	0.302	0.178	0.194	0.256	0.313
Substrate concentration	***350 g/l Whey Permeate***					
Enzyme concentration	***0.1%E***	***0.4%E***	***0.8%E***	***1.2%E***	***1.6%E***	***2%E***
Reduced Model RSM	0.567	0.767	0.710	0.1135	0.935	0.957

3.4.3 Fitting of enzymatic assay data with Maxilact and Whey Permeate as single experiments

Figures 3.16-3.21 show the enzymatic assays carried out with different Maxilact concentrations (0.1, 0.4, 0.8, 1.2, 1.6, 2%) and/or Whey Permeate (200 and 350 g/l) fitted as individual experiments using the JSim simulation program. From Figures 3.17-3.22 it is possible to observe that the proposed model explained not only the lactose hydrolysis, glucose and galactose release but also galactooligosaccharides synthesis by *Kluyveromyces lactis* β-galactosidase at various concentrations of enzyme and substrate. By comparison of the assays with different Whey Permeate concentration, it is clear that the fitting equations of the model for the assays with 350 g/l of Whey Permeate (Figures 3.20-3.22) show a tendency to underestimate the glucose concentration. Similar results were found by Boon *et al*., (1999).

Previously, GOS formation was observed for 5 hours by Iwasaki *et al*., (1996); for 12 hours by Neri *et al*., (2009); for 3.3 and 6.7 hours by Boon *et al*., (respectively 1999 and 2000); and for 5 hours Kim *et al*., (2004). The extent of reaction time

studied in this work of 5 hours is therefore within the context investigated previously from other authors and it was not considered worthwhile to study the reactions further since GOS reach a maximum and then started to be degraded (Figures 3.11-3.14).

Another parameter that changes within previous works in literature is the range of lactose concentrations studied. The reaction kinetics parameters were studied at different initial lactose concentration:

- From 0.39 to 1.67M by Iwasaki *et al*., (1996)
- 0.15, 0.28 and 0.88M by Kim *et al*., (2004);
- From 0.19 to 0.59 mol/kg (*ca* 0.19 to 0.59M) by Boon *et al*., (1999);
- Between 0.14 to 1.45M by Neri *et al*., (2009);

This study covered a wide range of Whey Permeate concentrations, whose initial lactose content is included between 0.58M and 1.012M. The levels considered, described the whole reaction up to completion and exhaustion of all lactose in most of the experiments (Figures 3.17-3.22). However, lactose was not fully depleted in the assay with 0.1% of Maxilact and 350 g/l of Whey Permeate (Figure 3.20). This is may be due to diffusional restrictions arising from the viscosity of the system and the small concentration of enzyme used, both of which delayed GOS formation. Furthermore, the previously cited works in the literature have covered different lactose concentrations (Iwasaki *et al*., 1996; Kim *et al*., 2004; Boon *et al*., 1999; Neri *et al*., 2009), whereas in the present study, different enzyme concentrations at two levels of Whey Permeate concentrations have been examined.

Due to the variety of the initial lactose concentrations, quantity enzyme used and assay conditions by other authors, the maximum GOS achieved in the synthesis reaction shifts from 0.1 mol/kg (Boon *et al*., 1999 and 2000) to 0.51M (Neri *et al*., 2009) and 0.05M (Kim *et al*., 2004). In this work, the maximum yield of 0.47M

(Results, section 3.2.1) was achieved and modelled using the equations proposed (equations 3.15-3.17).

The results obtained fall within similar conditions to previous investigations of the GOS synthesis studies carried out. However, differences in the nature of substrate (Whey Permeate rather then pure lactose), concentration levels of substrate and enzyme tested and assays conditions could influence the obtained results.

The effect of increasing WP concentration did result in slightly higher GOS maximum concentrations, with comparable reaction times. Normally higher enzyme concentrations would result in a shorter time to reach the maximum GOS concentration, with reaction times of around 50-60 minutes required to reach this maximum at the higher enzyme concentrations tested.

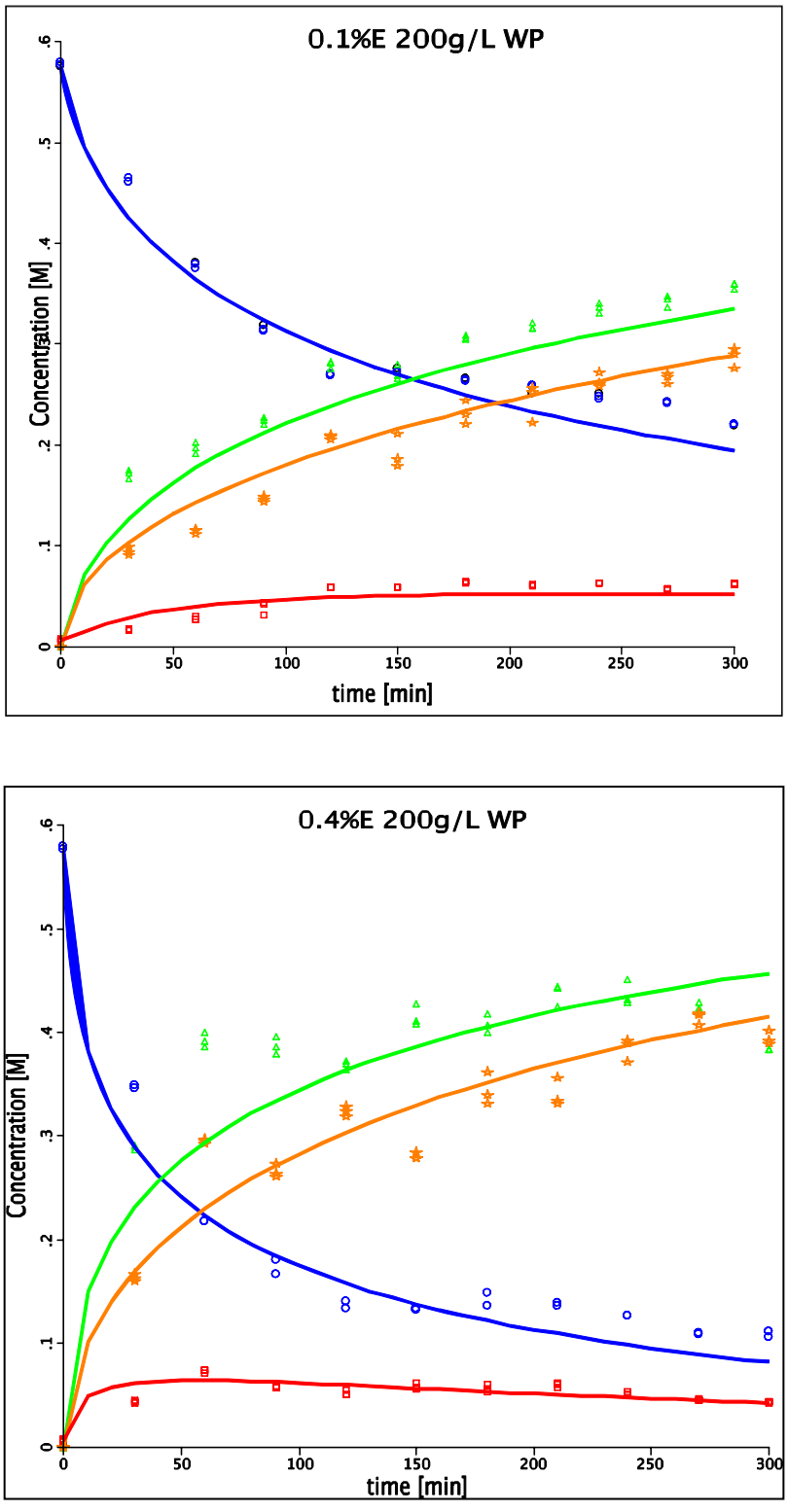


Figure 3.17: Experimental measurement of GOS synthesis and prediction of the individual fitting using the reduced reaction mechanism model. HPLC assays with 0.1 and 0.4% Maxilact in WP (200 g/l) are shown. Points represent experimental data, where ○: Lactose; Δ: Glucose; ☆: Galactose and □: GOS, and lines the individual fit. Assay conditions are described in Materials and Methods (Section 2.4).

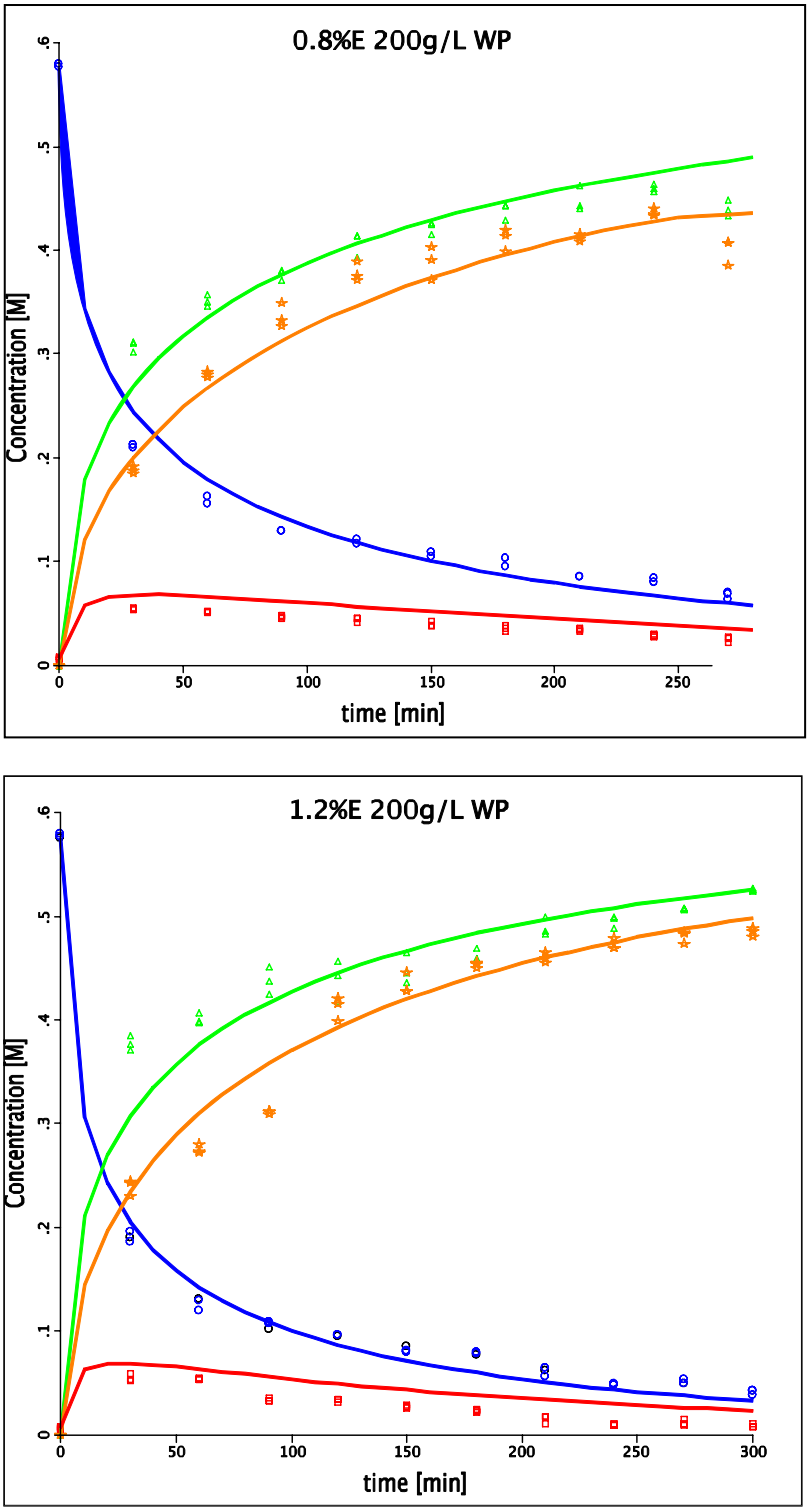


Figure 3.18: Experimental measurements of GOS synthesis and prediction of the individual fitting using the reduced reaction mechanism model. HPLC assays with 0.8 and 1.2% Maxilact in WP (200 g/l) are shown. Points represent experimental data, where ○: Lactose; Δ: Glucose; ☆: Galactose and □: GOS, and lines the individual fit. Assay conditions are described in Materials and Methods (Section 2.4).

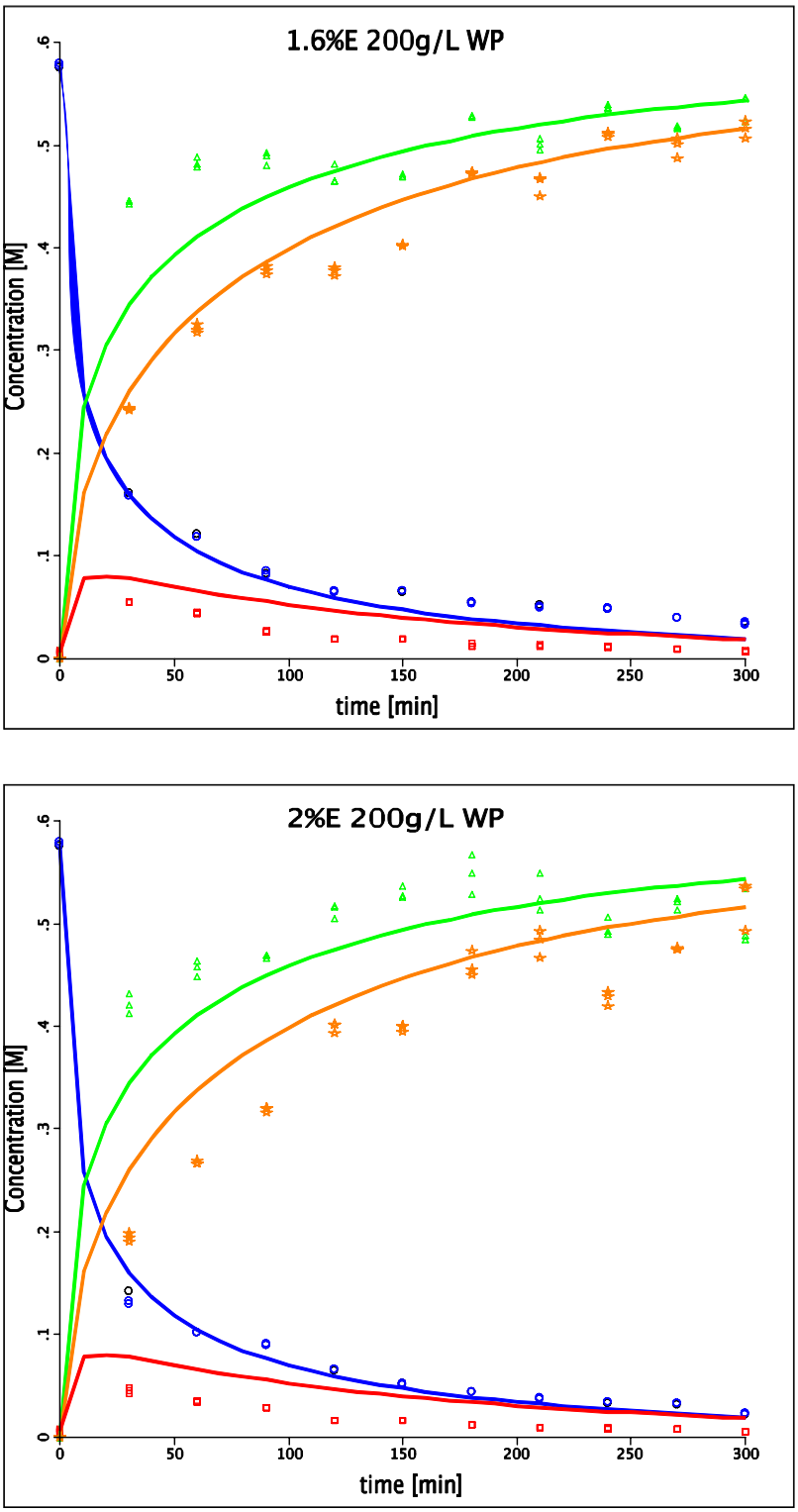


Figure 3.19: Experimental measurements of GOS synthesis and prediction of the individual fitting using the reduced reaction mechanism model. HPLC assays with 1.6 and 2% Maxilact in WP (200 g/l) are shown. Points represent experimental data, where ○: Lactose; Δ: Glucose; ☆: Galactose and □: GOS, and lines the individual fit. Assay conditions are described in Materials and Methods (Section 2.4).

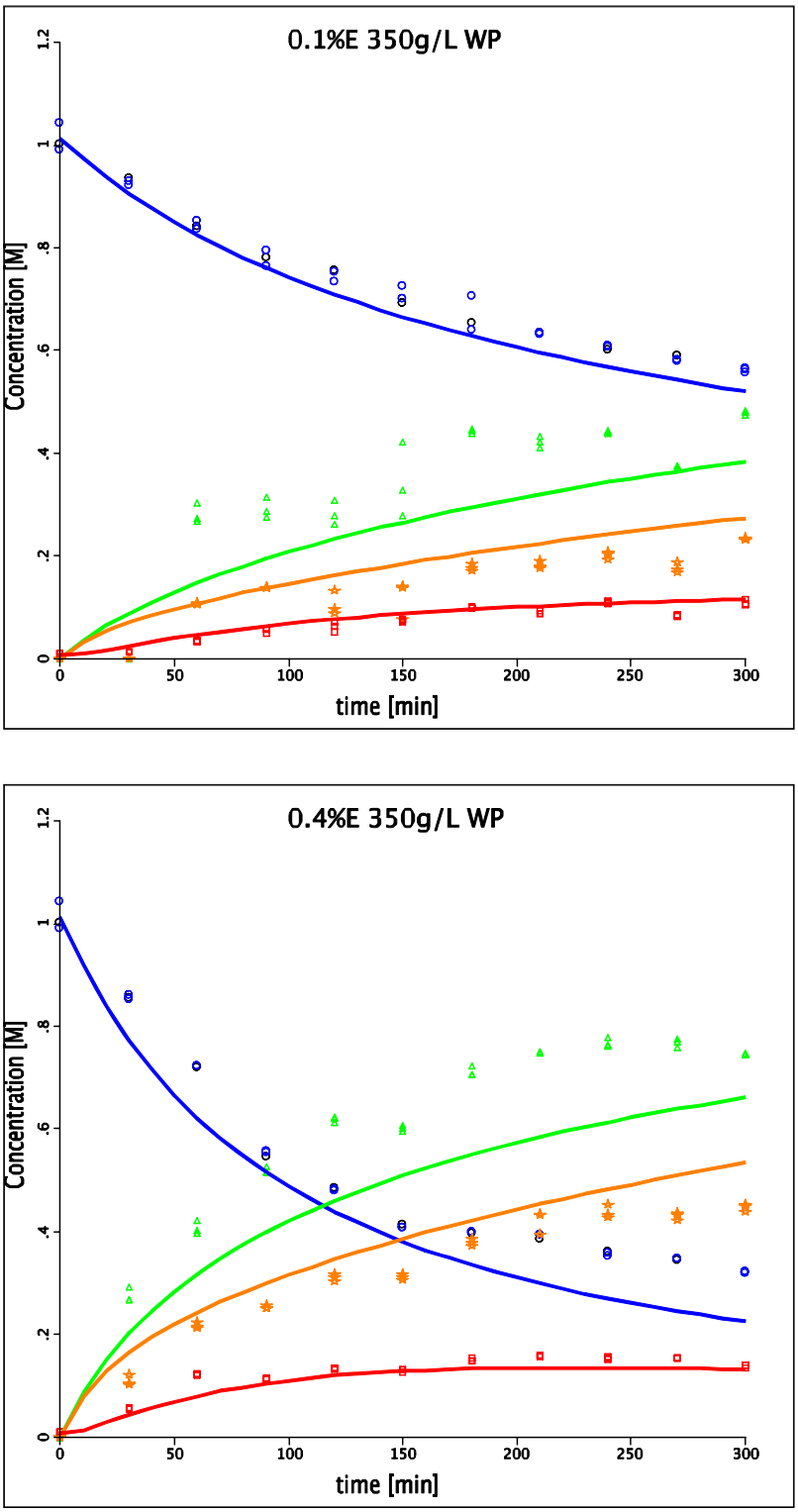


Figure 3.20: Experimental measurements of GOS synthesis and prediction of the individual fitting using the reduced reaction mechanism model. HPLC assays with 0.1 and 0.4% Maxilact in WP (350 g/l) are shown. Points represent experimental data, where ○: Lactose; Δ: Glucose; ☆: Galactose and □: GOS, and lines the individual fit. Assay conditions are described in Materials and Methods (Section 2.4).

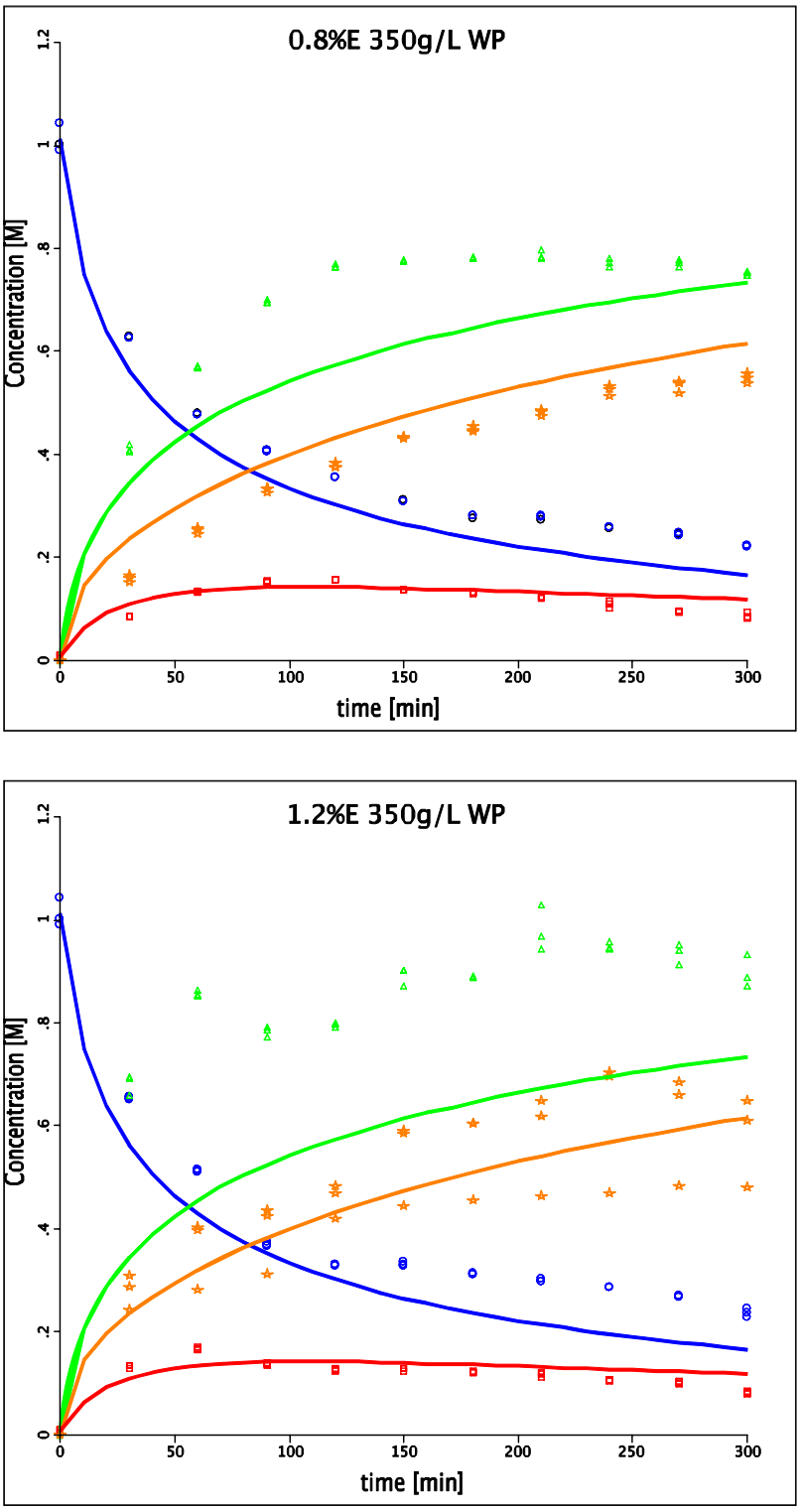


Figure 3.21: Experimental measurements of GOS synthesis and prediction of the individual fitting using the reduced reaction mechanism model. HPLC assays with 0.8 and 1.2% Maxilact in WP (350 g/l) are shown. Points represent experimental data, where ○: Lactose; Δ: Glucose; ★: Galactose and □: GOS, and lines the individual fit. Assay conditions are described in Materials and Methods (Section 2.4).

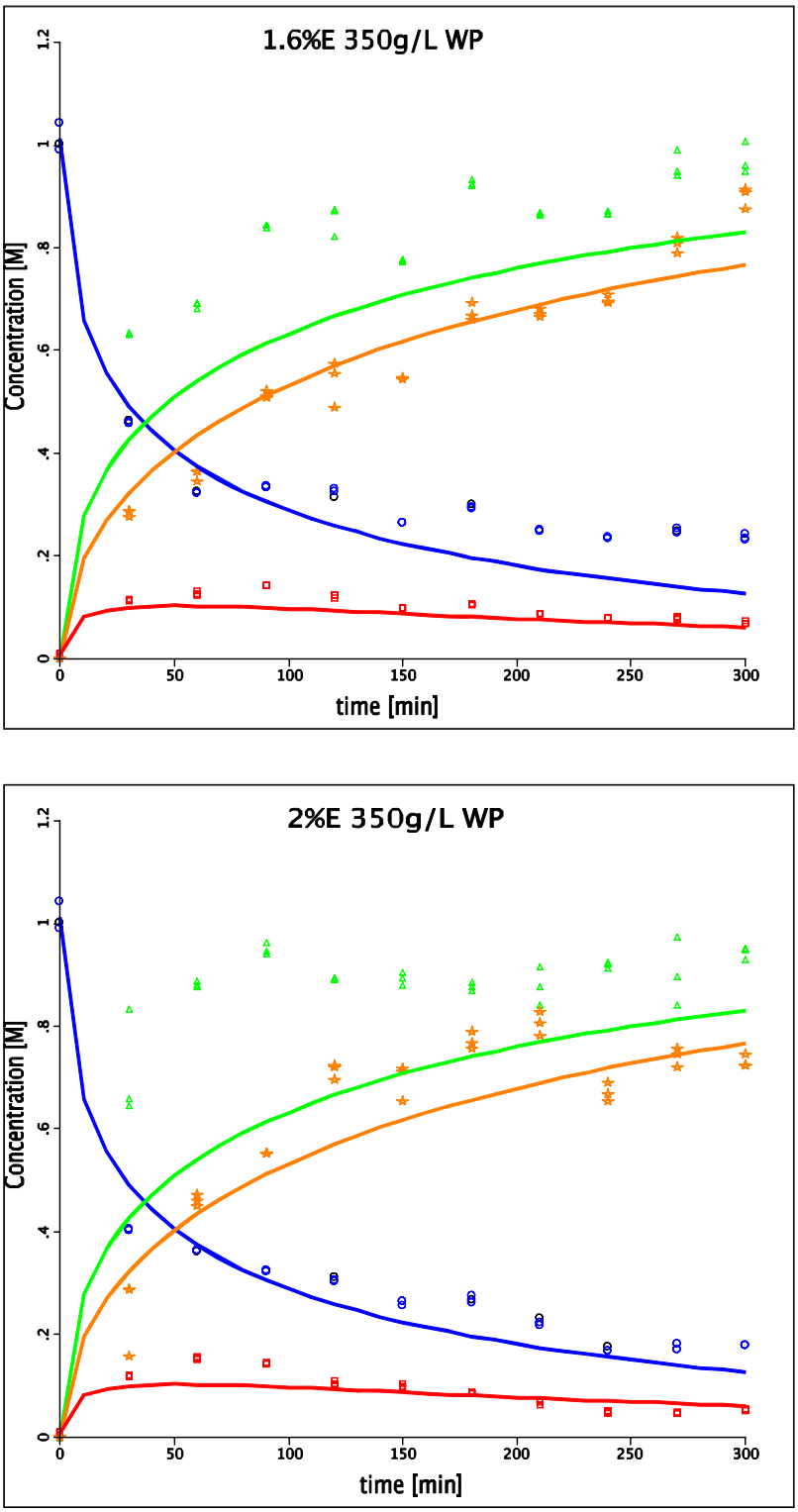


Figure 3.22: Experimental measurements of GOS synthesis and prediction of the individual fitting using the reduced reaction mechanism model. HPLC assays with 1.6 and 2% Maxilact in WP (350 g/l) are shown. Points represent experimental data, where ○: Lactose; Δ: Glucose; ✩ : Galactose and □: GOS, and lines the individual fit. Assay conditions are described in Materials and Methods (Section 2.4).

It can be seen in Figures 3.17 to 3.22 that the individual fitting of the model to the observed kinetics was good. However, it was noticed a poor prediction of the model for glucose kinetics was noticeable, especially at higher initial whey permeate concentrations. This may be due to experimental errors in the estimation of glucose due to HPLC peak overlapping and to the limitations of the model used, which ignore any further involvement of glucose in the reaction mechanism. Possible further improvements of the model and the analytical procedure should consider this, possibly improving the separation ability of the HPLC assay by the change of mobile phase.

The kinetic parameters of the reduced model were estimated by using triplicated data for the enzymatic assays with different concentration of Whey Permeate (200 and 350 g/l) and Maxilact (0.1, 0.4, 0.8, 1.2, 1.6 and 2%).

By definition, the parameters estimated should be positive since they are reaction constants in the given direction. Therefore the logarithms of the constants were estimated by fitting the time-course of lactose conversion data to the proposed reduced model with JSim (Materials and Methods, section 2.6) (Table 3.4). All the fitted parameters found were significant.

Table 3.4: Fitted parameter values of the enzymatic assays with Maxilact (0.1, 0.4, 0.8, 1.2, 1.6 and 2%) and Whey Permeate (200 -a- and 350 -b- g/l). All figures are mean ± standard deviations**.**

a

Parameters (log value)	**0.1%E 200 g/l WP**	**0.4%E 200 g/l WP**	**0.8%E 200 g/l WP**	**1.2%E 200 g/l WP**	**1.6%E 200 g/l WP**	**2%E 200 g/l WP**
$\ln(k_1)$ ($M^{-1}min^{-1}$)	4.3±0.7	10.3±1.1	8.4±0.5	7.9±0.8	18.3±1.5	8.2±1.8
$\ln(k_3)$ (min^{-1})	8.2±1.2	26.0±0.7	10.2±0.4	7.4±0.7	8.3±1.2	7.1±1.4
$\ln(k_{r3})$ ($M^{-1}min^{-1}$)	12.8±0.7	37.7±0.6	19.6±0.2	16.3±0.2	27.5±0.3	16.4±0.4
$\ln(k_5)$ ($M^{-1}min^{-1}$)	1.7±0.9	6±3	28.8±0.7	6.9±1.2	26.7±1.2	11.6±1.5
$\ln(k_{r5})$ ($M^{-1}min^{-1}$)	6.5±1.7	18±4	37.9±0.9	15.6±1.2	45.3±1.8	20±2

b

Parameters (log value)	**0.1%E 350 g/l WP**	**0.4%E 350 g/l WP**	**0.8%E 350 g/l WP**	**1.2%E 350 g/l WP**	**1.6%E 350 g/l WP**	**2%E 350 g/l WP**
$\ln(k_1)$ ($M^{-1}min^{-1}$)	1.6±0.6	1.2±0.2	7.3±1.9	10.4±1.9	11.3±1.7	6.1±1.8
$\ln(k_3)$ (min^{-1})	8.2±4	6±2	2.9±1.4	7.1±1.3	10.7±1.3	6.1±1.5
$\ln(k_{r3})$ ($M^{-1}min^{-1}$)	10.5±6	7±2	11.2±0.6	18.5±1.0	23±0.8	13.3±0.5
$\ln(k_5)$ ($M^{-1}min^{-1}$)	2.5±1.4	2.6±0.5	2.5±0.9	5.2±0.1	8±4	7±4
$\ln(k_{r5})$ ($M^{-1}min^{-1}$)	3.9±1.4	2.1±1.1	8.8±0.9	17±9	21±5	14±5

Figure 3.23 shows a schematic diagram of the reduced mechanism of GOS synthesis.

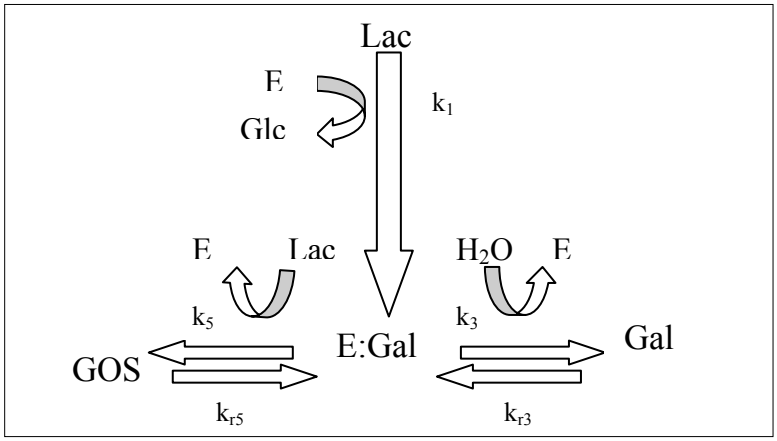


Figure 3.23: Schematic representation of reduced model GOS synthesis mechanism.

According to the reduced model mechanism of reaction (equations 3.15-3.17, Figure 3.23) the four rate constants can be described as follows:

- k_1 defines the Enzyme:Galactose complex production, which is the precursor of GOS. The higher this value is, the faster the GOS formation proceeds;
- k_3 and k_{r3} are a measure of hydrolysis of the Enzyme:Galactose complex. The higher the ratio k_3/k_{r3} is, the more inhibition of GOS formation will occur;
- k_5 and k_{r5} express the formation of GOS from the Enzyme:Galactose complex. The higher the ratio k_5/k_{r5} is, the faster GOS are synthesized.

In Figure 3.24 the estimated $\ln(k_1)$ for all experiments are compared. As can be seen, in general, experiments at 200g/l show a higher rate of conversion from lactose to E:Gal complex, indicating that it will take longer for the E:Gal complex to reach critical concentrations for GOS formation in the 350g/l experiments.

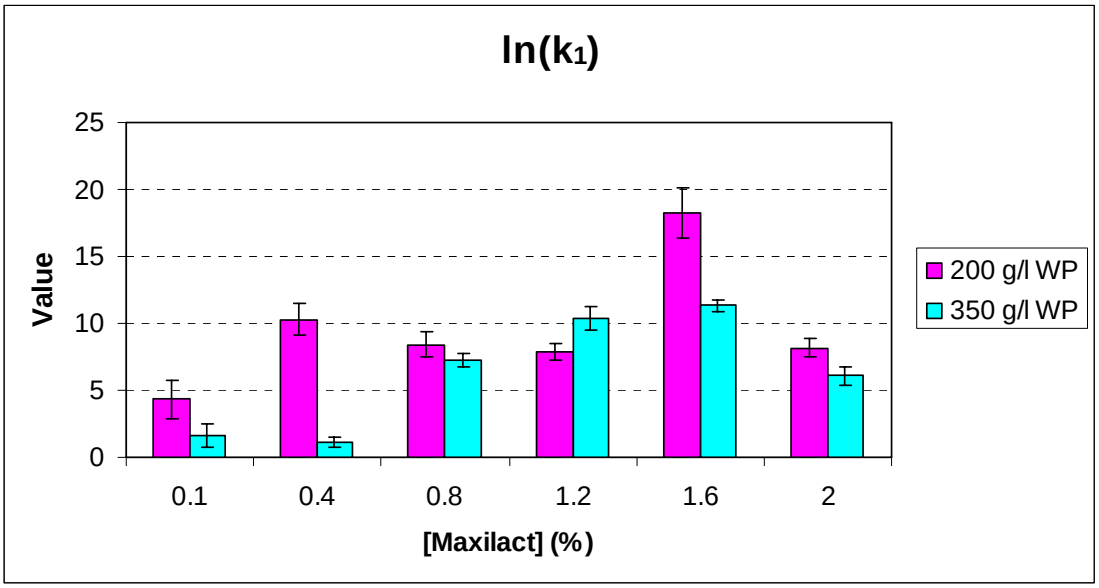


Figure 3.24: Comparison of estimated ln(k_1) kinetic parameter for different initial enzyme and Whey Permeate concentrations. Assay conditions described in Materials and Methods (Section 2.4). Error bars show the 95% CI for the estimated parameters from individual experiments.

In Figure 3.25 the ratio of the inhibitory step of GOS synthesis reaction is plotted for the different experiments performed. It can be seen that inhibition becomes more important at the lower Whey Permeate concentrations only when intermediate enzyme concentrations are used. Otherwise, the higher the concentration of Whey Permeate used, the faster the inhibition of GOS synthesis occurs.

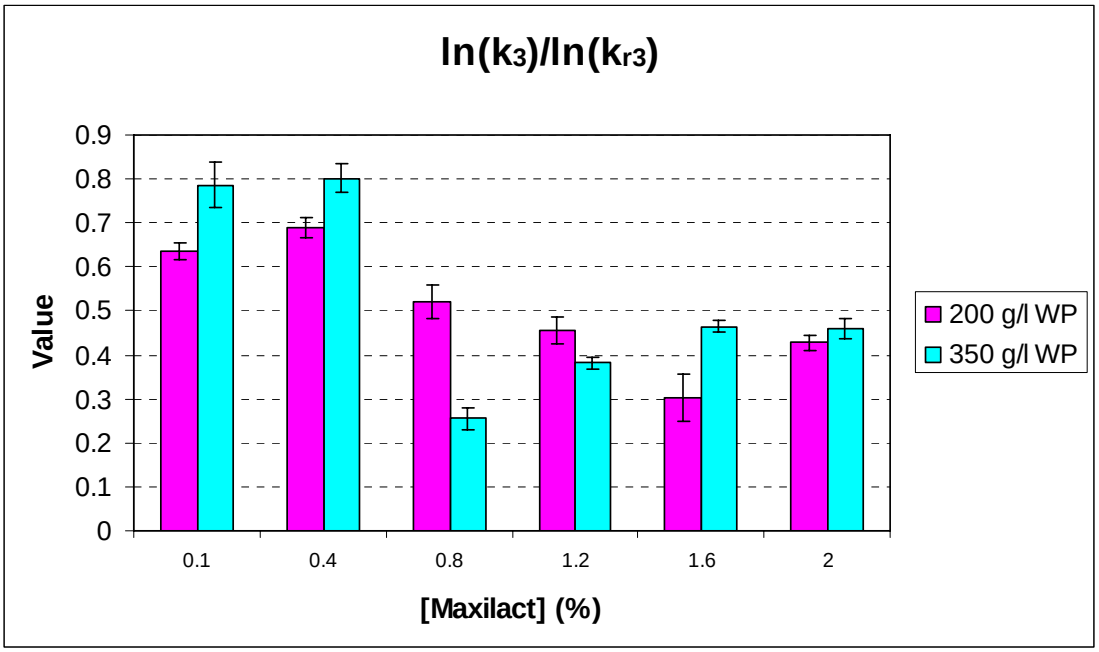


Figure 3.25: Comparison of estimated inhibition kinetics parameters $\ln(k_3)/\ln(k_{r3})$ for different initial enzyme and Whey Permeate concentrations. Assays conditions described in Materials and Methods (Section 2.4). Error bars show the 95% CI for the estimated parameters from individual experiments.

In Figure 3.26 the ratio of the GOS formation kinetic parameters is plotted for the different assays considered. Generally, the assay with the smallest concentration of Whey Permeate (200 g/l) has the higher ratio $\ln(k_5)/\ln(k_{r5})$, which indicates that the GOS synthesis reaction is happening faster than at higher concentrated Whey Permeate solutions (350 g/l). This is may be due to the increasing viscosity of the media that results from increasing the concentration of substrate.

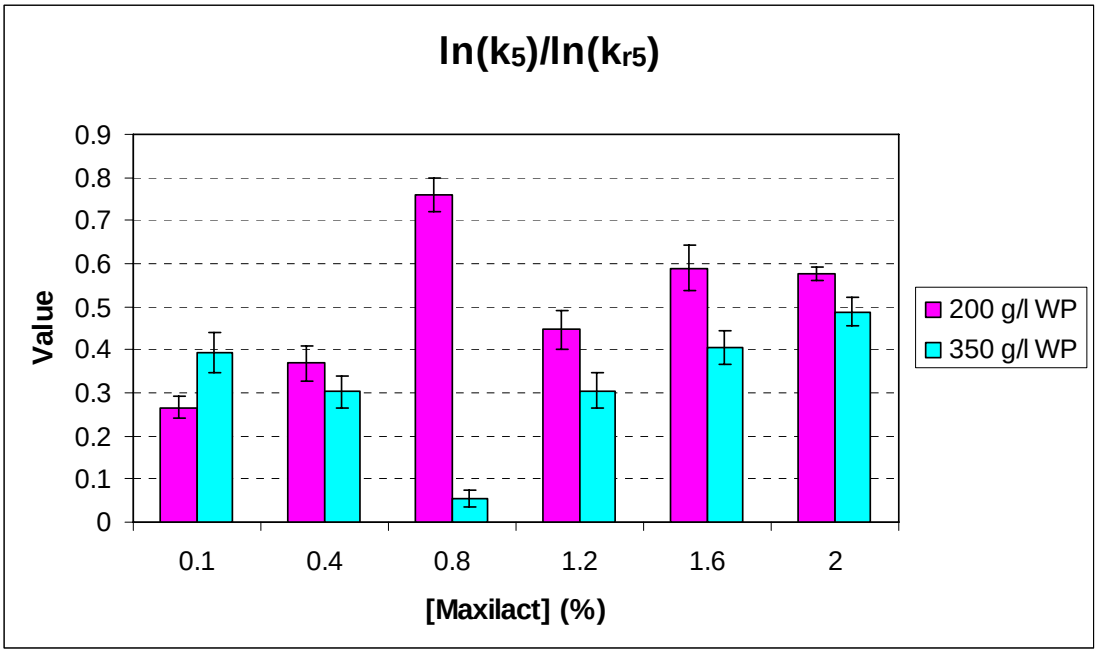


Figure 3.26: Comparison of estimated GOS formation kinetics parameters $ln(k_5)/ln(k_{r5})$ for different initial enzyme and Whey Permeate concentrations. Assays conditions described in Materials and Methods (Section 2.4). Error bars show the 95% CI for the estimated parameters from individual experiments.

3.4.4 Global fitting of enzymatic assays with Maxilact and Whey Permeate

3.4.4.1 Preliminary screening of data

After observing that the estimated parameters from individually fitted experiments, the whole set of data for 200g/l (and then 350g/l) was considered for modelling with a single set of parameter estimates. This will allow us to describe the mechanism of reaction as a whole and to obtain more precise information about the reaction parameters.

As a preliminary screening of data to obtain an estimate of a single set of parameters that would explain all the GOS synthesis performed at the same WP concentration was carried out. The average glucose (Glc) and galactose (Gal) residue ratio was calculated for all experiments, based on the sugar residue balance, as previously shown from Boon *et al*., 1999:

$$\frac{[Glc_{residue}]}{[Gal_{residue}]} = \frac{[Lac]+[Glc]+[GOS]}{[Lac]+[Gal]+2\cdot[GOS]} \tag{3.23}$$

The model used only lactose as substrate; as a consequence the ratio should be close to 1. An error of 10% was accepted; therefore all experimental data for which the average ratio was lower than 0.9 or higher than 1.1 the data from the peak with less confidence, glucose, were removed from the fitting data set.

3.4.4.2 Global fitting of the enzymatic assays with different Whey Permeate concentrations and Maxilact concentrations

All the enzymatic assays carried out with different Whey Permeate and Maxilact concentrations levels were modelled in one single fit using the reduced model previously described (Results, section 3.5.2). For that, the ODEPACK Fortran

library for simulation purposes and the ODRPACK Fortran library for fitting (see Materials and Methods, section 2.6) were used. This produced one set of parameters, which together with the proposed ODE set of equations would be able to describe all the kinetics of GOS formation.

Global fittings using firstly all data available at 200g/l, secondly all data available at 350g/l and finally using both sets of data together were carried out. The multiresponse nonlinear regression procedure converged to a single set of parameters for the 350g/l and for the global set of data. However, the 200g/l data set regression was found not to converge. Several attempts were made with different initial estimates, however no adequate final parameter estimate set was found.

In Figures 3.27-3.30 the global modelling of the assays with 200 and 350 g/l at increasing Maxilact concentrations are shown. The global fitting to the experimental data is good for both initial Whey Permeate concentrations employed. The model always describes accurately GOS synthesis and, in most of the cases, the lactose depletion and the galactose/glucose release. Because of the data deletion of glucose (Section 3.5.1) using the glucose/galactose ratio, there is a significant amount of experiments where the glucose seems to be underestimated, especially when using 350g/l of initial WP. This is not considered a problem of the model, rather a practical solution to the imbalance observed experimentally in the stoichiometric ratios of compounds during the reaction.

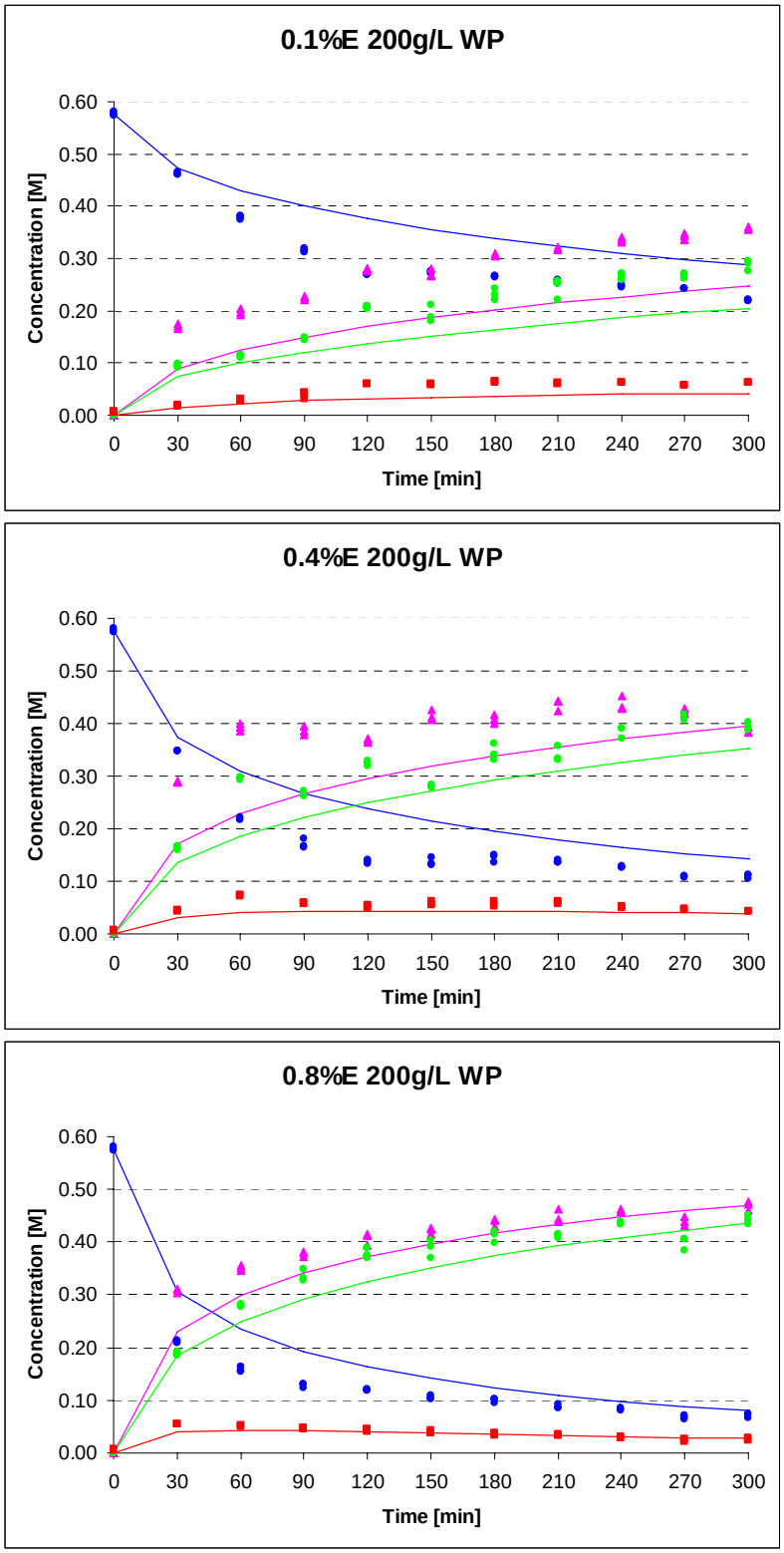


Figure 3.27: Experimental measurements of GOS synthesis and prediction of the global fitting using the reduced reaction mechanism model. HPLC assays with 0.1, 0.4 and 0.8% Maxilact in WP (200 g/l) are shown. Points represent experimental data, where ♦: Lactose; ▲: Glucose; ●: Galactose and ■: GOS, and lines the global fit. Assay conditions are described in Materials and Methods (Section 2.4).

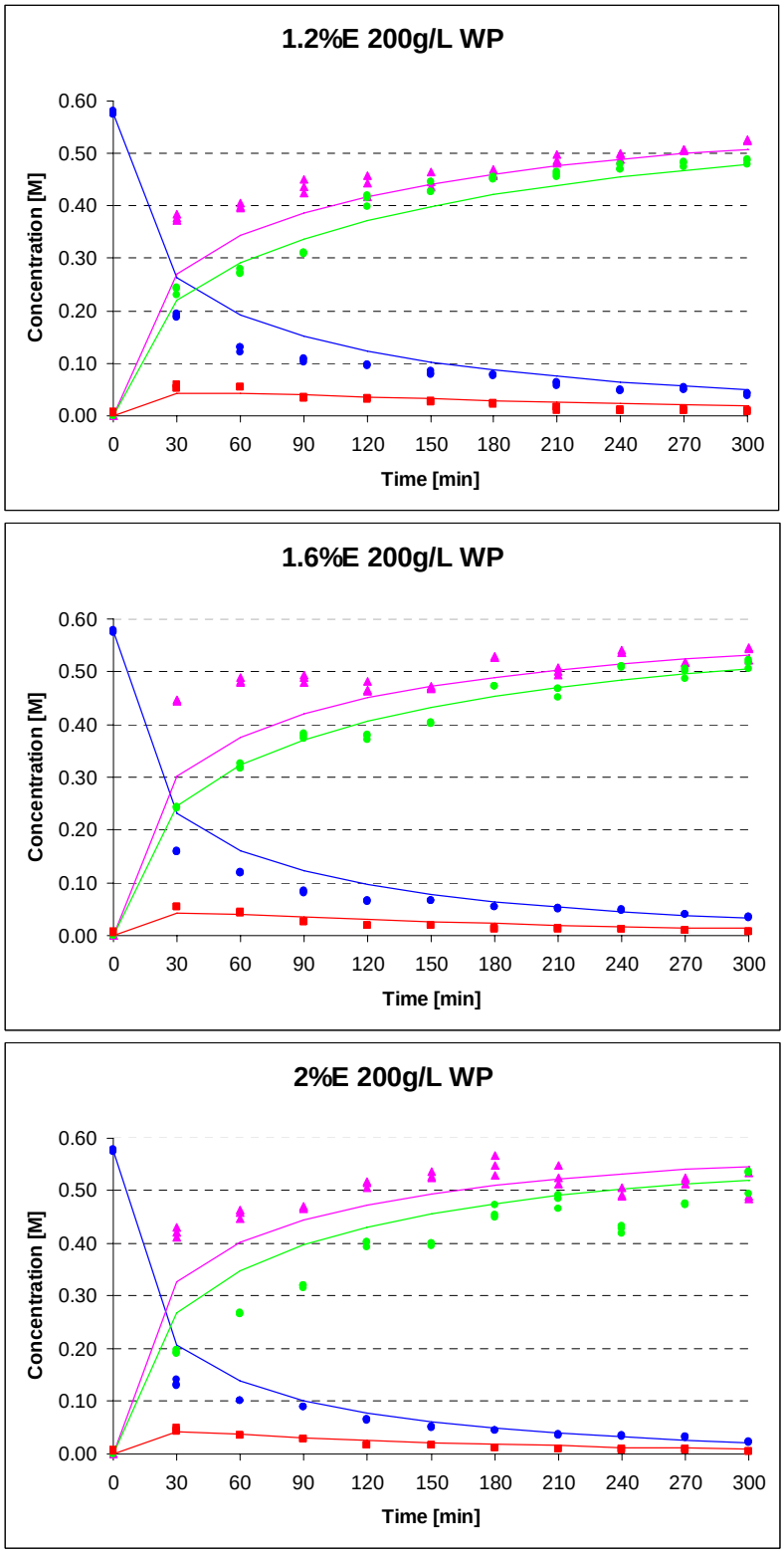


Figure 3.28: Experimental measurements of GOS synthesis and prediction of the global fitting using the reduced reaction mechanism model. HPLC assays with 1.2, 1.6 and 2% Maxilact in WP (200 g/l) are shown. Points represent experimental data, where ♦: Lactose; ▲: Glucose; ●: Galactose and ■: GOS, and lines the global fit. Assay conditions are described in Materials and Methods (Section 2.4).

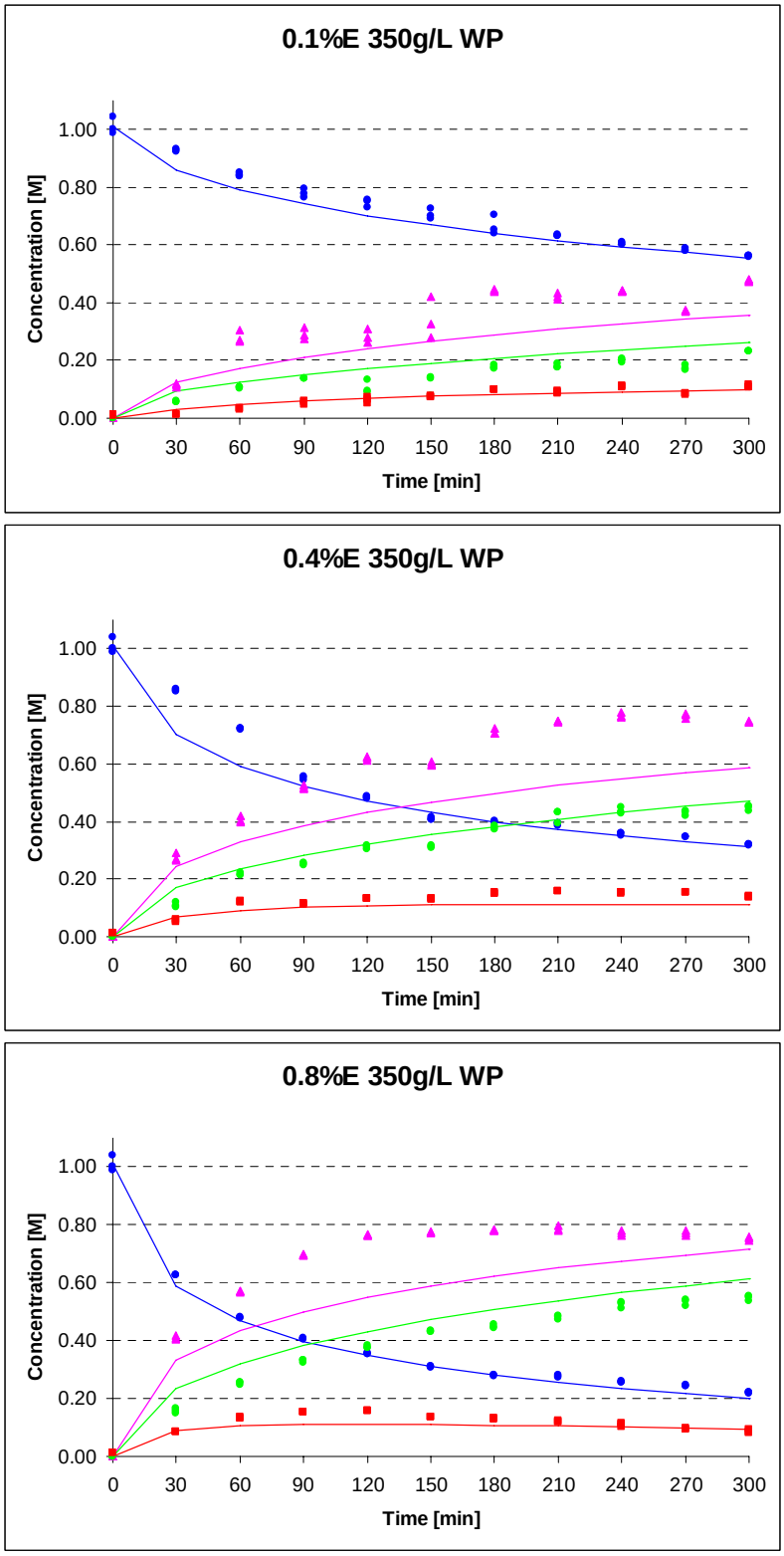


Figure 3.29: Experimental measurements of GOS synthesis and prediction of the global fitting using the reduced reaction mechanism model. HPLC assays with 0.1, 0.4 and 0.8% Maxilact in WP (350 g/l) are shown. Points represent experimental data, where ♦: Lactose; ▲: Glucose; ●: Galactose and ■: GOS, and lines the global fit. Assay conditions are described in Materials and Methods (Section 2.4).

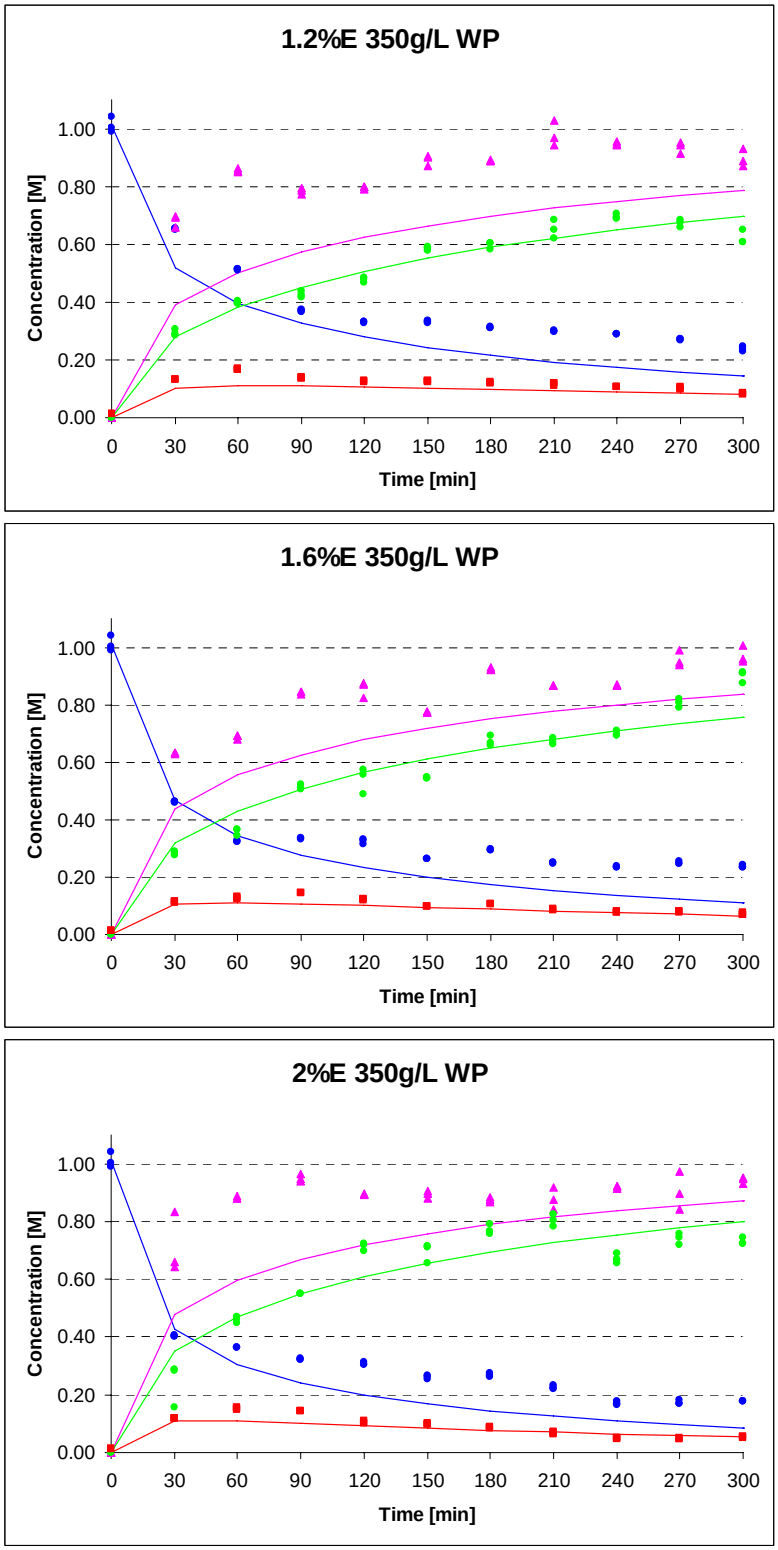


Figure 3.30: Experimental measurements of GOS synthesis and prediction of the global fitting using the reduced reaction mechanism model. HPLC assays with 1.2, 1.6 and 2% Maxilact in WP (350 g/l) are shown. Points represent experimental data, where ♦: Lactose; ▲: Glucose; ●: Galactose and ■: GOS, and lines the global fit. Assay conditions are described in Materials and Methods (Section 2.4).

The fitting kinetics parameter values of the global fitting of the assays with 200 g/l and 350 g/l are shown in Table 3.5.

Table 3.5: Global fitted parameter estimates of the enzymatic assays with different Whey Permeate (200 g/l and 350 g/l) and Maxilact concentrations (0.1, 0.4, 0.8, 1.2, 1.6 and 2%). No convergence was observed for the data at 200g/l. All figures are estimate ± standard error**.**

Parameters (log value)	**350g/l WP**	**200 & 350g/l WP**
$\ln(k_1)$ ($M^{-1}min^{-1}$)	5.84±0.06	7.89±0.05
$\ln(k_3)$ (min^{-1})	1.1±0.3	4±6
$\ln(k_{r3})$ ($M^{-1}min^{-1}$)	8.3±0.3	13±6
$\ln(k_5)$ ($M^{-1}min^{-1}$)	1.37±0.9	0.8±0.6
$\ln(k_{r5})$ ($M^{-1}min^{-1}$)	8.92±1.0	10.2±0.6

3.5 Use of solvents in the GOS synthesis reaction

The presence of solvents has been reported to alter the specificity of a number of enzymes. Thus, we examined the influence of addition of low amounts of solvents to the GOS synthesis reaction. While some solvents caused partial enzyme inactivation others did not markedly affect enzyme activity.

The solvents used were ethanol (EtOH), acetonitrile (ACN), dioxane, diethyl ether, and acetone.

The solvents also lower water activity which may favour GOS synthesis. However, the solvents influenced only slightly the water activity of the media (Table 3.6); probably due to the small amount added.

Table 3.6: Influence of different solvents (acetonitrile, ethanol, diethyl ether, dioxane, and acetone) at the same concentration (10%) in Whey Permeate (200 g/l) on water activity (a_w) in comparison to the control. All figures are mean ± standard deviations**.**

a_w WP Control	**a_w WP + ACN**	**a_w WP + EtOH**	**a_w WP + diethyl ether**	**a_w WP + dioxane**	**a_w WP + acetone**
0.988±0.001	0.987±0.001	0.987±0.001	0.983±0.001	0.987±0.001	0.985±0.001

It was noticed that some solvents, such as acetonitrile and dioxane, inhibited β-galactosidase activity. Some other solvents, such as acetone and diethyl ether, permitted β-galactosidase reaction. However, the yield of GOS synthesized in comparison to the control was not affected (Figure 3.31).

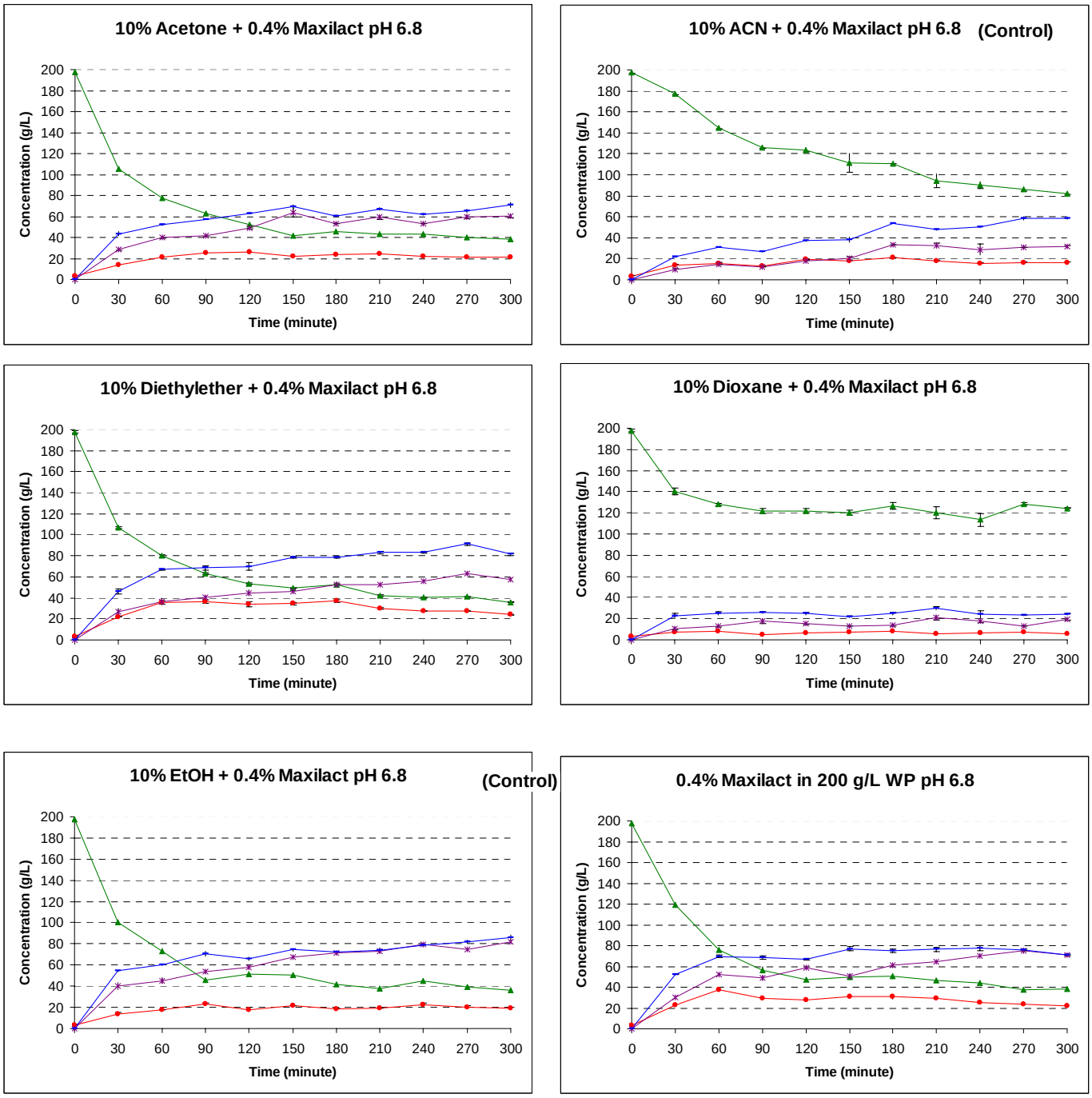


Figure 3.31: GOS synthesis in presence of different solvents. Average values of three replicates are reported with relative error bars. Assays were carried out in phosphate buffer (0.1 M, pH 6.8) with Whey Permeate (200 g/l), solvent (10%) and 0.4% Maxilact, at 40ºC, for 300 minutes reaction. Solvent added were: acetone, acetonitrile, diethyl ether, dioxane and ethanol. (Where ▲: Lac, —: Glc, *: Gal, and ●: GOS).

3.5.1 Fitting of the enzymatic assays carried out with solvents addition

Figures 3.32-3.37 shows the kinetic parameters of the enzymatic assays carried out with the addition of solvents (10%) in the reaction mixture, composed of Whey Permeate (200 g/l) and Maxilact (0.4%) fitted as single experiments with the JSim simulation program. The solvents used were ethanol, acetonitrile, acetone, diethyl ether and dioxane (see Results, section 3.2.2).

Figure 3.31 shows the changing of $\ln(k_1)$ between the assays carried out with solvents. All the solvents, within experimental error, allowed for the formation of E:Gal complex at the same rate. E:Gal is the precursor to GOS formation. However acetonitrile and dioxane had slower kinetics, which is consistent with the initial lactose depletion observed in these assays (Figure 3.31). In Figure 3.32 it can be seen that the introduction of small concentrations of acetonitrile and dioxane affected significantly ($p<0.05$) the initial step of precursor formation in the reaction.

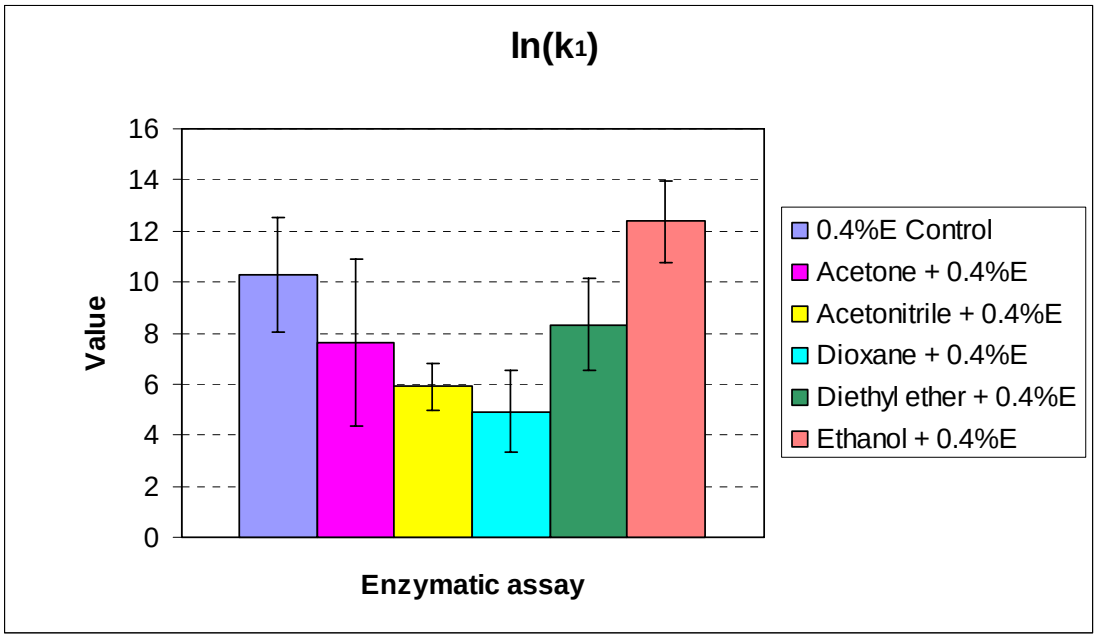


Figure 3.32: Comparison of estimated GOS formation kinetics parameters $\ln(k_1)$ for enzymatic assays carried out with the addition of solvents. Assay conditions described in Materials and Methods (Section 2.4.1). Error bars show the 95% CI for the estimated parameters from individual experiments.

The comparison of the kinetic parameter $\ln(k_3)$ and $\ln(k_{r3})$ between the different solvents can be seen in Figures 3.33 and 3.34. The use of organic solvents resulted generally in a reduction of both $\ln(k_3)$ and $\ln(k_{r3})$ compared to the control. This is expected to result in slower degradation of the E:Gal complex towards the formation of free Galactose. This might have the interesting result of displacing the reaction towards the formation of GOS, which is characterised by $\ln(k_5)$ and $\ln(k_{r5})$.

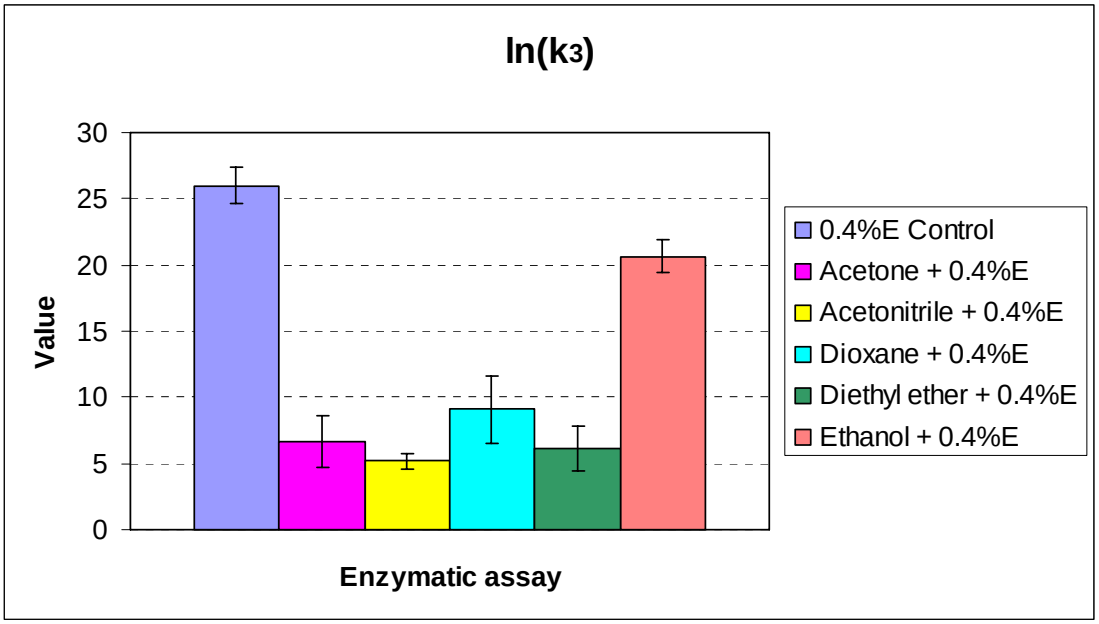


Figure 3.33: Comparison of estimated GOS formation kinetics parameters $\ln(k_3)$ for enzymatic assays carried out with the addition of solvents. Assay conditions described in Materials and Methods (Section 2.4.1). Error bars show the 95% CI for the estimated parameters from individual experiments.

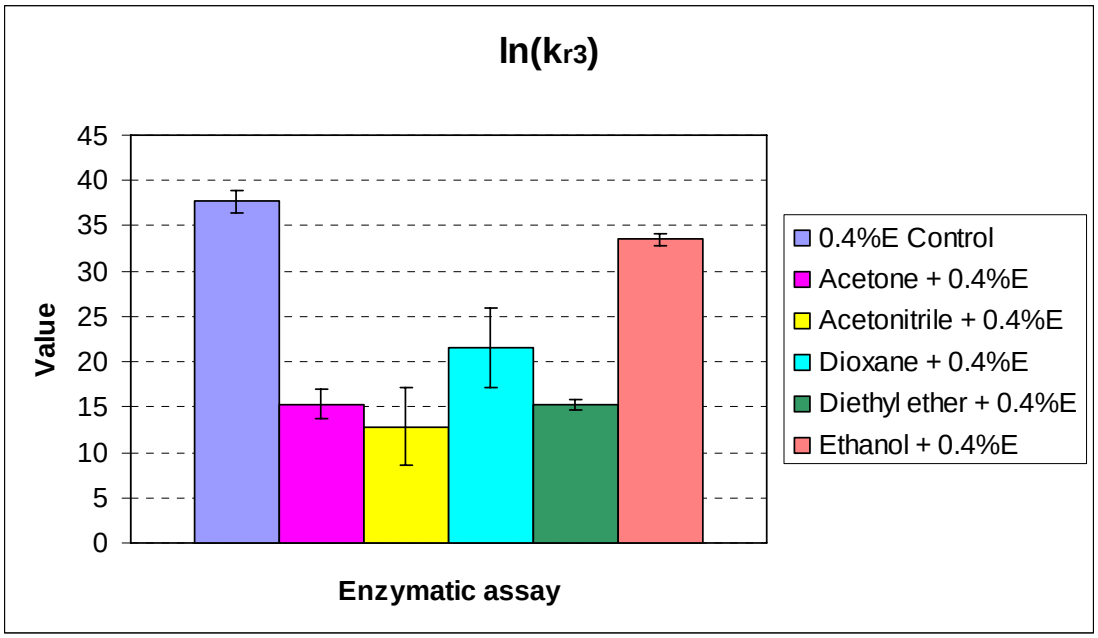


Figure 3.34: Comparison of estimated GOS formation kinetics parameters ln(kr_3) for enzymatic assays carried out with the addition of solvents. Assay conditions described in Materials and Methods (Section 2.4.1). Error bars show the 95% CI for the estimated parameters from individual experiments.

The ratio between $\ln(k_3)/\ln(k_{r3})$ (Figure 3.35) shows that the use of dioxane and diethyl ether reduced this ratio ($p<0.05$). Therefore the use of solvents had an observable effect in the balance of the reaction mechanism.

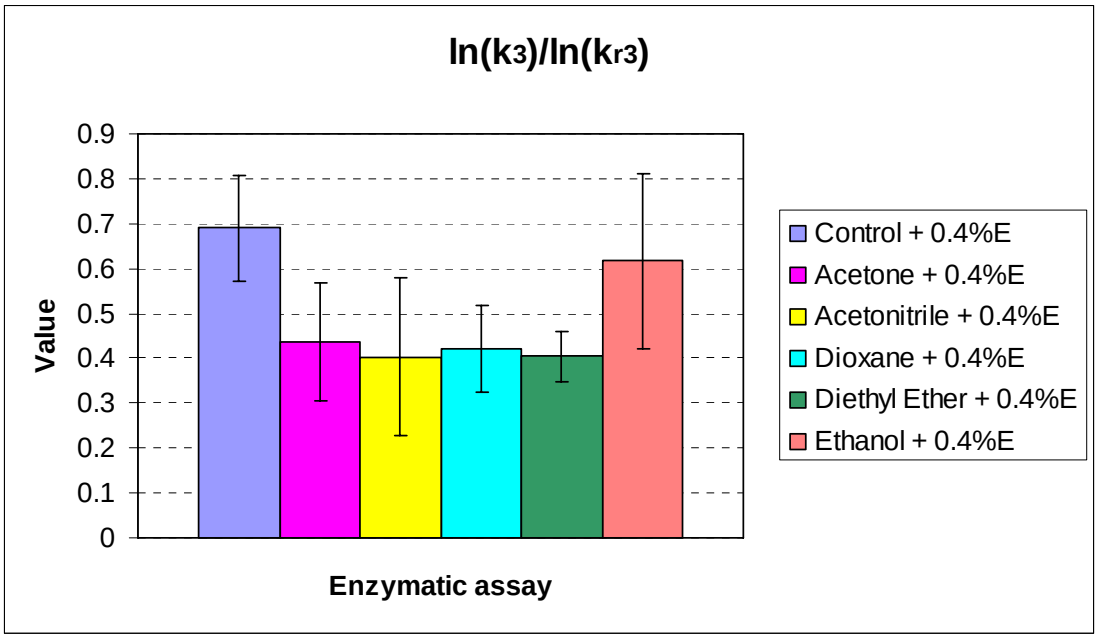


Figure 3.35: Comparison of estimated inhibition kinetics parameters $\ln(k_3)/\ln(k_{r3})$ for enzymatic assays carried out with the addition of solvents. Assay conditions described in Materials and Methods (Section 2.4.1). Error bars show the propagated 95% CI for the estimated parameters from individual experiments.

The kinetic parameter $\ln(k_5)$ (Figure 3.36) did not change between the experiments taken into consideration. No statistically significant difference between the different solvents was found.

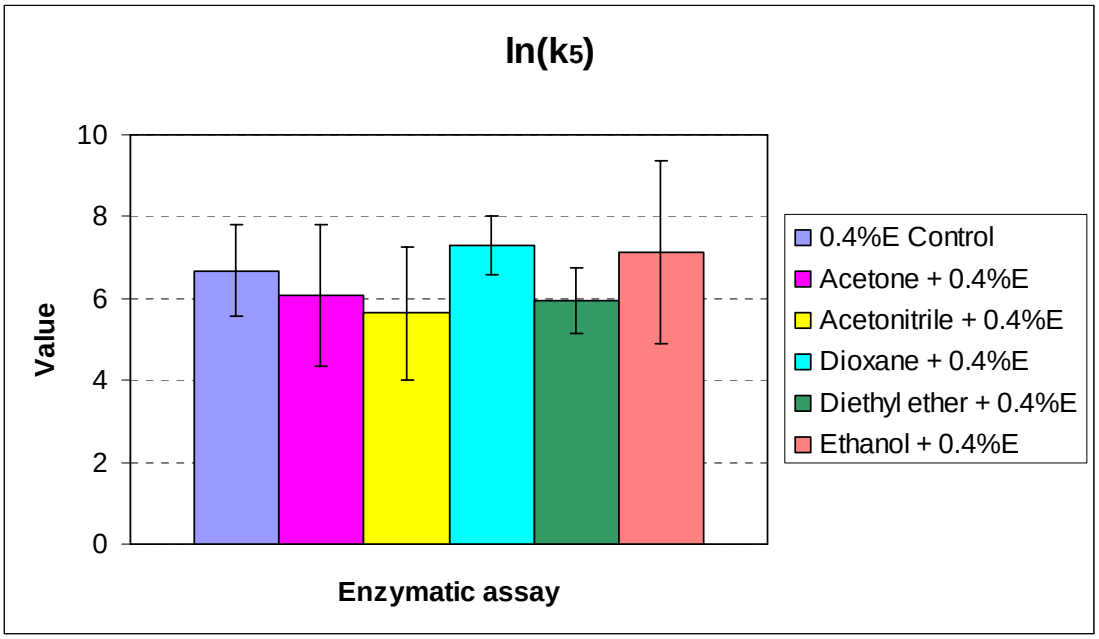


Figure 3.36: Comparison of estimated GOS formation kinetics parameters ln(k_5) for enzymatic assays carried out with the addition of solvents. Assay conditions described in Materials and Methods (Section 2.4.1). Error bars show the 95% CI for the estimated parameters from individual experiments.

In Figure 3.37 the kinetic parameter ln(k_{r5}) is represented. The assay carried out with dioxane addition showed a higher value then the other assays ($p<0.05$). Hence, in the presence of this solvent, the transglycosylation reaction is shifted towards the degradation of GOS rather then its synthesis. This is consistent with the kinetics shown in Figure 3.30.

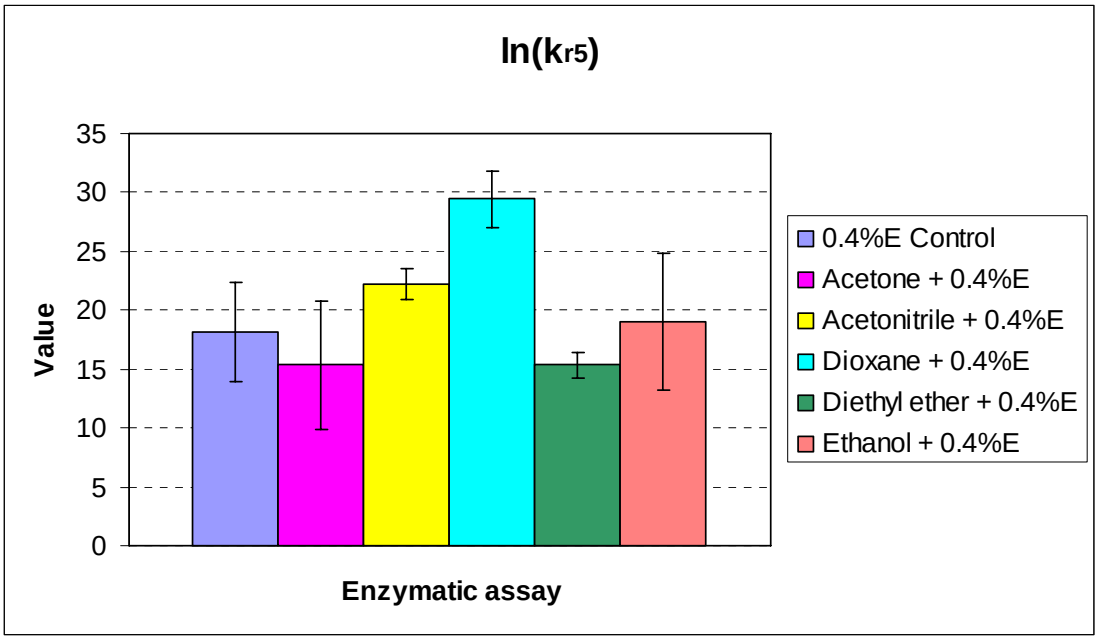


Figure 3.37: Comparison of estimated GOS formation kinetics parameters $\ln(k_{r5})$ for enzymatic assays carried out with the addition of solvents. Assay conditions described in Materials and Methods (Section 2.4.1). Error bars show the 95% CI for the estimated parameters from individual experiments.

The ratio between $\ln(k_5)/\ln(k_{r5})$ (Figure 3.38) showed that the assays carried out with acetone, diethyl ether and ethanol had the same GOS formation mechanism as the control.

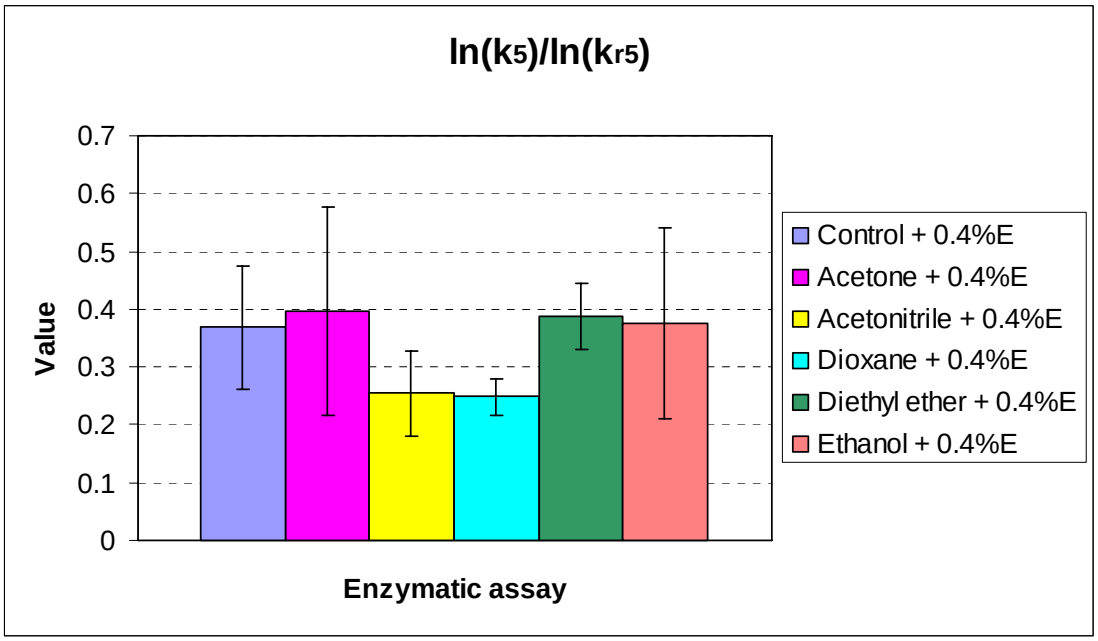


Figure 3.38: Comparison of estimated GOS formation kinetics parameters $\ln(k_5)/\ln(k_{r5})$ for enzymatic assays carried out with the addition of solvents. Assays conditions described in Materials and Methods (Section 2.4.1). Error bars show the propagated 95% CI for the estimated parameters from individual experiments.

3.6 Enzyme comparison

Many authors have sought to improve GOS synthesis by the selection of enzymes that are more efficient than others previously studied. It is generally believed that yeast enzymes are more suited for industrial processes than bacterial ones (Rustom *et al*., 1998; Cheng *et al*., 2006). However, a central problem with the data from the literature is that it is difficult to decide whether one enzyme source is better than another for GOS synthesis. This is due to the fact that different researchers have used different assay conditions such as temperature, pH, substrate concentration and enzyme concentration.

Many researchers have claimed that one enzyme preparation is better than another but have not used a standard that allows easy comparison between researchers. Where comparisons have been made between enzymes interpretation has been difficult since the enzymes were estimated at different pH, temperature, inclusion level –all factors that affect GOS synthesis.

We have explored this issue by attempting to compare two enzymes from widely different organism: *Kluyveromyces lactis* and *Esherichia coli*: one is eukaryotic, the other prokaryotic.

The DNA sequences are compared through a Basic Logical Alignment Search Tour (BLAST) search carried out via the ClustalW website. The β-galactosidases sequences were taken from BRENDA-enzymes database (Chang *et al*., 2009). As the *Escherichia co*li strain used in the Sigma Aldrich preparation is unknown, a consensus sequence was deduced via the ClustalW website. The alignment file was then plotted in GENEDOC© (2000) in order to show sequences identities through shading utilities (Figure 3.39).

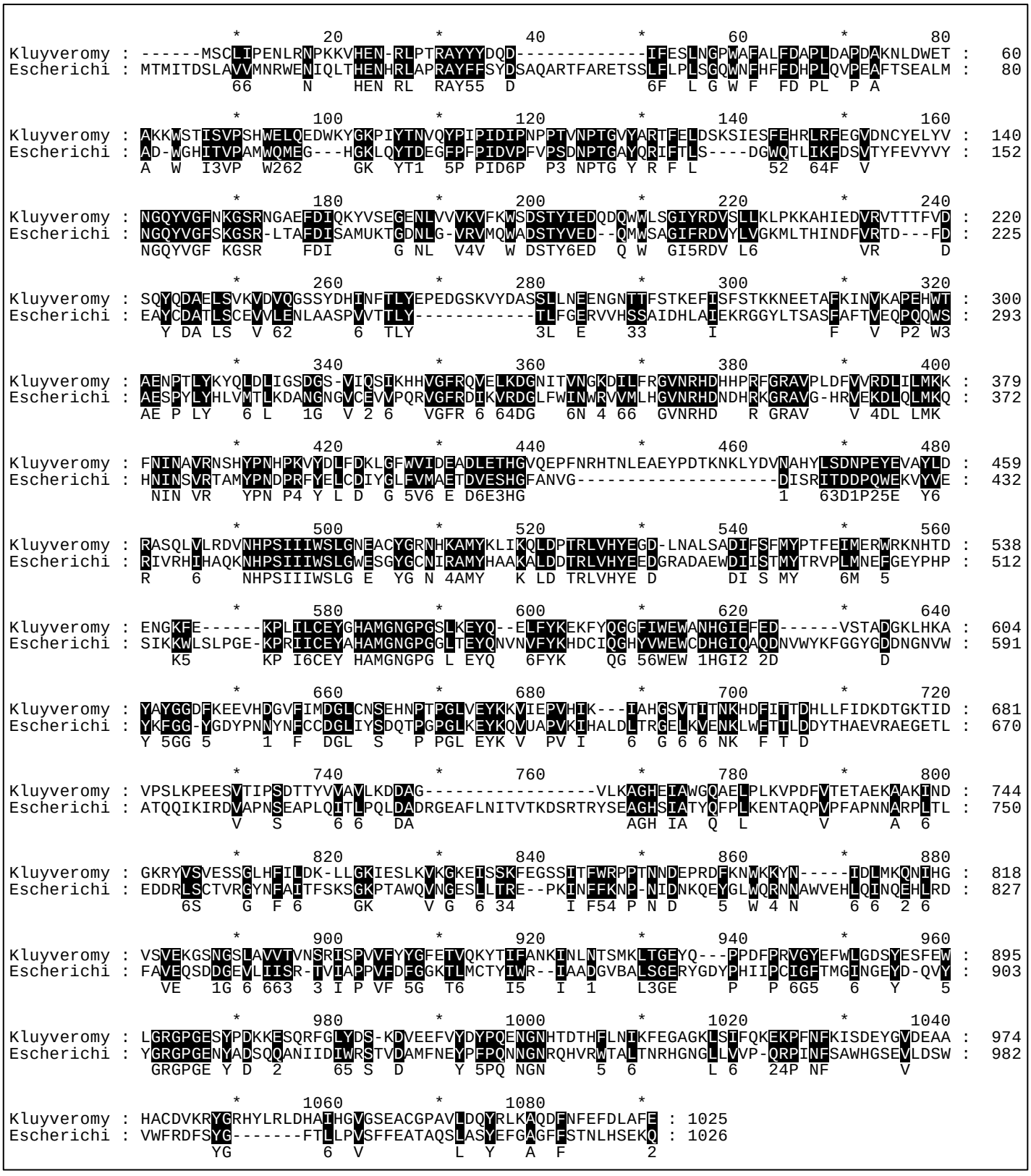


Figure 3.39 Alignments sequences of *Kluyveromyces lactis* and *Escherichia coli* consensus sequence via ClustalW website. Shadows show identities between the sequences.

The two enzyme preparations were assayed for GOS synthesis at:

- same pH (phosphate buffer 0.1 M, pH 7.0);
- same substrate concentration (Whey Permeate at 200 g/l);
- same temperature (37ºC);

The enzyme preparation were normalised to the same activity as measured by initial rate of lactose consumption in the first hour of reaction.

Under these conditions no significant difference in GOS synthesis profile was observed (Figure 3.40).

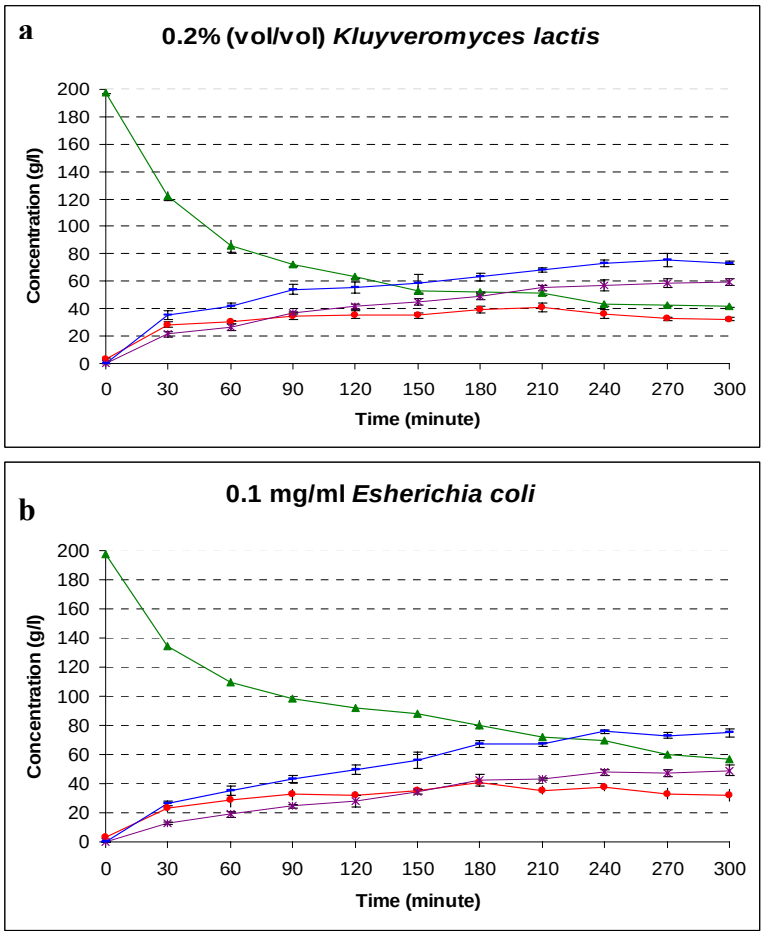


Figure 3.40: Comparison assays with different enzyme sources added (*Kluyveromyces lactis* -a- and *Escherichia coli* -b-). Average values of three replicates are reported with relative error bars. Assays were carried out in phosphate buffer (0.1 M pH 7.0) with Whey Permeate (200 g/l) at 37ºC, for 300 minutes reaction time. (Where ▲: Lac, —: Glu, *: Gal, and ●: GOS).

3.6.1 Fitting of the enzymatic assays carried out with different sources of β-galactosidase

The enzymatic assays carried out with different sources of β-galactosidase at the same conditions (substrate concentration, pH, temperature, time, ionic strength) have been fitted as single experiments. In Figure 3.41 the comparison of the kinetic parameters of the enzymatic assays carried out with different sources of β-galactosidase is shown. It can be seen that no parameters are different ($p<0.05$). Therefore, using different enzymes normalised to the same activity scale did not affect the maximum GOS formation, as was observed in the experimental results (Figure 3.40).

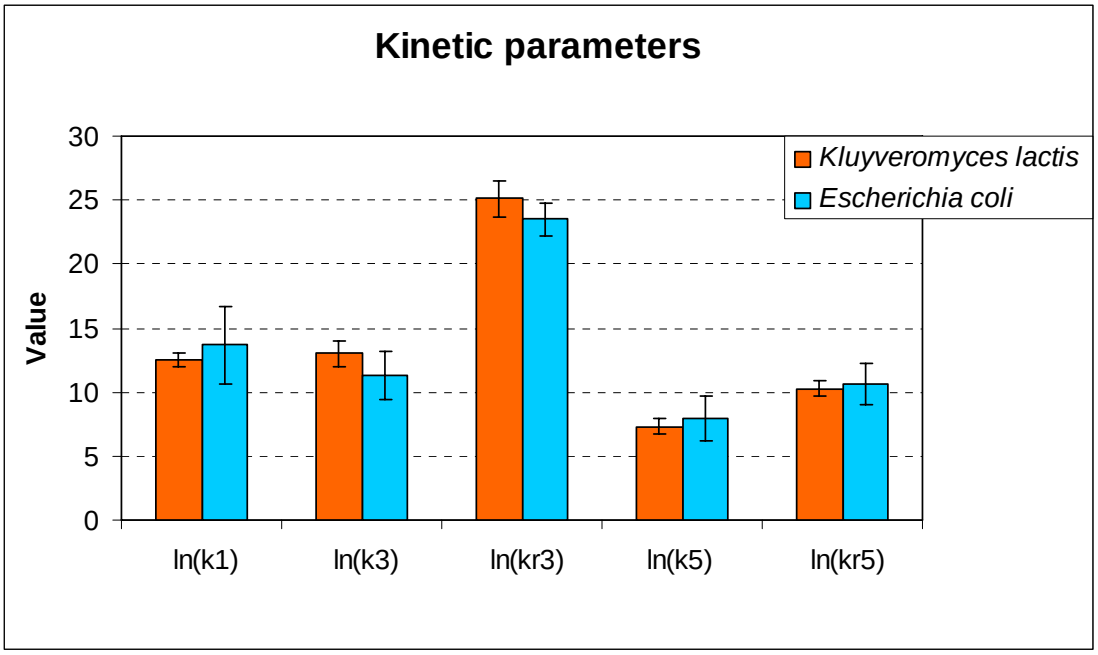


Figure 3.41: Comparison of estimated GOS formation kinetics parameters $\ln(k_1)$, $\ln(k_3)$, $\ln(k_{r3})$, $\ln(k_5)$ and $\ln(k_{r5})$ for different β-galactosidase sources at the same assay conditions. Error bars show the 95% CI for the estimated parameters from individual experiments. Assay conditions are described in Materials and Methods (Section 2.4.2)

Figure 3.42 shows the fitting of the enzymatic assays with the reduced model using the above kinetic parameters. From Figure 3.42 it is possible to see how the model fitted well the experimental data, especially for lactose degradation and GOS formation.

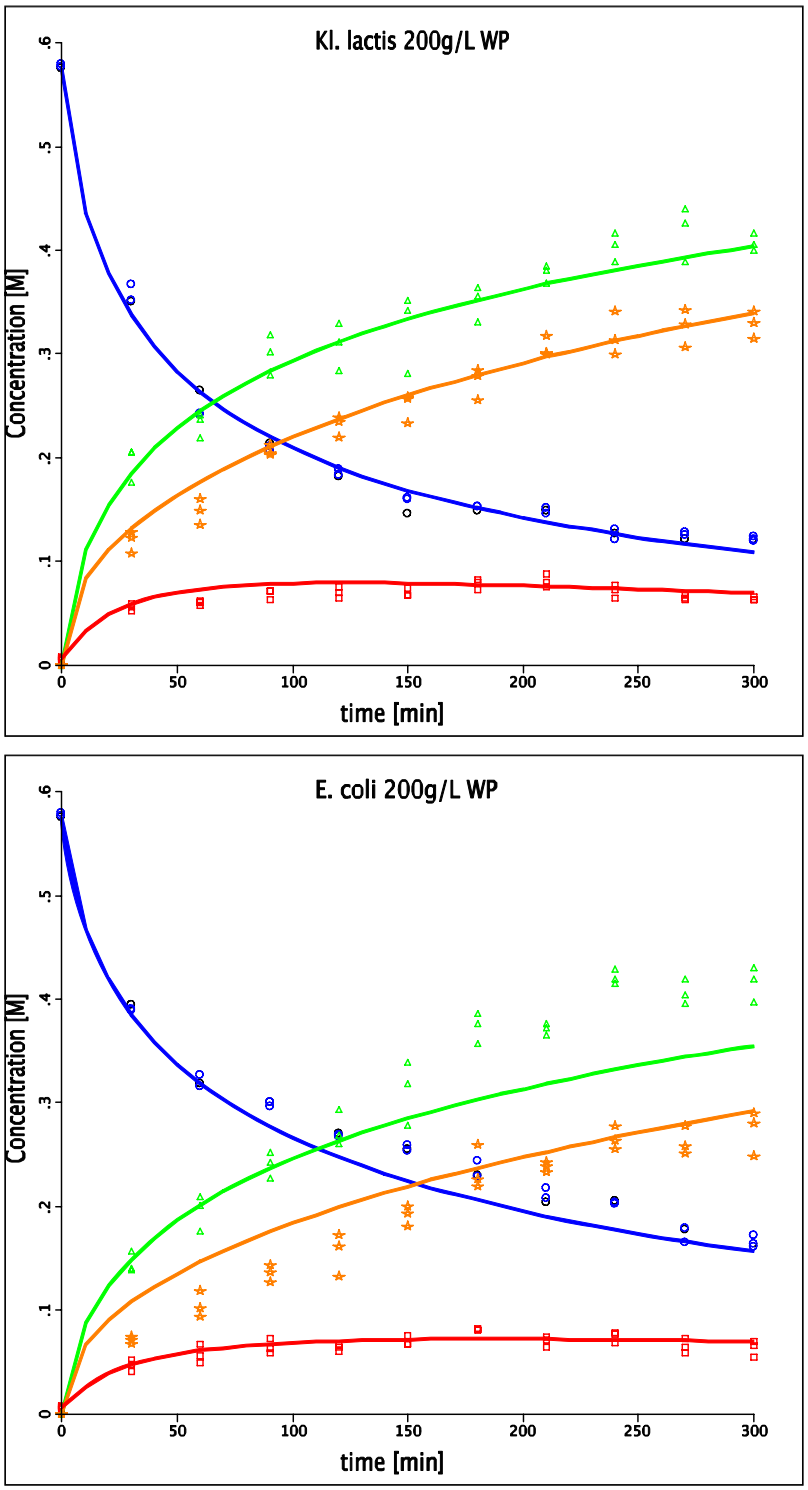


Figure 3.42: Experimental measurements of GOS synthesis and prediction of the global fitting using the reduced reaction mechanism model. HPLC assays with 0.2% (vol/vol) *Kluyveromyces lactis* and 0.1 mg/ml *Escherichia coli* in WP (200 g/l) are shown. Points represent experimental data, where ○: Lactose; Δ: Glucose; ☆: Galactose and □: GOS, and lines the best global fit. Assay conditions are described in Materials and Methods (Section 2.4.2).

3.7 Attempts to produce high GOS preparation

Synthesis of GOS reaches a maximum for all enzymes when about 60% lactose conversion has occurred and subsequently declines (Figure 3.15). It was not possible to obtain GOS yield higher than 24% (w/w) for any combination of enzyme or WP concentration assayed (Figure 3.15 b). Preparations high in GOS are of great industrial interest due to the increasing interest in probiotic preparations for their functional properties (see introduction, section 1.4). After characterising the reaction mechanism in previous experimental work, an attempt was made to produce a carbohydrate fraction enriched in GOS by further processing the reaction mixture. The following experiments were carried out:

1. Selective enrichment of the GOS mixture by lactose crystallisation.
2. Increase of GOS yield by addition of WP during the reaction mixture.

3.7.1 Lactose crystallization

The first series of experiments attempted to selectively crystallise lactose from GOS synthesis reaction mixtures. An assay with 0.8% Maxilact and 350 g/l of Whey Permeate was chosen as the one that gave the highest GOS yield of the all experiments. At the maximum point of GOS synthesis (90 minutes), the reaction was quenched by immersing the reaction medium in boiling water. The GOS synthesis reaction mixture was concentrated slowly using a Rotary evaporator. The objective of this step was to attempt to induce lactose to crystallise. When 50% of the water was removed, the solution was divided into 100 ml beakers and stored at 4 ºC and 25 ºC in order to allow lactose crystals to grow. Further water removal was difficult due to the formation of viscous syrup. A few seeds of lactose were added to stimulate crystal formation (Gänzle *et al*., 2008). The solutions were kept without agitation, until

further analysis, for three weeks. Contrary to what was expected, none of lactose or other sugars in the two solutions did crystallise. Prolonged evaporation was used concentrate the GOS synthesis reaction mixture. This caused the thickening of the syrup and no crystalline material was observed. The negligible lactose crystallization might have been due to presence of other sugars in the solutions and the high viscosity of the solution itself (Ibarz *et al*., 2002). At the moment, there are no studies regarding the effects of glucose, galactose and GOS, simultaneously, on lactose crystallization.

A HPLC analysis of the concentrated solutions was carried out. The concentrated solutions presented a higher concentration of GOS, as well as the other species in solutions, *i.e.* lactose, glucose and galactose but the ratio of species was the same as for the control (Table 3.7).

Table 3.7: Influence of water removal on GOS content. Where Lac: lactose; Glc: glucose; Gal: galactose.

Sample	GOS (g/l)	Lac (g/l)	Glc (g/l)	Gal (g/l)	GOS Yield
0.8% E + 350 g/l WP (Control)	68.614	100.266	119.389	70.797	23.623
0.8% E + 350 g/l WP After rotavapor stored at 4 ºC	93.152	184.716	211.550	93.152	19.033
0.8% E + 350 g/l WP After rotavapor stored at 25 ºC	93.839	190.026	213.549	93.840	18.865

From Table 3.7 it is possible to observe that the two different storage temperatures did not influence lactose crystallization.

It was assumed that lactose crystallization in the analysed systems will eventually occur; however the time scale necessary to let lactose crystals form is not compatible with industrial application. Therefore other approaches to increase GOS yield were examined.

3.7.2 Addition of Whey Permeate

The results obtained comparing assays with different Whey Permeate concentrations indicated that high concentrations of substrate enhanced GOS yield (Results, section 3.2.1). Therefore an attempt to add Whey Permeate to the synthesis mixture at the maximum point of GOS synthesis was made. The reaction mixture used for this study was 0.8% Maxilact and 350 g/l of Whey Permeate. A quantity of Whey Permeate solids was added to that synthesis mixture (70 g in 100 ml) after maximum GOS concentration was achieved (90 minutes) and stirred to facilitate its dissolution. It was not possible to dissolve all the Whey Permeate powder, due to the low solubility of lactose and the fact that it was not possible to bring the solution to its boiling point since this would have caused complete enzyme denaturation. The reaction component profile of the sugar species for the assay with addition of Whey Permeate are presented in Figure 3.43.

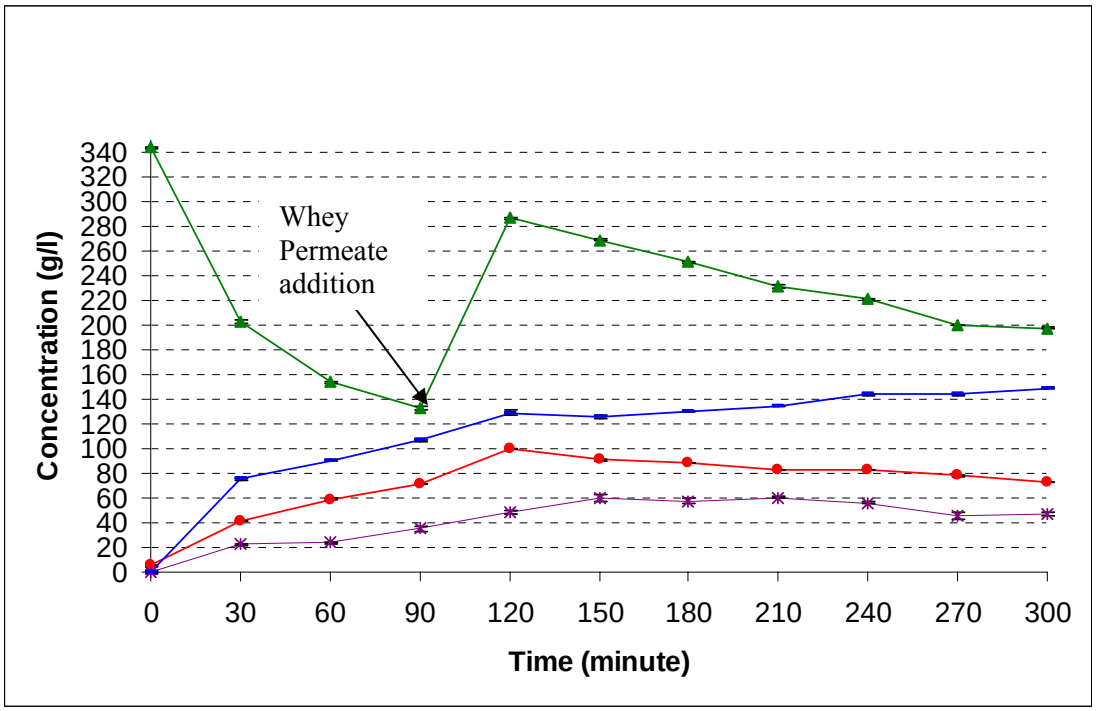


Figure 3.43: WP addition at 90 minutes of the enzymatic assay. Assay was carried out in phosphate buffer (0.1 M, pH 6.8) with Whey Permeate (350 g/l) and 0.8% Maxilact, at 37 ºC, for 5 hours reaction time. After 90 minutes from the starting of the reaction Whey Permeate (70 g/100 ml) was added (arrow) (Where ▲: Lac, ▬: Glu, *: Gal, and ●: GOS).

The Figure 3.43 shows that by adding more substrate to the reaction mixture at the point of maximum GOS synthesis initially induces an increase in GOS levels, which thereafter slowly decreases. The maximum GOS yield achieved was 22% (w/w) in comparison to the 24% (w/w) of the control.

4 Discussion

The synthesis of galactooligosaccharide from lactose, using β-galactosidases, has been extensively investigated over the last 50 years due to the functional properties of GOS as prebiotics (Tanaka *et al*., 1983; Hidaka *et al*., 1986 and 1988; Kunz *et al*., 1993; Champ *et al*., 2003). The interest in GOS synthesis has increased since its inclusion in Japanese legislation regarding foods for specified health use (Farnworth, 1997).

Many researchers have investigated the GOS synthesis reaction, with the objective of increasing the final GOS yield by changing β-galactosidases sources and/or synthesis conditions (Tables 1.5-1.10). However, most of the literature on GOS synthesis achieves a maximum GOS yield of 20-24% and only a few laboratories report yields higher than 40% (Tables 1.5-1.10). Variability in GOS yield may be influenced by several factors:

- competition between hydrolysis and transglycosylation at the β-galactosidase active site (Figures 1.4 and 1.6);
- low lactose aqueous solubility (Hunziker *et al*., 1962);
- the inhibitory effects of monosaccharides released during the reaction (Bakken *et al*., 1992; Shin *et al*., 1998);
- the viscosity of concentrated lactose solutions that slow the reaction kinetics (Iwasaki *et al.*, 1996).

In the present work, optimisation of GOS synthesis was examined for application on an industrial scale. A TLC and a HPLC assay method were used for analysing GOS synthesised by the enzymatic assays with β-galactosidases. A literature review showed that these analytical techniques are amongst the most widely

used in the field of GOS analysis, due to their short analysis time and the simple laboratory instrumentation required. This makes bench scale application and the study of reaction kinetics possible.

4.1 Analysis of GOS synthesis by TLC assay method

At the outset of these studies we attempted to devise a rapid, quantitative assay based on separation of GOS synthesis reactants and products by TLC.

We showed this analytical method could be successfully used for quantitation of individual components. However, problems with the precision and accuracy of this analytical method were encountered:

- The precision of analysis of some components (e.g. GOS and glucose) were better for others (*i.e.* galactose and lactose) as can be seen in Table 3.1. This may be due to differences between TLC plates that produce variations in separation and staining.
- When comparing the results obtained with TLC analysis against similar results in the literature, it was observed that the TLC method produced a biased result, with overestimation of GOS production (Zhou *et al.,* 2003; Kim *et al*., 2004). The lack of complete separation between GOS components and lactose may have caused this loss of accuracy.

Attempts were made to improve TLC plate variation by taking into account the differences in background staining between plates by the inclusion of internal standards to correct for run to run variation. Attempts were also made to improve resolution of the TLC method by examining a wide variety of solvent systems to improve separation and by examining different spot visualisation methods. Despite

screening a number of different configurations of solvent systems and staining methods, significant problems in precision and accuracy remained.

However, TLC allowed the analysis of more than thirty samples per day and therefore facilitated the screening of trends in the kinetics of the enzymatic reactions. Using the TLC assay, GOS synthesis from Whey Permeate was confirmed through comparison with commercially available GOS preparation Vivinal GOS® (Figure 3.4). The effect of enzyme concentration on the maximum GOS yield was also observed in these TLC studies (Figure 3.5). This has been previously reported by other authors (Čurda *et al*., 2006; Chockchaisawasdee *et al*., 2005).

The TLC method could have been improved by increasing the length of the TLC plate or by running the TLC plate twice. However, this would have doubled the analysis time, reducing the sample throughput. It is possible that this method may be used quantitatively in the future if conditions for separation of components can be improved. The method does accurately measure release of monosaccharides and might be useful for kinetic studies of the GOS synthesis reaction. Further attempts at optimising this assay method were abandoned in favour of a HPLC-based assay.

4.2 Analysis of GOS synthesis by HPLC

A HPLC solvent system was devised, that separated the components of the GOS synthesis reaction and allowed a more precise (Figure 3.9) and accurate (Figure 3.10) quantitation of GOS components. Despite many attempts, a baseline separation of glucose, galactose, lactose and GOS was not achieved. This was the best separation obtained considering the time and the resources available. However, separation between the components was sufficient to allow their accurate quantitation during GOS synthesis. A comparison between data derived by TLC analysis and data

obtained by HPLC analysis showed that the former greatly overestimated the level of GOS and galactose production and underestimated the lactose concentration.

Difficulties in accuracy of quantitation of carbohydrates in complex mixtures may account for some of the variation in GOS yield reported in the literature. The precision of yield data for GOS synthesis in the literature is difficult to assess since few reports show chromatograms of GOS synthesis mixtures and many do not give estimates of errors associated with GOS measurement which are presented in the form of error bars in only a few reports (Martínez-Villaluenga *et al*., 2008; Li *et al*., 2008). In our hands it was clear that TLC-based methods can greatly overestimate GOS synthesis yield compared to the HPLC method.

The differences between the two analytical methods were highlighted by attempts to model the reaction progress curves. High levels of error in parameters were associated with TLC derived data.

A study of enzymatic assays with different concentrations of Maxilact® β-galactosidase (0.1, 0.4, 0.8, 1.2, 1.6 and 2%) and Whey Permeate (200 and 350 g/l) was carried out. The results showed the clear influence of initial enzyme concentration on GOS yield as well as its speed of synthesis and degradation, as previously observed by other authors (Čurda *et al*., 2006; Chockchaisawasdee *et al*., 2005). High enzyme concentrations led to rapid GOS synthesis but also to its rapid degradation. Initial substrate concentration influenced GOS formation, consistent with previous studies (Goulas *et al*., 2007; Maugard *et al*., 2003). Higher initial lactose concentrations increased the transglycosylation reaction to favour GOS synthesis rather then monosaccharides formation. A yield analysis of GOS synthesis reactions led us to obtain an optimum GOS yield of 24% using 0.8% of Maxilact and 350 g/l, Whey Permeate (Figure 3.15).

4.3 Effect of solvent addition on GOS synthesis

A study of solvent addition on the enzymatic synthesis of GOS was carried out. Low solvent concentrations (10%) were tested in order not to inhibit the β-galactosidase action. The solvents used were acetone, acetonitrile, ethanol, diethyl ether and dioxane. These solvents were chosen after a review of previous work in the literature on the effect of solvents on β-galactosidase activity (Yoon *et al*., 2005; Giacomini *et al*., 2002). In comparison to the assay without solvent addition, it was observed that some solvents inhibited β-galactosidase activity (dioxane and acetonitrile) while other solvents (acetone, diethyl ether and ethanol) gave similar profiles to the control (Figure 3.31). Further work on the effects of solvents on the transglycosylation reaction, especially of ethers would be of considerable interest. The reduction of water activity by diethyl ether (Table 3.6) and the low polarity of those compounds may avoid β-galactosidase inactivation (Yoon *et al*., 2005).

4.4 Comparison of different sources of β-galactosidase

A significant effort, as judged by the number of literature reports, has gone into finding enzymes that will enhance the yield of GOS. It is generally believed that certain enzymes (from yeast) are better than others (from bacteria) at catalysing GOS synthesis (Rustom *et al*., 1998).

In selecting the best enzyme for GOS synthesis the studies in the literature are confusing (Table 1.5-1.10). Thus, it is almost impossible to compare studies with different enzymes since they are carried out under different conditions of pH, substrate concentration, enzyme activity, temperature and ionic strength, all of which might be expected to influence GOS production. To address this issue we have compared GOS synthesis for two enzymes under identical conditions of pH, ionic

strength, and temperature and substrate concentration. Crucially, the two enzymes were compared at the same level of catalytic activity. This was achieved by using a quantity of each enzyme that gave the same initial rate of lactose depletion using the HPLC assay method. The enzymes were selected such that they had a significant difference in primary sequence. Despite β-galactosidases having different origins and properties, the active site of β-galactosidases are highly conserved. It has been shown that the key residues of the catalytic site for microbial β-galactosidases are a pair of glutamic acid residues (Table 1.11, Zhou *et al*., 2003). BLAST analysis revealed the two enzymes used in this study, one prokaryotic, the other eukaryotic, were *ca.* 40% identical (Figure 3.39).

Under the conditions used here we found that when these enzymes were compared directly, there was no significant difference between them in terms of GOS synthesis profile (Figure 3.39). This study is preliminary and should be extended to a greater number of enzymes. Nonetheless, it raises the possibility that the differences between enzymes in GOS synthesis profiles may be related more to factors such as assay conditions and the specific activity of the enzyme preparations rather than inherent differences in selectivity.

GOS synthesis is clearly, from Figure 3.23, a competitive process with hydrolysis. Thus, the enzyme-galactosyl complex caused by lactose binding to β-galactosidases may either react with water or an alternative acceptor. It is difficult to envisage how an enzyme might be selected that favoured the alternative acceptor. Water is a less bulky and more mobile species than dissolved carbohydrates. The active site is necessarily open in order to accommodate the bulk of lactose. Therefore, it seems likely that competition between these reaction pathways is mainly influenced by thermodynamic factors such as lactose concentration and water activity. Many

enzymes may catalyse this reaction with equal efficiency. Further analytical developments in addition to further enzymatic studies are needed to establish this point but preliminary evidence supports this conclusion.

4.5 Optimisation of GOS yield

From the arguments above, it is clear that manipulation of water activity, lactose concentration and reaction products may enhance GOS synthesis. Many studies have shown that elevated lactose concentrations increase GOS yield (Goulas *et al.*, 2007). Unfortunately, the attainable lactose concentrations are limited by its low water solubility. Furthermore, increased viscosity of the reaction medium at high lactose levels may slow the reaction kinetics, delaying the reaction time at which maximum GOS is achieved. Relatively few studies have attempted to reduce water activity as a method to enhance GOS synthesis. The effect of agents such as solvents on GOS synthesis has also been little explored and was mostly focused on β-galactosidase inactivation (Yoon *et al.*, 2005). Another strategy: the removal of products by separation techniques has been attempted, but methods for separation are complex and expensive and are not suitable for large scale industrial application.

We attempted to isolate a high GOS fraction for commercial application by selective crystallization of lactose. This sugar is known to crystallise readily in a number of food systems. This work did not yield crystals due to the formation of an high viscosity syrup when water was evaporated from GOS synthesis reaction mixtures. This syrup seemed to inhibit crystal formation. This work was preliminary and did not consider a wide variety of crystallisation conditions. Further efforts in this area might prove rewarding.

4.6 Mathematical modelling of GOS synthesis reaction

4.6.1 Full model applied on TLC data

The GOS synthesis reaction was modelled based on a mechanism (equations 3.1-3.4) described by Kim *et al*. (2004). The GOS synthesis reaction model had been previously modelled in the literature using the King-Altman transformation (Boon *et al*., 1999 and 2000; Neri *et al*., 2009). However, in the present work, the King-Altman transformation could not be used since an effect of enzyme concentration on GOS yield was observed (Figure 3.5). Therefore, the model of Kim *et al*. (2004) was chosen to fit the experimental data, since it considered enzyme concentration as part of the mechanism and had the most complete set of hypotheses. The model fitted the experimental data well (Figure 3.7), but precision problems arising from high standard deviations in the estimates of parameters and kinetic parameters with large correlation led to the search for a reduced model. Generally, a correlation higher than 0.95 between kinetic parameters will generate doubts about their estimation and associated errors (Donaldson *et al*., 1987).

4.6.2 Reduced model applied to HPLC data

The experimental data obtained with the HPLC assay were initially fitted using the aforementioned mathematical model (equation 3.15-3.17), based on that of Kim *et al*. (2004). Some GOS synthesis models previously proposed in the literature, *i.e.* Iwasaki *et al*. (1996), Zhou *et al*. (2003), used a high number of parameters in the model - up to nine parameters in some cases. In the present work, a simplified mathematical model was proposed, using a smaller numbers of reaction steps and with five parameters, to explain the transglycosylation reaction. This reduced reaction mechanism avoided an ill-conditioned model. The reduced model was based on some

considerations that emerged on the basis of a literature review of GOS reaction modelling (Boon *et al*., 1999 and 2000; Zhou *et al*., 2003). The simplifications we employed considered a rapid interaction between lactose and the active site of the enzyme, which was considered as an irreversible step. Allolactose formation, as an intermediary step of GOS formation (Jobe *et al*., 1972), was assumed to be of negligible influence. The reduced model obtained, and its simplifications, was similar to other models in literature (Boon *et al.*, 1999, 2000; Neri *et al.*, 2009).

The reduced model was shown to have the same precision as the full model and also a considerably lower degree of correlation between parameters (Table 3.3). The experimental data obtained from GOS synthesis with different Maxilact and Whey Permeate concentrations was fitted both as single experiments and as a global set of data.

The individual fitting of the enzymatic assays with the reduced model (Figure 3.17-3.22) gave a good prediction of the reaction mixture changes during GOS synthesis by β-galactosidase. The kinetic parameters obtained through the data modelling as single experiments were always significant (Table 3.4). From a study of the individual fits, excluding some outliers, there might be a unique set of parameters that would fit all the experimental data obtained.

To estimate general kinetic parameters for the whole transglycosylation reactions studied, a global fitting of the experimental data available was carried out. Data screening was applied by considering the ratio between the residual glucose and galactose present in the synthesis mixture, as previously shown by other authors (Boon *et al*., 1999). This system allowed the elimination of experimental data with an error greater than 10%, in order to yield a more homogeneous set of data.

The global fitting was firstly applied by separating the data sets on the basis of initial WP concentration, and then by considering all the data together. The estimation of the assays with 200 g/l Whey Permeate did not converge to a meaningful result. The global fitting of all the data showed a good fit for both of the initial WP concentrations used, especially for the synthesised GOS (Figures 3.27-3.30). In the fitting of all the data, two kinetic parameters gave a high standard error (Table 3.5). In order to obtain more information about these parameters further experiments are needed using changing substrate/enzyme concentrations levels. The set of fitting kinetic parameters obtained with 350 g/l WP is not significantly different to the 200 and 350 g/l in the global model, with the exception of $\ln(k_1)$. This result might indicate the necessity to analyse the data using a model that could consider differences between assays with 200 g/l WP and 350 g/l in $\ln(k_1)$.

4.6.3 Reduced model applied to experiments in the presence of solvents

The reduced model GOS synthesis mechanism (equation 3.15-3.17) was used to fit the GOS synthesis profiles in the presence of solvents. The ratio between $\ln(k_3)/\ln(k_{r3})$ (Figure 3.35) indicated that the relative importance of the hydrolysis of E:Gal complex (a GOS precursor) had been reduced in the presence of dioxane and diethyl ether. The ratio between $\ln(k_5)/\ln(k_{r5})$ (Figure 3.38) for the experiment with dioxane was found to be significantly smaller than for the other assays carried out. This may explain the lower level of GOS production in the presence of dioxane (Figure 3.31). The ratio between $\ln(k_5)/\ln(k_{r5})$ (Figure 3.38) showed that reactions in the presence of acetone, ethanol and diethyl ether are not significantly different from the control (Figure 3.31). Considering the predicted kinetic parameters for hydrolysis of the E:Gal complex (Figure 3.35) versus GOS formation (Figure 3.38), it is clear

that GOS formation is enhanced in organic solvents such as acetone and diethyl ether. On the basis of these results, future work at increased solvent concentrations may be of interest. The effect of low polarity solvents on the transglycosylation reaction may also be interesting to explore (Yoon *et al*., 2005).

4.6.4 Application of reduced model to comparison of β-galactosidases

The fitting of the experimental data for both β-galactosidases using the reduced model (equations 3.15-3.17) showed no significant difference between them for kinetic parameters of the GOS synthesis reaction. A fundamental point to note is that the β-galactosidases have been employed under *identical* conditions. If two enzymes are compared at different pH/temperature, it is not possible to deduce whether any improvement in the GOS yield may be ascribed to the enzyme source or to the pH/temperature or other differences in assay conditions.

5 Conclusions

The present work showed that GOS synthesis from β-galactosidase activity on Whey Permeate is possible. Enzyme and substrate concentration together with solvent were factors which were shown to significantly affect the level of GOS synthesised, while the enzyme source did not. GOS reaction kinetics could be described by a simple reaction mechanism, which allowed the prediction of GOS synthesis kinetics. This may have future industrial applications.

TLC analysis proved to be an adequate screening technique, but when quantitative methods were required, HPLC with RI was found to be the more appropriate method for monitoring GOS synthesis.

A simplified reaction mechanism model, that would take into consideration the enzyme concentration, was proposed and used to fit GOS synthesis experimental data. The estimated parameters from experiments were successfully used to compare and interpret changes in the reaction profile. Changes in model parameters due to the use of different lactose or enzyme concentrations, the use of solvents in the reaction media or due to the employment of different enzyme sources were investigated.

A maximum GOS yield of 24% was achieved, which is comparable with the present literature results. Although higher yields of GOS have been reported in the past, they do not seem to be reproducible by most of the studies on galactooligosaccharides synthesis from different β-galactosidases sources (Tables 1.5-1.10). Thus, recent reports in the literature do not claim higher than 25% GOS production despite the wide variety of enzymes and reaction conditions used. It is possible that this is due to the fact that this reaction is governed by thermodynamic factors that are not greatly influenced by reaction conditions.

Increasing enzyme concentration resulted in faster synthesis and degradation of GOS for a given WP concentration. Increasing WP concentration increased the level of GOS synthesised and increased the time taken to reach maximum GOS concentration.

Using 10% acetone, diethyl ether and ethanol influenced the profile of the GOS reaction progress, apparently shifting the kinetics towards GOS production and away from hydrolysis.

Comparison of two β-galactosidases under identical conditions showed that they had the same GOS synthesis profile when initial rates were normalised. From this work it is clearly necessary to have a common protocol to compare β-galactosidases from different sources. This would ameliorate the difficulties in obtaining any meaningful benchmark from previous results in the literature.

6 Further work proposed

On the basis of the results obtained in this work, it would be interesting to carry out further research to investigate the effect of higher WP concentration on GOS maximum yield and to study the effect of viscosity of the reaction medium on the enzymatic reaction. Particularly, the effect of 0.4 and 0.8% Maxilact on concentrations of 450 and 550 g/l Whey Permeate should be tested.

Considering the results with solvent addition, work should be carried out to examine increasing the level of the solvent to 20 and 30%, for example. The solvents most interesting for further study are acetone and ethanol, as these were shown to positively influence the kinetic parameters of the GOS synthesis reaction. Furthermore, it may be interesting to study solvent addition to concentrated Whey Permeate solutions. This would provide the possibility to combine the favourable effect of reduced water activity due to the solvent with high substrate concentration, which might improve GOS final yield.

A standard GOS assay for β-galactosidase should be adopted in order to have a comparable set of conditions to benchmark different literature studies present on the subject.

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